Evaluation of methods for chemical and biological carbohydrate oxidation

Jan Pieter van der Lugt
Evaluation of methods for chemical and biological carbohydrate oxidation

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College voor Promoties aangewezen,
op dinsdag 24 november 1998, te 16.00 uur door

Jan Pieter VAN DER LUGT

doctorandus in de scheikunde

geboren te Berkel en Rodenrijs
Dit proefschrift is goedgekeurd door de promotoren:

Prof. dr. ir. J.A. Duine en Prof. dr. ir. H. van Bekkum

Samenstelling promotiecommissie:

Rector Magnificus
Prof. dr. ir. J.A. Duine
Prof. dr. ir. H. van Bekkum
Prof. dr. R.A. Sheldon
Prof. dr. ir. C.T. Verrips
Prof. dr. N.C.M. Laane
Prof. dr. L. Dijkhuizen
Dr. A.C. Besemer

voorzitter
Technische Universiteit Delft, promotor
Technische Universiteit Delft, promotor
Technische Universiteit Delft
Universiteit Utrecht
Landbouwuniversiteit Wageningen
Rijksuniversiteit Groningen
SCA Research Zeist

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Van der Lught, Jan Pieter

Evaluation of methods for chemical and biological carbohydrate oxidation / Jan Pieter van der Lught.


Copyright © 1998 by J.P. van der Lught

All rights reserved. No parts of the material protected by this copyright notice may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying, recording or by any information storage and retrieval system, without written permission of the author.

Het werk beschreven in dit proefschrift werd uitgevoerd bij TNO Voeding in Zeist met financiële steun van het ministerie van Economische Zaken, TNO Voeding en SCA Research (Zeist).
Opgedragen aan Laurie en Pieter
Contents

Page

1 Chapter 1
Chemical and enzymatic oxidation methods for carbohydrates

37 Chapter 2
Bio-electrochemical production of aldobionic acids from the corresponding aldose sugars

57 Chapter 3
To a large scale purification and proteolytic activation of hexose oxidase from the red seaweed Chondrus crispus

79 Chapter 4
Probing the substrate specificity of hexose oxidase for biosensor and biocatalytic applications

95 Chapter 5
Improved production of hypohalous acids or halogens by vanadium-containing haloperoxidases

115 Chapter 6
Oxidation of dialdehyde inulin with bromine

139 Summary

142 Samenvatting

146 Curriculum vitae

147 Nwoord
CHEMICAL AND ENZYMATIC OXIDATION METHODS FOR CARBOHYDRATES

1. Introduction

Many carbohydrates, in monomeric as well as in polymeric form, are applied on a large scale. However, for certain applications, in, e.g., the detergent, pharmaceutical, paper, food, chemical, textile, and oil drilling industries, improvement of existing or introduction of new properties is required. Functional properties are in this respect: water binding, metal binding, gelation, surface sizing, adhesion and flocculation. In this ongoing process to develop better products and new applications of products, there is a constant need for new modification methods. Recent developments which also stimulate the latter are the exploration of green, renewable chemicals, and the replacement of chemical modifications by biochemical modifications in the food industry.

Modification of carbohydrates can be accomplished by either enzymatical, physical or chemical means. Physical modifications comprise processes like extrusion, gelatinization, blending and drying. Chemical modifications may consist of acid hydrolysis, cross-linking, substitution, oxidation and grafting. These modifications are connected to applications in different market segments (Table 1). In this thesis attention is focused on modification of carbohydrates by oxidation.

Oxidation of carbohydrates leads to enhanced gelatinization strength, increased interaction with other carbohydrates and peptides, increased swelling properties and higher solubility of the polymer [1-3]. The functionalized polymers can be used in the paper industry as sizing agents and water binders, in the laundry industry as sequestering agents, and in the food industry as gelating and thickening agents or as emulsifiers (Table 2). Oxidized monosaccharides are used as binders in the pharmaceutical industry.
Table 1. Overview of modified carbohydrates and their applications

<table>
<thead>
<tr>
<th>Modification</th>
<th>Product</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis</td>
<td>starch amylodextrins</td>
<td>food, pharmaceuticals</td>
</tr>
<tr>
<td>Oxidation</td>
<td>oxidized starch</td>
<td>food, paper, textile, detergents</td>
</tr>
<tr>
<td></td>
<td>oxidized inulin</td>
<td>detergents</td>
</tr>
<tr>
<td>Esterification</td>
<td>acetylated starch</td>
<td>food, textile, paper</td>
</tr>
<tr>
<td></td>
<td>starch succinate</td>
<td>food, paper, pharmacy</td>
</tr>
<tr>
<td></td>
<td>starch phosphate</td>
<td>food, paper, textile, pharmaceuticals</td>
</tr>
<tr>
<td></td>
<td>propylene-glycol alginate</td>
<td>food</td>
</tr>
<tr>
<td>Etherification</td>
<td>carboxymethyl starch/inulin</td>
<td>paper, textile, oil, food, detergents</td>
</tr>
<tr>
<td></td>
<td>hydroxethyl starch/cellulose</td>
<td>food, paper, textile</td>
</tr>
<tr>
<td></td>
<td>ethyl, methyl starch/cellulose</td>
<td>paper, textiles, detergents,</td>
</tr>
<tr>
<td></td>
<td>cationic starch</td>
<td>paper</td>
</tr>
<tr>
<td>Nitration</td>
<td>nitrocellulose</td>
<td>membranes, coatings, explosives</td>
</tr>
</tbody>
</table>

Table 2. Examples of industrially produced oxidized carbohydrate derivatives

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Product</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>oxidized starch</td>
<td>food, paper, textile, detergents</td>
</tr>
<tr>
<td>inulin</td>
<td>carboxymethyl inulin</td>
<td>pharmaceuticals</td>
</tr>
<tr>
<td>cellulose</td>
<td>6-carboxy cellulose</td>
<td>food, pharmaceuticals</td>
</tr>
<tr>
<td>lactose</td>
<td>lactobionic acid</td>
<td>food, pharmaceuticals</td>
</tr>
<tr>
<td>glucose</td>
<td>gluconic acid</td>
<td>pharmaceuticals, food</td>
</tr>
<tr>
<td>sorbitol</td>
<td>L-sorbose</td>
<td>pharmaceuticals (vitamin C)</td>
</tr>
<tr>
<td>gluconic acid</td>
<td>5-ketogluconic acid</td>
<td>food, fragrance</td>
</tr>
<tr>
<td>sucrose</td>
<td>sucrose monocarboxylic acids</td>
<td>food</td>
</tr>
</tbody>
</table>

In the oxidation of carbohydrates and carbohydrate derivatives, a distinction can be made between different types of oxidations:
- \( C_1 \)-oxidation (reducing end) on mono-, oligo-, and polysaccharides,
- oxidation at the glycosidic bond, to reduce molecular mass and viscosity and to enhance biodegradability,
- oxidation of a diol group (usually \( C_2-C_3 \) of pyranoses and \( C_3-C_4 \) of furanoses),
- the oxidation of the primary alcohol group (usually \( C_6 \)).

This chapter covers the oxidation techniques for carbohydrates in general. Emphasis is put on the homogeneous catalysis of chemical carbohydrate oxidation as well as on biochemical oxidation. This chapter will not discuss non-oxidative means to
introduce carboxylate groups into carbohydrates like, e.g., carboxymethylation. For this type of modification, the reader is referred to [4-6].

2 Chemical oxidation routes

2.1 C2,C3-oxidation of polysaccharides

Research on glycolic oxidation of polysaccharides has been focused on two different routes (Figure 1). The first route is a two-step cleavage of the C2,C3-diol moiety consisting of periodate oxidation to dialdehyde polysaccharides, followed by chlorite oxidation [7-10] of the resulting dialdehyde to a dicarboxylate moiety (see 2.1.1). The second route is a one-step oxidation with alkaline hypochlorite [11,12] (see 2.1.2).

![Chemical oxidation route diagram](image)

**Figure 1.** Schematic overview of two oxidation methods of starch to C2,C3-dicarboxy starch [13]

The two-step procedure is more selective, gives higher yields of carboxylic acid groups than the oxidation with hypochlorite and less chain degradation, so higher molecular weight products. It may be noted however, that fully glycolic oxidized polysaccharides are not biodegradable. Therefore, partial oxidation should be applied for application in detergent formulations. Selective heterogeneous oxidation of carbohydrates with oxygen of secondary hydroxyl groups can be achieved using a noble metal catalyst, but, in general,
primary alcohol groups are preferably oxidized. A review on this issue has been published recently [14].

2.1.1 Periodate/chlorite oxidation

Periodate oxidation of carbohydrates can be performed economically by chemical (hypochlorite [15,16]) or electrochemical [17,18] oxidation of the iodate formed. The major drawback of the second step in the oxidation (the chlorite oxidation of the dialdehyde) is the ineffective use of the oxidant; while one equivalent of chlorite is formally required for the oxidation of an aldehyde group, three equivalents are actually consumed, due to formation of chlorine dioxide gas as a side reaction:

\[
\text{R-CHO + ClO}_2^- \rightarrow \text{R-COO}^- + \text{HOCl} \tag{1}
\]

\[
\text{OCl}^- + 2 \text{ClO}_2^- \rightarrow 2 \text{ClO}_2 \uparrow + \text{Cl}^- + \text{OH}^- \tag{2}
\]

Floor et al. [15] used hydrogen peroxide as an inexpensive HOCl scavenger, preventing the consumption of ClO\(_2\), and the possible oxidation of carbohydrates with HOCl. In this way, the oxidation of dialdehyde polysaccharides can be carried out with two moles of sodium chlorite per dialdehyde moiety (Figure 2).

*Figure 2. Oxidative glycol cleavage of α-1,4-linked carbohydrate polymers [13]*

The procedure was found to yield dicarboxy polysaccharides selectively, with somewhat better calcium-complexing properties than those of the products obtained in the conventional procedure using six moles of chlorite. The improvement in calcium sequestering capacity was attributed to the higher molecular weight of the products and to the high oxidation degree. The maximum conversion of aldehyde to carboxyl groups was 80-85%, which was not further improved by the use of three
moles of either sodium chlorite or hydrogen peroxide per mole dialdehyde unit. This partial resistance to oxidation was ascribed to the intramolecular formation of relatively stable hemiacetals of aldehyde groups [13,19]. Veelaert et al. [20,21] developed a semi-continuous process comprising the batchwise oxidation of starch with a catalytic amount of periodate, together with a continuously iodate-periodate recycle through a separate electrochemical cell for instant regeneration. In this design, high performances combined with low periodate to starch levels were achieved, which makes large scale production of dialdehyde starch feasible from a technical and economical point of view. The disadvantage of the periodate oxidation (2.1.1) is that the process has to be carried out in two steps and that relatively expensive reagents such as sodium periodate ($7.50/kg) and sodium chlorite ($3.00/kg) are required. The hypochlorite process to produce C5,C6-dicarboxy polysaccharides is therefore of interest.

2.1.2 Hypochlorite oxidation
The hypochlorite oxidation has been described by Whistler et al. [11,12, 22] and the results have been confirmed by Floor et al. [13] and Nieuwenhuizen et al. [19]. Other advantages of this method are the relatively low price of hypochlorite, and the reduced degree of polymerization of the oxidized polysaccharides, which leads to a better biodegradability of the product. Besemer et al. [23-25] reported a series of studies where a considerable improvement of this reaction was achieved by a reduction of oxidative degradation. Bromide was found to exert a catalytic effect on the rate of sodium hypochlorite oxidation of glucans and fructans (Figure 3).

![Figure 3. Oxidation of polysaccharides with hypochlorite, and bromide as catalyst](image)

This HOBr/OBr⁻ oxidation proceeds about 25 times faster than with HOCl/OCl⁻. The reaction rate is highest between pH 6-11. In the pH region 9-10, the reaction rate does not vary, suggesting that both oxidizing species, OBr⁻ and HOBr contribute to
the reaction to a comparable extent. In the hypochlorite oxidation of starch, using bromide as the catalyst, a higher calcium sequestering capacity of the products was obtained compared to the non-catalyzed reaction. The pH optimum is at pH 10, which is somewhat higher than that of the non-catalyzed hypochlorite reactions. The better performance of the products may be ascribed to the fact that at high pH the decomposition of the oxidant is prevented, leading to a higher degree of oxidation.

![Chemical structures](image)

**Figure 4.** Hemiacetal and lactone structures obtained in the oxidation of starch

Moreover, at low pH the formation of hemiacetal groups (Figure 4) is enhanced, which may cause a low reactivity for further oxidation. Oxidation of inulin with a stoichiometric amount of sodium hypochlorite (3 moles per fructose unit) leads to the formation of 3,4-dicarboxy inulin in high yields (80-95%). These products prepared in the disodium form exhibit an excellent calcium sequestering capacity (2.0-2.5 mmol Ca/g). In contrast to the oxidation of starch, the calcium sequestering capacity of the dicarboxy inulin is not improved when bromide is used as a catalyst [22].

### 2.2 C₆-oxidation of carbohydrates

For the selective oxidation of primary alcohol groups (uronic acids production) in carbohydrates, mainly NO₂(N₂O₄) has been used [26-28]. A high degree of primary oxidation is obtained, but the reaction is characterized by considerable degradation of the polymer and other non-selective oxidation at the secondary alcohol groups. By reduction with NaBH₄, the polyol configuration may be restored, obviously accompanied by some epimerization. De Nooy et al. [29] greatly improved the method by applying nitrate as the oxidant with a catalytic amount of nitrite and by applying 85% phosphoric acid as the medium. In several carbohydrates the primary alcohol group could be completely (>95%) oxidized to the carboxylic acid. By conducting the reaction at low temperature (4°C), degradation of the polymers was
reduced significantly. Smaller substrates (monomers, oligomers) can be selectively oxidized with Pt/O₂ [30]. This reaction proceeds only very slowly with a low degree of oxidation when is applied to polysaccharides because of the heterogeneous character of the catalyst [31]. The metal-catalyzed C₆-oxidation of polysaccharides suffers from steric hindrance, resulting in low selectivity and yield. Much effort is being put in the optimization of the C₆-oxidation with noble metal catalysts of mono- and disaccharides. Reviews on this issue have been published recently [14, 41]. Casciani et al. [32] described the oxidation of an alkyl polyglucoside in water with the nitrogen oxide radical TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, Figure 5, 1) in which the oxidant was regenerated with OCl⁻/HOCl at pH 8.6. Davis and Flitsch [33] used TEMPO for the selective oxidation of partially protected monosaccharides and suggested the use of TEMPO for the oxidation of polysaccharides. Recently, the Nooy et al. [34-36] introduced the use of TEMPO in polysaccharide chemistry for the selective oxidation of primary alcohol groups. In this approach the stable organic nitroxy radical is used as a mediator between the oxidant (HOCl/HOBr) and the carbohydrate substrate. The actual oxidant is the nitrosonium ion 2, which is formed by one-electron oxidation of TEMPO. During the oxidation of the primary alcohol group of the carbohydrate, 2 is reduced to the hydroxylamine 3 which is reoxidized by HOBr/HOCl, upon which again TEMPO is obtained.

![Figure 5](image)

1. TEMPO and the species involved in the specific C₆-oxidation of carbohydrates

This radical has been applied in several studies on the oxidation of carbohydrate polymers [35,36]. The hypochlorite/bromide system was the regenerating oxidant in water. Primary alcohol groups in glucans and derivatives thereof are oxidized rapidly and completely. For pyranosides, the selectivity is higher than 98% and no side products are detected. The pH optimum for the reaction was between pH 9 and 10. The oxidation is found to be first order in TEMPO and Br⁻. Recently, this technology has been used in the synthesis of α- and β-D-glucopyranuronate 1-phosphate and
α-D-glucopyranuronate 1-fluoride and with the use of immobilized TEMPO [37], but it has not yet been applied on a large scale. In the Organon-Sanofi synthesis (55 steps) of a pentasaccharide with heparin properties, two TEMPO-catalyzed steps are applied [38].

2.3 C1-oxidation of carbohydrates

The C1-oxidation of carbohydrate polymers seems to be of limited interest. Oxidation of only the reducing end in a polymer has hardly any effect on the functional properties of the oligomers or polymers. This is not the case when relatively small oligomers are considered [39]. In the literature, no examples are found of the specific chemical oxidation of oligomers at the C1-position. In contrast, glucose as a monomer, and also other mono- and disaccharides have been subjected to oxidation studies.

Due to the large amount of glucose produced annually in the EU, and the production regulation, (approx. 1.7 million ton/year) glucose modification has been extensively studied in biotechnological and chemical oxidation routes. Worldwide, approx. 50,000 tonnes/year of gluconates are produced.

Gluconic acid

Among the different gluconates, the sodium salt is the most important for applications as metal chelating agent in conditions of extreme alkalinity, e.g., bottle washing. Pharmaceutical applications (calcium supplementation) as well as the use of glucono-δ-lactone as acidulant in food processing (preservation) are also important. Moreover, glucono-δ-lactone is an intermediate in a new class of green surfactants (Procter and Gamble, Hoechst).

Chemical oxidation of glucose to gluconic acid can selectively be achieved by air over heterogeneous catalysts. Especially specific supported bimetallic catalysts (Pd-Bi) performed well. High yields (98-99%) can be obtained [40-41]. Other sugars which can be oxidized by heterogeneous catalysis are sorbose resulting in 2-keto-L-gulonic acid (52% yield) [42], lactose to lactobionic acid [43] and gluconic acid to 2-ketogluconic acid (98% yield) [14].
**Glucaric acid / 2-keto-D-glucaric acid**

Glucaric acid can be obtained by oxidation of glucose at the C₅ and C₆-positions. Using HNO₃ as the oxidizing agent, yields of about 30% of crystalline K-glucarate are reported. With Pt/O₂, yields of around 60% are obtained. Due to the limited selectivity an economic chemical process for D-glucaric acid is not yet feasible [44].

3 Enzymatic oxidation routes

**Biological oxidations**

The use of chemical agents for modifying carbohydrates is not allowed in the application in food products, unless these products are legally accepted as food grade after, e.g., intensive toxicological investigation. Based on the seniority and proven safety polypropylene glycol alginate and oxidized starch are still used. However, a general trend exists to replace chemical oxidation methods by biochemical ones. In principle, biological oxidations are catalytic and clean, using mild oxidants, e.g., O₂ and H₂O₂. However, most of the enzymes shown in Table 3 have not been applied so far, and in a few examples bacterial cell mass is used instead of the soluble enzyme. Since the former can have a drawback for certain processes (contamination, consumption of the product by the organism), the use of the enzyme as such would be desirable. However, there are three important reasons why this has not been achieved so far: - lack of commercial availability; - the problem of cofactor-regeneration (the enzymes are respiratory-chain-linked dehydrogenases or NAD-dependent); - the narrow substrate specificity of most enzymes (e.g., glucose oxidase, galactose oxidase) precluding their use as a multi-purpose tool.

Numerous carbohydrate oxidizing enzymes are known (Table 3), most of which are active on monomers like glucose. Glucose is the most common source of carbon and energy for cellular growth. The common, but not universal pathway of its metabolism is first phosphorylation and subsequently degradation in a number of steps (glycolysis, citric acid cycle) to H₂O and CO₂ (Figure 6). Some of the intermediates, or derivatives of them produced in these pathways, have found application in the food industry (e.g., ethanol, lactic acid, citric acid).

Other products based on glucose as a building block in fermentation are cellular material or secondary metabolites (e.g., antibiotics). Beside the phosphorylative route, several bacteria are also (or only) able to dissipilate glucose in a non-
phosphorylative route. Some bacteria only carry out the first step of this route. This step is catalyzed by dehydrogenases or oxidases which oxidize glucose directly at the C₁, C₂, C₃ or C₆ position. In the first case, gluconic acid is formed which can subsequently be oxidized by specific dehydrogenases at the C₂ and / or the C₅-position to ketogluconates.

**Table 3. Overview of most important carbohydrate oxidizing enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC number</th>
<th>Substrate</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose oxidase</td>
<td>1.1.3.4</td>
<td>glucose</td>
<td>C₁</td>
</tr>
<tr>
<td>hexose oxidase</td>
<td>1.1.3.5</td>
<td>glucose oligomers</td>
<td>C₁</td>
</tr>
<tr>
<td>galactose oxidase</td>
<td>1.1.3.9</td>
<td>galactose</td>
<td>C₆</td>
</tr>
<tr>
<td>pyranose oxidase</td>
<td>1.1.3.10</td>
<td>glucose</td>
<td>C₂</td>
</tr>
<tr>
<td>glucose dehydrogenase¹</td>
<td>1.1.99.17</td>
<td>glucose</td>
<td>C₁</td>
</tr>
<tr>
<td>glucose-3-dehydrogenase</td>
<td>1.1.99.13</td>
<td>glucose</td>
<td>C₃</td>
</tr>
<tr>
<td>oligosaccharide oxidase</td>
<td>-</td>
<td>glucose oligomers</td>
<td>C₁</td>
</tr>
<tr>
<td>D-gluconate-2-dehydrogenase</td>
<td>1.1.1.215 / 1.1.99.3</td>
<td>gluconate</td>
<td>C₂</td>
</tr>
<tr>
<td>2-keto-D-gluconate dehydrogenase</td>
<td>1.1.99.4</td>
<td>2-keto-D-gluconate</td>
<td>C₅</td>
</tr>
<tr>
<td>cellobiose oxidase</td>
<td>1.1.3.25</td>
<td>cellobiose</td>
<td>C₁</td>
</tr>
<tr>
<td>cellobiose dehydrogenase²</td>
<td>1.1.5.1</td>
<td>cellobiose</td>
<td>C₁</td>
</tr>
</tbody>
</table>

¹ Industrial enzyme  
² Commercially available

The oxidation of the primary (C₆) alcohol group in glucose has been reported for cells from *Ustulina deusta* [45] or *Bacterium industrium* var. *hoshigaki* [46] under aerobic conditions. However, these data have not yet been confirmed by other workers. Only one enzyme (galactose oxidase) has been shown to oxidize a sugar constituent in a polymer (guar gum) [47]. This enzyme has its limitations with respect to application since only D-galactopyranosyl residues can be oxidized.

Recently, in the patent literature, an organism called *Pseudogluconobacter* (not found in taxonomic lists), has been claimed to oxidize glucose and oligomers at 3 different positions [48-50]. So far, the enzymes required for this oxidation have not been identified. Up to now, no other enzymes have been found which are able to oxidize carbohydrate polymers. As far as is known, carbohydrate polymers containing keto/aldo or carboxylic acid groups are synthesized from the corresponding monomer.
Carbohydrate polymers which are used as a source for cellular carbon and energy are first hydrolyzed to monomers and oligomers before oxidation to sugar acids or transformation into other sugars (by epimerases) occurs. As a consequence, oxidative modification of carbohydrate polymers can so far only be achieved by chemical methods. However, chemo-enzymatic oxidation may occur in nature in the extracellular environment of wood rotting fungi where, as is known, peroxidases generate HOCl which may also oxidatively attack carbohydrate polymers. The most important types of enzymatic oxidations (applied in vitro, or used in vivo) described in the literature are presented (Figure 6).

Figure 6. Schematic representation of the presence of carbohydrate oxidizing enzymes as produced by several micro-organisms (POX = pyranose oxidase, CPO = chloroperoxidase, CBO = cellobiose oxidase, GDH = D-glucose dehydrogenase, GADA = D-glucurate dehydrogenase, 2-KGDH = 2-keto-D-glucuronate dehydrogenase, 2,5-DKGR = 2,5-diketo-D-glucuronide reductase, GOX = glucose oxidase, HOX = hexose oxidase, GAX = galactose oxidase, G-3-DH = glucose-3-dehydrogenase, FDH = D-fructose dehydrogenase, 5-DF = 5-dehydro-D-fructose, 2-KG = 2-keto-D-glucuronic acid, 2,5-DKG = 2,5-diketo-D-glucuronic acid, 2-KLG = 2-keto-L-gulonic acid)
3.1 Oxidation of hexoses at the C₃ position

a. Glucose oxidase

Numerous micro-organisms, but especially fungi, are able to oxidize glucose to gluconic acid. The fungus *Aspergillus niger* is one of the best known, but also species belonging to *Penicillium*, *Gliocladium*, *Scopulariopsis* and *Gonotrobotis* are known to produce gluconic acid. The key enzyme involved in gluconate production by fungi is glucose oxidase (E.C. 1.1.3.4, GOX). GOX catalyzes the oxidation of β-D-glucose to glucono-δ-lactone, utilizing O₂ as electron acceptor, with the production of hydrogen peroxide [51]. The lactone hydrolyzes to gluconic acid spontaneously, but the hydrolysis can be accelerated by lactonase (present in commercial preparations of GOX). The enzyme has a very narrow substrate specificity and any alteration in the glucose molecule reduces the rate of oxidation. Upon any change on the C₂-position, (except for 2-deoxy-glucose) or on the C₃-position the activity is nearly lost. GOX is reported to be of interest for use in biosensors [52] or for the removal of oxygen in processed food. Research has been carried out on the immobilization of GOX, together with catalase to remove the hydrogen peroxide, for the enzymatic production of gluconic acid [53]. GOX is commercially exploited in amperometric biosensors to detect glucose in body fluids [54]. *A. niger* as well as the bacterium *Gluconobacter* (see below) are used for large scale gluconate production.

b. Glucose dehydrogenases

The group of dehydrogenases can be subdivided in those containing a cofactor (e.g., a flavin, a quinone, like pyrroloquinoline quinone (PQQ)) and those containing a coenzyme (NADP(H)) [55]. Two types of quinoprotein glucose dehydrogenases (GDH) exist, one soluble and the other membrane-bounded [56]. The soluble type has so far only been detected in *Acinetobacter calcoaceticus* strains [57,58], whereas the membrane-bound enzyme is widely distributed among Gram-negative bacteria like *Gluconobacter*, *Acetobacter*, *Pseudomonas*, *Acinetobacter*, *Escherichia* and *Erwinia*. The enzyme catalyzes the oxidation of glucose via glucono-δ-lactone (GDL) to gluconic acid (Figure 7).

Until now, the NAD(P)-dependent GDH is not commercially exploited, due to the high price of the coenzyme [60]. For analytical purposes, or for the production of high added value pharmaceutical products, this enzyme is studied for the regeneration of
NADP$^+$ or NAD$^+$ [61,62], thereby using mannitol dehydrogenase as a second enzyme, catalyzing the formation of mannitol from fructose.

Figure 7. Oxidation of glucose by PQQ dependent glucose dehydrogenase in the periplasmic space of Gram-negative bacteria. The presence of membrane bound lactonase in fungi (Lase, EC 3.1.1.17) accelerates the conversion of glucono-δ-lactone to gluconic acid. CM = cell membrane [59].

Table 4. Substrate specificity (relative $V'_m/K_m$) of GDH (membrane bound, soluble and immobilized on a redox polymer film) isolated from Acinetobacter calcoaceticus [57, Chapter 2]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble$^1$</td>
</tr>
<tr>
<td>D-glucose</td>
<td>100</td>
</tr>
<tr>
<td>D-allose</td>
<td>66</td>
</tr>
<tr>
<td>2-deoxy-D-glucose</td>
<td>4</td>
</tr>
<tr>
<td>D-galactose</td>
<td>18</td>
</tr>
<tr>
<td>D-mannose</td>
<td>4</td>
</tr>
<tr>
<td>D-xylose</td>
<td>10</td>
</tr>
<tr>
<td>D-ribose</td>
<td>1</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>11</td>
</tr>
<tr>
<td>lactose</td>
<td>24</td>
</tr>
<tr>
<td>maltose</td>
<td>60</td>
</tr>
<tr>
<td>cellobiose</td>
<td>48</td>
</tr>
</tbody>
</table>

$^1$ Conditions: 100 mM potassium phosphate pH 7.0 containing 10 mM magnesium sulfate, at 30°C. Substrate concentrations unknown.

$^2$ Conditions: 250 mM sodium phosphate pH 7.0 at 25°C. The cell potential was 600 mV vs. Ag/AgCl. Oxidation rate as $V_{max}/K_m$.

n.m., not measurable below substrate concentration of 50 mM, n.d. = not determined
The soluble PQQ-dependent GDH is also studied for biosensor applications [63-66] and may be effectively immobilized in a redox polymer network, consisting of a poly(vinyl)pyridine polymer, functionalized with osmiumbis(bipyridine) chloride [67, Chapter 2]. Immobilization on a larger scale was also carried out on rigid carbon foil and carbon felt, showing the possibility to use the device as a bioreactor for the bi-electrochemical production of gluconic acid or lactobionic acid [Chapter 2].

The enzyme has been over expressed in E. coli [68]. Soluble PQQ-GDH is commercially used in test strips for the determination of glucose [69]. Table 4 shows its specificity. In contrast to glucose oxidase, the absence of the 2-OH group of glucose is not detrimental for the activity of the membrane bound enzyme. The latter enzyme has a broad substrate specificity and oxidizes monosaccharides as well as disaccharides after solubilization.

c. Hexose oxidase

Hexose oxidase (EC 1.1.3.5, HOX) oxidizes D-glucose at the C1-position to glucono-\(\delta\)-lactone with the formation of hydrogen peroxide.

**Table 5. Substrate specificity of soluble and immobilized hexose oxidase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Soluble HOX (Chapter 3)(^1)</th>
<th>Immobilized HOX (Chapter 4)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V(_{\text{max}}) ((\text{U} \cdot \text{mg}^{-1}))</td>
<td>K(_{\text{m}}) ((\text{mM}))</td>
</tr>
<tr>
<td>D-glucose</td>
<td>72</td>
<td>4.8</td>
</tr>
<tr>
<td>D-galactose</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>cellobiose</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>D-mannose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>lactose</td>
<td>16</td>
<td>50</td>
</tr>
<tr>
<td>maltose</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>maltotriose</td>
<td>0.8</td>
<td>7.4</td>
</tr>
<tr>
<td>maltotetraose (22.5)</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>maltopentaose (25)</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>maltoheptaose (25)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>D-xylose (100)</td>
<td>0.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>rhamnose (200)</td>
<td>1.2</td>
<td>70</td>
</tr>
<tr>
<td>tetrasaccharide (10)(^3)</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) For conditions, see Chapter 3. n.d. = not determined
\(^2\) Conditions: substrates in 50 mM MES buffer, pH 6.1, cell potential is 500 mV vs. Ag/AgCl.
\(^3\) \(\Delta\)4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc
In comparison with glucose oxidase, hexose oxidase has a much broader substrate specificity, oxidizing a wide range of aldose sugars (Table 5).

The enzyme has been found in red seaweeds [70-74]. From citrus fruits, an enzyme with similar characteristics is capable of oxidizing several saccharides [76]. However, this enzyme has not been studied in detail yet. Incubation of a crude extract with a mixture of protease (Pronase) improved the specific activity and stability of HOX in the extract [70, Chapter 3]. Besides the already known effect of Pronase concerning the liberation of HOX from particles in the homogenate [70], another effect could be the modification of HOX by proteolytic attack [Chapter 3]. Purification of HOX by an isolation procedure incorporating a Pronase treatment step gave HOX of which the fragments had somewhat shorter N-terminal amino acid sequences than those derived from the gene sequence [77]. The enzyme may be used in the analysis of a broad spectrum of carbohydrates [72], and application of the enzyme as a biosensor by immobilization on a conducting polymer showed that the substrate specificity can even be broadened [Chapter 4].

