PRODUCTION AND LOCALIZATION OF INULINASES
IN KLUYVEROMYCES YEASTS

Proefschrift

ter verkrijging van de graad van doctor aan de
Technische Universiteit Delft, op gezag van de Rector
Magnificus, prof. drs. P. A. Schenck, in het openbaar te
verdedigen ten overstaan van een commissie
aangewezen door het College van Dekanen op
dinsdag 9 oktober 1990 te 16.00 uur

door

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geboren te Hengelo (Gld)
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This study was carried out at the Department of Microbiology
and Enzymology of the Delft University of Technology, The Netherlands,
and supported by Unilever Research Laboratory, Vlaardingen,
The Netherlands.
Laten we eerst goed nadenken, zei de een.
Da's geen probleem zei de ander.
Daarna gaan we aan het werk, zei de een.
nu zonder de ander.

Kamagurka, 1987
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 General Introduction</td>
<td>7</td>
</tr>
<tr>
<td>2 Production, Distribution, and Kinetic Properties of Inulinase</td>
<td>29</td>
</tr>
<tr>
<td>in Continuous Cultures of <em>Kluyveromyces marxianus</em> CBS 6556</td>
<td></td>
</tr>
<tr>
<td>3 Production and Distribution of β-Fructosidase in Synchronous and</td>
<td>53</td>
</tr>
<tr>
<td>Asynchronous Chemostat Cultures of Yeasts.</td>
<td></td>
</tr>
<tr>
<td>4 Structure and Properties of the Extracellular Inulinase of</td>
<td>71</td>
</tr>
<tr>
<td><em>Kluyveromyces marxianus</em> CBS 6556.</td>
<td></td>
</tr>
<tr>
<td>5 Determination of Protein Concentration by Total Organic Carbon</td>
<td>97</td>
</tr>
<tr>
<td>Analysis.</td>
<td></td>
</tr>
<tr>
<td>6 Localization of Inulinase and Invertase in <em>Kluyveromyces</em> Species.</td>
<td>111</td>
</tr>
<tr>
<td>7 The Discovery of β-Galactosidase.</td>
<td>135</td>
</tr>
<tr>
<td>8 Summary</td>
<td>139</td>
</tr>
<tr>
<td>Samenvatting</td>
<td>144</td>
</tr>
<tr>
<td>Dankwoord</td>
<td>149</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
CHAPTER 1

GENERAL INTRODUCTION

Historically, the yeast *Saccharomyces cerevisiae* has been the microorganism most widely used in the food and beverage industry. Over the years, methods for its application in large-scale and low-cost production have been developed, and knowledge on its molecular genetics is rapidly expanding. During the last decade, another application of *S. cerevisiae* has become a reality: the use of this yeast as a host organism in the production of heterologous proteins (2,15). However, the physiological features of *S. cerevisiae* are not in all respects favourable for its application in the industrial production of heterologous proteins. For instance, *S. cerevisiae* has a limited metabolic versatility and its Crabtree-positive character affects the manageability of industrial fermentation processes. In this respect, some amongst the other 500 yeast species might be more versatile than *S. cerevisiae*. Yeasts now regarded as alternatives to *S. cerevisiae* for this purpose are, for example, the methylotrophic yeasts *Pichia pastoris* (50) and *Hansenula polymorpha* (45), and the lactose-utilizing yeast *Kluyveromyces marxianus* var. *lactis* (15).

Yeasts from the genus *Kluyveromyces* are especially attractive for the production of heterologous proteins since they belong to the group of GRAS (Generally Recognized As Safe) organisms and are acceptable for pharmaceutical and food production. Two varieties of the species, *K. marxianus* var. *lactis* and *K. marxianus* var. *marxianus*, are well known for their ability to ferment whey, a major waste stream from dairy industry. Thus far, a genetic transformation system has only been developed for the variety *lactis*. However, the growth characteristics of the variety *marxianus* are by far superior to those of the variety *lactis*. For example, *K. marxianus* var. *marxianus* shows a broader range of substrate utilization, has a much shorter doubling time, and grows at much higher temperatures (44). Thus, for industrial use the variety *marxianus* is, on physiological grounds, a more obvious choice than the variety *lactis*.

A general comparison of the physiological characteristics of *K. marxianus* var. *marxianus* and *S. cerevisiae*, is presented in Table 1. For industrial processes, important parameters are characteristics such as sensitivity of cultures to contamination, utilization of low-cost substrates, and manageability of the process. Industrial fermentation at
CHAPTER 1

TABLE 1. Comparison of physiological characteristics of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* var. *marxianus*.