Recently, the possible application of hexose oxidase in the baking industry has been mentioned [71,78]. The broad substrate specificity compared to glucose oxidase allows the oxidation of a wide range of sugars, thereby generating hydrogen peroxide as oxidant which is used to optimize dough performance. The HOX-gene from *Chondrus crispus* has been brought to expression in *Pichia pastoris*, *Saccharomyces cerevisiae* and *E. coli* [77]. However, the production levels of active enzyme are still poor at this moment and have to be improved. For this reason, and due to the fact that *C. crispus* has a long standing tradition as an edible organism, efforts have been made to develop a large scale production method with the use of carrageenanase, which reduces the viscosity of the crude extract [Chapter 3].

Recently, an enzyme with similar properties as HOX, isolated from *Acremonium strictum* T1, has been reported in literature [79,80]. The enzyme is called an oligosaccharide oxidase, and is able to oxidize several di- and oligosaccharides at the anomeric site, yielding the corresponding di- and oligobionic acids (Table 6). Differences in oxidation rate between the oligomers are rather small. A detailed comparison between HOX and oligosaccharide oxidase has not been made yet.
Table 6. Substrate specificity of glucooligosaccharide oxidase [79]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>maltose</td>
<td>100</td>
</tr>
<tr>
<td>maltotriose</td>
<td>98</td>
</tr>
<tr>
<td>maltotetraose</td>
<td>74</td>
</tr>
<tr>
<td>maltopentaose</td>
<td>46</td>
</tr>
<tr>
<td>maltohexaoase</td>
<td>66</td>
</tr>
<tr>
<td>maltolheptaose</td>
<td>56</td>
</tr>
<tr>
<td>D-glucose</td>
<td>59</td>
</tr>
<tr>
<td>lactose</td>
<td>64</td>
</tr>
<tr>
<td>cellobiose</td>
<td>47</td>
</tr>
</tbody>
</table>

1) Substrate concentration is 10 mM  
2) Conditions: 50 mM Tris-HCl buffer pH 7.8 at 30°C. Assay with 0.1 mM 4-amino antipyrine and horse radish peroxidase and 1 mM phenol, or with an oxygraph

d. Cellobiose oxidoreductases
The enzymes cellobiose dehydrogenase (EC 1.1.5.1, CDH) and cellobiose oxidase (EC 1.1.3.25, CBO) oxidize cellobiose to cellobiono-1,5-lactone with concomitant reduction of quinones, oxygen, or Fe(III). The lactone hydrolyzes spontaneously or with lactonase into cellobionic acid (Figure 8). The enzymes are found in wood-degrading fungi such as P. chrysosporium [75,76], Coriolus versicolor [83], Fomes annosus [84], Coniophora puteana [85]. However, also non-ligninolytic fungi like Monilia [86], and Sporotrichum thermophile [87] are found to produce this enzyme.

![Figure 8. Oxidation of cellobiose to cellobionic acid by CDH or CBO](image)

The role ascribed to both enzymes is to accomplish a synergistic link between cellulose and lignin degradation [78]. In the oxidation of cellulose, CBO and CDH reduce different types of quinones and phenoxy radicals, which are released during lignin degradation by lignin peroxidases, manganese peroxidases and laccases. Apart from the oxidative cellulose degradation, an extra function therefore is proposed, namely the prevention of repolymerization of phenoxy radicals, formed
when phenol oxidases (peroxidases and laccases) oxidize lignin and lignin degradation products. The functional role is still a matter of debate, since results have been reported in which the presence of CBO/CDH with lignin peroxidase and laccase reduces the rate of oxidation of lignin degradation products [90].

**Table 7.** Substrate specificity of CBO and CDH [92 - 95]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CBO(^1)</td>
</tr>
<tr>
<td>cellulose</td>
<td>100</td>
</tr>
<tr>
<td>cellobiose</td>
<td>88</td>
</tr>
<tr>
<td>cellotriose</td>
<td>68</td>
</tr>
<tr>
<td>cellotetraose</td>
<td>79</td>
</tr>
<tr>
<td>cellopentaose</td>
<td>102</td>
</tr>
<tr>
<td>cellohexaose</td>
<td>62</td>
</tr>
<tr>
<td>lactose</td>
<td>31</td>
</tr>
</tbody>
</table>

\(^1\) Conditions: 50 mM sodium acetate buffer, pH 5.0, with o-dianisidine and horse radish peroxidase at 30°C. Substrate concentrations unknown

\(^2\) Conditions: 100 mM sodium acetate buffer, pH 4.5, 0.7 mM substrate, 0.3 mM 3-methoxy-5-tert-butyl-1,2-benzoquinone at 24°C. n.m. = not measured

It has been demonstrated that CBO can be proteolytically cleaved into FAD- and heme-containing domains. The FAD fragment of CBO seems to have all the properties of CDH, suggesting that CDH is a cleavage product of CBO [89]. The substrate specificity is broad, oxidation of up to cellohexaose has been observed (Table 7) [88]. Disaccharides such as lactose can also be oxidized at C\(_5\) [91].

### 3.3 Oxidation at the C\(_6\) - position

**Galactose oxidase**

Galactose oxidase (EC 1.1.3.9, GAX) is a copper and organic cofactor (a tyr-cys adduct) containing monomeric protein with a molecular weight of about 68 kDa [96]. GAX is produced by a number of fungal species, but is usually obtained from *Dactilium dendroides*. Recently, this species has been shown to be similar to *Fusarium* [97]. It catalyzes the oxidation of primary alcohol groups to the corresponding aldehyde groups (Figure 9). An industrial application for the oxidation of various aliphatic alcohols [98] may not be expected yet, due to the low specific
activity of the enzyme. Glycerol is oxidized to optically pure S(-)-glyceraldehyde, but other data about the stereospecific effect (apart from the primary alcohol oxidation of various unusual L-sugars, leading to enantiomeric pure products [99]) are not yet known. Various aspects of research have been published in several reviews [47,96,100 -102]. The enzyme is studied for measuring galactose and its derivatives in biological fluids, or, in immobilized form, in the detection of stachyose, raffinose, melibiose and galactose in agro-raw materials and food products [103-105].

![Chemical structure](image)

**Figure 9.** The oxidation of galactose by galactose oxidase (GAX)

**Table 8.** Substrate specificity of galactose oxidase [47,106]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation (%)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-galactose</td>
<td>100</td>
</tr>
<tr>
<td>D-talose</td>
<td>52</td>
</tr>
<tr>
<td>methyl α-galactopyranoside</td>
<td>118</td>
</tr>
<tr>
<td>methyl β-galactopyranoside</td>
<td>340</td>
</tr>
<tr>
<td>3-O-methylgalactose</td>
<td>172</td>
</tr>
<tr>
<td>2-O-methylgalactose</td>
<td>50</td>
</tr>
<tr>
<td>melibiose</td>
<td>109</td>
</tr>
<tr>
<td>stachyose (Gal-(1→6)-Gal-(1→6)-Gal(1→1)-Fruc)</td>
<td>610</td>
</tr>
<tr>
<td>raffinose (Gal-(1→6)-Gal(1→1)-Fruc)</td>
<td>145</td>
</tr>
<tr>
<td>dihydroxyacetone</td>
<td>360</td>
</tr>
</tbody>
</table>

\(^1\)Conditions: 7 mM phosphate buffer pH 7.0, with o-tolidine and horse radish peroxidase at 30°C. Substrate concentration 0.5 mM

The use of galactose oxidase for the production of 5-C-hydroxymethyl aldoheoxes as intermediates has been patented by Procter and Gamble [107-108]. In a batchwise procedure, several D-aldohexoses were oxidized at C\(_6\), although relatively much enzyme is needed due to its low effectiveness. Some examples described are the oxidation of methyl β-D-galactopyranoside and lactitol. The products obtained can be used as sugar substitutes with a reduced caloric value. In 1987, the use of galactose oxidase in the synthesis of aldehyde groups containing heteropolysaccharides was patented by National Starch and Chemical [109]. These
products can be produced by the enzymatic oxidation of hydroxypropyl galactoglycoside starch ethers or ethyl galactoglycoside starch ethers with galactose oxidase. Recently the same company patented the use of galactose oxidase for the production of aldehyde-group containing cationic polysaccharides (e.g., from polygalactomannan, guar gum, locust bean gum, tamarind gum or gum arabic). The products are able to improve paper strength properties [110].

3.3 Oxidation at the C₂ - position

a. Pyranose oxidase

Enzymatic oxidation of D-glucose at the C₂-position, yielding D-glucosone (D-arabino-2-hexosulose), has been reported already in 1937 (Figure 10), [111]. The responsible enzyme, pyranose oxidase (EC 1.1.3.10, POX) was purified from Polyporus obtusus, uses oxygen as electron acceptor and produces hydrogen peroxide.

![Figure 10. Oxidation of D-glucose to 2-keto-D-glucose by pyranose oxidase (POX)](image)

Presently, pyranose oxidase has been purified to homogeneity of five different organisms: Coriolus versicolor, Polyporus obtusus, Phanerochaete chrysosporium, Peniophora gigantea, and a Bacidiomycetous fungus No. 52 [113-117]. These strains are members of the white rot fungi, and similarity exists in the characteristics of the enzyme although they appeared immunologically unrelated [118]. The substrate specificity of pyranose oxidase is shown in Table 9. In general, hexose substrates with the same structural and conformational features on C₂, C₃, and C₄ as glucose (the hydroxyl groups oriented in the equatorial positions) are the best.

The chemo-enzymatic conversion of D-glucose, via D-glucosone as the intermediate, in fructose (reduction of D-glucosone) has been considered some time ago as an industrially interesting process [118-121]. In the literature, it is referred to as the CETUS process, in which the hydrogen peroxide is removed by catalase, or utilized
in a co-process (with a haloperoxidase) in which propylene is halogenated to give propylene halohydrin. The latter might be enzymatically converted by a halohydrin epoxidase into propylene oxide. This process is not being used industrially.

Table 9. Substrate specificity of pyranose oxidase from Peniophora gigantea [108]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation ($V_{max} = 100%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>100</td>
</tr>
<tr>
<td>D-glucono-δ-lactone</td>
<td>1.5</td>
</tr>
<tr>
<td>L-sorbose</td>
<td>109</td>
</tr>
<tr>
<td>D-xylose</td>
<td>50</td>
</tr>
<tr>
<td>D-galactose</td>
<td>7.7</td>
</tr>
<tr>
<td>L-fucose</td>
<td>2.9</td>
</tr>
<tr>
<td>maltose</td>
<td>8.2</td>
</tr>
<tr>
<td>methyl β-D-glucoside</td>
<td>9.6</td>
</tr>
<tr>
<td>methyl α-D-glucoside</td>
<td>1.9</td>
</tr>
</tbody>
</table>

$^1$ Conditions: 100 mM potassium phosphate buffer pH 6.5 with ABTS and horse radish peroxidase at 30°C

Recently, an enzymatic process was suggested with the use of pyranose oxidase for the production of D-tagatose [122] (a non-caloric sweetener) starting from galactose (Figure 11). So far, the yield after reduction is, for unknown reasons, poor (30%).

![D-galactose](image1) → 1. POX 2. H$_2$ / Pd → ![D-tagatose](image2)

**Figure 11.** Conversion of D-galactose into D-tagatose by pyranose 2-oxidase (POX), and subsequent chemo-catalytic reduction (for details of rearrangement see Figure 12)

The basidiomycete Oudemansiella mucida was screened for the presence of pyranose oxidase and pyranose dehydratase (dehydration of glycosulosates at C$_3$ and C$_4$) [123]. This species did not produce the latter enzyme, although glucosone was converted into a new product (Figure 12, 2b). Structural analysis revealed the C$_2$ hydration of glucosone and a chemical rearrangement of the sugar (re cyclization).
Pyranose oxidase oxidized this molecule into a cyclic hemiacetal, which, in its linear form, has three adjacent carbonyl groups (Figure 12, 2b).

\[ \text{CHO} \quad \text{C=O} \quad \text{C=O} \]
\[ \text{HCC-OH} \quad \text{HCC-OH} \]

\[ \text{2b} \quad \text{CH}_2\text{-OH} \]

\[ \begin{array}{c}
\text{2a} \\
\text{POX} \\
\text{H}_2\text{O}_2 \quad \text{O}_2
\end{array} \]

\[ \begin{array}{c}
\text{2b} \\
\text{CH}_2\text{-OH}
\end{array} \]

\[ \begin{array}{c}
\text{1b} \\
\text{H}_2\text{O}
\end{array} \]

\[ \begin{array}{c}
\text{1a} \\
\text{POX}
\end{array} \]

\[ \begin{array}{c}
\text{1c} \\
\text{H}_2\text{O}_2
\end{array} \]

Figure 12. The 2-step oxidation of D-glucose to D-urethra-heros-2,3-dailies (1a,b,c = D-arabino-heros-2-ulose, C-2 hydrated and recyclized respectively, 2a,b = D-urethra-heros-2,3-dailies in cyclic hemiacetal and linear form, respectively) [123].

b. Pyranose 2-dehydrogenase

Recently, a novel C\textsubscript{2}-specific sugar oxidoreductase, designated as a pyranose 2-dehydrogenase, has been reported [124]. The enzyme is isolated from the fungus Agraricus bisporus and uses 1,4-benzoquinone, 3,5-di-t-butyl-1,2-benzoquinone or 2,6-DCIP as electron acceptors.

In contrast to oligomeric intracellular pyranose oxidase, this enzyme is secreted into the extracellular fluid. The enzyme is rather nonspecific (Table 10). Similar to the function of celllobiose oxidase and celllobiose-dehydrogenase in lignin degradation, this enzyme probably might also be involved in the prevention of repolymerization of phenoxy radical and quinone degradation products formed during ligninolysis [125,126].
Table 10. Substrate specificity of pyranose 2-dehydrogenase [124]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (%)(^{1})</th>
<th>Substrate</th>
<th>Activity (%)(^{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>100</td>
<td>cellobiose</td>
<td>94</td>
</tr>
<tr>
<td>D-xylose</td>
<td>116</td>
<td>maltose</td>
<td>93</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>113</td>
<td>maltotriose</td>
<td>70</td>
</tr>
<tr>
<td>D-galactose</td>
<td>88</td>
<td>sucrose</td>
<td>63</td>
</tr>
<tr>
<td>L-glucose</td>
<td>61</td>
<td>(\alpha,\alpha)-trehalose</td>
<td>54</td>
</tr>
<tr>
<td>D-ribose</td>
<td>34</td>
<td>lactose</td>
<td>21</td>
</tr>
<tr>
<td>D-allose</td>
<td>30</td>
<td>methyl (\alpha)-D-glucopyranoside</td>
<td>117</td>
</tr>
<tr>
<td>D-arabinose</td>
<td>18</td>
<td>methyl (\beta)-D-glucopyranoside</td>
<td>69</td>
</tr>
<tr>
<td>L-sorbose</td>
<td>10</td>
<td>methyl (\alpha)-D-galactopyranoside</td>
<td>23</td>
</tr>
<tr>
<td>D-arabino-2-hexosulose</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucono-1,5-lactone</td>
<td>118</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\) Conditions: 50 mM sodium phosphate (pH 6.5) with 1,4-ber zoquinone as electron acceptor at 25°C. Substrate concentration is 25 mM.

3.4 Oxidation at the C₃ - position

Glucose-3-dehydrogenase
Specific oxidation at the C₃-position of glucosides has been reported for only two bacteria. The enzyme is named glucose-3-dehydrogenase (EC 1.1.99.13), or D-aldohepxopyranoside-3-dehydrogenase, and oxidizes a wide range of sugars at the C₃-position. The enzyme is found in the cytoplasmic fraction of *Agrobacterium tumefaciens* [127,128], and in the membrane fraction of *Flavobacterium saccharophillum* [129,130]. The substrate specificity of the enzyme isolated from *A. tumefaciens* is broad. The substrate specificity isolated from *F. saccharophillum* has not been determined in detail as compared to the *A. tumefaciens* dehydrogenase (Table 11).

No specific applications of 3-keto-glucosides are known at this moment. One might consider the use of these 3-oxidized sugars for the controlled production of carbohydrate-protein conjugates, via the reductive amination of the keto-group or by Maillard-type reactions via the tautomerization involved in the imine group.
Table 11. Substrate specificity of two glucose-3-dehydrogenases [127,129]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. tumefaciens(^2)</td>
</tr>
<tr>
<td>cellubiose</td>
<td>100</td>
</tr>
<tr>
<td>maltose</td>
<td>70</td>
</tr>
<tr>
<td>maltobionate</td>
<td>46</td>
</tr>
<tr>
<td>sucrose</td>
<td>12</td>
</tr>
<tr>
<td>α,α-trehalose</td>
<td>30</td>
</tr>
<tr>
<td>leucrose</td>
<td>12</td>
</tr>
<tr>
<td>lactose</td>
<td>84</td>
</tr>
<tr>
<td>lactobionate</td>
<td>100</td>
</tr>
<tr>
<td>lactulose</td>
<td>85</td>
</tr>
<tr>
<td>melezitose</td>
<td>10</td>
</tr>
<tr>
<td>raffinose</td>
<td>7</td>
</tr>
<tr>
<td>D-glucose</td>
<td>84</td>
</tr>
<tr>
<td>methyl α-D-glucoside</td>
<td>84</td>
</tr>
<tr>
<td>methyl β-D-glucoside</td>
<td>85</td>
</tr>
<tr>
<td>glucose-1-phosphate</td>
<td>62</td>
</tr>
<tr>
<td>anhydro-1,6-D-glucose</td>
<td>4</td>
</tr>
<tr>
<td>2-deoxy-D-glucose</td>
<td>22</td>
</tr>
<tr>
<td>D-galactose</td>
<td>56</td>
</tr>
<tr>
<td>methyl β-D-thiogalactoside</td>
<td>10</td>
</tr>
<tr>
<td>D-mannose</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^1\) Conditions: 0.1 mM phosphate buffer, pH 6.0, 2 μM 2,6-DCIP at 30°C. n.d. = not determined  
\(^2\) Substrate concentration = 10 mM  
\(^3\) Substrate concentration = 0.26 mM

3.5 Oxidation at the C\(_2\) and C\(_5\) - positions

2-keto and 5-ketogluconate dehydrogenases  
2-Keto-D-gluconic acid is a precursor in the industrial production of D-arabino-ascorbic acid. It is also an intermediate for the production of 2-carboxy-D-gluconic acid and 2-amino-D-gluconic acid (laundry- and surfactant applications, respectively) [44]. Several bacteria (Acetobacter, Pseudomonas, Xanthomonas) convert D-glucose into 2-keto-D-gluconic acid. Acetobacter species also produce 5-keto-D-gluconic acid, a compound which is of much interest for the production of 5-methyl-4-hydroxy-3-furanose, an important fragrance. 2,5-diketo-D-gluconic acid is an interesting intermediate for the production of L-ascorbic acid. There are a number of processes commercially operative for the production of L-ascorbic acid. Some of these processes result in the preliminary production of 2-keto-L-gulonic acid (2-KLG) which
can then be rather simply converted to ascorbic acid through acid- or base-catalyzed cyclization (Figure 13). Accordingly, 2-KLG has become, in itself, a material of considerable economic and industrial importance. The traditional production of ascorbic acid occurs according to the so-called Reichstein synthesis. This process consists of 8 steps, in which diacetone-2-keto-L-gulonic acid is utilized as a precursor for 2-KLG. This intermediate is generated through a series of reductive and oxidative steps involving hydrogenation of glucose to D-sorbitol, microbiological conversion of D-sorbitol into L-sorbose (A. suboxydans) and, e.g., permanganate oxidation of diacetone-2-keto-L-gulonic acid. Such a sequence is more complex than the microbiological conversion of glucose into 2,5-diketo-D-gluconic acid. Research has been focused on the microbiological fermentation with strains from Acetobacter, Gluconobacter and Pseudomonas [131, 132]. The reduction of 2,5-diketo-D-gluconic acid into 2-keto-D-gulonic acid (2-KLG) can be carried out chemically or enzymatically [133] with the enzyme 2,5-diketo-D-gluconate reductase (Figure 13). The latter procedure has been developed by Genentech [134,135] making use of recombinant micro-organisms (Erwinia herbicola, Acetobacter cerinus, Gluconobacter spp.) equipped with an expression vector containing the gene for 2,5-DKG reductase from Corynebacterium sp. ATCC 31090. Glucose is then converted directly into 2-KLG. However, the production level of 2-KLG is still too low to justify industrial production [136].

![Diagram](image)

**Figure 13.** Microbiological production of 2-keto-L-gulonic acid (5), through enzymatic conversions, including D-glucose dehydrogenase (a), D-gluconate dehydrogenase (b), 2-keto-D-gluconate dehydrogenase (c) and 2,5-diketo-D-gluconic acid reductase (a). 1 = D-glucose, 2 = D-gluconic acid, 3 = 2-keto-D-gluconic acid, 4 = 2,5-diketo-D-gluconic acid, 5 = 2-keto-L-gulonic acid, 6 = L-ascorbic acid
Recently, the enzymatic synthesis of L-tagatose from galactitol with the enzyme galactitol dehydrogenase has been published [137]. As this enzyme is NAD$^+$-dependent, continuous regeneration of NADH is required with NAD-dependent lactate dehydrogenase. This route has to be further optimized in future because pyruvate is an expensive substrate.

4 Carbohydrate oxidation: current status and perspectives

Chemical methods
Although many chemical methods for carbohydrate oxidation have been described in the literature, only the random chemical oxidation of starch and the Pt-catalyzed C$_1$-oxidation of glucosides (Südzucker, D) are applied on an industrial scale. In general, heterogeneous oxidation is not applied on a large scale, due to fouling of the catalyst and the low specificity. The homogeneous diol oxidation yields dicarboxy polysaccharides. At this moment these products are not competitive with existing calcium sequestrants (polyacrylates). Cheaper carbohydrate sources (e.g., B-starch) might be of interest to develop dicarboxy polysaccharides with a better price-performance ratio.

Based on the experience gained with the specific diol oxidation of starch and inulin, carboxymethylated inulin, as a cheaper alternative to produce, has recently been introduced as co-builder in laundry applications on the Belgian market (Cosun, NL). The TEMPO-mediated oxidation of the primary alcohol groups in polysaccharides appears to be a general and highly selective reaction. Hardly any degradation of the carbohydrate polymer occurs, which makes this type of reaction, in principle, suitable for production of various classes of carboxylated polysaccharides. Also, these products can be envisaged as precursors for further derivatization (partial diol-oxidation, cross-linking, esterification, etherification, succinate coupling and reductive amination). Based on the current attention for this type of oxidation by various research groups, and a number of (non-disclosed) application patents filed by several industries, it is expected that industrial application of the TEMPO-mediated C$_1$-oxidation of carbohydrates will be realized in the near future [138]. An example in the area of fine chemical synthesis is a pentasaccharide with heparin properties (Organon, NL and Sanofi, F), where two synthetic steps are TEMPO-catalyzed oxidations.
Chapter 1

The use of peracetic acid for the in situ generation of bromine, might be a step forward in the development of a salt free oxidation process for polysaccharides. With this technique, dialdehyde polysaccharides can be oxidized, yielding products with aldehydes as well as carboxylic groups in the polymers. These products can be used for further derivatization for application in non-food products.

**Biological methods**

Although many enzymes or activities have been described catalyzing the oxidation of sugars at several specific positions, only a few enzymes and micro-organisms are used in industry for that purpose. Glucose oxidase is used in the baking industry for the improvement of the quality of bread dough and biscuits, caused by the in situ generation of H₂O₂ from glucose and O₂ by the enzyme. In this respect, hexose oxidase (HOX) or oligosaccharide oxidase could be much better candidates as they have broad substrate specificities so that H₂O₂ can be generated from the large spectrum of aldose (oligo)saccharides present in dough. Since successful steps have been made for the production of HOX at a relatively large scale, either via recombinant organisms containing the hox gene [77] or by a method enabling isolation of HOX from seaweed at large scale [70, Chapter 3], this enzyme seems to have potential in this field. Since the action of peroxidases requires the presence of H₂O₂, these oxidases might also become important in the future where the application of the peroxidase requires in situ generation of H₂O₂ (biopulping, laundry industry). When cellulose sugars serve as the substrate for the oxidase, also cellobiose oxidase could be a candidate.

Since determination of glucose concentrations is of utmost importance in the medical field and in food and fermentation industries, much effort has been made with respect to rapidity, reliability and on-line aspects of these determinations. With respect to enzymatic methods, several biochemical companies provide commercial test kits based on glucose oxidase or NAD-dependent glucose dehydrogenase. So far, determination of glucose in body fluids was mainly based on diagnostic test strips containing glucose oxidase as the sensing enzyme. Recently, a strong improvement has been achieved by developing test strips based on sGDH. Since sGDH is also superior compared to GOX in amperometric biosensors based on the wired enzyme principle [67], sGDH may also replace GOX in this field. It is to be expected that research aimed at obtaining stable enzyme electrodes with adequate
electron transfer from cofactor to electrode may provide spin off for bio-electrochemical carbohydrate oxidations (see below).

Production of gluconic and lactobionic acid from the corresponding aldose sugars nowadays occurs (Avebe, NL) via the bacterium *Gluconobacter oxydans*, the enzyme catalyzing this being the membrane-bound, PQQ-containing glucose dehydrogenase, also present in many other Gram-negative bacteria. Production of other aldobionic acids is hampered by the fact that the enzyme has a rather narrow substrate specificity. Thus, to achieve this, either the enzyme should be modified or other enzymes should be used. With respect to the latter, again HOX and the oligosaccharide oxidase but also soluble, PQQ-containing glucose dehydrogenase could be attractive enzymes. It should also be realized that the enzymes could be used as such for the conversion processes, circumventing the problems caused by the use of whole cells. So far, in most cases the use of redox enzymes as such was problematic due to cofactor regeneration. However, as shown in chapters 2 and 4, electrochemical cofactor regeneration has now been achieved for HOX and sGDH, respectively, in conversion experiments. Preliminary data (Chapter 2) indicate that such a bio-electrochemical reactor could be economically feasible. Since HOX has been shown to have a broad substrate specificity which even broadens upon immobilization on the electrode (chapter 4), HOX could be used for the oxidation of a large number of sugars at the free C₁ position in a way much more specific than is possible with chemical methods.

Several enzymes have been described which oxidize a sugar molecule at different positions yielding keto sugars. So far, except for the production of 2,5-diketo-D-gluconic acid and 2-keto-D-gulonic acid, no applications of these enzymes or organisms containing them are known. The reason could be the non-commercial availability of these enzymes or the too low availability of the ketosugars, hampering studies on their application. The enzyme galactose oxidase has been subject of research for many years, but despite the fact that the gene has been over-expressed and patent applications for modification of galactose or galactose-containing polysaccharides have been filed, no industrial production of this enzyme is known yet.

Limited papers have been published on the selective oxidation of sugars at the C₂- and C₃- position by pyranose oxidase and glucose-3-dehydrogenase. However, the production of keto-sugars has recently gained industrial interest for the production of
flavor precursors [139,140] and the search for carbohydrate-protein conjugates as functional ingredient (emulsifiers, surfactants, water binders, thickening agents). These type of conjugates can be applied in food and non-food applications when produced by reductive amination or Maillard-type reactions of the keto-group. Therefore, these enzymes might be of interest in future when the functional behavior of these conjugates has been studied in more detail.

**Chemo-enzymatic methods**

Oxidation of sugars is possible with hypohalous acids as well as halogens. Since it is known that certain peroxidases form these hypohalous acids from the corresponding halide and $\text{H}_2\text{O}_2$, and the fact that oxidation of the carbohydrate converts the acid into halide, implicate that such oxidations could be carried out with a catalytic amount of halide (compared with the stoichiometric amount formed with the chemical method). However, it appears that in the pH range in which these hypohalous acids exist, the rate of decomposition with $\text{H}_2\text{O}_2$ is much larger as compared with that of carbohydrate oxidation with the hypohalous acids (chapter 5 and 6). On the other hand, since $\text{Br}_2$ is reasonably stable at low pH, carbohydrate oxidation with $\text{Br}_2$ generated at low pH and high $\text{Br}^-$ concentrations with chloroperoxidase seem feasible. However, also under these conditions the rate of carbohydrate oxidation should not be too slow since otherwise decomposition of $\text{Br}_2$ as well as enzyme inactivation by the oxidizing species take place, as shown in the case of DAI (Chapter 6). Since generation of $\text{Br}_2$ from $\text{Br}^-$ and peracetic acid was found to be successful, and peroxidases have been described which generate peracetic acid from acetic acid and $\text{H}_2\text{O}_2$, this process could provide another chemo-enzymatic system which could be explored.

5 Aim and outline of the thesis

The aim of the research carried out for this thesis was to investigate the possibilities to develop more specific, less salt producing, catalytic processes for the oxidation of carbohydrates. In this chapter, a general overview of the relevant chemical and biological modifications is presented. Although cofactor regeneration by electrochemical oxidation via electron conducting polymers has been investigated in the field of amperometric biosensors [142], so far
this principle has not been explored in the field of conversion processes. Here we investigate this possibility for the application of glucose dehydrogenase (Chapter 2). To explore the potential of application of the recently characterized enzyme hexose oxidase [70], investigations were carried out to enable the large scale isolation of the enzyme (Chapter 3) and the possible application and substrate specificity of HOX in biosensors (Chapter 4).

It is known that carbohydrates can be oxidized by hypohalites and halogens [13,23]. To avoid the production of stoichiometric amounts of salt, a cyclic regeneration could, in principle, be applied using catalytic amounts of haloperoxidase and halide and stoichiometric amounts of H₂O₂. To investigate the feasibility of this chemo-enzymatic approach, the kinetics of the conversion of Br⁻ into Br₂/Br₃⁻ with a vanadium-containing chloroperoxidase were studied in Chapter 5.

In Chapter 6, the feasibility of oxidizing dialdehyde inulin with Br₂ as such, with Br₂ formed from peracetic acid, and Br₂ generated by vanadium-containing chloroperoxidase from Br⁻ and H₂O₂, is described.

6 Literature

54. Boehringer Ingelheim (G).
69. Glucotrend, Roche Diagnostics (G)
77. WO. patent 96/40935 (1996).
78. WO. patent 96/38851 (1996).
     167, 493.
     214, 795.
118. Liu, T.-N. E., Wolf, B., Geigert, J., Neidleman, S.L., Chin, J.D., Hirano, D.S.,
120. US. patent 4,569,910 (1986).
121. Eur. patent 0,228,274 A2 (1986).
     Chem. 15, 115.
     Res. 278, 59.
     167, 119.
     Phytochemistry 25, 2537.
129. Takeuchi, M., Ninomiya, K., Kawabata, K., Asano, N., Kameda, Y., Matsui, K.
     52, 1905.
140. Flavor Technology; Physical Chemistry, modification and process (1995), ACS Washington, DC (USA)
BIO-ELECTROCHEMICAL PRODUCTION OF ALDOBIONIC ACIDS FROM THE CORRESPONDING ALDOSE SUGARS

Summary

Soluble quinoprotein glucose dehydrogenase (s-GDH, EC 1.1.99.17) from *Acinetobacter calcoaceticus* was effectively immobilized in a redox polymer network, consisting of a poly(vinyl)pyridine polymer functionalized with osmiumbis(bipyridine) chloride, on a carbon disk electrode (6 mm²). The resulting enzyme electrode operated optimally at a cell potential of 600 mV vs. Ag/AgCl in 250 mM sodium phosphate buffer, pH 7.0, with glucose as substrate. Under these conditions, a maximum current density of 24 A·m⁻² was measured. The temperature optimum of the immobilized enzyme was 37°C, and the pH optimum was between pH 6.0 and 8.0. The electrode was used to convert glucose and lactose into gluconic acid and lactobionic acid, respectively.

Enzyme electrodes of larger dimensions were prepared with flat plate rigid carbon (1.5 x 4 x 0.2 cm). With the resulting electrode a current density of 3.3 A·m⁻² (99 A·m⁻³ volumetric productivity) could be obtained. This is 14% of the activity obtained with the carbon disk electrode. Using linear extrapolation of the productivity obtained at small scale, a production of approximately 14 g gluconic acid or 25 g lactobionic acid/m³ reactor volume per hour is predicted. Assuming an operational life time for the electrochemical reactor of one month, the enzyme costs and redox polymer costs are negligible (less than NLG 0.02/kg of product). The cost price determining factors for this process at industrial scale, are the reactor costs and the electricity costs. Economic evaluation of a large scale biocatalytical oxidation with this type of enzyme electrode indicates that this process may be economically feasible, since the indicative cost price of aldonic acids is competitive with the present one. To increase the volumetric productivity, enzyme immobilization on a 2 cm² carbon felt electrode was carried out. A very stable, although low current density of 0.75 A·m⁻² (213 A·m⁻³ volumetric productivity) suggests that the major part of the enzyme has no effective contact with the polymer, or that the overall contact is poor. Although the volumetric productivity with carbon felt is increased, further research on the optimization of immobilization procedures should be carried out to evaluate the feasibility of this approach.
Oxidoreductases are enzymes that catalyze the oxidation and/or reduction of their substrates. This class of enzymes catalyzes reactions which are of potential interest for a wide variety of processes in the chemical, pharmaceutical and food industries. However, processes in which these enzymes are exploited are quite rare. This is caused by the poor availability of these enzymes (high costs) as well as by the need for cofactor regeneration. Cofactor regeneration is possible by chemical, enzymatical or electrochemical methods. In this chapter, research is described to evaluate the electrochemical cofactor regeneration at an economical feasible way.

The development of immobilized enzyme electrodes has received much attention recently [1,2]. In most cases applications with biosensors are studied, mainly focused on the measurement of glucose. Emphasis is put on the development of systems to oxidize glucose into glucono-δ-lactone, and to transfer the electrons from the reduced enzyme to an electrode. One of the latest developments is the immobilization of redox enzymes in a network of electron conducting polymers, coupled to an electrode [2-6] (Figure 1).

![Diagram](image)

**Figure 1.** Simplified scheme for the direct electrochemical regeneration of enzymes (E = enzyme, CG = conducting group of polymer, - = polymer network)

The theory behind this principle is the reaction of the redox centers of the polymer network with the reduced cofactor in the immobilized enzyme, resulting in an electron transfer from the reduced redox centers to the electrode, regenerating the cofactor in this way. To achieve an efficient electron transfer to the electrode, it is
necessary to minimize the reaction of the reduced cofactor with oxygen. Therefore, the use of oxidases is not optimal, although most of the research is conducted with glucose oxidase. Another drawback of this enzyme is its low operational stability, because the reduced enzyme reacts with \( \text{H}_2\text{O}_2 \) [7]. Therefore, this reaction has to be carried out in the presence of catalase. Alternative electron acceptors like benzoquinone have been used in attempts to circumvent the inactivation and oxygen-dependence of glucose oxidase [7,8]. The operational stability of the enzyme was increased 50 times, and an overall productivity of 0.1 kg gluconic acid·h\(^{-1}\)·L\(^{-1}\) reactor was achieved. However, disadvantages of this method are the absolute absence of oxygen which is required as well as the necessity of an additional purification step to remove the mediator from the final product.

Also NAD(P)-dependent dehydrogenases are not attractive, because the reduced coenzyme NAD(P)H is not covalently bound to the enzyme and diffuses away from the enzyme [9-13]. Moreover electrochemical oxidation of NAD(P)H causes formation of inactive derivatives. Thus, a cofactor-containing glucose dehydrogenase would be most suitable for the development of an amperometric glucose sensor. A very promising system for developing an amperometric biosensor has recently been described [14]. Soluble quinoprotein glucose dehydrogenase (s-GDH, EC 1.1.99.17) from \textit{Acinetobacter calcoaceticus} [15-18] was immobilized on the redox polymer poly(vinylpyridine) functionalized with osmiumbis(bipyridine) chloride (POs-EA), and coupled to a carbon electrode. With this amperometric oxygen insensitive glucose sensor a current density of 16 A·m\(^{-2}\) has been achieved [14].

The enzyme has a firmly bound quinone cofactor (PQQ, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]quinoline-4,5-dione) [19], and is active with numerous artificial electron acceptors but not with oxygen. The catalytic activity is very high: per milligram of protein, approximately 3-8 mmoles of glucose are oxidized per minute with 2,6-dichlorophenol indophenol and Wurster's Blue, respectively [20,21], which is up to 20 times the activity of pure glucose oxidase [14]. In view of the high specific activity and the successful electrochemical cofactor regeneration achieved in the investigations on biosensor development [14], this system was chosen to investigate the economic feasibility of bioelectrochemical production of aldonic acids.

At this moment, gluconic acid and aldobionic acids are produced by fermentation, using \textit{Gluconobacter oxydans} [22]. The oxidation of glucose occurs with a membrane bound GDH, and oxidation of oxygen (cofactor regeneration) takes place
via the respiratory chain of the organism. The cost price determining factors of this process are the microbiological production of the biocatalyst, the microbiological conversion and the downstream processing of the final product. The cost price of these products is estimated between NLG 2/kg for sodium gluconate to NLG 5/kg for glucono-δ-lactone (pharmaceutical quality) and NLG 10/kg for lactobionic acid (70%, sirup). Using these values and the extrapolated data of the results here described, the economic feasibility of bio-electrochemical production of aldobionic acids was evaluated.

In this chapter, the immobilization of s-GDH in electrodes based on earlier work from Ye et al. [14] is described. The optimal conditions for enzyme immobilization are evaluated with respect to the current density. Based on these data, small scale oxidations of glucose and lactose into their respective alcobionic acids were carried out. Based on the current densities obtained with s-GDH immobilized on flat plate rigid carbon and carbon felt, possibilities for upscaling and economic feasibility for the bio-electrochemical production of aldonic acids are discussed.

2 Materials and Methods

Materials

Purified apo-s-GDH was a gift from Professor J.A. Duine (Delft University of Technology, The Netherlands) and was reconstituted with PQK essentially according to Van der Meer et al. [23]. The activity of s-GDH was determined in 990 μL of 50 mM potassium phosphate buffer (pH 7.0) containing 50 μM 2,6-dichlorophenol indophenol, 50 mM glucose (in anomic equilibrium) and 1 mM phenazine methosulfate. To this reaction mixture 10 μL holo-s-GDH (ca 0.5 μg·mL⁻¹) was added and the activity was determined spectrophotometrically with DCPIP at 600 nm (ε = 21 mM⁻¹·cm⁻¹). The protein content was determined according to Bradford [24] using bovine serum albumin as the standard. The enzyme preparation used under these conditions had a specific activity of 1000 U·mg⁻¹ protein.

The redox polymer, poly(vinylpyridine) functionalized with osmiumbis(bipyridine) chloride (POs-EA), was synthesized essentially according to the method of Gregg and Heller [5,6]. Polyethylene glycol diglycidyl ether (PEGDE) was obtained from Polysciences (USA). BSA, MOPS, Bis-TRIS-propane, TAPS and CAPS were obtained from Sigma (USA). Enzyme kits for the determination of glucose, gluconate
and lactose were purchased from Boehringer (G). All other chemicals were from Merck (G) and of analytical grade. The potentiostat was purchased from Electronica, B-Fac, University of Nijmegen (NL).

**Preparation of the s-GDH electrodes**

s-GDH carbon disk electrodes were prepared according to the method of Stigter *et al.* [25] using 5 μL of 2 mg·mL⁻¹ solution of s-GDH in 5 mM MOPS buffer (pH 7.5), 5 μL POs-EA (8 mg·mL⁻¹ in demineralized water), 2 μL PEGDE (2.3 mg·mL⁻¹ in demineralized water) and 5 μL of buffer (50 mM CAPS, pH 10). Preliminary experiments had shown that these conditions were optimal with respect to maximum current density. The solutions were mixed and the mixture was applied to a polished circular carbon disk (Amor, diameter 0.8 cm) and distributed over the electrode with a spatula. The electrode was dried overnight (16 h) at 4°C over silica in an desiccator in vacuo. Control electrodes without s-GDH were made according to the same procedure.

When indicated, additional cross-linking of the s-GDH electrodes was performed by incubating the enzyme electrode in 5 mL of a 1, 2 or 5 (w/w)% glutaric dialdehyde (GDA) solution in 50 mM MOPS buffer pH 7.0 for 3 hours, after which the electrode was washed thoroughly with milli-Q water to remove excess of glutaric dialdehyde. The electrode was dried and stored in a desiccator over silica at 4°C until used.

**Electrochemical measurements**

Electrochemical measurements were performed as described by Stigter *et al.* [25] using an Amor electrochemical flow-through cell (Figure 2) fitted with a spacer with a surface of 6 mm²[28]. The disk was placed in the cell with the uncoated surface on the working electrode (glassy carbon). A control electrode without GDH was used as a reference electrode. Buffer was pumped through the cell with a peristaltic pump (1 mL·min⁻¹). After a stable background was obtained, substrate solutions were pumped through the cell. Unless otherwise stated, the cell potential was 600 mV vs. Ag/AgCl, and the temperature of the cell was 25°C.

For the determination of enzyme kinetic constants, in discrete concentration steps, glucose (0.1 - 100 mM), maltose (0.5 - 200 mM), galactose (1 - 200 mM), xylose (5 - 100 mM) and fructose (0.1 - 100 mM) were pumped through the cell. If necessary, the data were corrected for the decline of the electrode activity using the time related
current density for the reference solution (100 mM glucose). Experiments were conducted at 25°C with buffers containing 250 mM sodium phosphate (pH 7.0), unless otherwise mentioned. Electrode stability was determined every 5 minutes with 2 mL of 50 mM glucose in 250 mM phosphate buffer. Half-life times of the enzyme electrodes were calculated by fitting a first order (inactivation) equation (ln I/I₀ = -kt) to the data points.

The pH optimum of the immobilized enzyme was determined in the range of pH 5.0 and 10.0, with the use of "Universal Buffer" consisting of citric acid (31.3 mM), potassium phosphate (28.4 mM), boric acid (28.6 mM), and barbituric acid (40.9 mM). The pH was adjusted with 10 mM sodium hydroxide.

![Schematic view of the electrochemical flow cell (Amor)](image)

**Figure 2. Schematic view of the electrochemical flow cell (Amor)**

**Bio-electrochemical production of gluconate and lactobionate from glucose and lactose**

Conversion of glucose and lactose was carried out by recycling a fixed volume of substrate solution (3 mL) with 5 mM substrate in 250 mM phosphate buffer (pH 7.5) containing 10 mM calcium chloride over the electrode. The electrode was prepared as described above, however, to prevent any liberation of the enzyme from the electrode, the concentration of PEGDE as cross-linking agent was increased from 2.3 to 50 µg·mL⁻¹. At various time intervals samples of 100 µL were withdrawn for analysis. Analysis of the substrates and products was carried out using Boehringer
enzyme kits and High Performance Anion Exchange Chromatography (HPAEC-DIONEX, Chrompack NL).

**Electrodes with a higher specific surface area**

Immobilization of s-GDH was carried out with electrode material which can be obtained for preparative purposes. For the immobilization, the same procedure as described above was used. Flat plate rigid carbon with a dimension of 1.5 x 4 x 0.2 cm (Sigratemp PR, Sigri GmbH, G) was used. The electrode was dried overnight (16 h) at 4°C over silica in a desiccator in vacuo. The electrode was tested with 0.1 M glucose solution in 250 mM phosphate buffer pH 7.5, with a platinum reference electrode. A small part of the electrode was also placed in the Amor flow cell, and tested under the same conditions as applied in the flat plate rigid carbon cell. Immobilization in a material with a higher specific surface area was carried out by applying 4 mL of an enzyme / polymer mixture to a 1 x 1 x 2 cm carbon felt electrode (Sigratemp GFA-5, Sigri GmbH, G). The electrode was dried overnight (16 h) at 4°C over silica in a desiccator in vacuo. Preliminary experiments were carried out in a flow cell (2 mL volume, see Figure 3) with 100 mM glucose in 250 mM phosphate buffer, pH 7.5 at 0.6 V.

**Figure 3.** *Schematic view of the flow cell with carbon felt*
3 Results and Discussion

Characterization of the s-GDH disk electrode and optimization of the current density

The current density is an important parameter since it is directly related to the amount of substrate converted at the electrode. The current density of s-GDH electrodes immobilized at pH 10.0 appears to be high and depends on the potential at which the electrodes are operated (Figure 4). A maximum current density of 25-26 A·m² is obtained at a potential of 600 - 900 mV versus Ag/AgCl. No direct oxidation of glucose with electrodes without enzyme was observed. Thus the increase in current density is due to an electron transport from the enzyme through the redox polymer to the electrode. The electron transfer between the reduced cofactor PQQ and the osmium-bipyridyl groups in the redox polymer, or between the redox polymer and the electrode, or the redox polymer itself, might be the limiting factor for current density at a potential below 600 - 900 mV versus Ag/AgCl.

![Graph showing current density vs. working potential](image)

**Figure 4.** Current density of the s-GDH disk electrode as a function of potential (vs. Ag/AgCl at 30 °C with glucose as substrate)

The high current density found is comparable with the data found by Ye et al. [14]. The current density also depends on the concentration of the sodium phosphate buffer used in this study. The maximum current density is obtained using a 250 mM
sodium phosphate buffer of pH 7.5 (Figure 5). This effect might be explained by an improved conductivity in the solution and thus higher effective cell potential, or an improved direct communication between the electron conducting group of the polymer and the active site of the enzyme due to an increased ionic strength.

Figure 5. Current density of the s-GDH disk electrode as a function of buffer concentration of the solution

This effect has been observed earlier with the same type of electrodes [28]; optimal conductivity with these electrodes, containing galactose oxidase was achieved at 200 - 250 mM phosphate buffer. When operated from pH 6.0-8.0 (with an optimum at pH 7.0), the current density of the enzyme electrode is nearly constant (Figure 6). Although a high pH at immobilization is optimal (data not shown), hardly any activity at pH 10 and higher is found. This is in contrast with earlier observations where a graphite electrode was used [14,18], showing no variation in activity in the pH range 6.3-8.8. The pH optimum found, corresponds with the pH optimum for s-GDH when the electron acceptor is phenazine methosulfate [14]. The s-GDH electrode shows an increase in current density up to a temperature of 37°C. Maximum current density is obtained when the electrode is operated at 37°C in 250 mM phosphate buffer (Figure 7). The current density decreases when operated at temperatures higher than 40°C (data not shown).
Figure 6. Activity of the s-GDH disk electrode as a function of pH

Figure 7. Temperature profile of the s-GDH disk electrode

Enzyme electrodes made according to the standard procedure were additionally cross-linked in glutaric dialdehyde (GDA) solutions of various concentrations at pH 7.0. The experimental data on the half-life time of s-GDH immobilized in the redox polymer are listed in Table 1.
When no GDA is used, the initial current density is very high (Table 1). However, the half-life time of the electrode is low, due to some release of the enzyme from the electrode, which could be detected by assaying the outlet stream of the cell on activity. When GDA is used, the enzyme deactivates, but the half-life time of the electrode increases. Any release of the enzyme from the electrode could not be detected.

<table>
<thead>
<tr>
<th>Glutaric dialdehyde (%)</th>
<th>Half life time (hours)</th>
<th>$I_0$ (µA·cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.5 +/- 0.8</td>
<td>2500</td>
</tr>
<tr>
<td>1</td>
<td>100 +/- 5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>53 +/- 3</td>
<td>9.6</td>
</tr>
<tr>
<td>5</td>
<td>16.5 +/- 1</td>
<td>16.5</td>
</tr>
</tbody>
</table>

When the GDA concentration is increased (1-5%), the initial current density increases (Table 1). This is in contrast with results found earlier for other enzymes, in which GDA has no influence on the initial activity on alcohol dehydrogenase and galactose oxidase, except at concentrations higher than 2.5 % [25-27]. The opposite is seen for the stability of the electrode; the use of 1% GDA leads to a ten fold stability increase compared to the non cross-linked GDH. However, increasing the GDA concentration leads to a decrease in half-life time. The use of GDA thus leads to a higher stability of the GDH electrode, but a lower current density of the electrode.

**Substrate specificity and bio-electrochemical conversions**

The immobilized s-GDH shows Michaelis-Menten type kinetics. The apparent kinetic constants of several known substrates [21] were determined (Table 2). Typical $K_{m\text{(app)}}$ values of 5 mM were observed for sugars with a glucose unit at the anomeric site. For other monosaccharides the immobilized enzyme has a $K_{m\text{c}}$ of approximately 10 mM. In these cases, The $V_{\text{max\text{(app)}}}$ values decrease when
compared to the $V_{\text{max}}(\text{app})$ of glucose. The values of the immobilized enzyme are comparable to the values of the free enzyme [21].

**Table 2.** Substrate specificity of the s-GDH disk electrode and free s-GDH ($V_{\text{max}}(100\%) = 2.4 \text{ mA cm}^{-2}$ or $24 \text{ U mg}^{-1}$ for the free enzyme)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme electrode</th>
<th>Free enzyme [21]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m(\text{app})(\text{mM})$</td>
<td>$V_{\text{max}}(\text{app})(%)$</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.5</td>
<td>100</td>
</tr>
<tr>
<td>Maltose</td>
<td>5.2</td>
<td>62</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.1</td>
<td>72</td>
</tr>
<tr>
<td>Galactose</td>
<td>9.8</td>
<td>80</td>
</tr>
<tr>
<td>Xylose</td>
<td>10.0</td>
<td>64</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

The rate of conversion of lactose and glucose as a function of time by the s-GDH disk electrode is shown in Figures 8 and 9, respectively. A solution (3 mL) of 250 mM sodium phosphate buffer with 5 mM substrate was continuously circulated through the Amor cell. The conversion was carried out at 22°C.

![Graph](image)

**Figure 8.** Bio-electrochemical conversion of lactose (5 mM) using the s-GDH disk electrode, $\Delta =$ glucose, $\circ =$ gluconic acid.
Figure 9. Bio-electrochemical conversion of glucose (5 mM) using the s-GDH disk electrode, \( \triangle \) = lactose, \( \circ \) = lactobionic acid

From the current density, the amount of glucose and lactose converted could be calculated (see below). The amount of substrate converted and product formed was determined by enzymatic determination or by HPLC. The figures found were in agreement with the integral of the current measured during the reaction, which indicates that the substrates were converted into the aldonic acid and no other electrochemical conversions took place.

In approximately 20 hours, 4 mM (80%) of a 5 mM lactose solution is converted into lactobionic acid (Figure 8). The initial current density of the electrodes was 12 A·m\(^{-2}\). This current density is measured at the \( K_m \) value of the substrates, so the current density of these electrodes with enzyme saturating substrate conditions will be 24 A·m\(^{-2}\). The enzyme electrode at which glucose was converted, had a lower operational activity, causing a lower oxidation rate of glucose (Figure 9).

In both cases, product inhibition appeared to be absent. The current density of a control electrode without immobilized enzyme is lower than 0.01 A·m\(^{-2}\), indicating that no significant non-enzymatic oxidation of glucose has occurred.
Chapter 2

**Volumetric productivity of the electrodes**

The bio-electrochemical conversion efficiency can be determined by deriving this from the electrical current, which is related to the amount of product formed by the following equation:

\[
\text{moles of product} = \left( \frac{l \times t \times 60}{F \times n} \right),
\]

in which \(l\) is the current (A), \(t\) = time (min), \(F\) is the Faraday constant (96,494 C·mol\(^{-1}\)) and \(n\) is the number of electrons transferred per mole of product. To correlate the current density measured with a volumetric productivity, the specific surface / volume ratio is used, assuming that the efficiency of the enzyme immobilization and the thickness of the enzyme-polymer layer on the electrodes is the same with the different electrodes used. Using the reactor volume, the enzyme activity can be expressed as volumetric productivity (A·m\(^{-3}\)) as derived from the current density (A·m\(^{-2}\)) measured. For the carbon disk electrode prepared with s-GDH, the volumetric productivity would be 24 A·m\(^{-2}\) (current density) \(\times\) 6\(\times\)10\(^{-6}\) m\(^2\) (electrode surface) \(/\) 0.2\(\times\)10\(^{-6}\) m\(^3\) (reactor volume) = 720 A·m\(^{-3}\). The current density of 24 A·m\(^{-2}\) also corresponds with a molar productivity of 0.007 mol·min\(^{-1}\)·m\(^{-2}\) (2 electron reaction). As the reaction volume in the cell is 0.2 mL, the surface / volume ratio of the disk electrode is 30 m\(^2\)·m\(^{-3}\), and the volumetric productivity is 0.22 moles·min\(^{-1}\)·m\(^{-3}\), equivalent to a theoretical production rate of 6 and 3.4 kg·hour\(^{-1}\)·m\(^{-3}\) of lactobionate and gluconate respectively.

As the material used in our experiments (polished carbon disk electrode) is not available for industrial purposes, flat plate rigid carbon was used as an industrial feasible alternative. This material is useful for applications in an industrial electrolyzer, in which the use of parallel plates is from an electrochemical point of view technically feasible. Experiments were carried out on a flat plate rigid carbon electrode (1.5 x 4 x 0.2 cm). Enzyme immobilization was carried out according to the procedure for the carbon disk electrode. Although a very low current density was obtained in a glucose solution with a platinum reference electrode, a relatively high current density (330 μA·cm\(^{-2}\) = 3.3 A·m\(^{-2}\)) was measured when the electrode was placed in the Amor flow cell. This indicates that, in principle, the immobilization on a flat plate rigid carbon electrode can be carried out effectively, but that the configuration of the electrochemical cell (effective cell potential, buffer mixing and flow characteristics) also determines the performance of the cell. As the reactor volume was 0.2 mL (0.2\(\times\)10\(^{-6}\) m\(^3\)), and the effective electrode surface was 6 mm\(^2\) (6\(\times\)10\(^{-6}\) m\(^2\)), the volumetric productivity on a flat plate electrode can be expressed as (3.3 \(\times\) 6\(\times\)10\(^{-6}\) /
We tried to improve the volumetric productivity by the use of carbon felt. The immobilization of the enzyme on a 2 cm³ carbon felt, resulted in a maximum current of 0.24 mA. The measured signal was very stable, and hardly any decrease in activity of the immobilized enzyme in the cell could be detected during several hours. According to the specification of the supplier, the specific surface of the carbon felt is 160 m²·m⁻³. The current can therefore be expressed as current density (0.75 A·m⁻²) or as volumetric productivity (213 A·m⁻³). In Table 3, the results are summarized.

Table 3. Activity of immobilized s-GDH at several electrodes

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Device</th>
<th>Volume (mL)</th>
<th>Current density (A·m⁻²)</th>
<th>Volumetric productivity (A·m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon disk</td>
<td>Amor flow cell</td>
<td>0.2</td>
<td>24</td>
<td>720</td>
</tr>
<tr>
<td>Flat plate rigid carbon</td>
<td>Amor flow cell</td>
<td>0.2</td>
<td>3.3</td>
<td>99</td>
</tr>
<tr>
<td>Carbon felt</td>
<td>TNO flow cell</td>
<td>2.0</td>
<td>0.75</td>
<td>213</td>
</tr>
</tbody>
</table>

The current density obtained with carbon felt is only 3% of the current density obtained with the disk electrode. Although these experiments show that the volumetric productivity using carbon felt instead of a flat plate rigid electrode can be increased, further studies will be needed to increase the immobilization efficiency, or the electron transfer rate from the enzyme/polymer to the electrode e.g., in the electrode itself. When performing the immobilization, we noticed that the addition of an enzyme / polymer solution led to an inhomogeneous distribution of the enzyme on the electrode. As parts of the electrode are not covered with enzyme / polymer and other parts are covered with a relatively thick layer of enzyme / polymer, this might explain the low current density obtained. Therefore, apart from the improvement of the electron conductivity in the carbon felt electrode, alternatives (e.g., electrochemical polymerization of the enzyme with polymer present in solution) to improve the immobilization yield on carbon felt need to be developed and studied in the future.

Evaluation of bio-electrochemistry on large scale

Enzyme electrodes made with flat plate rigid carbon resulted in a current density of 3.3 A·m⁻². This is 14% of the current density found with the carbon disk electrode.
Linear extrapolation of these data in a flat plate electrochemical reactor leads to a production rate of approximately 14 g gluconate and 25 g lactobionic acid/m³ reactor volume per hour, respectively.

Several parameters determine the economic feasibility for industrial application of this process. These parameters are the enzyme cost price per unit activity, the enzyme stability and the immobilization yield. The polymer used is not commercially available, but the backbone of the polymer (poly 4-vinylpyridine) is a commodity chemical. The functional group which is attached first, osmium complex \([\text{cis-bis} (2,2'\text{-bipyridine-N,N'}) \text{dichloroosmium (II)})], is not commercially available, however, a manufacturing protocol has been described \([5,6]\). Toxicity aspects of liberation of this polymer from the electrode into the product solution have not been studied either. The second functional group added to the PVP, 2-bromoethylamine hydrobromide is commercially available.

Van den Brink \([28]\) has reviewed electrochemistry as an industrial technology and gives a number of examples of organic synthesis in industry. These include organic compounds for various markets, including fine chemicals for plastics, food preservation and precursors for pharmaceuticals. Based on cost price calculations, he concluded that electrochemistry as a production tool will only be feasible for products with high added value. When enzymes are used, the replacement of traditional chemical synthesis is attractive since a number of side-products arising during production is likely to be low, and a low cell voltage is required. On an industrial scale, flat plate electrochemical reactors are used in the form of series of coupled flat electrodes. In principle, this type of equipment, coated with the enzyme polymer, might be used for bio-electrocatalysis. The final dimensions of the reactor will be determined by the volumetric productivity of the electrode and the required capacity.

Based on the calculations of Van den Brink, the total costs for bio-electrocatalysis can be estimated.

**Reactor costs** (anode, cathode, membranes and auxiliary equipment) are determined by production parameters (current efficiency, current density), investment costs and return on investment criteria. For a typical product with a molecular mass of 200 D, an average of NLG 0.20 - 1.00/kg may be used. This cost price is based on stainless steel, and standard operating conditions (temperature,
pressure, type of chemical used). The conditions with bio-electrochemistry are milder (room temperature, atmospheric pressure, moderate pH). Under these conditions, a reactor made of glass can be used. It is therefore expected that the reactor costs for bio-electrochemical applications will be lower.

*Electricity costs* are based on the consumed power (cell potential x current) and the production/ m³. In our case, the electricity costs will be in the order of NLG 0.50/kg of product.

*Maintenance costs* increase linearly with the reactor surface area. This implies that the reactor surface area has to be kept as low as possible, even when this leads to a decreased efficiency or occurrence of side reactions when the temperature increases. A general accepted number would be NLG 1.00/kg of product.

*Enzyme costs* are determined by the commercial availability of the enzyme. Using s-GDH, linear extrapolation from the immobilization data used in these experiments shows a need for 58 mg enzyme/m³ reactor. This amount is very low, and a total cost price of NLG 5000,= is estimated. Assuming that the operational life time of the enzyme-electrode has been optimized to one month, the enzyme costs/kg are in the order of NLG 0.01/kg of product.

*Polymer costs* are based on the amount of polymer used throughout this study (66 mg/m³). For a production of 14 g gluconic acid / hour and an operational life time of one month, a reactor of 1 m³ would require a surface area of 0.3 m² and usage of 20 mg of polymer. With an estimated cost price of the polymer of NLG 150,000,=, these costs are negligible.

Based on these considerations, the total costs per kg of product are determined mainly by the reactor costs and the electricity costs, and not by the enzyme costs. Therefore, optimization of the reactor with respect to volumetric productivity is justified. In the case of sugar oxidation as described here, these costs are estimated to be in the order of NLG 2.50/kg. Optimization of the process can be achieved by an optimal configuration of the reactor by increasing the volumetric productivity. The costs calculated here are in the same order as those in the microbiological production route of lactobionate and glucono-δ-lactone (NLG 2.30 - 10.00/kg). This means that with existing electrochemical technology using flat plate electrodes, bio-electrochemistry could be attractive as an alternative for chemical (environmental issues) or microbiological (variance in quality of final product) oxidations of sugars.
However, it is clear that further optimization studies will be required before final conclusions can be drawn.

4 Conclusions

The construction of s-GDH electrodes according to the method by Gregg and Heller, resulted in a reactor with a standard half-life time under continuous operation of approximately 9 hours, which is in agreement with the data found by Ye et al. [14]. The half-life time can be extended to at least 100 hours (continuous operation) by further treatment of the electrode material with 1% GDA. The cross-linking procedure, however, resulted in a considerable loss of current density compared with non cross-linked enzyme electrodes.

s-GDH electrodes with a current density of 24 A·m⁻² can be constructed, a number which is comparable with the data found by Ye et al. [14]. The cell potential in this experiments is 600 mV vs. Ag/AgCl. The pH dependency between pH 5-10 is similar to that of the free enzyme. The optimum is rather broad and lies between pH 6.0 and 8.0. Different mono-sugars can be oxidized, and the apparent $K_m$ values are comparable to the soluble enzyme.

Both glucose and lactose could be converted on small scale. When the enzyme reactor is used at the level of $K_m$ substrate concentration, no substrate or product inhibition was observed. The operational loss in activity was the same as compared to the pulsed substrate mode. Preliminary experiments with flat plate rigid carbon- and carbon felt enzyme electrodes showed that these materials can be used for upscaling of the immobilization. With the current densities obtained on the flat plate electrode (3.3 A·m⁻²) calculations were made for bio-electrocatalysis on large scale. Estimated cost prices of NLG 2.50/kg for gluconic acid or lactobionic acid shows that this process might be economically feasible in the future. It should be investigated whether optimization of the volumetric productivity is possible by alternative immobilization methods of the enzyme on carbon felt or improvement of the electron transfer from the polymer to the electrode, $c_{eq}$, in the electrode itself.
Acknowledgement

We thank Prof. Dr. J.A. Duine (Delft University of Technology) for the generous gift of purified s-GDH and of PQQ.

5 Literature cited

TO A LARGE SCALE PURIFICATION AND PROTEOLYTIC ACTIVATION OF HEXOSE OXIDASE FROM THE RED SEAWEED CHONDrus CRISPUS

Summary
The existing purification protocol for hexose oxidase (HOX) from the red seaweed Chondrus crispus was improved with respect to upscaling. Incubation with cellulase and carrageenase lowered the troublesome viscosity of the cell homogenate to such an extent that a normal first concentration/chromatographic step could be applied. The enzyme yield obtained with the procedure was comparable to that of the old one but the specific activity was lower. Therefore, an isolation procedure on a large scale is available now but if the higher specific activity is required, modification of the new procedure should be attempted to see whether this can be achieved. Incubation of crude extract with a mixture of proteases (Pronase) improved the specific activity and stability of HOX in the extract. Thus, Pronase liberates HOX from particles in the homogenate, and by proteolytic attack. Investigation of HOX prepared by a procedure incorporating a Pronase treatment step gave HOX of which the subunits had somewhat shorter N-terminal amino acid sequences than observed for the N-terminal sequence predicted from the gene sequence, but no additional cleavages. Except for the removal of amino acids which may lead to a conformational change of HOX, no other clues are available which could explain the effect of Pronase treatment in an early stage of the purification protocol on the increase of specific activity and stability. Analysis of HOX for the presence of bound sugars revealed that the enzyme contains glucose and galactose, in line with the glycoprotein nature already proposed in the literature. Glycanases were ineffective in the removal of the sugars from HOX and an immunological assay failed to detect sugars bound to HOX. This suggests an unusual type of binding of the sugars; Perhaps basic amino acids are involved, in line with the extremely low pI of the enzyme. Further studies of the HOX substrate specificity, showed that many aldoses (except mannose) can function as substrates, and that also a relatively large oligosaccharide such as maltopentaose (but not maltohexaose) can be oxidized.
1 Introduction

As expressed by its name, hexose oxidase (HOX, EC 1.1.3.5) oxidizes a broad spectrum of hexose sugars to their corresponding lactones under simultaneous formation of $H_2O_2$. These properties give the enzyme potential for analysis of sugars (including glucose because the affinity of HOX for this sugar is 10 fold higher than that of glucose oxidase), for in situ $H_2O_2$ generation, for O$_2$ removal, and for oxidative conversions of sugars in which chemical oxidations cannot be applied. The enzyme has been found in the red seaweeds Chondrus crispus [1,2,3,4,5], Iridophycus flaccidum [6], and Eutora cristata [1,3]. A similar enzyme from citrus fruits is capable of oxidizing several saccharides [7]. The Chondrus crispus enzyme is reported to be a glycoprotein, and contains flavin as a cofactor [1].