<table>
<thead>
<tr>
<th>Physiological feature</th>
<th><em>S. cerevisiae</em></th>
<th><em>K. marxianus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum growth rate</td>
<td>0.53 h(^{-1})</td>
<td>0.89 h(^{-1})</td>
</tr>
<tr>
<td>Optimum temperature of growth</td>
<td>30(^{\circ})</td>
<td>40(^{\circ})</td>
</tr>
<tr>
<td>Substrate range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon sources</td>
<td>few</td>
<td>many</td>
</tr>
<tr>
<td>Whey (lactose)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hemicellulose (xylose)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Molasses (sucrose, raffinose)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrogen sources</td>
<td>few</td>
<td>many</td>
</tr>
<tr>
<td>Affinity for sugars</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Crabtree effect</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flocculation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Production of extracellular,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glycosylated proteins</td>
<td>few</td>
<td>many</td>
</tr>
<tr>
<td>Genetic accessibility</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Elevated temperature reduces the risk of contamination with other microorganisms as well as the costs for cooling facilities. *K. marxianus* var. *marxianus* is known for its growth at elevated temperatures. On the other hand, a high growth rate is only a relative advantage. Indeed, this yeast can outcompete most of contaminating microorganisms on the basis of its higher maximum specific growth rate. However, in an industrial process a high growth rate is hard to maintain: at high cell densities and high growth rates, the oxygen supply will become insufficient to keep up with oxygen consumption. Sensitivity to contamination is also reduced when, in sugar-limited cultures, the yeast has a high affinity for sugars. Due to a low affinity for sugar (37), *S. cerevisiae* cultures are easily contaminated by wild yeasts. Moreover, the substrate range of *S. cerevisiae* is very restricted as compared to that of *K. marxianus*. This affects the possibility
of using waste streams, such as whey and wood hydrolysate, as low-cost growth substrates. *Kluyveromyces* can grow and ferment both the lactose in whey (24) and the hemicelluloses (xylose) in wood hydrolysate (33). The manageability of a yeast depends on the nature of its metabolism. *S. cerevisiae* belongs to the group of Crabtree-positive yeasts, i.e. at elevated glucose concentrations the yeast shows a fermentative metabolism even under fully aerobic conditions (52,54). The latter physiological feature negatively affects both the cell yield and the yield of products other than ethanol. Again from this point of view *K. marxianus* seems preferable as it is a Crabtree-negative yeast. Nevertheless, a great advantage of *S. cerevisiae* over *K. marxianus* is its well-established molecular genetics.

In most cases, the choice of an organism for industrial utilization and especially for the production of heterologous proteins, firstly depends on its genetic accessibility and secondly on physiological characteristics. With the continuing rapid developments in molecular biology, it is to be expected that many more microorganisms will become accessible to genetic manipulation. Hence, it might now become more important to look for yeasts with optimal physiological properties. In this respect, the choice of *K. marxianus* var. *marxianus* is an obvious one: short generation time on glucose (about 45 min), growth on a variety of sugars including the polyfructoside inulin, growth at a broad pH range (2.6 to 7), growth at elevated temperatures (up to 45°C), growth without vitamins, and growth without floc formation.

In the production of extracellular proteins by yeasts an important physiological feature is the post-secretary localization of the protein. Externitized proteins in yeasts are usually retained by the cell wall but, depending on the yeast strain and culture conditions, in some cases they are released into the surrounding medium (2,23,31). Knowledge of the conditions that affect the localization of a homologous protein may be useful as a model for the production and localization of heterologous proteins. The down-stream processing of intracellular proteins is more difficult than that of proteins that are either excreted into the culture fluid or retained in the cell wall. Purification and concentration of an intracellular protein requires disruption of the cells, resulting in the additional solubilization of large amounts of contaminating proteins. If a protein is excreted in the culture liquid, it is present at low concentration but at higher purity.
CHAPTER 1

Induced release of a protein from the cell wall without disruption of the cells may lead to a preparation of the desired protein in a concentrated as well as a relatively pure form.

The extracellular invertase has been extensively used as a model in research on production, secretion, extracellular localization, and downstream processing of proteins from *S. cerevisiae*. By using regulatory and secretory signals of the yeast, and production processes developed for homologous proteins such as invertase, successful secretion of heterologous gene products by *S. cerevisiae* has now been accomplished (29,53,55). Like the invertase, these heterologous proteins are often completely retained in the cell wall of *S. cerevisiae* (53,55). Due to the structure of the cell wall, release of the wall-retained protein can only be accomplished by disruption of the cells. In view of the potential attractiveness of *K. marxianus* for fermentation processes, the question is whether this organism might be an alternative. Little is known about the factors that influence the localization of extracellular proteins in the yeast *K. marxianus*. In this thesis a physiological study on production of extracellular inulinase of *K. marxianus* var. *marxianus* CBS 6556 is presented. The extracellular glycoprotein inulinase catalyzes the hydrolysis of the polyfructoside inulin outside the cell wall, in the culture liquid. Inulinase also hydrolyzes sucrose, both within the cell wall and outside the cell wall. In this respect, it strongly resembles the invertase of *S. cerevisiae* (23,43,51). Thus, analogous to the invertase of *S. cerevisiae*, inulinase may serve as a model enzyme for the production and localization of recombinant proteins in *K. marxianus*. The main objective of this thesis was to make an inventory of the environmental factors that influence production and post-secretory localization of inulinase in *K. marxianus* CBS 6556.