As indicated in a recent patent [8], applications of HOX are foreseen in the bakery industry. The isolation procedure, using the red seaweed Chondrus crispus as a source for the enzyme, has been published in the past [3,4] but the enzyme is not commercially available yet. However, as indicated in a recent report on purification and characterization of the enzyme [1], the yield obtained with the original procedure is variable and usually very low. Substantial improvement in these respects was achieved by introducing an incubation step with Pronase (a commercially available mixture of bacterial proteases), liberating HOX from particles present in the cell homogenate in a reproducible way [1]. However, as was indicated by the authors, the incubation step also liberates carrageenan, leading to a very viscous solution, requiring extensive dilution in order to allow application of the subsequent chromatography/concentration step.

Recently, it was reported that the structural gene for HOX has been cloned, sequenced, and brought to expression in several recombinant organisms [9,10]. Although this provides a possibility for large scale production of HOX, the use of the recombinant enzyme will not be allowed for all applications, especially those in the food industry. Moreover, since it has been concluded that HOX is a glycoprotein and has a covalently bound flavin as a cofactor [1] (although this was not found by others [9]), post-translational modification of the proenzyme form is required, which could mean that much effort has to be made before satisfactory expression levels of active, mature recombinant enzyme can be achieved. For these reasons and in view of the fact that C. crispus has a longstanding tradition as an edible organism, production of substantial amounts of HOX by a large scale isolation procedure using this source as
the starting material still is an attractive alternative. However, as indicated above, the liberation of carrageenan during HOX extraction forms a serious problem that needs to be dealt with.

The cell wall of *Chondrus crispus* gametophytes is composed of cellulose microfibrils embedded in a matrix of carrageenans [11-13]. The polysaccharides of the matrix are predominantly κ-carrageenan and they also comprise a small but significant proportion of ι-carrageenan, either as ι-chains mixed with the κ-chains, or as ι-sequences within κ-chains. Studies performed on red seaweed cells using enzymes to hydrolyze the polysaccharides, improved the accessibility to the protein fraction. For instance, a combination of carrageenase and cellulase proved to be efficient to produce protoplasts from seaweed species [14]. We therefore studied the use of carrageenase, to reduce the viscosity of the Pronase extract. Since application of a proteolytic step in the isolation of an enzyme is very unusual and difficulties exist to explain the origin of the fragments of HOX observed with SDS-PAGE [1,9], the effect of Pronase on purified HOX was also investigated. When glycosylation was investigated by applying glycanases to HOX, negative results were obtained [1], just as had been found by others [9]. However, a colorimetric assay gave a positive result [1]. Therefore, to solve this problem, a direct method consisting of acidic hydrolysis of the protein and determining the sugars in the hydrolysate was applied to HOX.

2 Materials and methods

2.1 Materials

*Chondrus crispus* was a gift from Prof. J.A. Duine (Delft University of Technology, The Netherlands). The collected weed was washed with tap water, dried in air at 40°C, grounded to a size < 0.5 mm in a blender (Culatti Mikro-Schlagmuhle) and the resulting powder was stored at 20°C. Pronase E was obtained from Merck (G). Carrageenase was a gift from Dr. P. Potin (CNRS UPR, France) and was isolated from the marine bacterium *Cytophaga drebachienis* (strain Dsij) [15-16]. It consists of a mixture of κ-carrageenase (1200 U·mL⁻¹) and ι-carrageenase (500 U·mL⁻¹). Cellulase (*Trichoderma spp.*) was a gift from Gist brocades (NL). All materials needed for purification were obtained from Pharmacia (Uppsala, Sweden). All chemicals were of analytical grade. The sugar substrates used were obtained form
Merck (G), Sigma (USA), Fluka (G), Janssen Chimica (B), and BDH (G) (analytical grade), except tetrasaccharide \(\Delta-4,5\text{-GlucA}(1\rightarrow4)\text{Gluc}(1\rightarrow4)\text{Rha}(1\rightarrow4)\text{Gluc}\), which was obtained by hydrolysis of Gellan gum (Gelrite, Kelco, USA) and prepared at TNO [17].

2.2 Enzyme purification

2.2.1 Method 1 (adapted from Groen et al. [1]).

**Extraction**
Seaweed (1.2 kg) powder was mixed with 2 gram Pronase powder and suspended in a solution of 5 L tap water and 5 L demineralized water. The suspension was incubated under stirring for 7 hours at 22°C. Subsequently, the suspension was diluted with 30 L demineralized water and stored at 4°C overnight. After the particles had settled, the viscous fluid above the sediment layer was removed by suction, and centrifuged for 30 minutes at 1500 \(x\) \(g\). The supernatant was stored at 4°C. The pellets were suspended in tap water (total volume 18 L), and the Pronase treatment was repeated. Clear supernatant was obtained by a second centrifugation step for 30 minutes at 13,000 \(x\) \(g\). In the first and second extraction procedure, approx. 8000 U of activity were obtained. However, the last part of the second supernatant (app. 2000 U) was highly viscous, and was not used in the isolation procedure.

Further purification steps were carried out according to the procedure described in [1]. Anion exchange chromatography was performed with Source Q columns.

**Gel filtration**
Gel filtration was conducted with 1800 mL Superdex 200 Prep grade, packed in a XK 50/100 column, equilibrated with 20 mM Bis-Tris pH 6.5. Elution took place with the same buffer containing 100 mM NaCl, with a flow of 2 mL-min\(^{-1}\). The collected fractions were stored at -20°C. The enzyme obtained with this procedure will be referred to as: HOX type 1.
2.2.2 Method 2, adapted from Ikawa [4].

Extraction
Dry seaweed powder (300 g) was suspended in a solution consisting of 4 L tap water and 4 L demineralized water. The suspension was stirred for several hours, and subsequently homogenized in 500 mL portions with a Moulinex mixer for 2 minutes. The mixture was stored overnight at 4°C under stirring. The mixture was centrifuged for 30 minutes at 1500 x g. Centrifugation was repeated during 30 minutes at 13,000 x g. The pellet was resuspended in 8 L water and the extraction process was repeated. This procedure was carried out 4 times. Further purification steps were carried out according to the procedure described in [1]. The purified enzyme will be referred to as: HOX type 2.

2.2.3 Method 3, Extraction with carrageenase, cellulase and Pronase (extraction adapted from Fleurence et al. [14])

Extraction
Dry seaweed powder (300 g) was suspended in a solution consisting of 3 L 10 mM Bis-Tris/HCl, pH 6.5. The suspension was incubated with 0.5 mL carrageenase solution and 1 g cellulase powder for 7 hours at 30°C. Pronase (500 mg) was added and the mixture was stored overnight at 30°C, and subsequently diluted to 10 L with demi water to ensure the efficacy of the second enzyme treatment. The mixture was centrifugated for 30 minutes at 1500 x g. Centrifugation was repeated during 30 minutes at 13,000 x g. The supernatant was stored at 4°C. The pellet was resuspended in water (total volume 4 L) and the extraction process was repeated (1 mL carrageenase solution and 1 g cellulase powder). This procedure was carried out 2 times. After 3 extractions, the pellet volume was reduced significantly. Further purification steps were carried out according to the procedure described in [1]. This purified enzyme will be referred to as: HOX type 3.
2.2.4 Method 4, Extraction with carrageenase and cellulase (extraction adapted from Fleurence et al. [14])

Extraction
Dry seaweed powder (150 g) was suspended in a solution consisting of 1.5 L, 10 mM Bis-Tris/HCl, pH 6.5. The suspension was incubated at 30°C with 0.50 mL carrageenase solution and 500 mg cellulase powder for 9 hours. The mixture was stored overnight (this enzyme preparation will be referred to as HOX type 4) and was diluted with 1.5 L demi water and centrifugated for 30 minutes at 1500 x g. Centrifugation was repeated during 30 minutes at 13.000 x g. The pellet was resuspended in water (total volume 1.5 L) and the extraction process was repeated (0.5 mL carrageenase solution and 500 mg cellulase powder). The pellet volume was reduced significantly after 2 extractions. Further purification steps were carried out according to the procedure described in [1].

2.2.5 Method 5, Extraction with carrageenase, cellulase and Pronase, interrupted with Protease inhibitor (extraction adapted from Fleurence et al. [14])

Extraction
Dry seaweed powder (50 g) was suspended in a solution consisting of 0.5 L, 10 mM Bis-Tris/HCl, pH 6.5. The suspension was incubated with 0.17 mL carrageenase solution and 166 mg cellulase powder for 9 hours at 30°C. The mixture was then incubated after adding 83 mg Pronase for a further 2 hours at 25°C. The Pronase treatment was stopped with 4 Protease inhibitor cocktail tablets (Boehringer Mannheim, G). During the extraction samples were taken and the HOX activity was determined.
The mixture was diluted with 0.5 L 20 mM Bis-Tris pH 6.5 and centrifugated for 30 minutes at 1500 x g. Centrifugation was repeated during 30 minutes at 13.000 x g. The pellet was resuspended in water (total volume 1.5 L) and the extraction process was repeated (0.17 mL carrageenase solution and 166 mg cellulase powder).
Further purification steps were carried out according to the procedure described in [1].
2.3 Enzyme characterization

**Enzyme assays**

Activities were determined in air-saturated mixtures containing 0.1 M citrate buffer (pH 3-7) or 0.1 M pyrophosphate buffer (pH 6.5-11), 0.18 mM ABTS (2,2'-azino-bis(3-ethylbenzathiazolone-6-sulfonic acid) diammonium salt, molar extinction coefficient = 36.8 mM⁻¹cm⁻¹ at 410 nm), 40 mM glucose and 10 μg·mL⁻¹ horseradish peroxidase (HRP, Sigma).

Control experiments with HRP at the measured pH and temperature ranges were carried out to be sure that the HOX activity found is not influenced by any decrease in HRP activity. The coupled enzyme assay could be used accurately in the pH range 3 - 10. To determine the activity of the enzyme at pH > 8.0, a Clark electrode (YSI Instruments) was used measuring the oxygen concentration. The protein content was determined according to Bradford [18]. The influence of 100 mM NaCl was determined in the pH range between 4.0 - 6.0. The temperature stability of the enzyme was determined by 15 and 30 minutes pre-incubation of the enzyme over the temperature range of 4.5-70°C. The activity was measured at 25°C. The temperature optimum of the enzyme was determined at pH 6.0 in 100 mM citrate buffer, 100 mM NaCl between 15 and 50°C.

**Molecular weight and identity of the glycosyl residues**

The native molecular weight was determined on a Superdex 200 HR 10/30 column (250 μL sample, 0.4 mL·min⁻¹, 50 mM phosphate buffer pH 7.0 + 0.1 M NaCl). The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), IgG (160 kDa), human transferrin (81 kDa), ovalbumin (43 kDa) and ribonuclease (13.7 kDa).

Native PAGE was carried out on a 20% homogeneous gel using LMW markers (Pharmacia) comprising phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Activity staining was carried out with a Phast dry gel, incubated in the standard assay mixture. The development of a green color is an indication for enzyme activity.

SDS-PAGE was carried out on a Pharmacia Phast System apparatus with gradient gels (10-15%) and the low molecular weight calibration kit (Pharmacia), and on a 12.5% SDS gel (Biorad II Miniprotein) according to Laemmli [19], with reduced
(DTT), non-reduced, boiled and non-boiled hexose oxidase. The carbohydrate composition of purified HOX type 1 was also determined by HPAEC (Dionex, NL).

**Isoelectric focusing**

HOX type 1 and 2 were analyzed by isoelectric focusing (IEF) between pH 3-10, according to the instructions of the supplier. A mixture of pl markers was run parallel with the HOX samples. The mixture consisted of cytochrome C (pl 10.2), myoglobin (pl 7.4 and 7.0), carbonic anhydrase (pl 6.1), lactoglobulin A/B (pl 5.4 and 5.5), ovalbumin (pl 4.8), glucose oxidase (pl 4.2) and amyloglucosidase (pl 3.6). The gels were stained with CBB.

**Amino acid sequence**

N-terminal protein of the hexose oxidase type 1 subunits was determined by using the PVDF subunit blots. The analysis was carried out according to the Edman degradation [20], on an Applied Biosystems mode 477 A gas-phase sequencer, connected to an on-line 120 A PTH.

**Substrate specificity**

The purified enzyme was characterized with respect to substrate specificity, using a wide range of different substrates in 50 mM MES buffer at pH 6.1. For the substrates which were oxidized at sufficient rate, the apparent kinetic parameter values were determined.

3 Results

3.1 Purification and characterization

The results of the different extraction methods are summarized in Table 1. When the extraction is carried out without Pronase (HOX type 2), the extraction yield is low (2800 U·kg⁻¹ seaweed). During purification of the enzyme, much enzyme activity is lost, the final activity yield being only 12% of the activity obtained after ammonium sulfate precipitation (set at 100%). When Pronase is used during the extraction, the yield increases, compared with the extraction without Pronase (5700 U·kg⁻¹ seaweed). Also the final activity yield of HOX type 1 after purification is 3 times
higher (42%) than that of HOX type 2 (12%). In the extraction of HOX type 3 with carrageenase and cellulase, the HOX activity obtained in the extract was 2800 U·kg⁻¹ of seaweed. This amount increased to 6300 U·kg⁻¹ within 30 minutes after addition of Pronase. After two subsequent extraction steps, hardly any insoluble residue was left. The total protein extraction yield was very high (2.5 g·100 g⁻¹). However, further purification of HOX type 3, resulted in a low activity yield (9.3%).

**Table 1.** Protein and activity extraction yield with different methods

<table>
<thead>
<tr>
<th>HOX Type</th>
<th>Enzymes applied in extraction</th>
<th>Yield (after extraction)</th>
<th>Viscosity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carr. Cell. Pronase</td>
<td>Total activity (U·kg⁻¹)²</td>
<td>Total protein (g·100 g⁻¹)</td>
</tr>
<tr>
<td>type 1</td>
<td>- - +</td>
<td>5700</td>
<td>0.6</td>
</tr>
<tr>
<td>type 2</td>
<td>- - -</td>
<td>2800</td>
<td>1.1</td>
</tr>
<tr>
<td>type 3</td>
<td>+ + +</td>
<td>6300³</td>
<td>2.5</td>
</tr>
<tr>
<td>type 4</td>
<td>+ + -</td>
<td>2150⁴</td>
<td>1.3</td>
</tr>
<tr>
<td>type 5</td>
<td>+ + +⁵</td>
<td>4300³</td>
<td>2.0</td>
</tr>
</tbody>
</table>

¹⁺ is highly viscous, - is nonviscous
² Total activity determined before purification
³ Measured 30 minutes after Pronase addition
⁴ Activity loss overnight at 4°C
⁵ Pronase added as a second treatment after 9 hours of incubation with carrageenase and cellulase

During the extraction, HOX type 4 activity was released in the medium (see Figure 1) up to 4300 U·kg⁻¹ after 9 hours. As the activity of HOX type 2 declined after prolonged incubation, the incubation was stopped to prevent further decrease by endogenous proteases. The activity decreased to 50% (2150 U·kg⁻¹) during storage overnight at 4°C. The extraction procedure was repeated (method 5) and extended by addition of Pronase (2 hours). Immediately after addition of Pronase, the HOX type 5 activity in the medium increased from 2200 U·kg⁻¹ to 4300 U·kg⁻¹. The Pronase treatment was stopped with an inhibitor cocktail. Further purification of HOX types 4 and 5 was facilitated by the reduced viscosity of the supernatant. This effect was achieved with a one-step treatment of the seaweed with carrageenase and cellulase. A large dilution of the supernatant before anion exchange chromatography was no longer necessary anymore, and a considerable reduction of supernatant volume and purification time was achieved.
Figure 1. Extraction of HOX type 4 (■) and volume of supernatant (●) during incubation with carrageenase and cellulase

Further purification of HOX type 4 resulted in a significant loss of final activity yield (18.6%). Similar to HOX type 1, in which also Pronase was used, HOX type 5 seems more stable, and the final enzyme activity yield after purification is 40%. A high protein extraction yield (2.0 g·100 g⁻¹) was obtained after the two-step enzymatic incubation of the fronds with carrageenase/cellulase and Pronase. After the extraction, hardly any pellet was left after centrifugation. Total hydrolysis of the protoplasts caused the release of all the proteins present in the seaweed. Clear indications were found that the use of Pronase enhances the liberation of protein from the cell wall (Table 1). Although the highest amount of HOX-activity was extracted with a combination of carrageenase/cellulase and Pronase, the final purity of HOX type 3 is low (9.4 U·mg⁻¹ protein). Taking into account the final enzyme activity yield (Table 2), it is shown that only HOX types 1 and 5 show reasonable yields (40%). HOX types 2,3 and 4 are not stable, probably due to the presence of endogenous proteases. These proteases are inactivated during the isolation of HOX type 1 and HOX type 5 by Pronase. In HOX type 3 nearly all the proteins (including proteases) were released due to the very intensive enzyme treatment. In this case, the inactivation by Pronase was not sufficient.
### Table 2. Purification data of hexose oxidase

<table>
<thead>
<tr>
<th>Step</th>
<th>Type Activity</th>
<th>Protein (mg)</th>
<th>S.A. (U-mg⁻¹)</th>
<th>Yield (act) (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amm. sulfate</td>
<td>HOX 1</td>
<td>6080</td>
<td>1.07</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>3520</td>
<td>498</td>
<td>7.07</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td>Final preparation</td>
<td>2840</td>
<td>36.2</td>
<td>78.4</td>
<td>42</td>
<td>73</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>HOX 2</td>
<td>850</td>
<td>0.27</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>850</td>
<td>359</td>
<td>2.37</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>Final preparation</td>
<td>94</td>
<td>3.8</td>
<td>25.0</td>
<td>12</td>
<td>93</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>HOX 3</td>
<td>1810</td>
<td>0.24</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>560</td>
<td>920</td>
<td>0.72</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Final preparation</td>
<td>310</td>
<td>33</td>
<td>9.4</td>
<td>9</td>
<td>39</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>HOX 4</td>
<td>200</td>
<td>0.10</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>216</td>
<td>256</td>
<td>0.84</td>
<td>95</td>
<td>8</td>
</tr>
<tr>
<td>Final preparation</td>
<td>42</td>
<td>16.2</td>
<td>2.6</td>
<td>19</td>
<td>93</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>HOX 5</td>
<td>185</td>
<td>0.18</td>
<td>87</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>144</td>
<td>79</td>
<td>1.82</td>
<td>68</td>
<td>10</td>
</tr>
<tr>
<td>Final preparation</td>
<td>85</td>
<td>3.3</td>
<td>25.8</td>
<td>40</td>
<td>143</td>
</tr>
</tbody>
</table>

Apart from this effect, we investigated the effect of Pronase on the HOX-activity during the extraction. A sample of HOX type 4 extract was centrifugated (13,000 x g), and incubated with Pronase during 1 hour. Within 30 minutes, a significant increase in HOX-activity (approx. 75%) was observed (Figure 2). Even after prolonged incubation of 24 hours, this activation could be detected (data not shown), whereas activity loss of the non-Pronase-treated sample was found. This shows that a Pronase treatment also stabilizes the enzyme. However, when HOX type 2 (isolated without Pronase and fully purified) was used, no activation could be detected anymore. This indicates that the Pronase activation is only possible during or just after the extraction. These data clarify that:

* with a Pronase treatment, the enzyme is activated and stabilized within 30 minutes.
* with a Pronase treatment during the extraction, HOX is stabilized by inactivation of endogenous proteases, or by specific modification in HOX so that it is no longer susceptible to inactivation by endogenous proteases.
Figure 2. Activation of HOX type 4 (1.3 mg·mL⁻¹) by Pronase (0.17 mg·mL⁻¹) at 30 °C. ■ = with Pronase, ▲ = control sample

Molecular weight and pl
The HOX preparations isolated have a very low pl, between 3.2-3.5. This value is lower than found earlier (4.4) [10], but higher than reported by Groen et al. (2.8) [1]. The molecular weight values of the different HOX preparations were determined (Table 3).

Table 3. Molecular weight of HOX type 1 - 5

<table>
<thead>
<tr>
<th>Type (Method)</th>
<th>SDS PAGE</th>
<th>(subunits, kDa)</th>
<th>Gel filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>reduced</td>
<td>non reduced</td>
<td>(kDa)¹⁾</td>
</tr>
<tr>
<td>HOX 1 (Pronase)</td>
<td>25 / 36</td>
<td>72¹⁾ / 25 / 36</td>
<td>107</td>
</tr>
<tr>
<td>HOX 2 (No Pronase)</td>
<td>25 / 36</td>
<td>80 ± 4</td>
<td>107</td>
</tr>
<tr>
<td>HOX 3 (Car+Cell+Pro)</td>
<td>25 / 36</td>
<td>72 / 25 / 36</td>
<td>n.d.</td>
</tr>
<tr>
<td>HOX 4 (Car+Cell)</td>
<td>25 / 36</td>
<td>80 ± 4</td>
<td>107</td>
</tr>
<tr>
<td>HOX 5 (Car+Cell/Pro)</td>
<td>25 / 36</td>
<td>72 / 25 / 36</td>
<td>107</td>
</tr>
<tr>
<td>[10]</td>
<td>25/29/40/60</td>
<td></td>
<td>110-120</td>
</tr>
<tr>
<td></td>
<td>55-57²⁾</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹⁾ The 72 KDa subunit was active
²⁾ The 55-57 kDa subunit was an impurity, and was β-glycosylated
³⁾ n.d. = not determined
On a gel filtration column, the molecular weight of hexose oxidase isolated in the presence of Pronase (HOX, type 1), is approx. 107 kDa, the same as that of the enzyme isolated without Pronase. The molecular weights of the native enzyme and subunits differ slightly compared to the data from others [10].

**HOX type 1**

On SDS PAGE, bands of 25 and 36 kDa were found. Based on the intensity of the bands in the gel, the enzyme probably consists of two subunits each with a molecular weight of 36 kDa and one subunit of 25 kDa. Since the band at 36 kDa is rather broad, the two 36 kDa subunits are probably not identical (Figure 3). However, care must be taken in the correct determination of the molecular weight of hexose oxidase, since it has been found that the enzyme is glycosylated [1].

![Figure 3. SDS PAGE, 12.5 %, CBB in trichloroacetic acid staining of HOX type 1, 2 and 3](image)

*a = non-reduced, non-boiled, b = reduced, non-boiled, c = non-reduced, boiled, d = reduced, boiled.*

Using native-PAGE (non reduced) a molecular weight of 90 kDa for HOX type 1 was determined. With SDS-PAGE, an extra distinct band of about 72 kDa was found, indicating that this band dissociates into a 36 and 25 kDa subunit under reducing conditions (see Figure 3). The total molecular weight of the subunits (36 kDa and 25 kDa) does not correspond with the molecular weight of the band of 72 kDa. This difference might be explained by differences in behavior during electrophoresis of the subunits, caused, e.g., by glycosylation. Activity staining of the gel showed that the 72 kDa subunit still contained hexose oxidase activity, while the 25 kDa subunit was...
not active (data not shown). Boiling for 10 minutes results in dissociation of the 72 kDa unit. The presence of 2% DTT has no effect. This indicates that the 36 kDa subunits are not covalently bound by S-S bridges.

**HOX type 2**
The isolation of hexose oxidase without Pronase (HOX, type 2) yielded a less pure enzyme as judged by SDS-PAGE. The molecular weight of the enzyme seems to be somewhat higher than that of HOX type 1, and estimated to be 94 kDa using native PAGE.

*With SDS-PAGE, the same phenomenon is observed as with HOX type 1: a strong band of approx. 76-84 kDa was visible, hydrolyzing into subunits of 25 and 36 kDa after 10 minutes boiling (Figure 3). Using trichloroacetic acid (TCA) in the staining method, more impurities are found than with CBB in acetic acid. Using both techniques reveals the difference in specific activity obtained for both oxidases: HOX type 2 has much more impurities which are made visible with CBB in TCA. These impurities have a molecular weight of 65 kDa and 56 kDa, and do not dissociate into subunits after boiling (Figure 3).*

**HOX type 3**
With SDS-PAGE, a similarity between HOX type 3 and HOX type 1 can be observed. HOX type 3 also consists of 2 subunits, with a molecular weight of 36 and 25 kDa. Under non-reducing conditions, a similar pattern as found for HOX type 1 is observed: A distinct band of 72 kDa, which disappears after boiling, and bands of 25 and 36 kDa are found (Figure 3). The molecular weight as determined with native PAGE was 90 kDa (data not shown). Although the specific activity of HOX type 3 is lower than that of HOX type 1, no significant impurities of other proteins were observed.

**HOX type 4 and type 5**
The molecular weight of the subunits of HOX type 4 and HOX type 5, as determined with SDS-PAGE, are similar to the bands obtained by HOX type 1 and HOX type 3, respectively. Because the Pronase treatment with HOX type 5 has not been as intensive as with HOX type 3, endogenous pronases were inactivated by Pronase and the addition of a protease inhibitor cocktail. This resulted in a higher specific activity of the HOX type 5.
**N-terminal amino acid sequence**

The N-terminal sequences of the two subunits (25 en 36 kDa) from hexose oxidase type 1 were determined. The following amino acid sequences were obtained:

36 kDa subunit: Thr-Leu-Pro-Gln-Lys-Asp-Pro-Gly-Tyr-Ile-Val-Ile-Asp-Val-Asn-Ala.

25 kDa subunit: Met-His-Asp-Glu-Thr-Met-Asp-Tyr-Pro-Phe-Tyr-Ala-Leu-Thr-Glu. His-Asp-Glu-Thr-Met-Asp-Tyr-Pro-Phe-Tyr-Ala-Leu-Thr-Glu-?-
Ile.

The 36kDa subunit showed uniformity, the 25 kDa subunit showed two sequences, of which one sequence missed the amino acid Met.

Recently, the complete amino acid sequence for hexose oxidase was published [9,10]. The sequence was derived from the DNA sequence of the HOX gene. A total of 546 amino acids was found, representing the complete enzyme. Comparison of these sequences with the hexose oxidase sequence published, revealed that the 36 kDa subunit is the original N-terminus of the protein. Compared with the data from the HOX gene [9, 10] the sequence found in the 36 kDa subunits, is in accordance with amino acids Thr3 - Ala18 in the HOX gene. Apparently, Pronase has removed two amino acids (Met-Ala) from the original enzyme. The (not uniform) N-terminal sequence of the 25 kDa unit is equivalent with amino acids 341-356 from the HOX gene. Hansen et al. [9] found an amino acid sequence similar to a sequence which is known to be sensitive to proteolytic activation of zymogens. This sequence is present in the region Val329 - Asp344. The N-terminus of the 25 kDa unit starts at Met341, indicating that Pronase could have activated hexose oxidase by cleavage in this region. By proteolytic cleavage, the enzyme remains active, and apparently becomes less susceptible to other proteolytic activity. This would also explain the increase in intensity of the bands of HOX isolated with Pronase using SDS- PAGE.

**Glycosylation of HOX**

HPAEC-Dionex was used to determine glycosylation of HOX type 1. In the purified sample, galactose (7% (w/w)) and glucose (19% (w/w)) were detected. The enzyme thus contains 26% (w/w) carbohydrates. This is equivalent to approx. 0.15 mol/mol enzyme.
3.2 Enzyme characteristics

The addition of NaCl had a significant positive effect on the hexose oxidase activity, the activity increased by 20-65%. This is in accordance with [1]. The pH optimum of hexose oxidase is broad and lies between pH 5.5-6.5 (Figure 4).

![Graph showing pH optimum of HOX-activity type 1 with different buffers](image)

**Figure 4.** pH optimum of HOX-activity type 1 (+ = 100 mM citrate buffer, ○ = 100 mM citrate buffer with 100 mM NaCl, △ = 100 pyrophosphate buffer)

A second pH optimum is found at pH 10 when the enzyme assay is carried out with a Clark cell (data not shown). This optimum was also found earlier [1]. However, during this study further activity measurements were carried out at pH 6, unless otherwise stated.

The temperature optimum of the enzyme is 30-35°C (Figure 5). The optimum is rather broad, and the activity is > 95% of the maximum in the range of 25-40°C. The activation energy for the reaction = 30 kJ/mol (calculated from the Arrhenius plot). Above 40°C the enzyme activity decreases. The enzyme is stable for at least 30 minutes at 35°C (Figure 6). Prolonged incubation (30 minutes) has hardly any effect on the activity profile. It can be concluded therefore that the enzyme is temperature stable up to 40°C. No activity is found anymore above 70°C. As a coupled enzyme assay has been used, care must be taken with the interpretation of the activity data obtained above 60°C.
Figure 5. Temperature optimum of HOX-activity type 1. Activities were determined with the coupled assay, containing 100 mM NaCl.

Figure 6. Temperature stability of HOX type 1. Activities were determined with the coupled assay after incubation for 15 (+) or 30 (∆) minutes at the indicated temperature.

The substrate specificity of HOX type 1 was studied by measuring activities with different substrates in 50 mM MES buffer pH 6.1. The results are shown in Table 4. Kinetic evaluation of the purified enzyme shows a higher affinity for glucose ($K_m^{(app)}$).
is 3.3 mM) and cellobiose (Kₘ (app) is 2 mM) as compared to Groen et al. [1] (Kₘ (app) is 8.5 and 12.5 mM respectively). However, the Kₘ (app) for maltose and lactose are higher. The Kₘ (app) for maltotriose is 7.4 mM with a Vₘₚ (app) of 0.8 U·mg⁻¹. The enzyme is able to oxidize oligomers up to maltopentaose. Although mannose is a reducing sugar, it is not oxidized by the enzyme. Other oligomers than glucose-oligomers, containing glucose at the reducing end (melibiose, tetrasaccharide) can not be oxidized.

**Table 4.** Substrate specificity of HOX type 1 (apparent values are shown)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Vₘₚ (U·mg⁻¹)</th>
<th>Kₘ (mM)</th>
<th>Substrate</th>
<th>Vₘₚ (U·mg⁻¹)</th>
<th>Kₘ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>72</td>
<td>3.3</td>
<td>ribose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>galactose</td>
<td>30</td>
<td>12</td>
<td>sucrose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>lactose</td>
<td>16</td>
<td>50</td>
<td>fructose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>cellobiose</td>
<td>12</td>
<td>2</td>
<td>sorbitol</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>maltose</td>
<td>18</td>
<td>40</td>
<td>tetrasaccharide ¹</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>maltotriose</td>
<td>0.8</td>
<td>7.4</td>
<td>melibiose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>maltotetraose</td>
<td>0.2</td>
<td>1.5</td>
<td>stachyose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>maltopentaose</td>
<td>0.5</td>
<td>5</td>
<td>arabinose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>maltohexaose</td>
<td>0</td>
<td>0</td>
<td>mannose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>rhamnose</td>
<td>1.2</td>
<td>70</td>
<td>raffinose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>xylose (140 mM)</td>
<td>0.1</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Δ4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc

4 Discussion and conclusions

The recently published [1] procedure for isolation of HOX, starting with particles obtained from the fronds of *C. crispus*, yields HOX of high specific activity and purity in a reproducible way. However, probably related to the release of HOX from the particles by application of Pronase, polysaccharides are also solubilized, leading to a very viscous extract. Here we show that the viscosity is lowered by application of carrageenase and cellulase, to such an extent that chromatography of the extract without excessive dilution can be carried out. Since the isolation yield of HOX by this new procedure (Table 2, method 5) is comparable to that achieved by the method with Pronase alone (Table 2, method 1), upscaling seems possible now. However, the specific activity of the final preparation prepared by the new procedure was 3
times lower than that obtained with the original one, implying that in case high specific activity is a prerequisite, modifications of the new procedure need to be developed in order to achieve this (see also below).

During the cellulase/carrageenase treatment, the enzyme is released within several hours. However, the enzyme is not stable, probably due to the presence of endogenous proteases. Pronase treatment was originally introduced to liberate HOX from particles in a reproducible way [1]. However, assuming that no particles were present in the experiment described in the legend of Fig. 2 (they should be removed by the centrifugation step applied before), Pronase treatment leads to a higher specific activity and to stabilization of HOX. In addition, the Pronase based procedure [1] yielded an 8 times higher specific activity (about 4 times when corrected for the higher activity caused by 0.1 M NaCl in the assay [1]) than that without Pronase [9], although it was claimed for both procedures that a homogeneous final preparation was obtained. This suggests that Pronase treatment activates the enzyme.

Activation of hexose oxidase after final purification is not possible, probably due to endogenous protease cleavage during the purification procedure. Treatment of HOX with Pronase (ratio 1:8 w/w%) during 24 hours does not influence the activity, indicating that Pronase does not deactivate the enzyme at this ratio. Therefore, suitable conditions for isolating hexose oxidase on a large scale, seem to be a cellulase/carrageenase treatment to hydrolyze the cell walls and to reduce the viscosity of carrageenan, followed by a Pronase treatment before endogenous proteases deactivate hexose oxidase. As no significant impurities of other proteins were determined in HOX type 3, we conclude that during the extraction, endogenous proteases have also been active, resulting in a decrease in HOX activity in the crude extract. The non-active HOX with similar protein characteristics as the active HOX might still be present in the final enzyme preparation, resulting in a lower specific activity of HOX type 3, compared to HOX type 1. To obtain hexose oxidase with a high specific activity, a treatment with Pronase only is preferred. In that case, the activity of endogenous proteases is prevented, and HOX can be activated. Although much progress has been made to facilitate isolation of HOX on large scale, further research is required to optimize the hydrolysis of the carrageenan to further minimize the volume of the supernatant. Also, the amount of Pronase added vs. the amount of HOX present, needs more detailed investigation to prevent any deactivation of HOX by Pronase during the purification.
Even when no deliberate proteolytic treatment is incorporated in the isolation procedure [9], the protein encoded by the HOX gene (61.9 kDa) is largely fragmented to peptides with theoretical sizes of 25 and 36 kDa. Sequencing of the N-terminal end of the small fragment (that of the large fragment could not be determined since the N-terminal amino acid was blocked) revealed that this fragmentation is due to a scission between amino acids Lys and Thr in the stretch Lys-Thr-Ser-Tyr-Met-His$^{342}$. Here it is shown that HOX obtained by a procedure including a Pronase treatment step yields a large fragment having an N-terminal sequence starting with Thr$^3$- (the first two amino acids, Met and Ala are lacking) and a smaller fragment starting with Met-His$^{342}$- . Since neither a decrease in size of the two fragments nor smaller fragments were observed, it seems that Pronase treatment neither causes other cleavages in the protein chain of HOX than that already present nor removes large stretches of amino acids from the C-terminal ends. On the other hand, the Pronase treatment seems to decrease the molecular mass of the native enzyme, as observed with PAGE. This could indicate, however, that removal of the N-terminal amino acids from the fragments by Pronase (6 in total), induces a conformational change in native HCX. If this view is correct, it implies that natural activation (either by endogenous proteases or those excreted by contaminating microbes) is less effective in activating the enzyme than one of the proteases in the Pronase mixture. Assuming that the same HOX was present in the starting material for both procedures (but see below), further investigations are required to explain why this presumed conformational change leads to activation and stabilization of the enzyme.

Although HOX was discovered about 25 years ago, its identity is still a matter of debate. Thus the question can be raised whether this is due to disagreement about some properties of the enzyme or to the possibility that different HOX enzymes exist in _C. crispus_. For example, controversy exists with respect to the question whether HOX is glycosylated or not. Negative results were obtained with an immunological assay [9], and with an assay using glycanases [1,10] but positive results were obtained by a staining procedure [1] and in the present study with direct hydrolysis of HOX and determining sugars in the hydrolysate. This could indicate that HOX is always glycosylated but in an unusual way, explaining why certain assay methods gave a negative result (although possible sites for glycosylation are present in the amino acid sequence of HOX) and why N-terminal sequencing of the two fragments, as shown here, and of internal fragments [9], did not reveal unusual amino acids.
Further structural elucidation of the tertiary structure of HOX will be needed to determine the possible presence of these glycosylation sites at the surface of the protein. On the other hand, it could also be imagined that *C. crispus* produces a glycosylated and a non-glycosylated form of HOX, just as occurs with glucose oxidase, the fungus producing a non-glycosylated, peroxisomal form and a glycosylated external form. If the hypothesis of the existence of two HOX enzymes is correct, it could explain the variation in reported isoelectric points of HOX, the glycosylated form having a very low value (2.8 [1], and 3.2 and 3.5 here) whereas the non-glycosylated form has a higher one (4.3 and 4.5 [9]). Further investigations are required to corroborate these hypotheses.

Already, it is known that the substrate specificity of HOX is very broad [1]. The data presented here confirm this, although differences are found in the affinity for the substrates. Further insight is provided into the scope of the substrate specificity: neither mannose nor maltohexaose are substrates, although maltopentaose is.

**Acknowledgements**

We thank Dr. Philippe Potin (CNRS, France) and Prof. dr. J.A. Duine (Delft, University of Technology, The Netherlands) for generous gifts of carrageenase and of *Chondrus crispus*, respectively.

**5 Literature cited**

PROBING THE SUBSTRATE SPECIFICITY OF HEXOSE OXIDASE FOR BIOSENSOR AND BIOCATALYTIC APPLICATIONS

Summary

Hexose oxidase, isolated from the seaweed *Chondrus crispus*, was immobilized on a poly(vinyl)pyridine redox polymer functionalized with osmiumb(bipyridine)chloride, and studied with respect to efficiency of electron transfer to an electrode and to substrate specificity. An electrode with optimal activity was achieved when using an enzyme solution of 1.5 mg·ml⁻¹ in the electrode preparation procedure, resulting in a current density of 0.67 A·cm² at 22°C. The operational half-life time of such an electrode was 25 hours. The optimal cell potential for this was 550 mV vs. Ag/AgCl. Maximum activity was obtained at 38°C. The activity of the immobilized enzyme was very sensitive to the type of buffer used. Addition of 100 mM NaCl decreased the activity with 30-50% at a certain MES concentration.

A broad activity optimum was found between pH 5.5 - 7.0. The immobilized enzyme showed a much broader substrate specificity with respect to aldose sugars than the free enzyme, and was able to oxidize malto-oligomers up to maltoheptaose. In contrast with the free enzyme, mannose and the tetrasaccharide Δ-4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc were also suitable substrates for the enzyme electrode. To achieve the broadening of substrate specificity on the electrode the complete immobilization procedure has to be applied. The reason for this is unknown yet.

1 Introduction

During the past few years, the development of biosensor applications in the biotechnological, pharmaceutical, and food processing industry has been of increasing interest [1]. This is due to the need for fast and sensitive (on-line) methods for the detection of certain compounds. Based on synthetic redox polymers designed to shuttle electrons between the enzyme/cofactor and the electrode, a large number of devices has recently been
described in the literature. Various sensors based on ferrocene derivatives [2,3], and quinone derivatives [4-6] coupled to organic conducting polymers [7-10] have been reported. Most probably, a short distance between the active site of the enzyme and the conducting polymer enables a direct electron transfer between the enzyme and the electrode. Although a current can be measured, evidence for electron transfer via the redox polymer has not been given.

An important class of compounds to be determined by means of biosensors are mono- and disaccharides, which nowadays are analyzed by HPLC or enzymatic detection kits. Different enzymes are being studied, of which the enzyme glucose oxidase for the determination of glucose is the most popular one. The main disadvantage of glucose oxidase is the susceptibility to oxygen [3]. An alternative is the use of glucose dehydrogenase. NAD(P)-dependent glucose dehydrogenases have a broad substrate specificity, but their coenzyme-dependency limits practical application as the coenzyme diffuses away from the electrode and is inefficient in electron shuttling [7,11]. Since several years another class of dehydrogenases, containing PQQ as a cofactor, has gained interest. A striking example is the NAD(P)-independent PQQ-containing soluble glucose dehydrogenase (PQQ-GDH), which is used commercially in diagnostic strips for the determination of glucose in blood. This enzyme is of interest, because it lacks all disadvantages mentioned above [12-14].

Other NAD(P)-independent dehydrogenases tested in biosensors are fructose dehydrogenase for the determination of D-fructose [7,15], sorbitol dehydrogenase for the determination of D-fructose, L-sorbitose, D-xyllose, L-xyllose and D-sucrose [16]; mannitol dehydrogenase for the determination of D-fructose [17]; and oligosaccharide dehydrogenase for the determination of maltose [18].

Other oxidases than glucose oxidase have not been tested in biosensors in detail until now, although galactose oxidase [19] has been studied for the detection of galactose, lactose, melibiose, raffinose and stachyose and glucose-2-oxidase [20] for the detection of glucose.

Recently, a new enzyme, hexose oxidase, with similarities to oligosaccharide oxidase [21,22] has been described [23, Chapter 3]. The enzyme is found in red seaweeds and is capable to oxidize a wide range of sugar monomers and oligomers. It has also been tested in an enzyme reactor for the determination of mono- and oligosaccharides [24]. The possible use of this enzyme in the baking industry was published in 1996 [25]. As oxidases are inactivated by hydrogen
peroxide formed, the use of electron-conducting polymers is advantageous, because electron uptake via the electrode diminishes the "oxidase activity" [26]. Other studies have shown that the half-life time of an oxidase increases when the production of hydrogen peroxide is prevented by using benzoquinone instead of oxygen [6]. The use of electron conducting polymers has been described in literature as a promising technique for biosensor applications, because of the relatively high current densities obtained with several enzymes, e.g., PQQ-dependent glucose dehydrogenase, galactose oxidase and alcohol dehydrogenase [14, 26-30].

The enzyme hexose oxidase might be of interest for use in biosensor applications for the determination of specific sugars [23] and can also be applied for mild enzymatic conservation of food products [31]. Preliminary experiments in our laboratory with immobilized hexose oxidase on the electron conducting polymer poly(vinyl)pyridine, showed a difference in substrate specificity compared to the specificity of the free enzyme. This is of interest, because this indicates the possibility to oxidize sugars which could not have been enzymatically oxidized until now, and the oxidation of sugars containing double bonds [34] could be feasible now. Here we describe the immobilization of purified hexose oxidase (isolated from the seaweed *Chondrus crispus*) on a poly(vinyl)pyridine-based conducting polymer. The immobilization conditions were optimized with regard to current density. In addition, the influence of type and concentration of several buffers, the pH optimum and temperature stability of the immobilized enzyme were determined. Finally, the substrate specificity compared to that of the soluble enzyme is compared.

2 Materials and Methods

2.1 Materials

Hexose oxidase (HOX) was purified from the seaweed *Chondrus crispus* (Chapter 3, HOX type 1) according to the method described by Groen et al. [23]. The activity of hexose oxidase was determined in air saturated buffer containing 0.18 mM ABTS, 40 mM glucose and 0.5 U horseradish peroxidase (Sigma). The activity was measured at 410 nm with a PE lambda 5 spectrophotometer. One unit of enzyme corresponds with the formation of 1 μmol hydrogen peroxide per minute at 22°C. The enzyme preparation used had a specific activity of 60 U·mg⁻¹ protein at pH 6.1 in
this assay. The protein content was determined using bovine serum albumin (BSA) as the standard [32]. The redox polymer, a poly(vinyl)pyridine substituted with osmium(bipyridine)chloride equipped with aminoethyl groups (POs-EA), was synthesized according to the method described by Gregg and Heller [9,33]. Polyethylene glycol diglyceridyl ether (PEGDE) was obtained from Polysciences (USA). BSA, MOPS, BIS-Tris-propane, TAPS and CAPS were obtained from Sigma (USA). The sugar-substrates used were obtained from Merck (G), Sigma (USA), Fluka (G), Janssen Chimica (B), and BDH (G) and are of analytical grade. Tetrasaccharide, \(\Delta-4,5\)-GlucA(1\(\rightarrow\)4)Gluc(1\(\rightarrow\)4)Rha(1\(\rightarrow\)4)Gluc, was prepared by an enzymatic treatment of Gellan gum (Gelrite, Kelco, USA) [34]. The potentiostat was purchased from Electronica, B-Fac, University of Nijmegen, (NL).

2.2 Construction of HOX electrodes

HOX-electrodes were prepared according to the method described by Stigter et al. [24] and Smolander et al. [14] using 5 \(\mu\)L of 1.5 mg\(\cdot\)mL\(^{-1}\) (unless otherwise stated) solution of HOX in 5 mM MOPS buffer (pH 7.5), 5 \(\mu\)L redox polymer, (POs-EA, 8 mg\(\cdot\)mL\(^{-1}\) in milli-Q water), 2 \(\mu\)L poly(ethylene glycol) diglyceridyl ether (PEGDE, 2.3 mg\(\cdot\)mL\(^{-1}\) in milli Q water) and 5 \(\mu\)L of buffer (50 mM phosphate buffer pH 7.2). The mixture was applied to a polished carbon disk (Amor, diameter 0.8 cm) and distributed over the surface with a spatula. The enzyme electrodes were dried overnight (16 hrs) at 4°C in a desiccator in vacuo over activated silica. In order to determine the background current density, control electrodes were prepared as described above, using MOPS buffer without HOX. To study the effect of PEGDE on the enzyme, electrodes were prepared without addition of PEGDE. HOX-electrodes were also prepared according to the procedure described by Smolander et al. [14]. The disks were modified, prior to the immobilization of the enzyme, with dimethylferrocene (17 \(\mu\)L, 0.4 mg in toluene).

2.3 Electrochemical measurements

Electrochemical measurements were performed according to the method described by Stigter et al. [28] using an Amor electrochemical flow-through cell fitted with a spacer with a working surface of 6 mm\(^2\). The enzyme coated electrode was placed in the cell with the uncoated surface directed towards the working electrode. Buffer (20
mM sodium phosphate buffer with 100 mM NaCl, pH 7.2) was pumped through the cell by a peristaltic pump (1 mL·min⁻¹). After a stable background was obtained, a pulse of substrate solution (2 mL 0.1 M glucose in buffer) was applied. Unless otherwise stated, the cell potential applied was 500 mV vs. Ag/AgCl. Electrode stability was determined by pulsing the enzyme electrode every 5 minutes with the substrate solution. Half-life times of the enzyme electrodes were calculated by fitting a first order inactivation equation (ln I/I₀ = -kt) to the data points.

The pH optimum of the immobilized enzyme was tested at room temperature between pH 4.5 and 11.5, with a combined buffer, containing 40 mM MES, 40 mM Tris-HCl and 1 mM citric acid. Before experiments were carried out, a solution of 50 mM MES buffer pH 6.1 was pumped through the cell until a constant background current was obtained (this took usually several hours). To determine the substrate specificity, a wide range of sugars in a concentration range between 2.5-200 mM were tested. Experiments were conducted at 22°C with buffers containing 50 mM MES buffer, pH 6.1, unless otherwise mentioned. When necessary, the data were corrected for the decline of the electrode activity by measuring the current density with the reference solution (100 mM glucose) during the experiments after each series of substrate.

3 Results and discussion

3.1 Characterization of the HOX-electrodes

Immobilization of HOX to the standard procedure, resulted in a maximum current density of 67 μA·cm⁻² for the 0.1 M glucose solution. The current density depends on the enzyme concentration (Table 1). No detectable current was observed for an electrode without HOX. A relatively sharp optimum in activity is found when an enzyme concentration of 1.5 mg·mL⁻¹ is used during preparation of the enzyme electrode. This phenomenon cannot be explained by differences in the pH during immobilization, since the pH of the mixture was kept constant.

This effect was found in different cells immobilized in the same way and indicates that the cells could be prepared in a reproducible way. The half-life time of the electrode varies, and is the highest at 1.5 mg·mL⁻¹ of HOX. Possibly, these
parameters are correlated to the type of distribution and cross linking of the enzymes at the electrode.

Attempts to further optimize the current density and half-life time of the HOX-electrode were made by varying the amount of PEGDE added. The addition of PEGDE as epoxy cross linker might also have, apart from an encaging effect, a negative effect on the activity as it could inactivate the enzyme. (This was found for immobilized glucose oxidase, Stigter, unpublished results). Absence of PEGDE resulted in electrodes with the same initial activity, but with a half-life time of 12 ± 2 hours, compared to a half-life time of 25 hours when PEGDE is used. Thus, using PEGDE at the standard concentration, no inactivation of HOX occurs and the half-life of the electrode is increased, probably by immobilizing HOX.

**Table 1.** Current density and stability of immobilized HOX as a function of initial enzyme concentration. (HOX immobilization and activity measurements were carried out in duplicate)

<table>
<thead>
<tr>
<th>Enzyme concentration (mg·mL⁻¹)¹</th>
<th>Activity (%) ²</th>
<th>Half-life time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>28</td>
<td>9</td>
</tr>
<tr>
<td>1.0</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>1.5</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>2.0</td>
<td>42</td>
<td>10</td>
</tr>
<tr>
<td>3.0</td>
<td>37</td>
<td>11</td>
</tr>
</tbody>
</table>

¹) in the polymer solution prior to drying
²) 100% = 67 μA·cm⁻²

Smolander et al. [14] also carried out optimization experiments by pretreatment of a glassy carbon electrode with 1,1-dimethylferrocene in the carbodiimide coupled PQQ-dependent glucose dehydrogenase. This resulted in a current density of 10 μA·cm⁻², as compared to 0.1 μA·cm⁻² obtained with the enzyme immobilized in a POs-EA polymer. Since the difference could be due to better electron transfer between the enzyme and the electrode, a HOX-electrode was prepared in the normal way, except that the bare electrode was pretreated with ferrocene. However, the resulting electrode showed the same activity and stability as the standard one.
**Effect of cell potential**

As shown in Figure 1, the HOX electrode in the presence of glucose needs a certain potential before a measurable current density is observed. Since the conducting polymer has a redox potential of 350 mV (against Ag/AgCl) [33], it is to be expected that the current decreases strongly below this value. Using potentials above this value, no further increase but a constant current is observed until 500 mV. Above the latter value, a slight increase is observed which may be due to an additional current, originating from the oxidation of hydrogen peroxide by the electrode (generated by HOX).

![Graph showing current density vs cell potential](image.png)

**Figure 1.** Current density of a HOX electrode as a function of the applied potential under standard conditions with 0.1 M glucose as substrate, in 20 mM phosphate buffer containing 100 mM NaCl, pH 7.2. (Data points are the average values of duplicate experiments)

**Effect of temperature**

HOX in solution shows a temperature optimum between 30 - 35°C, and the enzyme is stable up to 30°C (Chapter 3). Because immobilization of the enzyme might affect the temperature stability, the activity of the HOX-electrode was tested between 5 and 50°C. As shown in Figure 2, the immobilized enzyme has a maximum of activity at 38°C. At this temperature, the enzyme is fully stable for at least 15 minutes.
Figure 2. Current density of a HOX electrode as a function of temperature for 0.1 M glucose in 20 mM sodium phosphate buffer containing 100 mM NaCl, pH 7.2. 100% = 78 μA·cm⁻². (Data points are the average value of duplicate experiments)

Figure 3. Current density of a HOX electrode in a glucose solution containing different buffers as a function of the buffer concentration at pH 6.1, except for TRIS-HCl buffer pH 10.1. (● = MES buffer, + = MES buffer with 100 mM NaCl, ▲ = Phosphate buffer, ♦ = Phosphate buffer with 100 mM NaCl, ■ = Citric acid, ■ = Tris-HCl, at 25°C. Data points are a mean of duplicate experiments)
A further increase in temperature leads to a fast decrease of activity. It is concluded, therefore, that immobilization of HOX brings about insignificant temperature stabilization.

**Effect of buffer and pH**

When comparing the different buffers used (Figure 3), it appears that Tris-HCl gave the highest activity. However, since the pH of this buffer was 10.1 and 6.1 for the others, the difference could be due to a pH effect. To eliminate the possible effect of the type of buffer used, the pH dependency of the immobilized enzyme was determined using a combination of buffers simultaneously. The combination was based on the results obtained in the former experiments. As shown in Figure 4, the difference in activity is not due to a pH effect since a 4-times lower activity is obtained with a mixture of buffers (40 mM MES, 40 mM Tris-HCl and 1 mM citric acid and NaOH) at pH 10.1.

![Graph showing pH vs. Current density (μA/cm²)](image)

**Figure 4.** Activity of the HOX electrode as a function of pH. (Data points are the average values of duplicate experiments in different cells)

Although the effect of the buffer concentration may be one of the causes of the variations observed, this effect is not straightforward: variation of the MES concentration gives a bell-shaped curve whereas for the other buffers more or less straight lines are observed; addition of 0.1 M NaCl to MES buffers gives a
stimulation at low, but a decrease at high MES concentrations. Instead of a possible stimulatory effect as found for the soluble enzyme [23, Chapter 3], 100 mM NaCl in sodium phosphate buffer (5-20 mM) inhibits the activity with 30-50%. Both phosphate and citrate (pH 6.1) lowers the immobilized enzyme activity at concentrations higher than 5 mM as compared to MES buffer. Although the results might indicate that a cationic (Tris-HCl) or a zwitterionic (MES) buffer are better for the HOX electrode than anionic buffers (phosphate and citrate), more systematic investigations are required to elucidate this. To ensure practical usefulness, also the stability of the HOX electrode should be studied under various conditions. The large influence of the type of buffer applied in an enzyme electrode has not been found before [27-30]. Therefore, the differences found in the relationship between the ionic strength of the buffer and the current density measured, show that care has to be taken in the selection and strength of the buffer applied in the cell. Using a combination of different type of buffers, the soluble enzyme is active in a broad pH range, with maxima at pH 6 and 10 (Chapter 3). The immobilized enzyme has a broad pH optimum between pH 5.5 and 7, at higher pH no specific optimum as compared to the soluble enzyme [23] is found anymore, although the enzyme activity at pH > 8.5 is more or less stable. The half-life time of the electrode is the same in the pH range 4.5-11.4.

3.2 Substrate specificity of the HOX electrode

When comparing the values of the apparent specificity constants for HOX immobilized on the electrode and free in solution (Table 2), it appears that sugars other than glucose are much better substrates for the immobilized than for the free enzyme (except cellobiose). Also certain substrates, which did not show any activity with free enzyme, did so with immobilized enzyme (mannose, maltohexaose and tetrasaccharide). Since this difference suggested that the properties of the immobilized enzyme were changed, experiments were carried out to prove this and to reveal the underlying reason(s) for it. The standard enzyme preparation procedure was followed, except for the addition of the conducting polymers. Immobilized HOX, thus with and without the addition of the conducting polymer, was tested after drying, with the spectroscopical method using ABTS. At set time intervals, the solution in the cuvette
was gently mixed by stirring with the electrode and the extinction was measured after removing the electrode.

During the reaction, the development of green color was clearly visible for glucose, mannose and the tetrasaccharide Δ-4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc (Table 3). Oxidation of fructose was not observed.

**Table 2.** Substrate specificity of immobilized hexose oxidase (substrates in 50 mM MES buffer, pH 6.1. Values are apparent values)

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Immobilized HOX</th>
<th>Soluble HOX (Chapter 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>($\mu$mol-cm$^{-2}$)</td>
<td>(mM)</td>
</tr>
<tr>
<td>glucose</td>
<td>29.4</td>
<td>16</td>
</tr>
<tr>
<td>galactose</td>
<td>28.6</td>
<td>32.5</td>
</tr>
<tr>
<td>cellobiose</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>mannose</td>
<td>21.0</td>
<td>0.2</td>
</tr>
<tr>
<td>lactose</td>
<td>9.0</td>
<td>50</td>
</tr>
<tr>
<td>maltose</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>maltotriose</td>
<td>4.9</td>
<td>50</td>
</tr>
<tr>
<td>maltotetraose (22.5)</td>
<td>4.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>maltopentaose (25)</td>
<td>5.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>maltotriaose (25)</td>
<td>12.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>maltotriose (25)</td>
<td>5.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>xylose (100)</td>
<td>3.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>rhamnose (200)</td>
<td>3.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>tetrasaccharide (10)$^1$</td>
<td>12.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>melibiose (100)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>stachyose (25)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>arabinose (100)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>fructose (200)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>sorbitol (200)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>sucrose (200)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>raffinose (200)</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$ the tetrasaccharide Δ-4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc has a low solubility
n.d. = not determined

As shown in Table 3, the phenomena are not due to electrochemical artifacts, since the broadening of the substrate specificity (towards mannose and tetrasaccharide) was also observed when the immobilized HOX used $O_2$ as electron acceptor instead of the conducting polymer. Since this indicated that some step(s) in the immobilization procedure changed the enzyme, the effect of omission of each step was studied.
Table 3. Oxidase activity of HOX immobilized HOX on an carbon disk electrode with and without redox polymer

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Activity (U·cm⁻²) With polymer</th>
<th>Activity (U·cm⁻²) Without polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>40</td>
<td>0.0050</td>
<td>0.12</td>
</tr>
<tr>
<td>Mannose</td>
<td>40</td>
<td>0.0050</td>
<td>0</td>
</tr>
<tr>
<td>Tetrasaccharide¹</td>
<td>10</td>
<td>0.0004</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹  Δ-4,5-GlucA(1→4)Gluc(1→4)Rha(1→4)Gluc

As shown in Table 4, application of the complete electrode preparation procedure is crucial for inducing the effect, even the drying step. It even appears that the underlying surface (carbon disk) is essential since applying the complete procedure on a hydrophilic (filter paper) or a hydrophobic (plastic polypropylene tubes) surface resulted in immobilized HOX which showed oxidase activity towards glucose but not to mannose (or tetrasaccharide).

Table 4. Activity of HOX on mannose and glucose, immobilized on the electrode under different conditions

<table>
<thead>
<tr>
<th>Entry</th>
<th>PEGDE</th>
<th>Redox polymer</th>
<th>Drying¹</th>
<th>Activity² with Mannose</th>
<th>Activity² with Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

¹ Without the drying step, the enzyme (and polymer when present) solubilize into the buffer within 60 seconds
² ABTS assay

Since all compounds and steps used in the electrode preparation appear to be essential for broadening the substrate specificity of HOX, it is difficult to envisage the underlying reason for this. No examples for this can be found in the literature and
such phenomena have not been observed for other enzyme electrodes (GAX, GOX, s-GDH) prepared in the same way as for HOX [27-30].

4 Conclusions

Like Q-GDH, hexose oxidase can effectively be immobilized on a redox conducting polymer and applied as a biosensor. The immobilized enzyme showed activity when tested under amperometric conditions as measured by direct electron transfer to the electrode. No explanation could be given for a relatively sharp optimum in activity and stability of the HOX-electrode in the enzyme concentration applied at the electrode. Although the substrate specificity for glucose oligomers is comparable to that of the soluble enzyme, maltohexaose and even maltoheptaose can also be oxidized. Substrates with an anomic site like mannose, raffinose and the tetrasaccharide $\Delta$-4,5-GlucA(1-4)Gluc(1-4)Rha(1-4)Gluc can also be oxidized, while the soluble enzyme is not active towards these substrates. The broadening of the substrate specificity of HOX could be attractive in cases where oxidation by other methods fails, for instance in the oxidation of partially modified oligosaccharides, or sugars which can not be oxidized by other enzymes. However, for this purpose, the current density obtained with the present electrode is too low so that further optimization studies are required. Attempts to reproduce the effect of substrate broadening on other materials like filter paper and on the wall of a plastic cuvette failed. A combined effect of a rigid carrier and drying of the enzyme with the redox polymer appears to be needed to obtain this effect. Further studies to define if the osmium group in the redox polymer is responsible for this effect, requires further immobilization studies with derivatives of this polymer. As no other examples of substrate broadening after immobilization are described in literature, further research will be required to elucidate the underlying mechanism responsible for this effect.

The substrate specificity of the immobilized hexose oxidase for a wide range of reducing mono sugars can be compared to s-GDH (Chapter 2). Because with immobilized GDH a very high current density can be obtained, this enzyme is probably more suitable for application as a biosensor and in biocatalytical conversions. Immobilized HOX could be applied as a universal biocatalyst for the specific oxidation of sugars or oligomers with functional groups present, like, e.g., the tetrasaccharide as used in these studies. Oxidation of this substrate would result
in an oligosaccharide with hydroxyl groups, a double bond and a carboxylic group, and this product could be used for further derivatization to obtain carbohydrate based cross linking agents.

5 Literature

IMPROVED PRODUCTION OF HYPOHALOUS ACIDS OR HALOGENS BY VANADIUM-CONTAINING HALOPEROXIDASES

Summary

Chloroperoxidase (CPO) from Curvularia inaequalis was used to evaluate the enzymatical and chemical constraints of employing haloperoxidases in the generation of halogens and the corresponding hypohalous acids from halides with the aim to oxidize carbohydrates with these species. In general, at high pH (pH > 8), the net result of chemical equilibria is the formation of hypohalous acids, whereas at low pH (pH < 4), the formation of halogens is favored. The hypohalous acids formed by CPO decompose easily with hydrogen peroxide. This reaction is very fast for HOCl at pH values > 2, in line with the fact that no HOCl was detected under the conditions used. It will therefore depend on the ratio of the reactivity and concentration of the organic substrate, the presence and concentration of hydrogen peroxide, and the pH whether or not oxidation (eq., halogenation) or decomposition of HOCl / HOBr takes place. This reaction is very fast for HOCl at pH > 2 and no formation of any HOCl could be detected. Since the reactivity of carbohydrates with HOCl is very low (as compared to that of MCD), enzymatic generation of HOCl with CPO is not suited for that purpose. Br₂ and Br₃⁻ generation from bromide and H₂O₂ with CPO was investigated at pH 4.0-4.5 (10-100 mM bromide) as well as pH 5.0 (1 mM bromide). Under these conditions, Br₂ as well as Br₃⁻ will be present. The net formation of Br₃⁻ is higher at pH 4.0-4.5, due to the high bromide concentration. Because Br₃⁻ does not directly react with H₂O₂, carbohydrates can be oxidized with Br₂ at these pH values, application of CPO for oxidation of carbohydrates at low pH seems feasible.