PROTEIN SECRETION IN YEAST.

Importance of secretion for protein production. An important factor in the production of proteins for industrial use, and especially of heterologous proteins, is to obtain concentrated, purified proteins. As mentioned above, production of secreted proteins may offer the advantage of an initial protein separation by the organism itself, thus facilitating downstream processing of the desired protein. Moreover, many naturally occurring secretory proteins from animals and plants, such as calf
prochymotrypsin, interferon, and thaumatin, are not fully active unless they are glycosylated and proteolytically processed through the secretory pathway (15,55). Yeasts are particularly interesting for the production of heterologous proteins as they possess a secretory and glycosylation pathway that, at least in part, is homologous to the one found in higher eukaryotes. The production and secretion of a number of heterologous proteins by yeasts have recently been reviewed by Esser and Kamper (15). Most of these results were obtained by fusion of homologous leader or signal sequences, such as the invertase signal sequence, to the amino-terminal portion of the heterologous gene.

The secretory process. Externalization of a secretory protein in yeast appeared to be topologically related to growth of the cell surface. By using fluorescein-labeled antibodies, it was observed that newly synthesized invertase and acid phosphatase are mainly deposited around the developing bud (16,48). This localized distribution of glycoproteins was found to coincide with the appearance of transport vesicles in the vicinity of the bud (48). The post-translational secretory pathway and carbohydrate assembly of extracellular proteins in yeast were elucidated by Schekman and associates using temperature-sensitive mutants of S. cerevisiae, with invertase as the model glycoprotein (12,14,35,39,40). These mutants are

![Secretory Pathways in Yeast Diagram](image)

defective in one of the stages of secretion of glycoproteins at the non-permissive temperature. A schematic diagram of invertase secretion from the yeast cell is shown in Figure 1. The polypeptides destined to be secreted, possess leader sequences consisting of predominantly hydrophobic amino acid residues at the amino terminal end. These leader sequences route the protein to the endoplasmic reticular membrane, where it is translocated into the lumen of the endoplasmic reticulum. During this translocation, the polypeptide acquires its core oligosaccharides at the proper asparagine residues in Asn-X-Ser (Thr) tripeptides, in which X may be any other amino acid (36,47). In the case of S. cerevisiae invertase, 9 to 10 of these N-glycosidically-linked core oligosaccharides with a composition equal to the mammalian high-mannose type (Glc$_3$Man$_9$GlcNAc$_2$), are attached per subunit (14,36,39). The protein is then transported from the endoplasmic reticulum to the Golgi complex via specific vesicles. In the Golgi, the core oligosaccharides are elongated through addition of mannose residues that form the outer chain. In the case of invertase this can comprise as many as 150 mannose moieties (29,32,39). This compartmentalized oligosaccharide assembly on invertase is shown in Figure 2. The secretory proteins are packaged by vesicles in the Golgi complex, transported to the budding

![Diagram of invertase secretion](image)

CHAPTER 1

portion of the cell, and delivered outside the plasma-membrane by exocytosis (14,39). Most of the secreted proteins in yeast are active as multimeric forms; invertase is excreted into the yeast cell wall as an octamer. The formation of these higher multimeric complexes has been shown to occur very early in the secretory process (i.e. in the endoplasmic reticulum), before assembly of outer chains by glycosylation takes place (13,46).

The physiological significance of glycosylation. The extracellular proteins of yeasts are all glycosylated, although the degree of glycosylation may differ considerably. The physiological significance for glycosylation to secretion of glycoproteins in yeasts is still unclear. The difference between non-glycosylated and glycosylated proteins in yeasts is their localization. Glycoproteins are extracellular, or membrane-bound, or are localized in separate cellular compartments like the vacuole. However, a direct proof for involvement of the carbohydrate chains in secretion has not been found. On the contrary, it appears that the secretory events can proceed in the absence of outer-chain glycosylation (29). Glycosylation may influence the rate of processing and turnover of the protein (12,29,36), or it may influence the stability of the protein (9,34). It may also prevent the occurrence of irreversible protein aggregation (41) and may play a role in retention of the protein in the cell wall (13,26,31).