1 Introduction

As earlier shown by Besemer et al. [1], hypobromite and hypochlorite oxidize the glycol moiety of carbohydrates like starch and inulin into the corresponding dicarboxy derivatives. This oxidation is carried out at high pH (8-11). The mechanism of this
reaction is not exactly known, but has been reasoned to proceed via HOCl/OCl\(^-\) and HOBr/OBr\(^-\) as oxidants. Painter et al. [2-6] used bromine at low pH (pH 5) to oxidize aldehyde groups of different carbohydrates. The products contained a mixture of carboxylic acids and lactones [2-5,7]. This type of oxidation possibly proceeds via the presence of bromine (Br\(_2\)) in solution. A disadvantage of these types of chemical oxidations is the use of chlorine and the stoichiometric production of the salts (NaCl in the hypochlorite oxidation and NaBr in the bromine oxidation) in the product solution, resulting in the need for a separate purification step of the product. A possible solution to overcome this problem is the enzymatic generation of oxidizing halogen species with haloperoxidases, using a catalytic amount of halide and a stoichiometric amount of hydrogen peroxide (H\(_2\)O\(_2\)). Haloperoxidases catalyze the incorporation of halogen atoms into organic molecules according to the following overall reaction [8,9]:

\[ AH + H_2O_2 + H^+ + X^- \rightarrow AX + 2 H_2O \]  

(1)

where AH is an organic substrate, X is a chloride, bromide or iodide ion, and AX is the halogenated product. Apart from the oxidation of carbohydrates by halogen species, these species halogenate reactive organic compounds (e.g., monochlorodimedone, MCD (Figure 1) or the dye phenol red), usually used to detect haloperoxidase activity [10]. Both reactions are fast, and can be monitored by UV-spectroscopy.

![Figure 1. Haloperoxidase standard assay: MCD oxidative halogenation, X = Br, Cl](image)

To obtain an effective polysaccharide oxidation, the conditions at which optimal production of halogenating species are enzymatically produced (Br\(_2\) at low pH and HOBr/OBr\(^-\) and/or HOCl/OCl\(^-\) at high pH) have to be known. Complicating factors in this respect are the reported catalase activity of haloperoxidases, resulting in sub-optimal conditions of the enzymatic reaction by decomposition of H\(_2\)O\(_2\), and the inhibition of the enzymatic reaction by halides at specific conditions [11-13]. However, it can be questioned (see below) if these factors are caused by the haloperoxidase, or by
chemical equilibria of the halogenating species. Although much research on halogenation with haloperoxidases has been carried out, in most cases the difference between the chemical rate of decomposition of H$_2$O$_2$ and the real catalase activity of an enzyme (metal ion-containing oxidases or heme-containing peroxidases), and the influence of the dissociation of the hypohalous acids, are not taken into account. The latter reactions are pH-dependent [14]:

\[
\begin{align*}
\text{Br}_2 + \text{Br}^- & = \text{Br}_3^- \quad (2) \\
\text{Br}_2 + \text{H}_2\text{O} & \rightarrow \text{HOBr} + \text{H}^+ + \text{Br}^- \quad (3) \\
\text{H}_2\text{O}_2 + \text{HOBr} & \rightarrow \text{H}^+ + \text{Br}^- + \text{H}_2\text{O} + \text{O}_2 \quad (4)
\end{align*}
\]

When no organic substrate such as monochlorodimedone (MCD) is present, peroxidases catalyze the formation of oxygen in a reaction described as "bromide or chloride assisted catalytic disproportionation of hydrogen peroxide" [15,16] (Figure 2).

![Diagram](Image)

**Figure 2.** The reaction of HOBr with MCD or with H$_2$O$_2$[15]

The formation of oxygen becomes evident as the pH increases (pH 5 and higher). This bromide-mediated catalase reaction as well as the Br$_3^-$ formation was completely "inhibited" by the presence of 100 µM MCD [17]. The kinetics of peroxidases has also been measured by oxygen formation [12,18]. Although the actual bromine and the tribromide concentration vary as a function of the sodium bromide concentration (equations 2-4), these studies did not take into account that the difference in oxygen formation can be explained as a difference in actual tribromide (Br$_3^-$) concentration, and thus the actual concentration of HOBr [19-21]. Kinetic studies therefore have to be interpreted carefully, before conclusions on the kinetics can be drawn in a correct way. Recently, the first study on the thermodynamics and the mechanism of catalysis by haloperoxidases was reported by Shevelkova et al. [22]. These authors provided evidence that chloroperoxidase from *C. fumago* is only needed for a rapid equilibrium between halogens which are capable of enzyme-independent halogenation. In order to discriminate between the above-mentioned aspects, the optimal conditions for the
formation of halogens and hypohalous acids as a function of the pH have to be determined. These data can be incorporated into a kinetic reaction model, taking into account the chemical equilibria 1-4.

The haloperoxidases known so far, can be classified into 3 groups, containing iron in the form of heme group or vanadium as prosthetic group, or without a prosthetic group in the active site (Table 1). The name of the enzyme is given to the most electrophilic halide that can be oxidized in the reactivity order of \( I^- > Br^- > Cl^- \).

Large scale applications, have been limited to the use of peroxidases from *Coprinus cinereus* [23] in the bleaching of denim jeans because of the instability of these enzymes under non-physiological conditions. However, recent developments give rise to expectations that in the near future stable peroxidases (temperature > 60°C and hydrogen peroxide concentration > 100 mM) can be produced cost-effectively by protein engineering [24]. A recent review on selective oxidations catalyzed by peroxidases has been published by Van Deurzen et al. [25]. The crystal structure of a vanadium chloroperoxidase from *Curvularia inaequalis* azide complex has recently been resolved [26]. The non-heme peroxidases are much more suitable for the oxidative halogenation of organic compounds; They are very stable and are not inactivated during the halogenation reaction [52,53]. They have little or no catalase activity (when used as peroxidase in the absence of halides) [34,54]. We therefore studied the enzymatic generation of halogens and hypohalous acid by vanadium-containing chloroperoxidase from *Curvularia inaequalis* by differentiation between the enzymatic activity of the enzyme (MCD chlorination and bromination), and the influence of the chemical equilibria of the halogen formation and decomposition of \( H_2O_2 \). By modeling of the enzymatic kinetic parameters and the equilibria of the halogen species, the feasibility to oxidize carbohydrates with enzymatically generated hypochlorite (at high pH) or bromine (at low pH) can be evaluated.

The reaction mechanism of vanadium containing enzymes can be described as a Ping-Pong BiBi reaction mechanism, where first hydrogen peroxide is bound to the vanadium. Since several inorganic vanadium-hydrogen peroxide complexes are known, it is reasonable to assume that in the first reaction step hydrogen peroxide binds covalently to the vanadium ion [27] presumably as V-O-O-H. The next step is the oxidation of the bromide ion with simultaneous cleavage of the oxygen-oxygen bond [8]. The resulting vanadium-hypobromite complex reverts to the native enzyme and releases hypobromous acid or an equivalent active species (see below) which is
responsible for all reactions of the enzyme (Figure 3).

\[
\begin{align*}
E + H_2O_2 & \rightarrow E\cdot H_2O_2 \\
E\cdot H_2O_2 + Br^- & \rightarrow E\cdot HOBr \\
E\cdot HOBr + OH^- & \rightarrow HOBr + E
\end{align*}
\]

**Figure 3.** Schematic reaction mechanism of formation of HOBr by vanadium-containing peroxidases [2]. \((E = \text{enzyme})\)

**Table 1.** Sources and prosthetic groups of haloperoxidases

<table>
<thead>
<tr>
<th>Enzyme (halide ion used)</th>
<th>Source</th>
<th>Prosthetic group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(iodide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>horseradish peroxidases</td>
<td>horseradish roots (plants)</td>
<td>heme</td>
<td>[28]</td>
</tr>
<tr>
<td>thyroid peroxidases</td>
<td>thyroid glands (vertebrates)</td>
<td>heme</td>
<td>[29]</td>
</tr>
<tr>
<td>(bromide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromoperoxidase</td>
<td><em>Streptomyces aureofaciens</em> (bacterium)</td>
<td>absent</td>
<td>[30-32]</td>
</tr>
<tr>
<td></td>
<td><em>Penicillus capitatus</em> (green alga)</td>
<td>heme</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td><em>Corallina pilulifera</em> (red alga)</td>
<td>vanadium</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td><em>Ascophyllum nodosum</em> (brown alga)</td>
<td>vanadium</td>
<td>[35,36]</td>
</tr>
<tr>
<td></td>
<td><em>Xanthoria parietina</em> (lichen)</td>
<td>vanadium</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td><em>Macrocystis pyrifera</em> (brown alga)</td>
<td>vanadium</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td><em>Fucus distichus</em> (brown alga)</td>
<td>vanadium</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td><em>Ceramium rubrum</em> (red alga)</td>
<td>vanadium</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td><em>Laminaria saccharina</em> (brown alga)</td>
<td>vanadium</td>
<td>[27,36]</td>
</tr>
<tr>
<td>lactoperoxidase</td>
<td>milk, saliva, tears (mammals)</td>
<td>heme</td>
<td>[39]</td>
</tr>
<tr>
<td>lignin peroxidases</td>
<td><em>Phanerochaete chrysosporium</em> (fungus)</td>
<td>heme</td>
<td>[40,41]</td>
</tr>
<tr>
<td>(chloride)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chloroperoxidase</td>
<td><em>Caldariomyces fumago</em> (fungus)</td>
<td>heme</td>
<td>[42,43]</td>
</tr>
<tr>
<td></td>
<td><em>Curvularia inaequalis</em> (fungus)</td>
<td>vanadium</td>
<td>[11,44]</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas pyrocinia</em> (bacterium)</td>
<td>absent</td>
<td>[45,46]</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces lividans</em> (bacterium)</td>
<td>absent</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td><em>Serratia marcescens</em> (bacterium)</td>
<td>absent</td>
<td>[48]</td>
</tr>
<tr>
<td>myeloperoxidase</td>
<td>white blood cells (vertebrates)</td>
<td>heme</td>
<td>[49-51]</td>
</tr>
</tbody>
</table>
2 Theory of equilibria of halogen species and kinetic modeling

As described in Chapter 1, the oxidative glycolic cleavage of carbohydrates can be carried out by the use of hypochlorite/hypochlorous acid (pKa=7.5) and bromide/hypobromous acid (pKa=8.6). Chloride and bromide are the species used as substrates in the peroxidase reaction. Figure 4 shows the importance of the pH in the relative concentration of bromine species in solution. It appears that the behavior of these species is quite complex, since depending on the pH, different active species may be formed.

![Diagram showing relative concentrations of bromine species as a function of pH](image)

**Figure 4.** Relative concentrations of a mixture containing Br₂ (1), Br⁻ (2), HOBr (3) and OBr⁻ (4) as a function of pH at 20 °C in water [14]. The initial bromide concentration is 10 mM, based on equilibria 2,3,5 and 6, (see below)

The same type of relationship exists for the presence of chlorine species as a function of pH [1]. At pH < 2, chlorine is mainly present, while between pH 2-7, the main species present is HOC\. At pH > 8, the species are mainly present as OCl⁻. The following strategy to evaluate the kinetics of the bromine formation can be followed:

a. The determination of the kinetics of the decomposition of bromine species (HOBr) by H₂O₂,

b. The determination of kinetic reaction constants of vanadium chloroperoxidase (CPO) in the oxidation of chloride and bromide at low pH (pH 3-6), using MCD as the substrate (initial rate kinetics),

c. Set-up of the reaction model, including the kinetics of CPO, chemical equilibria
and the decomposition reaction of H₂O₂,
d. Validation of the reaction model by fitting the measured data with respect to Br₃⁻ formation.

For the bromine equilibria, the following equations are used [14]:

\[ \text{Br}_2 + \text{Br}^- \rightleftharpoons \text{Br}_3^- \]  \hspace{1cm} (2)
\[ \text{Br}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HOB}r + \text{H}^+ + \text{Br}^- \]  \hspace{1cm} (3)
\[ \text{HOB}r \rightleftharpoons \text{H}^+ + \text{OBr}^- \]  \hspace{1cm} (5)
\[ \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^- \]  \hspace{1cm} (6)

The equations have the following thermodynamic data [14]: \( K_2 \) (association constant of \( \text{Br}_3^- = 17 \text{ M}^{-1}, \) equation 2), \( K_3 \) (the equilibrium constant of \( \text{Br}_2 = 8.09 \times 10^{-11} \text{ M}, \) equation 3), \( K_4 \) (the dissociation constant of \( \text{HOB}r = 2 \times 10^6 \text{ M}, \) equation 5), \( K_6 \) (dissociation constant of water = \( 1.8 \times 10^{-16} \text{ M}, \) equation 6). The actual rate constants for these reactions are not known, but it is generally accepted that these equilibria are obtained within seconds at room temperature.

The chemical decomposition of hydrogen peroxide with HOBr has already been formulated as [55]:

\[ \text{H}_2\text{O}_2 + \text{HOB}r \rightarrow \text{H}^+ + \text{Br}^- + \text{H}_2\text{O} + \text{O}_2 \]  \hspace{1cm} (4)

For the enzymatic reactions the following equations are formulated:

\[ \text{H}_2\text{O}_2 + \text{E} \rightleftharpoons \text{E-H}_2\text{O}_2 \]  \hspace{1cm} (7)
\[ \text{E-H}_2\text{O}_2 + \text{Br}^- \rightleftharpoons \text{Ecomplex} \]  \hspace{1cm} (8)
\[ \text{Ecomplex} \rightleftharpoons \text{E} + \text{HOB}r + \text{OH}^- \]  \hspace{1cm} (9)
\[ \text{Ecomplex} + \text{Br}^- \rightleftharpoons \text{Inhibited complex} \]  \hspace{1cm} (10)

The first step is the binding of hydrogen peroxide to the enzyme (equation 7). This binding step has been studied in detail for the chlorination reaction. The second order rate constant for this reaction is in the order of \( 1 \times 10^6 - 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) (compared to the backward reaction set at \( 1\text{s}^{-1} \)), which has also been reported for other peroxidases [56] and implicates that in diluted conditions, the concentration of the total enzyme-\( \text{H}_2\text{O}_2 \) complex is equal to the starting concentration of enzyme.

The binding of bromide and the formation of HOBr are the reactions usually referred to
when the Michaelis-Menten kinetics ($V_{\text{max}}$ (app) and $K_m$ (app)) are applied (used in equation 8 and 9). The rate constants in the reaction model are derived from the kinetic data from the bromination of MCD. Assuming that no chemical decomposition of hydrogen peroxide occurs, the decrease of hydrogen peroxide is equivalent to the fast formation of brominated MCD.

Using the chemical equilibria of bromine species (pH 3-5) and the rate of decomposition of hydrogen peroxide with HOBr, insight can be obtained in the concentration profile of the species (Br$_2$, Br$_3^-$ and HOBr) during the reaction. In general, due to the decomposition of H$_2$O$_2$ with HOBr, the bromide concentration is nearly equal to the starting concentration. This means that this concentration remains constant and the reaction rate will be first order in hydrogen peroxide.

At higher pH (pH > 7) bromate formation ($K_{11} = 15$ M$^{-2}$·s$^{-1}$, [14]) might lead to a loss of reactive species:

$$2\text{HOBr} + \text{OBr}^- = \text{BrO}_3^- + 2\text{H}^+ + 2\text{Br}^- \quad (11)$$

However, this reaction is third order and will hardly contribute to the concentration profiles of the species in dilute systems as the concentration of OBr$^-$ is very low.

Until now, no reaction rate for the formation of tribromide ion is known, although the equilibrium constant is known [14]. The reaction rate is assumed to be very high.

In the absence of MCD, it will be possible to measure the amount of Br$_3^-$ at known bromide concentrations. These data can be used to fit the reaction rate of chloroperoxidase with bromide and hydrogen peroxide.

3 Materials and methods

Vanadium chloroperoxidase (Curvularia inaequalis, V-CPO) was a gift from the Unilever Research Laboratory (NL). The hypochlorite solution was a gift from Akzo Nobel (NL). All chemicals used were of analytical grade. Kinetic experiments were carried out with solutions prepared from milli-Q water. The enzyme was desalinated on a PD-10 column prior to use, to prevent any possible interference of halides present in the enzyme solution during kinetic experiments, and stored in Milli-Q water. Monochlorodimedone (MCD) was obtained from Sigma (USA). Hydrogen peroxide solutions were freshly made before experimental use by dilution from a 30% stock
solution (Merck, G). For computer simulations, Facsimile software from AEA Technology plc (Harwell, UK) was used.

Oxidations with (vanadium) chloroperoxidase
Vanadium chloroperoxidase (CPO) was supplied in 50 mM Tris/H₂SO₄ with 90 mM NaCl and 50 μM sodium orthovanadate pH 8.3. The enzyme is stable at room temperature for at least 5 months, but can also be stored at -20°C. [57]. Kinetic experiments were carried out at room temperature on a lambda 5 PE spectrophotometer by measuring the rate of chlorination of 2-chlorodimedone (50 μM) at 287 nm (ε = 20.2 mM⁻¹ cm⁻¹) to 2,2-dichlorodimedone, or the rate of bromination of 2-chlorodimedone (50 μM) at 287 nm (ε =18.2 mM⁻¹ cm⁻¹ at pH 4.0 and 19.9 mM⁻¹ cm⁻¹ at pH values higher than 4.5) [56]. The solutions were buffered with 50 mM sodium acetate. Oxidation of bromide (1, 25, 50 and 200 mM) with CPO (10 and 100 mM H₂O₂) was carried out at pH 4.0, 50 mM sodium acetate, without the addition of MCD. UV-Vis spectra measuring Br₃⁻ were recorded at 287 nm (ε = 3.64·10⁴ M⁻¹ cm⁻¹). To determine if HOCl is the active species in the chlorination reaction, the enzymatic chlorination of phenol (which might proceed via HOCl, [58]) was carried out with CPO (1.23 μg·mL⁻¹) at pH 4.0 in 20 mM sodium acetate buffer, 10 mM NaCl and 8 mM phenol and 10 mM H₂O₂. At 0, 5 and 10 minutes a (chloro)phenol spectrum was recorded between 190 and 400 nm. Spectral measurements with CPO (9.2 μg·mL⁻¹) in the oxidation of NaCl (20 mM) were carried out at room temperature with 10 mM H₂O₂ in 20 mM sodium acetate at pH 3.8, 4.8 and 5.2. To measure the formation of HOCl, wavelength scans between 190 and 450 nm were recorded at 1 minute time intervals during 10 minutes. Spectral measurements of HOCl (5 mM in 100 mM sodium acetate buffer) were recorded in the pH range of 3.0-5.0 between 190 - 400 nm. From these experiments the molecular extinction coefficient (ε) of HOCl as a function of pH was calculated.

Chemical oxidations and halogenations
The rate constants of the reaction of HOCl and H₂O₂ with phenol, MCD and glucose respectively, were determined in the concentration range of 0-250 mM HOCl in 10 mM sodium acetate at pH 4 and 6 (and pH 7 for phenol). The experiments were carried out at room temperature. At fixed time intervals the concentration of HOCl was determined. Samples were taken and analyzed for the hypochlorite content, adding the sample to an aqueous solution of KI-acetic acid and titrating with 0.1 M thiosulfate. The decomposition rate of HOB₃ with H₂O₂ was determined at room temperature by
measuring the oxygen production during the reaction by a Clark cell (oxygen monitor, YSI Instruments) at varying concentrations of Br₂ (10-80 μM), Br⁻ (8-62 μM) and H₂O₂ (10-80 μM). The solution was purged with nitrogen at the start of the reaction. The reaction was started by addition of one of the substrates, and the rate of oxygen formation was calculated from the plot using calibrated values for oxygen. The measured values have been corrected for a background of oxygen formation or a drift in the electrode.

4 Results and discussion

Kinetics of chloroperoxidase (chlorination)
Kinetic experiments with CPO in the chlorination of MCD were carried out at a fixed concentration of 10 mM NaCl, which is far above the Kₘ [44]. The results are summarized in Table 2. The data are in accordance with data reported earlier [11]. However, the activity measured is based on the chlorination of MCD, while in our case, the formation of HOCl as an oxidant in carbohydrate oxidation is of much more interest. The formation of free HOCl has been studied [11] at pH 3.8 (100 mM sodium citrate), in which the formation of HOCl was measured spectroscopically (absorption maxima at 245 and 314 nm). These experiments were repeated in 20 mM sodium acetate and spectra were recorded in the region of 200-500 nm in order to reproduce these results. It was indeed possible (Figure 5) to obtain the same type of data as reported earlier [11].

Table 2. Apparent kinetic parameter values of CPO for MCD at varying pH values (10 mM NaCl in 50 mM sodium acetate)

<table>
<thead>
<tr>
<th>pH</th>
<th>3.5</th>
<th>4.5</th>
<th>5.0</th>
<th>5.5</th>
<th>6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kₘ (H₂O₂) (μM)</td>
<td>240</td>
<td>190</td>
<td>94</td>
<td>67</td>
<td>6</td>
</tr>
<tr>
<td>Vₘₐₓ (U·mg⁻¹)</td>
<td>16.0</td>
<td>20.8</td>
<td>18.4</td>
<td>19.1</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Upon addition of CPO, the extinctions at 235 and 315 nm increase. This might be ascribed to formation of HOCl, but the signal obtained after 10 minutes could easily be destroyed by only shaking the reaction mixture. In that case, the control signal was obtained again.
Figure 5. Absorption spectra of HOCl formation by CPO in the presence of NaCl (20 mM) and H₂O₂ (10 mM) at pH 3.8 in 20 mM sodium acetate buffer

Figure 6. Spectrum of 5 mM HOCl in 100 mM sodium acetate buffer pH 3.8

It is likely that the formation of oxygen has been measured, as it is known that HOCl reacts easily with H₂O₂ at pH > 2. When the spectrum of HOCl in sodium acetate buffer is recorded (Figure 6), it can be observed that the maxima found (240 and 300 nm) are
not exactly the same as the spectrum recorded earlier [11]. In sodium acetate buffer, HOCI appeared to be stable. We therefore tried to measure a spectrum of HOCI in sodium citrate. However this was not possible, due to a very fast reaction between HOCI and sodium citrate (having a less stable carboxylic group at the 3-position then sodium acetate), yielding carbon dioxide. This explains the spectrum recorded earlier [11], where HOCI was tested in sodium citrate at pH 3.8. In this experiment as well, gas formation instead of the HOCI spectrum must have been measured. Additional evidence is based on the difference in the molar extinction coefficient found. Based on earlier data [11], ε (314 nm) is approx. 0.0080 M⁻¹·cm⁻¹. In this study ε (314 nm) in 100 mM sodium acetate buffer pH 3.8 is 23±0.5 M⁻¹·cm⁻¹. In conclusion: At pH 3.8, HOCI reacts very fast with sodium citrate buffer to yield carbon dioxide (with or without H₂O₂) and could, in our opinion, not have been measured as presented earlier [11]. HOCI decomposes in the presence of H₂O₂ under formation of oxygen in acetate buffer at low pH, but is stable in this buffer without H₂O₂.

**Chemical reactivity of HOCI**

Because of the inability to detect HOCI formation in the presence of hydrogen peroxide and the absence of MCD, the question might arise whether HOCI is really formed as an intermediate (also in the MCD reaction) [59]. Chemical chlorination of phenol is suggested to occur through HOCI as the active reagent [58]. We therefore tested CPO for this type of chlorination: if phenol is chlorinated by the use of CPO, NaCl and H₂O₂, this would be an indirect evidence for the formation of HOCI. It appeared that phenol was indeed chlorinated indicating that HOCI is formed by CPO.

The reactivity of several substrates is shown in Table 3 (the reactivity is expressed as k-value (M⁻¹·min⁻¹), assuming the reaction to be first order in the substrate used, dc/dt = -k[S][HOCI]). It appears that the rate constant of MCD chlorination with HOCI is much higher than the reactivity of HOCI for H₂O₂. This explains why in a biochemical assay MCD is such a good (halogenation) substrate: the decomposition rate of HOCI at pH 4 with H₂O₂ is less than 2% of the halogenation rate of MCD. By contrast the oxidation rate of glucose by HOCI is low. This implies that in the enzymatic reaction with chloroperoxidase the reactivity of glucose is too low to "compete" with H₂O₂; HOCI will be decomposed by H₂O₂. It will therefore depend on the substrate to be halogenated or oxidized, whether or not halogenation / oxidation or decomposition of HOCI takes place in this system. In the case of phenol, the reactivities (of phenol and H₂O₂) are
comparable and both reactions occur. At higher pH phenol chlorination will be accelerated due to phenol ionization to phenolate.

**Table 3. Chemical reactivity of HOCl for several substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$k$ (M$^{-1}$·min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>4</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1615</td>
</tr>
<tr>
<td>MCD</td>
<td>4</td>
<td>&gt; 15,000</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&gt; 15,000</td>
</tr>
<tr>
<td>Phenol</td>
<td>4</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1257</td>
</tr>
<tr>
<td>Glucose</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.44</td>
</tr>
</tbody>
</table>

$^1$ Reactions in 10 mM sodium acetate at room temperature

**Kinetics of chloroperoxidase, (bromination)**

a. **Decomposition of bromine and hypobromous acid in the presence of H$_2$O$_2$**

Different concentrations of hydrogen peroxide were tested with respect to decomposition by bromine/hypobromous acid at pH 4.0 and 5.0. This, in order to predict the effective concentration in kinetic experiments and stability of Br$_2$ and HOBr in solution in the presence of hydrogen peroxide. The oxygen production was measured with an oxygen electrode, and the decomposition data obtained were fitted according to the following equation:

$$\text{H}_2\text{O}_2 + \text{HOBr} \rightarrow \text{H}^+ + \text{Br}^- + \text{H}_2\text{O} + \text{O}_2$$  

(4)

The reaction rate is first order with respect to the concentration of HOBr. The k-value (5700 M$^{-1}$·s$^{-1}$) for this reaction was derived by kinetic modeling of the HOBr concentration profile. The reaction is pH-dependent and is faster at higher pH [12,16].

b. **Kinetic reaction constants of CPO in the oxidation of bromide**

The kinetic constants of CPO were measured between 1- 200 mM of bromide and different concentrations of hydrogen peroxide. With initial rate measurements of
bromination of MCD, a Michaelis-Menten type of behavior was found. This behavior was influenced by the formation of non-reactive Br$_3^-$ at low pH (pH 4.0 and 4.5) and at higher bromide concentrations (> 25 mM). At pH higher than 5.5, the brominating activity decreased very quickly, and accurate determination of the kinetics was difficult. The results obtained are summarized in Table 4.

**Table 4. Apparent kinetic parameter values of CPO for MCD-bromination**

<table>
<thead>
<tr>
<th>Br$^-$ [mM]</th>
<th>pH 4.0</th>
<th></th>
<th>pH 4.5</th>
<th></th>
<th>pH 5.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (U·mg$^{-1}$)</td>
<td>$K_m$(H$_2$O$_2$) (mM)</td>
<td>$V_{\text{max}}$ (U·mg$^{-1}$)</td>
<td>$K_m$(H$_2$O$_2$) (mM)</td>
<td>$V_{\text{max}}$ (U·mg$^{-1}$)</td>
<td>$K_m$(H$_2$O$_2$) (mM)</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>10.7</td>
<td>98</td>
<td>16.6</td>
<td>131</td>
<td>8.4</td>
</tr>
<tr>
<td>2.5</td>
<td>15</td>
<td>10.7</td>
<td>77</td>
<td>13.8</td>
<td>79</td>
<td>5.0</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>13.5</td>
<td>75</td>
<td>16.4</td>
<td>65</td>
<td>3.3</td>
</tr>
<tr>
<td>25</td>
<td>27</td>
<td>18.1</td>
<td>53</td>
<td>10.8</td>
<td>40</td>
<td>2.6</td>
</tr>
<tr>
<td>100</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>200</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
<td>22</td>
<td>1.9</td>
</tr>
</tbody>
</table>

n.c. = not calculated, due to the strong interference of Br$_3^-$ formation during spectroscopic measurements.

In the region pH 4-5 large differences in kinetic behavior are found. At pH 4.0, a mixed type of inhibition by Br$^-$ and at pH 4.5 competitive inhibition (i.e., E + Br$^-$ = EBr$^-$) is observed. At pH 5.0 uncompetitive inhibition (i.e., Ecomplex + Br$^-$ = EcomplexBr$^-$) is found. In the latter case, $K_m$ and $V_{\text{max}}$ decrease at higher bromide concentration. In the reciprocal plot, parallel lines are found (data not shown). In general, CPO has a 3-4 times higher $V_{\text{max}}$ for bromide than for chloride at pH 4.5 and 5.0 (Table 2). The pH optimum for the chlorine and bromine formation is approximately the same. The differences found in the type of inhibition at different pH have been reported for the bromination with BPO from A. nodosum [13]. CPO has also been reported to have pH dependent inhibition kinetics [11]. For BPO and CPO the halide has been reported as an inhibitor at low pH [11]. As both enzymes contain vanadium, and hardly possess catalase activity, this inhibition is indeed determined by enzymatic constraints and not influenced by decomposition of HOBr and HOCl respectively, or by chemical equilibria of the halogens.

c. Bromine equilibrium and enzyme kinetics

Based on the data of bromine formation and dissociation cf hypobromous acid [14,22],
the kinetic parameters can be used for computer simulated equilibria and an enzymatic reaction model. This model calculates the relative concentration of species present in equilibrium, based on the pH, the initial effective concentration of substrates, the enzymatic reaction rate and the decomposition reaction with hydrogen peroxide.

Figure 7. Relative concentrations of a mixture containing \( \text{Br}_2 \) (1), \( \text{Br}_3^- \) (2), HOBr (3) and OBr\(^-\) (4) as a function of the pH at 20°C. The initial bromide concentration is 100 mM

By varying the reaction conditions such as pH and initial concentration of NaBr, the concentration of the different species present could be fitted. An example is given in Figure 7. When this figure is compared with Figure 4 (10 mM bromide) [14], it is shown that using a higher bromide concentration (100 mM), the concentration of \( \text{Br}_3^- \) significantly increases. This species does not react with hydrogen peroxide or organic molecules.

Figure 8 shows the formation of \( \text{Br}_3^- \) during the reaction of hydrogen peroxide (10 mM) and bromide (25 mM) with CPO at pH 4.0. Any HOBr formed immediately reacts according to the chemical equilibria (eq. 2-6) to give \( \text{Br}_2 \) and \( \text{Br}_3^- \). The latter species is measured spectrophotometrically at 287 nm [60]. With the parameters obtained in this study (enzymatic reaction constants and decomposition of HOBr with hydrogen peroxide) and the chemical equilibria of bromine species, the model is able to predict the effective concentration of \( \text{Br}_3^- \).
At pH 5, a significantly higher decomposition of hydrogen peroxide is found to occur (and is also predicted by the model) when bromide is oxidized by CPO at lower bromide concentration (10 mM, see Figure 4). This is caused by the fact that the effective concentration of bromine is pH dependent. At higher pH and low bromide concentration (10 mM), a higher concentration of HOBr is present compared to a higher bromide concentration (100 mM, Figure 7). This leads to a faster decomposition of hydrogen peroxide. From an enzymatic point of view, a higher concentration of bromide causes inhibition. However, from a chemical point of view this effect is advantageous, because the relative concentration of HOBr also decreases, and the decomposition rate with hydrogen peroxide is lower. This means that HOBr is “stabilized” via the formation of the tribromide ion (Br$_3^-$). According to equation 2, bromine can be generated and used as the oxidant in carbohydrate oxidation (see also chapter 6).

![Graph showing the formation of Br$_3^-$ by CPO at pH 4.0.](image)

**Figure 8.** $\text{Br}_3^-$ formation by CPO at pH 4.0. (25 mM bromide, 10 mM H$_2$O$_2$ in 50 mM sodium acetate) ■ = measured data, ◆ = computer fit

5 Conclusions

Vanadium-containing peroxidase is able to generate halogens with hydrogen peroxide in the absence of monochlorodimedone (MCD). These halogens are in chemical equilibrium, and decompose with H$_2$O$_2$. Since HOCl decomposes very fast at pH >2.0, we did not succeed in detecting the presence of any enzymatically generated HOCl in
solution due to the very fast decomposition with hydrogen peroxide. Whether halogenation with HOCl takes place depends on the reactivity of the substrate. MCD is a very reactive substrate, reacting with HOCl much faster than H$_2$O$_2$. In the absence of MCD, the HOCl formed decomposes immediately with hydrogen peroxide. In the presence of citrate buffer, carbon dioxide is formed. Therefore, HOCl formation could not have been detected spectrophotometrically, as was suggested in literature. Experiments with HOCl and different substrates at pH 4 and 6 show that the reaction rate for halogenation / oxidation depends on the reactivity of the substrate. If the reactivity of the substrate, in a specific pH range, is in the same order (phenol) or preferably higher (MCD) than the reactivity of HOCl for H$_2$O$_2$, enzymatic oxidative halogenation, $cq.$, oxidation by the peroxidase is expected to be feasible. As glucose (and other carbohydrates) has a very low reactivity compared to MCD for HOCl, the enzymatic vanadium peroxidase catalyzed HOCl oxidation of carbohydrates at alkaline pH is not feasible.

HOBr decomposes very fast at pH > 6.0 in the presence of H$_2$O$_2$. The decomposition rate of HOBr also depends on the amount of halide present. To define the conditions under which Br$_2$ (or Br$_3^-$) can be generated enzymatically for the oxidation of carbohydrates, a reaction model combining enzymatic reaction parameters, decomposition of HOBr and chemical equilibria was developed. It was shown, that during the reaction, the bromide concentration is nearly constant. In order to obtain a significant concentration of Br$_3^-$, it is necessary to use relatively high concentrations of bromide (10-100 mM). However, this high concentration will decrease the reaction rate due to (un)competitive inhibition at pH 4.5 and 5.0, but because of the equilibrium of Br$_3^-$ and bromine at this pH, the net formation of bromine will be higher at pH 4.0-4.5. Based on these data, it is expected that the enzymatic vanadium peroxidase catalyzed Br$_2$ generation might be able to oxidize carbohydrates under specific conditions.

Acknowledgement
We acknowledge the generous gift of chloroperoxidase from Dr. J.H.A. Boot from Unilever Research Laboratories, Vlaardingen (NL).
6 Literature cited

Biophys. Acta 872, 104.

113
OXIDATION OF DIALDEHYDE INULIN WITH BROMINE

Abstract
Oxidation of dialdehyde inulin (DAI) with Br₂ was investigated. Br₂ was also generated in situ by oxidation of Br⁻ with either peracetate or with H₂O₂ and chloroperoxidase from Curvularia inaequalis. A rather low second order oxidation rate of DAI at pH 3-6 with Br₂ was observed of 3.8×10⁻³ M⁻¹s⁻¹ at room temperature, based on the assumption that only one reactive aldehyde group exists per furanose subunit. At the reaction conditions applied (pH 3-6), half of the aldehyde groups are present in the hemiacetal form, as observed by ¹³C-NMR measurements. After about 50% of the total number of aldehyde groups had been oxidized, the oxidation rate decreased significantly. It is suggested that the presence of hemiacetals limits the oxidation rate of DAI between pH 3-6, the oxidation probably proceeds via keto-enol tautomerization of the free aldehyde, bromine addition and elimination of HBr. The use of peracetic acid and a catalytic amount of bromide enables a 100% oxidation of DAI into dicarboxy inulin at pH 5, but glycosidic cleavage lowers the yield of dicarboxy inulin. The Ca-sequestering capacity (SC) after extensive oxidation of DAI is reasonable. Attempts to oxidize DAI by chemo-enzymatic means failed, although chloroperoxidase is able to generate bromine from bromide at pH 4.0-5.0. The reasons are that the relatively slow oxidation rate of DAI with Br₂ cannot compete with the relatively fast decomposition of HOBr with H₂O₂ and inactivation of the enzyme with the oxidizing species present in solution.

1 Introduction

Oxidative cleavage of the glycol moiety of carbohydrates such as starch, inulin and cellulose affords the corresponding dialdehyde or dicarboxy derivatives. The periodate cleavage of 1,2-diol structures in carbohydrates [1-3] results in the production of two aldehyde groups per glycol unit and the reduction of periodate to iodate (Figure 1). For the subsequent oxidation to dicarboxy polymers, chlorite is used. The use of chlorite has its drawbacks, because chlorine dioxide gas is
produced as a side reaction, thereby consuming 2 equivalents of chlorite [4,5]. Floor et al. [6] made a considerable improvement by using hydrogen peroxide as an inexpensive HOCl scavenger. With the use of this scavenger, the oxidation can be carried out with two moles of sodium chlorite per dialdehyde moiety.

![Electrochemical Reaction](image)

**Figure 1.** The periodate oxidation of starch

Periodate oxidation on an industrial scale is limited due to the relatively high price of the oxidant. Processes have been developed to regenerate the periodate by means of electrochemical oxidation [7,8]. Several publications have shown that it is possible to produce dialdehyde starch with this technique [9-12]. Veelaert et al. [13,14] developed a semi-continuous process, comprising batch-wise oxidation of starch with a catalytic amount of periodate which is continuously recycled through a separate electrochemical cell. In this design, high performances combined with low periodate-to-starch ratios turned out to be possible.

![Chemical Reaction](image)

**Figure 2.** Formation of hemiacetals and hemialdol in the dialdehyde (a) derived from α-methyl glucopyranoside [15]
Aldehyde carbohydrate chemistry is quite complex [15] because the aldehyde functions can form hemiacetals with a hydroxyl group elsewhere in the molecule. The most studied methyl aldohexopyranoside is α-methyl glucopyranoside (Figure 2). Here, glycolic oxidation involves removal of C₃ and a C₂/C₄ dialdehyde results. Several structures are theoretically possible for this "dialdehyde" and equilibria between the various forms are possible [16,17]. The occurrence of hemiacetal formation in oxidation products of polysaccharides is very likely because these links may be inter-residue as well as intra-residue (Figure 3).

![Figure 3](image)

**Figure 3. Inter- and intra-residue hemiacetal formation in dialdehyde starch**

In our opinion, Floor et al. [6] were not able to obtain 100% oxidation of dialdehyde starch because of these hemiacetal structures. However, with dialdehyde sucrose a 100% oxidation with bromine water was achieved [18-20]. The kinetics of the oxidation of D-glucose by aqueous bromine solutions has been studied [21]. Molecular bromine (Br₂) is the oxidizing agent, tribromide (Br₃⁻) and hypobromous acid (HOBr) making only negligible contributions. Besemer et al. [22] compared the consecutive reaction with sodium chloride in the oxidation of dialdehyde starch and dialdehyde inulin. In the oxidation of dialdehyde starch the best results were obtained at pH 5, whereas the dialdehyde inulin oxidation required different reaction conditions (pH 2.5-3). This difference was explained by the fact that the hydrated acetal form of inulin is more stable than the acetal forms of dialdehyde starch.

The presence of inter-residue hemiacetal formation during oxidation of polysaccharides has been studied extensively by Painter et al. [23-26] and Ishak et al. [27] (equation 1-3). A strong decrease in oxidation rate during the reaction was noticed. This decrease was ascribed to the presence of 6-membered hemiacetal rings between the aldehyde groups of oxidized carbohydrate residues and the nearest hydroxyl group on neighboring unoxidized xylose residues. Here too,
indications were found for acetal formation during oxidation between the carbonyl groups of the oxidized sugar residues and the secondary hydroxyl groups of intact sugar residues in the same molecular chain. After decomposition of the acetals, the remaining glucose residues become oxidizable for periodate. After hydrolysis with alkali, treatment with aqueous acid or bromine did not regenerate the lactones [27]. These results show that complete oxidation of dialdehyde polysaccharides is difficult, due to the formation of relatively stable hemiacetals in the substrate. The chemical stability of dialdehyde carbohydrates, like dialdehyde starch, has been studied in alkaline [28-29] and in acidic [30] media.

The alkaline degradation mainly involves two different types of reactions, the Cannizzaro reaction and β-elimination. The Cannizzaro reaction is a disproportionation of two aldehyde groups into a carboxylic acid and an alcohol function. The β-elimination constitutes a series of reactions, although complete identification of all degradation products has not been realized yet. Veelaert et al. [31] studied the acidic degradation of dialdehyde starch at low pH during heating and concluded that degradation upon heating was minimized at pH 3. Oxidation of dialdehyde carbohydrates at low pH would therefore be favorable to obtain high molecular weight dicarboxyl derivatives.

Because of our interest in the enzymatic oxidation of carbohydrates, the use of the bromide/bromine couple as a catalyst for the oxidation of dialdehyde polysaccharides at low pH raised our interest. This chapter describes first an attempt to elucidate the mechanism of bromine oxidation of dialdehyde polysaccharides.

Oxidation of dialdehyde polysaccharides with Br₂ at pH 4.5 results in the formation of acid which might be formed according to the following reactions:

\[
\begin{align*}
\text{Br}_2 + (\text{CHO}) + \text{H}_2\text{O} & \rightleftharpoons (\text{COOH}) + 2\text{H}^+ + 2\text{Br}^- \quad (1) \\
\text{or} \\
\text{Br}_2 + \text{H}_2\text{O} & \rightleftharpoons \text{HOBr} + \text{H}^+ + \text{Br}^- \quad (2) \\
\text{HOBr} + (\text{CHO}) & \rightleftharpoons (\text{COOH}) + \text{H}^+ + \text{Br}^- \quad (3)
\end{align*}
\]

Because of the relatively good solubility, dialdehyde inulin was used as the substrate material in these studies (Figure 4).
Figure 4. Structure of inulin (a), of dialdehyde inulin (b) and of dicarboxy inulin (c) \((n = 1-50)\)

To avoid the use of bromine as such, alternative studies were carried out with the system peracetate/bromide. Bromide is oxidized into bromine at low pH, which can subsequently be used for the oxidation of dialdehyde inulin (equation 4-5).

\[
\begin{align*}
\text{AcOOH} + 2\text{Br}^- + \text{H}_2\text{O} &\rightarrow \text{HOAc} + \text{Br}_2 + 2\text{OH}^- \\
\text{Br}_2 + \text{H}_2\text{O} &\rightleftharpoons \text{HOBr} + \text{Br}^- + \text{H}^+ 
\end{align*}
\]

(4) \hspace{1cm} (5)

A second alternative was investigated, based on the enzymatic generation of bromine from bromide by the enzyme chloroperoxidase with hydrogen peroxide as the primary oxidant (Figure 5). Based on the kinetic studies of this enzyme (Chapter 5), the chemo-enzymatic oxidation of dialdehyde inulin was studied.

Figure 5. Oxidation of dialdehyde polysaccharides with chloroperoxidase (CPO) and bromide
2 Materials and methods

Potato starch was supplied by Avebe (NL). Inulin, isolated from chicory roots was obtained from Sigma (USA) and Cosun (NL), the average DP was 10. Sodium metaperiodate, acetic acid, 0.1 M sodium thiosulfate solution, potassium iodide, sodium hydroxide, sodium bromide, hydrogen peroxide, and acetic anhydride were obtained from Merck (G). Sodium hypochlorite was of a technical quality, containing about 150 g chlorine-L\(^{-1}\) and was a gift from AKZO Nobel (NL). Bromine (99.5% pure) was obtained from Aldrich (USA).

Dialdehyde inulin and dialdehyde starch (both 100 % oxidized) were prepared according to Floor et al. [6]. Inulin (10 g, 62 mmol) was dissolved in 150 mL water at 4°C. Periodate (13.5 g, 62 mmol) was added and the pH was adjusted to pH 4. The solution was stirred for 18 hours at 4°C and subsequently ultrafiltrated. After freeze drying, dialdehyde inulin was obtained as a white powder. The same procedure was used for the preparation of dialdehyde starch.

Peracetic acid was prepared according to Soedjak et al. [32] by reaction of acetic anhydride with 30% aqueous hydrogen peroxide. Acetic anhydride (4.87 g) was added dropwise to a stirred solution of 1 g of 30% aqueous H\(_2\)O\(_2\) containing concentrated H\(_2\)SO\(_4\) (approx. 0.1 g) over a period of 40 minutes, while the temperature was kept below 30°C. The peracetic acid solution obtained had a concentration of approx. 1.2 M, and was stored at 4°C. The titer was determined accurately before use.

The calcium sequestering capacity of the isolated products was measured with a calcium selective electrode (Radiometer, DK) [33]. NMR spectra were recorded on a Varian UNITY-400 spectrometer operating at a proton NMR frequency of 400 MHz and a carbon NMR frequency of 100 MHz. \(^{13}\)C-NMR spectra were recorded in the gated decoupling and APT modes. All oxidized products were dissolved in D\(_2\)O. Computer simulations for the determination of the bromine equilibria were carried out with the software "Facsimile" (AEA, Technology plc, Harwell, UK).

**Oxidation of dialdehyde inulin with Br\(_2\)**

Bromine solutions were prepared by mixing a small amount of bromine (5 mL) with a solution containing a small amount of sodium bromide (5 g·100 mL\(^{-1}\)). Before use the pH was adjusted to the desired pH value and the concentration was determined.
Oxidations were carried out in solutions (10 mL) with 10 and 20 mg dialdehyde inulin (DAI), with different concentrations of bromide at pH 4.5. The reaction was kept at a constant pH by addition of 0.02 M sodium hydroxide (pH-stat). The pH dependency of the reaction was measured by oxidation of 10 mg dialdehyde inulin (0.06 mmol of aldehyde groups assumed) in 10 mL solutions at pH 3.0 - 6.0, using approx. 1 mmol of bromine. The effect of the DAI-concentration on the reaction rate was measured with different amounts of dialdehyde inulin (5, 10 and 20 mg). During the latter experiments the pH of the reaction mixture was 3.0.

**Oxidation of dialdehyde inulin with peracetate and bromide**

Oxidations were carried out in solutions (10 mL) with 200 mg (1.23 mmol, 60 mM) dialdehyde inulin (DAI), 90 mM peracetic acid at pH 3 and pH 5. The concentration of sodium bromide was varied between 1 and 20 mM. During the reaction, the pH and the peracetic acid content were measured. All reactions were carried out at room temperature for 48 hours. Control experiments were carried out without DAI addition. The mixtures, incubated at pH 3, were desalinated by nanofiltration and freeze-dried. The calcium sequestering capacity was determined of the products thus obtained. Sodium sulfite was added to the reaction mixtures incubated at pH 5, the pH was adjusted to 11, and the solution was heated at 80°C for 1 hour. After complete hydrolysis of the lactones, the mixture was desalinated and freeze dried. The calcium sequestering capacity of the isolate was determined [33].

Another oxidation was carried out in solution (20 mL) with 500 mg DAI (3.1 mmol aldehyde equivalents, 75 mM), sodium bromide (0.5 mmol) and peracetic acid (10 mmol) at 45°C. The pH of the peracetic acid was adjusted to 5.0 prior to addition to the DAI. During oxidation the pH was kept constant. After 48 hours, the pH of the mixture was adjusted to 11, and the solution was subsequently heated at 45°C to decompose the lactones. After desalination by nanofiltration, the sample was freeze-dried, and a $^{13}$C-NMR spectrum was recorded.

In order to elucidate the reaction mechanism of the oxidation, a third series of experiments was performed, in which 250 mg DAI (3.1 mmol aldehyde equivalents) was oxidized with peracetic acid (6.2 mmol) and sodium bromide (0.5 mmol) during 48 hours at room temperature at pH 3, 4 and 5 (total volume was 20 mL). Control oxidations were carried out at pH 4.0 with a higher concentration of sodium bromide (12.4 mmol), and with peracetic acid alone. In order to compare this type of oxidation with the experiments performed by Ishak et al. [27], oxidation of DAI (3.1 mmol
aldehyde equivalents) was also carried out with a stoichiometric amount of bromine (3.1 mmol) at pH 3, 4 and 5 during 48 hours at room temperature. The oxidation at pH 5.0 was also carried out for 240 hours. All products were isolated by nanofiltration and freeze-drying.

**Partial oxidation of dialdehyde inulin and dialdehyde starch with bromine and with bromide/peracetic acid**

To study the effect of oxidation on the yield of the reaction and calcium sequestering capacity of the product, partial oxidation of DAI (250 mg, 3.1 mmol dialdehydes) was carried out at pH 5.0 with 1.5 mmol of bromine. After 17 hours of oxidation (50%, according to titration of NaOH), the sample was split into two parts. One part was oxidized further with bromine (0.8 mmol) at pH 5.0. The second part was oxidized with peracetic acid (0.8 mmol) at pH 5.0. After 48 hours, the pH of the samples was adjusted to 10.0. After 30 minutes, the samples were desalinated by nanofiltration and freeze dried. Experiments to elucidate the reaction mechanism, were conducted in a similar way unless otherwise mentioned.

Partial oxidation of dialdehyde starch (DAS, 2 gram, 24 mmol) was carried out at pH 5.0 in a total volume of 20 mL using 1 mmol sodium bromide and 1 mmol peracetic acid. After disappearance of the red/brown color, peracetate (1 mmol) was added again. This procedure was repeated until a total addition of 12 mmol of peracetic acid was added, and the sample was split into two parts. One part was oxidized further with 6 mmol peracetic acid at pH 5.0. The other part was further oxidized with 6 mmol sodium chlorite and 6 mmol hydrogen peroxide at pH 5. After the oxidation was completed, the samples were desalinated by nanofiltration and freeze-dried.

**Oxidation of dialdehyde inulin with chloroperoxidase**

Vanadium chloroperoxidase (CPO) was a gift from Unilever Research (Vlaardingen, The Netherlands). The enzyme originated from the fungus *Curvularia inaequalis*. The kinetics of this enzyme have been discussed in Chapter 5. Oxidations with CPO were carried out at room temperature in 20 mM sodium acetate pH 5.0, unless stated otherwise. Vanadium chloroperoxidase (CPO) was supplied in 50 mM Tris/H₂SO₄ with 90 mM NaCl and 50 μM sodium orthovanadate, pH 8.3, and was desalinated before use. Kinetic experiments were carried out at room temperature on a lambda 5 PE spectrophotometer by measuring the rate of chlorination of 2-chlorodimedone (MCD, 50 μM) at 287 nm (ε = 20.2 mM⁻¹·cm⁻¹) to 2,2-dichlorodimedone, or the rate of
bromination of 2-chlorodimedone (50 μM) at 287 nm (ε = 18.2 mM⁻¹·cm⁻¹ at pH 4.0 and 19.9 mM⁻¹·cm⁻¹ at pH values higher than 4.5 [34]). The solutions were buffered with 50 mM sodium acetate. Oxidations of bromide with CPO and H₂O₂ were carried out at pH 4.0, 50 mM sodium acetate, without the addition of MCD. Spectra measuring Br₃⁻ were recorded at 287 nm.

3 Results and Discussion

Oxidation with Br₂
As already described by Ishak et al. [27], the first part of the oxidation proceeds rapidly up to 50 % oxidation of the aldehyde. The second part of the oxidation occurs more slowly. This phenomenon can be explained by the assumption that the first part of the reaction is the oxidation of free hydrated aldehyde groups, while the second part of the reaction is the oxidation of hemiacetals [27]. The reaction rate for the latter reaction is much lower than the rate of reaction of a free aldehyde. The data obtained on the oxidation of DAI at pH 4.5 are shown in Table 1. The (initial) oxidation rate was measured with the pH-stat by titration with 50 mM NaOH.

Table 1. Oxidation of DAI with Br₂ at pH 4.5

<table>
<thead>
<tr>
<th>Entry</th>
<th>DAI (mM)</th>
<th>Br₂ (mM)</th>
<th>Oxidation rate (mmol Br₂·s⁻¹)</th>
<th>Total acid produced (μmol)</th>
<th>(%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.52</td>
<td>0.94</td>
<td>2.1 * 10⁻⁵</td>
<td>24</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>5.44</td>
<td>1.34</td>
<td>2.8 * 10⁻⁵</td>
<td>27</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>5.87</td>
<td>1.74</td>
<td>3.3 * 10⁻⁵</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>12.00</td>
<td>0.93</td>
<td>2.3 * 10⁻⁵</td>
<td>26</td>
<td>130</td>
</tr>
<tr>
<td>5</td>
<td>11.01</td>
<td>1.36</td>
<td>2.5 * 10⁻⁵</td>
<td>35</td>
<td>117</td>
</tr>
<tr>
<td>6</td>
<td>11.39</td>
<td>1.76</td>
<td>2.7 * 10⁻⁵</td>
<td>41</td>
<td>103</td>
</tr>
</tbody>
</table>

*1) based on bromine equivalents

At the start of the reaction, DAI was not completely dissolved, but during the reaction (10-15 minutes), DAI solubilized completely. Increasing the concentration therefore has no influence on the initial reaction rate. The oxidation rate of DAI is higher with increasing Br₂ concentration. Based on the total amount of equivalents of oxidant
added, the total acid formation is expressed in %. This formation is attributed to oxidation by bromine and the formation of free carboxylic acid functions. The pK value of the carboxylic acid groups is approx. 4.5-5.0. Small pH differences influence the relative presence of free carboxylic functions, and might explain the differences found in the total acid produced.

To determine the correct orders of the reaction, the relative concentrations of bromine and bromide present in solution have to be known. For the bromine equilibria, the following equations apply [33, 35]:

\[
\begin{align*}
\text{Br}_2 + H_2O & \rightleftharpoons \text{HOBr} + H^+ + Br^- \\
\text{HOBr} & \rightleftharpoons H^+ + OBr^- \\
H_2O & \rightleftharpoons H^+ + OH^- \\
\text{Br}_2 + Br^- & \rightleftharpoons \text{Br}_3^-
\end{align*}
\]

This set of equations is used for computer simulation using the following kinetic data [35]: \( K_1 \) (the equilibrium constant of \( \text{Br}_2 \), equation 5) = \( 8.09 \times 10^{-11} \) (M), \( K_2 \) (the dissociation constant for \( \text{HOBr} \), equation 6) = \( 2 \times 10^9 \) (M), \( K_3 \) (dissociation constant of water, equation 7) = \( 1.8 \times 10^{-16} \) (M), \( K_4 \) (association constant for \( \text{Br}_3^- \), equation 8) = 17 (M\(^{-1}\)). Based on these equilibria, the amount of bromine initially added is recalculated towards real concentrations of bromine and \( \text{HOBr} \). The results of the oxidations at different pH and with different concentrations of DAI are shown in Table 2.

**Table 2.** Defining the kinetics of DAI oxidations with bromine, the effect of pH and DAI concentration

<table>
<thead>
<tr>
<th>Entry</th>
<th>pH</th>
<th>DAI (mM)</th>
<th>( \text{Br}_2 ) (mM)</th>
<th>( \text{HOBr} ) (mM)</th>
<th>Oxidation rate ( \text{Br}_2 \cdot \text{s}^{-1} ) (mmol Br(_2) \cdot s(^{-1}))</th>
<th>( k ) (M(^{-1}) \cdot s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.98</td>
<td>5.05</td>
<td>54.9</td>
<td>0.01</td>
<td>2.63 ( \times ) 10(^4)</td>
<td>4.0 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>2</td>
<td>4.05</td>
<td>5.14</td>
<td>54.7</td>
<td>0.13</td>
<td>2.60 ( \times ) 10(^4)</td>
<td>3.9 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>3</td>
<td>5.00</td>
<td>4.76</td>
<td>54.9</td>
<td>1.15</td>
<td>2.15 ( \times ) 10(^4)</td>
<td>3.5 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>4</td>
<td>5.60</td>
<td>5.00</td>
<td>48.6</td>
<td>5.60</td>
<td>2.13 ( \times ) 10(^4)</td>
<td>3.3 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>5</td>
<td>2.97</td>
<td>2.36</td>
<td>54.7</td>
<td>0.13</td>
<td>1.17 ( \times ) 10(^4)</td>
<td>3.8 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>6</td>
<td>2.98</td>
<td>5.05</td>
<td>54.7</td>
<td>0.13</td>
<td>2.63 ( \times ) 10(^4)</td>
<td>4.0 ( \times ) 10(^{-3})</td>
</tr>
<tr>
<td>7</td>
<td>3.02</td>
<td>9.52</td>
<td>54.7</td>
<td>0.13</td>
<td>3.65 ( \times ) 10(^4)</td>
<td>n.c (^1)</td>
</tr>
</tbody>
</table>

\(^1\) not calculated; the value would not be correct due to the small excess of bromine.
Using excess of bromine, the second order (in aldehyde and bromine) constant for the oxidation of the free aldehyde function can be determined. It is assumed that in one sugar moiety, one aldehyde is present as hemiacetal, while one aldehyde is present in the free form (Ishak et al. [27]). The reaction constant calculated is valid for the oxidation of the free aldehyde. The oxidation rate of the hemiacetal is (based on the data from Ishak et al. [27]), approx. a factor of 10 lower. The amount of HOB present in the reaction medium varies by a factor of 500 between pH 3 and 5.8 (entries 1-4). The Br₂ concentration on the other hand is nearly constant in this pH range. The oxidation rate is therefore dependent on the concentration of bromine, and not on the concentration of HOB.

To determine the order of reaction rate in DAI, DAI was dissolved completely in the reaction medium by ultrasonic means. The oxidation rate was found to increase linearly (entries 5-7) with increasing DAI concentration. With increasing the pH from 3 to 6, the total alkaline consumption in the reaction increases. It can therefore be concluded that the reaction rate is first order in the concentration bromine and DAI, assuming that one aldehyde was present in the free form. By extending the reaction time, the second aldehyde (present as hemiacetal) will be oxidized. It is not known whether this oxidation depends on the equilibrium between the hemiacetal and the free form. It should be considered that the oxidation rate is experimentally determined by the addition rate with sodium hydroxide. In the range of pH 4-5, the pK value of the acid formed influences the titration. Lactone formation must also be taken into account. A higher pH favors the presence of free acid in the carboxylate form. This was shown by extended oxidation at low pH, which leads to acid formation when the reaction medium is adjusted to pH 6. This effect is enhanced at pH 11 and 45°C. Also in this case, sodium hydroxide titration showed the release of acid by lactone hydrolysis. β-elimination may also be responsible for acid formation, although at high oxidation degrees, this effect may become negligible. Several mechanisms in the oxidation of aldehydes can be envisaged. A radical mechanism based on bromine atoms can be excluded, because the oxidation also proceeds when the experiments are carried out in the dark. A second possibility is the oxidation of bromine with a substitution at the trigonal carbon. However, this mechanism is not likely, because Br⁻ has to be the reactive species, while in ionic bromine chemistry, Br⁺ is generally the reactive species. The third possible mechanism is hydride transfer to the bromine molecule from the hydrated aldehyde group under Lewis base catalysis [36] leading directly to the carboxylic acid. As a
strong difference is noticed between oxidation of the free aldehyde and the hemiacetal, the hydrate mechanism is not our first choice, because the hemiacetal contains a related structure, and, at first sight, both aldehydes would, in that case, be oxidized at approximately the same rate. A second reason is that the oxidations are carried out at pH 3-5, and water is not likely to abstract a proton from the hydrated aldehyde OH's under these conditions. A fourth mechanism for the oxidation proceeds via the tautomerization of the free aldehyde, followed by bromine addition, elimination of HBr and subsequent hydrolysis to the acid. It may be mentioned that incorporation of bromine in the product was not observed. The mechanism is formulated in Figure 6.

Figure 6. Proposed model for the oxidation of DAI with bromine (via tautomerization mechanism and addition of bromine/elimination of HBr)
Oxidation of dialdehyde inulin with peracetic acid as the primary oxidant

We have tested peracetic acid as a replacement for hydrogen peroxide in the chloroperoxidase catalyzed oxidation of halides [32]. However, in our experiments, it turned out that bromide was oxidized chemically by peracetic acid (AcOOH), yielding bromine and acetic acid (HOAc, see equation 4 and 5). Therefore, this system might be applied for the in situ generation of bromine, thereby using peracetic acid as the primary oxidant and bromide in a catalytic amount.

During the oxidation, carried out at room temperature, the solution turns yellow in a few seconds. This color is continuously present during the oxidation, indicating that the formation of bromine is much faster than the oxidation of DAI by bromine. At the start of the oxidation, the pH of the mixture increases somewhat, but remains constant during the reaction. This is in accordance with the reaction in equation 4, indicating the net formation of 2 OH\(^-\) upon oxidation of 2 Br\(^+\). Control incubations with peracetic acid showed that the oxidant was stable during the reaction. However, after some hours, the reaction stopped, and the yellow color disappeared. Addition of new bromide to the solution started the reaction again. This phenomenon is not due to evaporation of bromine, but to the equilibrium of bromine in the peracetate / bromide reaction (equation 4).

![Graph showing concentration of peracetic acid during oxidation of DAI (62 mM) with bromide and peracetic acid (● = pH 3.0, 20 mM NaBr, 130 mM peracetic acid, ■ = pH 5.0, 1 mM NaBr, 90 mM peracetic acid)]

Figure 7. Concentration of peracetic acid during oxidation of DAI (62 mM) with bromide and peracetic acid (● = pH 3.0, 20 mM NaBr, 130 mM peracetic acid, ■ = pH 5.0, 1 mM NaBr, 90 mM peracetic acid)
The excess of peracetic acid to bromide favors the formation of HOBr, even at pH 3-5. Because peracetic acid is a strong oxidant, even the chemical equilibrium of bromine (equation 5,6 and 8) is shifted towards HOBr formation. Two typical profiles of the peracetic acid concentration in the oxidation of bromide are shown in Figure 7. It should be noted that there was no difference in the peracetic acid decrease as a function of time in the bromide concentration used (1, 2, 5, 10 and 20 mM) between pH 3-5. The decrease of peracetic acid at pH 3, measured by titration, and corrected for the amount of bromide, was in the order of 17-22 mM / hour in all cases.

![Figure 8](image)

**Figure 8.** $^{13}$C-NMR spectrum of DAI (a) and 100% peracetate / bromide oxidized DAI (b), see Figure 4 for formulas
The second order reaction rate for the oxidation of DAI with peracetic acid is in the order of: \( \frac{d[\text{AcOOH}]}{dt} = \frac{[\text{AcOOH}]}{\text{time (s)} \cdot [\text{DAI}] = 0.020 / 3600 [0.06] = 9 \cdot 10^{-5} \text{M}^{-1} \text{s}^{-1} \) using bromide as the catalyst. As discussed above, the rate limiting step is also influenced by the equilibrium of bromine. When bromine is the oxidizing molecule, the effective concentration of bromine is kept low, because the oxidation of DAI by bromine yields bromide as new substrate reductant for peracetic acid.

The first series of oxidations of DAI with peracetic acid and bromide yielded materials which showed after pretreatment at pH 11, an increase in the calcium sequestering capacity, indicating that oxidation at C₃ and C₄ has taken place. The samples oxidized at pH 3 were not hydrolyzed at pH 11, and the calcium sequestering capacity was only 0.6 - 1.1 mmol Ca per gram. NMR analysis of the fully oxidized sample (Figure 8) showed complete oxidation of the DAI towards dicarboxy inulin (DCI). The signals from DAI are assigned as follows [22]: 59.5 and 60.3 ppm: methyl groups from C₁ and C₆, 71 ppm: C₅, 89.8 - 90.6 ppm: C₃ and C₄ in aldehyde form, 98.7 ppm: C₂. The signals from oxidized DAI are assigned as follows: 24.4 ppm: HAc, 64.3 - 64.4 ppm: methyl group from C₁ and C₆, 102.3 ppm: C₅, 173.7 and 178.5 ppm: C₃ and C₄ oxidized species. This indicates that the bromine formed is able to oxidize DAI for 100% at low pH. If lactones are formed, they can be hydrolyzed efficiently at 45°C and pH 11.

**Table 3.** Oxidation of 250 mg DAI (3.1 mmol aldehyde) at different pH with peracetic acid (PA) and bromide and with bromine (room temperature, 48 hours)

<table>
<thead>
<tr>
<th>Entry</th>
<th>pH</th>
<th>Bromide (mmol)</th>
<th>PA (mmol)</th>
<th>Bromine (mmol)</th>
<th>SC ¹) (mmol Ca/g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0.5</td>
<td>6.2</td>
<td>-</td>
<td>0.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>6.2</td>
<td>-</td>
<td>0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0.5</td>
<td>6.2</td>
<td>-</td>
<td>1.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>-</td>
<td>6.2</td>
<td>-</td>
<td>0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>12.4</td>
<td>6.2</td>
<td>-</td>
<td>1.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>1.3</td>
<td>73</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>1.1</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>1.2</td>
<td>88</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>2.0 ²)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

¹) SC = Ca sequestering capacity
²) after 240 hours reaction time
n.d. = not determined
In order to elucidate the reaction mechanism of the peracetate oxidation with bromide, several oxidations of DAI with peracetate/bromide and with bromine were carried out. The results are shown in Table 3.

The calcium binding capacity (SC) of the products obtained in entries 1-2 is low. This is apparently due to the fact that complete oxidation has not been achieved yet. When applying a higher pH, the calcium binding capacity increases, although the peracetic acid oxidation rate in this pH region is constant (entry 3). Whether this is due to glycosidic cleavage of the oligomer or increased acetal formation at lower pH is not yet known. The use of peracetic acid alone results in a very low calcium binding capacity (entry 4). This would indicate the need for bromine as oxidant. When an equivalent amount of bromide is used (entry 5, cf. equation 4), the equilibrium favors the formation of bromine. In a one step stoichiometric oxidation, bromide is converted to bromine by peracetic acid. This indeed results in a higher calcium binding capacity of the isolated product.

The oxidation of DAI with bromine added in a stoichiometric amount at pH 3-5 results in products with moderate calcium binding capacity (entries 6-8), as compared by the results obtained by Besemer et al. [22]. These authors found an SC of 2.5-2.6 mmol·g\(^{-1}\), and it seems that our oxidation is halfway. The oxidation is not yet complete, as could also be deduced from the results by Ishak et al. [27]. The oxidation of the second aldehyde was very slow, as this aldehyde is present in the hemiacetal form. This was confirmed in our studies when the reaction time was extended to 240 hours (entry 9), the Ca-SC increased to 2.0 mmol·g\(^{-1}\).

![Figure 9. \( ^{12}C \)-NMR spectrum of DAI, 25% oxidized with bromine](image_url)
NMR analysis of DAI, 25% oxidized with bromine showed that oxidation takes place in the molecule, although the carboxylic groups at C₃ or C₄ are not separated in the spectrum (see Figure 9) as was the case with completely oxidized DAI (Figure 8). The signals are assigned as follows: 58.9 - 59.5 ppm: methylene groups from C₁ and C₆, 98.3 ppm: C₂, 89.4 and 89.7 ppm: C₃ and C₄ in acetal form, 69.6 ppm: C₅. These signals are comparable with the chemical shift of DAI (Figure 8). Comparison of the shift obtained for the oxidized C₃ and C₄ species with the 100% oxidized sample (Figure 8) shows an unclear broad peak. This signal can be ascribed to an oxidized species in the free carboxylic form (sharp peak at 176.2 ppm), and an oxidized species, present in a lactone form (broad peak at 175 ppm). Taking into account the cyclic form of the dialdehyde, the C₃ aldehyde function is most likely to be oxidized and to be present in the lactone form. The carboxylic signal is (mainly) assigned to the oxidized C₄, which must be present as the free carboxylic function, because a 4 membered cyclic ring of the lactone is not likely to occur. However, both species should be present as separate signals in the spectrum. As this is not the case, it is suggested that, due to inter-residue acetal-formation, a “mixed type of oxidized polymer” is present with randomly oxidized C₃ and C₄ (Figure 10), the remaining aldehyde functions also being present as hemiacetals with a neighboring C₆, resulting in a broad signal in the NMR spectrum (Figure 9). If inter-residue hemiacetal formation takes place, this would explain the difficulty to oxidize DAI easily with bromine to 100%. The rate-limiting step is probably the formation of the free aldehyde, enabling bromine oxidation, but under these conditions, also glycosidic cleavage could reduce the molecular weight of the molecule and reduce the calcium sequestering capacity. To study this phenomenon in more detail, partial oxidation of DAI and dialdehyde starch (DAS) was carried out up to 50%. These products were further oxidized under different conditions. The results are shown in Table 4.

### Table 4. Oxidation of 50% oxidized DAI and DAS

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Oxidant</th>
<th>Degree of oxidation (%)</th>
<th>Yield (%)</th>
<th>SC (mmol Ca/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DAI</td>
<td>bromine</td>
<td>50</td>
<td>88</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>DAI, 50% ox.</td>
<td>bromine</td>
<td>100</td>
<td>68</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>DAI, 50% ox.</td>
<td>peracetate/Br⁻</td>
<td>100</td>
<td>48</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>DAS, 50% ox.</td>
<td>peracetate/Br⁻</td>
<td>100</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>DAS, 50% ox.</td>
<td>chlorite/H₂O₂</td>
<td>100</td>
<td>67</td>
<td>2.3</td>
</tr>
</tbody>
</table>

131
Figure 10. Postulated inter-hemiacetal and lactone formation in partially oxidized DAI (additional structures are possible, note that free carboxylic acid and aldehyde are not found in the NMR spectrum)

Further oxidation of 50% oxidized DAI results in a loss in yield; bromine and peracetic acid are responsible for glycolic cleavage of the molecule. It appears that further use of bromine does not increase the SC, whereas with a peracetic acid oxidation, probably due to the lower concentration of bromine in the solution, a reasonable SC can be obtained.

DAI is more resistant to glycosidic cleavage than DAS when comparing both substrates in this oxidation (entry 3 and 4). The oxidation of DAS with peracetic acid / bromide results in a large decrease in molecular weight as expressed by the yield,
and a poor SC is achieved. Chlorite is an oxidant, which is more specific for aldehyde groups; the yield and SC are in accordance with data obtained earlier [33].

**Oxidation of DAI with chloroperoxidase**

The use of chloroperoxidase (CPO) in the oxidation of DAI is based on the in situ generation of bromine at low pH from bromide, using hydrogen peroxide as the primary oxidant. At this low pH no considerable loss of HOBr/Br2 is expected. Because the behavior of CPO towards substrates with a low reactivity is unknown, several parameters had to be tested. Therefore, the stability of the enzyme in the presence of DAI (150 mM) was determined at pH 4.0. After 24 hours, the peroxidase activity was still present, indicating that the aldehyde functions did not interact with the vanadium in the active site, while incubation of the enzyme with 60 mM EDTA completely inhibited the enzyme.

To test the enzyme stability for hydrogen peroxide, when no reactive substrate like MCD is present, the enzyme (50 μL, 3.6 U) was incubated with different hydrogen peroxide concentrations. The results are shown in Table 5.

**Table 5.** *Activity of CPO (bromination of MCD) as a function of hydrogen peroxide concentration, incubated in the absence of MCD (room temperature, in 50 mM sodium acetate, pH 4.0)*

<table>
<thead>
<tr>
<th>H2O2 (mM)</th>
<th>Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 0 minutes</td>
</tr>
<tr>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

n.d. = not determined

The enzyme loses its activity at >10 mM hydrogen peroxide within several minutes. This means that the presence of a reactive substrate like MCD protects the enzyme against deactivation, because the active site is continuously blocked with the substrate to form an enzyme-substrate complex (see Chapter 5). Thus, using the enzyme under conditions without MCD or a similar reactive substrate will only be possible with a low hydrogen peroxide concentration, in the order of 1 mM.
Apart from the instability of the hydrogen peroxide (\(H_2O_2 + HOBr \rightarrow H_2O + O_2 + H Br\)), \(Br_2\) and \(Br_3^-\) might also inactivate the enzyme. However, when MCD is present, these species react very fast resulting in very low net concentrations in the solution. To test this possibility, the enzyme (72 U) was incubated with 400 \(\mu\)M \(Br_2\) during 10 minutes at room temperature and subsequently measured on its MCD bromination activity. The activity disappeared completely. Therefore, in the enzymatic oxidation of substrates with a lower reactivity than MCD, one has to prevent the production of too much \(Br_2\) and \(Br_3^-\), because these species inactivate the enzyme completely.

The first enzymatic oxidation was carried out at pH 4.5 with 62 mM DAI, 23.3 mM NaBr, 1 mM \(H_2O_2\) and 3.6 U chloroperoxidase. The original activity of the enzyme was 66 U·mg\(^{-1}\), while this activity decreased to 17 U·mg\(^{-1}\) in 15 minutes. After 2.5 hours, no activity was found anymore. With the original activity, within 15 minutes a total of 2 mM of \(Br_2\) could have been formed. These results indicate that the reaction time of bromine formed with the aldehyde functions of the inulin is not fast enough to prevent deactivation of CPO.

![Graph](image)

**Figure 11.** Activity of CPO with incubation of DAI and hydrogen peroxide added at set intervals (■ = pH 3.5, ♦ = pH 4.0, ▲ = pH 4.5). The symbols represent the moments of hydrogen peroxide addition.
A second experiment was carried out using 125 mg DAI (78 mM), 1.95 mM NaBr, 0.19 mM NaCl in 10 mM sodium acetate pH 4.0. At specific times, hydrogen peroxide (5.8 μmol = 0.6 mM) was added and the activity of the enzyme was determined spectrophotometrically with MCD. This experiment was repeated at pH 3.5 and pH 4.5. The results are shown in Figure 11.

Before the enzyme activity was determined, the presence of bromine species was determined spectrophotometrically by assaying the solution without the addition of bromide. At all measured time intervals, the hydrogen peroxide was consumed by the enzyme. Gas formation was visible during the experiments, but was more pronounced at higher pH, indicating that Br₂ / HOBr were formed and decomposed by reaction with hydrogen peroxide during the reaction. Because the decomposition of Br₂/HOBr is faster at higher pH, the stability of the enzyme at pH 4.5 is higher.

These data indicate that under very narrowly defined conditions, CPO could be used in the oxidation of DAI. These conditions are: a low pH at which the enzyme is active (pH 3.5-4.5), but hydrogen peroxide decomposition is prevented to a reasonable extent (pH < 4.5), the hydrogen peroxide concentration is low (< 1 mM), and the net formation of Br₂ relative to its direct addition to DAI is low, and does not exceed the μM range.

A third experiment was carried out at pH 4.0 under the conditions as described above. The addition of hydrogen peroxide was carried at set time intervals. After 1 hour, new enzyme was added. The addition of hydrogen peroxide and enzyme was repeated 10 times. At the end of the experiment, a total amount of 427 μmol hydrogen peroxide was added, which means that if no hydrogen peroxide decomposition has taken place, a theoretical amount of 25% of the aldehyde could have been oxidized. The solution was freeze-dried (yield 120 mg) and analyzed with ¹H and ¹³C-NMR. However, no oxidation could be detected.

4 Conclusions

Chemical oxidation of dialdehyde inulin (DAI) can be carried out at low pH, in which Br₂ is the active species. The reaction is suggested to proceed via tautomerization of the aldehyde and bromine addition, followed by a two step elimination of HBr. During the oxidation, lactones are formed, which can be hydrolyzed efficiently at pH 11. When peracetic acid is used for the in situ generation of bromine, the reaction rate is
affected by the equilibrium of the peracetic acid/bromide reaction, which favors the formation of HOBr. At a high concentration of bromine, DAI can be oxidized completely.

Partial oxidation with bromine or peracetic acid / bromide shows the formation of lactones as indicated by $^{13}$C-NMR analysis. This formation can be explained by inter-residue lactone formation. The aldehydes in DAI are mainly present in hemiacetal form, being oxidized to a carboxylic group. The lactones can be hydrolyzed at higher pH, as observed by the presence of two separate signals in the $^{13}$C-NMR spectrum of the 100% oxidized and hydrolyzed sample. A good calcium sequestering capacity can be obtained using peracetic acid, although the yield of dicarboxy inulin is moderate (48%). Peracetic acid is responsible for glycosidic cleavage, as was shown in the oxidation of dialdehyde starch.

Attempts to generate bromine with the use of chloroperoxidase failed. As the aldehydes are present as acetals, the bromine generated in the solution deactivates the enzyme within a few minutes. Besides this deactivation, hydrogen peroxide is decomposed by the hypobromous acid formed. This effect is stronger at increasing pH. Even with subsequent addition of fresh enzyme and hydrogen peroxide to the solution, no oxidation of DAI could be detected. Due to the absence of free aldehydes, the low overall reaction rate of the bromine oxidation of DAI, prevents the enzymatic oxidation of DAI.

Acknowledgements
We acknowledge the generous gift of chloroperoxidase from Dr. J.H.A. Boot from Unilever Research Laboratories (NL). We thank Joke Venekamp and Jaques Vogels for assistance in the NMR analysis.

5 Literature

Evaluation of methods for chemical and biological carbohydrate oxidation

Summary

The goal of this study was to investigate chemical and biological methods for the oxidation of carbohydrates. This thesis describes the results obtained with the enzymes quinoprotein glucose dehydrogenase and hexose oxidase, oxidizing different carbohydrates at the C₁-position (Chapter 2-4). Beside these studies, the enzymatical and chemical constraints for the chemo-enzymatic oxidation at secondary hydroxyl groups of carbohydrates by hypohalous acids or halogens, produced by haloperoxidases, are studied (Chapter 5). Finally, the reaction mechanism of the chemical oxidation with bromine, and the chemo-enzymatic oxidation of dialdehyde inulin with bromine produced by chloroperoxidase, are studied (Chapter 6).

Chapter 2 describes the immobilization of soluble quinoprotein glucose dehydrogenase (s-GDH) from Acinetobacter calcoaceticus in a redox polymer network, consisting of a poly(vinyl)pyridine polymer functionalized with osmiumbis-(bipyridine)chloride and aminoethyl groups, on a carbon disk electrode. The enzyme-electrode operated optimally at a cell potential of 600 mV vs. Ag/AgCl, and under specific conditions, a (high) maximum current density of 24 A·m⁻² was measured. The electrode was studied for the bio-electrochemical conversion of glucose and lactose into gluconic acid and lactobionic acid, respectively. It was demonstrated that enzyme immobilization at a flat plate rigid carbon electrode was feasible, although the current density was 14% of the activity obtained using a polished carbon disk. This activity is equivalent to a production rate of approximately 14 g gluconic acid or 25 g lactobionic acid per m² reactor volume per hour. Using process technological data from literature, the cost price determining factors of this process are the reactor costs and the electricity costs. Therefore, this process seems economically feasible when carried out on a large scale. Preliminary immobilization experiments to increase the volumetric productivity showed a very stable, although very low, current density of 0.75 A·m⁻² (213 A·m⁻³) on carbon felt. This improvement shows that further research to optimize the immobilization procedure is legitimate.

In order to develop a practical procedure for the large scale isolation of hexose oxidase (HOX) from the red seaweed Chondrus crispus, the application of cellulase
and carrageenase, to reduce the viscosity of the cell homogenate, was studied (Chapter 3). The enzyme yield obtained with the new procedure was comparable to that of a recently published yield, but the specific activity was lower. Incubation of crude extract with a mixture of proteases (Pronase) improved the specific activity and stability of HOX in the extract. Amino acid sequencing of the subunits showed that the fragments had somewhat shorter N-terminal amino acid sequences than normally observed in literature. As no additional cleavages were found, the removal of amino acids may lead to a conformational change of HOX and might explain the effect of Pronase treatment in an early stage of the purification protocol on the increase of specific activity and stability. Analysis of a HOX preparation for the presence of bound sugars revealed that it contains glucose and galactose. The enzyme is active on many aldoses (with the exception of mannose), and malto-oligosaccharides up to maltopentaose. Hexose oxidase has also been studied in an amperometric sensor under conditions which are comparable to the immobilization of GDH (see also Chapter 2). The immobilization procedure has been studied with respect to efficiency of electron transfer to an electrode and to substrate specificity. The activity of the immobilized enzyme appeared to be very sensitive to the type of buffer used. Addition of 100 mM NaCl decreased the activity with 30-50%. At concentrations higher than 5 mM, phosphate buffer and citrate buffer had an inhibitory effect on the enzyme activity. Within the pH range 4.5 - 11.5, a broad optimum was found between pH 5.5 - 7.0. The immobilized enzyme shows a broad substrate specificity, and is able to oxidize malto-oligomers up to maltoheptaose. In contrast with the soluble enzyme, it was shown that mannose and the tetrasaccharide Δ-4,5-GlucA(1→4)Gluc(1→4)Rha-(1→4)Gluc were also suitable substrates. The change in substrate specificity requires application of the complete immobilization procedure for the production of the electrode. The impact on the substrate specificity and whether this phenomenon also occurs with other enzymes is presently unknown (Chapter 4). As halogenating species such as HOCI and HOBr can be used for the specific oxidation of secondary hydroxyl groups of carbohydrates, the possibility of enzymatic generation of these hypohalous acids was evaluated. The enzyme vanadium-chloroperoxidase (CPO) from Curvularia inaequalis was used to evaluate the enzymatical and chemical constraints for the generation of the hypohalous acids from NaCl or NaBr (Chapter 5). At higher pH (pH > 8), mainly hypohalous acids are present, whereas at low pH (pH < 4), the presence of halogens is favored. The
hypohalous acids formed by CPO decompose with hydrogen peroxide. Therefore, it will depend on the ratio of the reactivity and concentration of the organic substrate and hydrogen peroxide, whether oxidation or halogenation of the substrate or decomposition of HOCl/HOBr takes place at a certain pH. Though the activity of CPO at pH 5.0 is higher than at pH 4.0-4.5 (1 mM bromide with monochloro-dim edone as substrate), a higher concentration of brominating species can be obtained between pH 4.0-4.5 (minimizing decomposition of hydrogen peroxide), using a high concentration of bromide (10-100mM).

Based on the results obtained in Chapter 5, the chemo-enzymatic oxidation of carbohydrates was studied in more detail in Chapter 6. Dialdehyde inulin (DAI) was used as the substrate because this compound has a higher reactivity for oxidation with bromine than inulin itself. Br₂ was also generated in situ by oxidation of Br⁻ with either perchlorate or with H₂O₂ and chloroperoxidase from Curvularia inaequalis. A rather low second order oxidation rate for bromine of 3.8·10⁻³ M⁻¹·s⁻¹ at room temperature was observed, based on the assumption that only 1 reactive (free) aldehyde per dialdehyde fructofuranose unit was present. This assumption is supported by a significant decrease in the oxidation rate, when 50% (based on titration) of the aldehyde groups has been oxidized. ¹³C-NMR analysis of DAI shows a strong favor for hemiacetal formation, which limits the oxidation rate of DAI. Peracetic acid can be used as the oxidant of bromide, generating bromine in situ and allowing the use of a catalytic amount of bromide. Complete oxidation of DAI with peracetic acid / bromide is possible but glycosidic cleavage lowers the yield of dicarboxy inulin. Attempts to oxidize DAI by chemo-enzymatic means failed, although CPO is able to generate bromine from bromide at pH 4.0-5.0. Due to the presence of hemiacetals in DAI, the oxidation rate with bromine is low, and the concentration of bromine/HOBr generated in solution increases quickly during the reaction. These species are subsequently decomposed by hydrogen peroxide (see also Chapter 5), or deactivate the enzyme.

J.P. van der Lught
November 1998
Samenvatting

Evaluatie van chemische en biologische methoden voor de oxidatie van koolhydraten

Samenvatting

Het doel van dit onderzoek was de bestudering van chemische en biologische methoden voor de oxidatie van koolhydraten. Dit proefschrift beschrijft de resultaten die zijn verkregen met het enzym quinoprotein glucose dehydrogenase en hexose oxidase. Deze enzymen oxideren verschillende koolhydraten op de C₆ positie (Hoofdstuk 2-4). Naast dit onderzoek zijn de enzymatische en chemische randvoorwaarden voor de chemo-enzymatische oxidatie van de secundaire hydroxyl-groepen van koolhydraten door middel van hypohalogeniden of halogenen die enzymatisch zijn geproduceerd door haloperoxidases, bestudeerd (Hoofdstuk 5). Tenslotte is het reactiemecanisme van de chemische oxidatie van dialdehyde inuline met broom en de chemo-enzymatische oxidatie, waarbij broom wordt geproduceerd door vanadium chloroperoxidase, bestudeerd (Hoofdstuk 6).

Hoofdstuk 2 beschrijft de immobilisatie van het niet-membraan gebonden quinoprotein dehydrogenase (s-GDH) van Acinetobacter calcoaceticus in een redox polymeric netwerk, dat bestaat uit een poly(vinyl)pyridine dat is voorzien van osmiumbis(bipyridine)chloride- en aminooethylgroepen, gecoat op een koolstof disk-elektrode. De enzymelektrode functioneert optimaal bij een celspanning van 600 mV met een zilver-elektrode als referentie. Onder optimale opstandigheden wordt een maximale stroomdichtheid gemeten van 24 A·m⁻². De enzym-elektrode is bestudeerd voor de bio-elektrochemische omzetting van glucose en lactose naar respectievelijk gluconzuur en lactobionzuur. Tevens wordt gedemonstreerd dat de enzym-immobilisatie op een vlakke-plaatelektrode mogelijk is, maar dat de stroomdichtheid slechts 14% bedraagt van de activiteit die wordt verkregen met een koolstof disk-elektrode. De verkregen activiteit is equivalent aan de produktie van ongeveer 14 gram gluconzuur of 25 gram lactobionzuur per m³ reactorvolume per uur. Gebruikmakend van procestechnologische gegevens uit de literatuur, wordt aangetoond dat de kostprijseffecten van deze omzettingen, de reactorkosten en de elektriciteitskosten zijn. Om de volumetrische productiviteit te verbeteren, zijn enkele voorlopige experimenten uitgevoerd met een koolstofvlies elektrode. Hierbij werd een stabiele, alhoewel nog lage stroomdichtheid van 0,75
A·m⁻² (213 A·m⁻³) gemeten. Deze verbetering geeft aan dat verder onderzoek naar de optimalisatie van de immobilisatieprocedure zinvol is.

Om een praktisch haalbare isolatie van hexose-oxidase (HOX) uit het rode zeewier *Chondrus crispus* mogelijk te maken, zijn cellulase en carrageenase gebruikt om de viscositeit van de ruwe enzymoplossing te verlagen (Hoofdstuk 3). De enzymopbrengst die met deze nieuwe procedure wordt verkregen is vergelijkbaar met die van een reeds eerder gepubliceerd opbrengst, maar de specifieke activiteit is lager. Wanneer een ruw enzymextract wordt geïncubeerd met een mengsel van proteases (Pronase), verbetert de specifieke activiteit en de stabiliteit van HOX in het extract.

Wanneer de aminozuurvolgorde van de subunits van het gezuiverde enzym worden vergeleken met literatuurgegevens, blijkt dat de fragmenten een wat kortere N-terminale aminozuurvolgorde bezitten. Omdat er verder geen hydrolyse-produkten worden gevonden, lijkt het er op of de verwijdering van enkele aminozuren leidt tot een conformatieverandering van HOX. Dit zou het effect van Pronase in een vroeg stadium van de zuivering op de toename van de specifieke activiteit en de stabiliteit van HOX kunnen verklaren. Analyse van HOX op de aanwezigheid van suikers toont aan dat het enzym geglycosileerd (glucose en galactose) is. Het enzym is actief op verschillende aldose suikers (behalve mannose), en oxideert oligosacchariden tot en met maltopentaose.

Hexose-oxidase is ook bestudeerd in een amperometrische sensor onder condities die vergelijkbaar zijn met die van GDH (zie Hoofdstuk 2). De immobilisatie van HOX is bestudeerd met het oog op de effectiviteit van de elektronenoverdracht naar de elektrode, en op de substratspecificiteit. De activiteit van het geïmmobiliseerde enzym blijkt sterk afhankelijk te zijn van het type buffer dat wordt gebruikt. Het gebruik van 100 mM NaCl doet de activiteit met 30-50% dalen. Concentraties van meer dan 5 mM fosfaatbuffer of citraatbuffer hebben een negatieve invloed op de enzymactiviteit. Tussen pH 4,5-11,5 vertoont het geïmmobiliseerde enzym een brede substratspecificiteit, waarbij malto-oligomeren tot en met maltohexaoese kunnen worden geoxideerd. In tegenstelling tot het vrije enzym blijkt dat ook mannose en het tetrasaccharide Δ-4,5-GlcA(1→4)Glc(1→4)Rha-(1→4)Glc geschikte substraten zijn. Het verschil in deze substratspecificiteit wordt alleen verkregen wanneer de gehele immobilisatieprocedure om de elektrode te bereiden wordt toegepast. Het effect van
Samenvatting

dezo substraatspecificiteit, en het voorkomen van dit verschijnsel bij andere enzymen is op dit moment nog niet bekend (Hoofdstuk 4).

Omdat hypocloriet (HOCl) en hypobromiet (HOBr) kunnen worden toegepast in de oxidatie van secundaire hydroxylgroepen van koolhydraten, is de enzymatische produktie van deze verbindingen onderzocht. Het enzym vanadium-chloroperoxidase (CPO) uit Curvularia inaequalis is gebruikt om de enzymatische en chemische randvoorwaarden voor de productie van deze hypohalogenieten uit NaCl en NaBr vast te stellen (Hoofdstuk 5). Bij pH > 8 zijn de hypohalogenieten aanwezig als zuren, bij pH < 4 zijn ze voornamelijk aanwezig als halogeen. De hypohalogenieten kunnen echter ontleden met waterstofperoxide. Het is daarom afhankelijk van de verhouding in activiteit en de concentratie van de te oxideren organische verbinding of er halogenering / oxidatie van het substraat of ontleding van HOCl of HOBr bij een bepaalde pH plaatsvindt. Alhoewel CPO bij pH 5,0 een hogere activiteit bezit dan bij pH 4,0 (met monochlooridemedon als substraat en 10-100 mM bromide) kan een grotere concentratie broom en tribromide worden verkregen tussen pH 4,0-4,5 wanneer een hogere concentratie bromide wordt gebruikt. Omdat bij een lage pH broom en tribromide met elkaar in chemisch evenwicht zijn wordt verwacht dat de chemo-enzymatische oxidatie onder specifieke omstandigheden mogelijk is.

Op basis van de resultaten die zijn verkregen in hoofdstuk 5, is de chemo-enzymatische oxidatie van koolhydraten met broom in hoofdstuk 6 verder bestudeerd. Als substraat is hierbij dialdehyde inuline (DAI) gebruikt, omdat dit substraat een grotere reactiviteit bezit dan inuline zelf. De oxidatie is ook bestudeerd met broom dat in situ wordt genegeerd uit bromide door behulp van perazijnzuur, en met behulp van CPO uit Curvularia inaequalis uit bromide en waterstof peroxide. Een nogal lage tweede orde oxidatiesnelheid van DAI met broom van $3.8 \cdot 10^{-3} \text{ M}^{-1}\text{s}^{-1}$ wordt bij kamertemperatuur gemeten. Deze constante is gebaseerd op de aannname dat er slechts 1 reactieve, vrije aldehydegroep per dialdehyde fructofuranose beschikbaar is. Deze aanname wordt ondersteund door een sterke daling in oxidatiesnelheid wanneer 50% (gebaseerd op titratie) van de aldehydegroepen is geoxideerd. $^{13}$C-NMR analyse van DAI toont tevens een sterke voorkeur voor de vorming van hemi-acetalen, welke de oxidatiesnelheid van DAI verlagen, aan. Perazijnzuur kan worden gebruikt als oxidant van bromide, waarbij in situ broom wordt geproduceerd. Op deze wijze kan bromide katalytisch in de oxidatie worden
toegepast. Een volledige oxidatie van DAI met perazijnzuur en bromide is mogelijk alhoewel oxidatieve hydrolyse de opbrengst van dicarboxy inuline verlaagt. Pogingen om DAI chemo-enzymatische te oxideren zijn niet gelukt, maar CPO is wel in staat om enzymatisch broom bij pH 4,0-4,5 te produceren. Door de afwezigheid van vrije aldehyden is de oxidatiesnelheid van DAI met broom laag, en neemt de concentratie broom en tribromide in oplossing gedurende de reactie snel toe. Deze verbindingen kunnen vervolgens met waterstofperoxide hydrolyseren (zie ook Hoofdstuk 5) of ze deactiveren het enzym CPO.

J.P. van der Lugt
November 1998
Curriculum vitae


Jan Pieter van der Lught is getrouwd en vader van twee kinderen.
Nawoord

Naast de samenvatting is voor veel lezers ongetwijfeld het nawoord één van de meest gelezen onderdelen van een proefschrift. Alhoewel het nawoord het enige onderdeel is van een proefschrift dat niet door anderen wordt gespeeld, gecorrigeerd of herschreven, is het gezien het grote aantal mensen dat bij de toestandkoming betrokken is geweest van groot belang hierbij alle zorgvuldigheid te betrachten. Er zijn dan ook groot aantal mensen die ik hier graag wil bedanken.

Het onderzoek dat beschreven is in dit proefschrift is gestart in 1993. In die periode was ik met Arie Besemer betrokken bij het onderzoek naar de chemische oxidatie van koolhydraten, en hij had het voorstel, om met de ideeën die we hadden een promotieonderzoek te starten. Als ik me toen had gerealiseerd wat hiervoor komt kijken was ik er waarschijnlijk niet aan begonnen, maar ja, als je eenmaal bezig bent….. Arie, ik wil je bedanken voor de steun, toewijding en begeleiding in al deze jaren. Ik wil mijn waardering uitspreken voor met name het laatste jaar, waarin je vanuit SCA het proefschrift tot een goed einde hebt helpen brengen.

Zeer veel dank ben ik verschuldigd aan Hans Duine en Herman van Bekkum die als promotoren optreden. Leek het of er de eerste periode weinig input van jullie nodig was, de laatste jaren hebben jullie dit allebei op een geheel eigen wijze ingehaald. Verbeterde inzichten, de kunst van het schrijven, de controle-experimenten en de opbouw van de hoofdstukken, voor dit leerproces wil ik jullie bedanken.


Alle (ex-)collega’s, bedankt voor de samenwerking, de inzet en motivatie om ook alle nieuwe vragen die steeds weer ontstonden te beantwoorden. We hebben wel eens te vroeg gejuicht, maar al met al zijn we toch heel ver gekomen.

De echte promotor (maar om in de terminologie te blijven ook een acceptor), heeft met name de laatste 2 jaar achter de schermen een heel grote rol gespeeld. Lieve Edith, ik weet dat je hier eigenlijk niet genoemd wilt worden, omdat je blij zal zijn.
wanneer het voorbij is. Toch wil ik je hier bedanken, omdat niet iedereen die mij kent zich zal realiseren dat dit boekwerk hier ligt dankzij jouw geduld! Lieve Laurie, bedankt voor je mooie tekening. Ook al begrijp je nu niet wat papa voor een boek aan het schrijven was “over suikers”, je wilde met alle plezier een bijdrage leveren. Pieter, ik zal mijn kamer binnenkort opruimen, zodat je, wanneer ik niet thuis ben, ongestoord aan de computer kunt zitten, zonder alle papieren en aantekeningen overhoop te halen!