Glycoproteins and the yeast cell wall. After exocytosis over the plasma membrane, part of the extracellular glycoproteins produced by yeast are retained in the cell wall. Models for the mode of retention of extracellular proteins in the yeast cell wall have been proposed by Lampen (31) and Kidby and Davies (26). Common to both models is a layered cell wall, with a mannan-protein component in the outer region and an inner region consisting of glucan (Figure 3). The models differ in the structure of the external region of the cell wall and in explaining the mode of retention of glycoproteins in the cell wall. Lampen (31) based his model on experiments on induced cell wall degradation and the concomitant release of invertase by the yeast S. cerevisiae (Figure 3A). He proposed that the outer layer of the cell wall is constructed of mannan components, held together by phosphodiester bonds. The glycoprotein invertase is then held just below this outer layer by either mannose-phosphodiester bridges between cell wall mannan and outer chain mannan moieties, or by hydrogen bonds, or by other hydrophilic forces. The mannan-type glycosylation thus serves as a means by which the protein is bound to the cell wall matrix. Contrary to the model of
CHAPTER 1

Lampen, the model of Kidby and Davies (26) proposes that the glycoprotein is not bonded within the cell wall but is soluble within the cell wall matrix. The retention of invertase in the cell wall of S. fragilis (renamed K. marxianus var. marxianus) appeared to be dependent upon a wall component

![Diagram A]

![Diagram B]

FIG. 3. Schematic structures of the yeast cell wall and the mode of glycoprotein retention according to (A) Lampen and (B) Kidby and Davies. Essential features are at the foot of figure B. Figure A from reference 31, figure B from reference 26.

that is reducible by sulfhydryl compounds like 2-mercaptoethanol. This finding prompted Kidby and Davies (26) to postulate that the enzyme is retained by a permeability barrier, whose structural integrity is maintained by disulphide linkages (Figure 3B). Retention thus is correlated to cell wall permeability. Increase in molecular weight of a protein by multimerization, as well as glycosylation may lead to a more efficient protein retention (13).

THE GLYCOPROTEIN INULINASE

Inulinases and their substrates. Of the 469 yeast species described by Barnett, Payne, and Yarrow (3), only ten species divided over seven genera
(Debaryomyces, Candida, Hansenula, Kluyveromyces, Pichia, Schwanniomyces, Schizosaccharomyces) are able to metabolize the fructan inulin. Among these species, representatives of *K. marxianus* (previously named *K. fragilis*) have been studied in most detail. The ability to use inulin as the carbon-and energy source depends on the production of an extracellular enzyme that hydrolyzes the fructan into fermentable monosaccharides. Fructans are polyfructose molecules that can be subdivided into two categories: the inulin type and the levan type. The inulin type fructans are mainly found as reserve carbohydrates in various plants, including chicory, dandelion, dahlia, and Jerusalem artichoke. They all consist of a linear chain of β(2-1)-linked fructose molecules and, depending on the plant source, with a degree of polymerization (DP) between 5-45 (4). At the reducing end the chain is terminated by a β(2-1)-linked glucose molecule (Figure 4). Thus the two terminal sugar residues form a sucrose moiety. The levan type fructans are produced by numerous bacterial species and consist of branched molecules.

<table>
<thead>
<tr>
<th>EC number</th>
<th>Recommended name</th>
<th>Systematic name</th>
<th>Other names used (compare Table 2)</th>
<th>Mode of degradation</th>
<th>Substrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inulin-degrading enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Transferases</td>
<td>2.4.1.93</td>
<td>inulin fructotransferase (depolymerizing)</td>
<td>inulin fructosyl-1,2-fructofuranosyltransferase (cycling)</td>
<td>exo: produces difructose anhydride III (= 1,2' : 2,3')</td>
<td>inulins of DP ≥ 3</td>
</tr>
<tr>
<td>b. Hydrolyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2.1.7</td>
<td>inulinase</td>
<td>2,1-β-α-fructan fructohydrolase</td>
<td>inulase</td>
<td>endo</td>
<td>inulin</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>exo</td>
<td>inulin ≈ sucrose</td>
</tr>
<tr>
<td>3.2.1.26</td>
<td>β-D-fructofuranosidase</td>
<td>β-fructofuranoside fructohydrolase</td>
<td>inverte</td>
<td>exo</td>
<td>sucrose ≈ inulin</td>
</tr>
<tr>
<td><strong>Levan-degrading enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Transferases</td>
<td>—</td>
<td>(levan fructotransferase; depolymerizing)</td>
<td>(levan fructosyl-6,2-fructofuranosyltransferase; cycling)</td>
<td>exo: produces difructose anhydride IV (= 2,6' : 6,2')</td>
<td>levans of DP ≥ 4</td>
</tr>
<tr>
<td>b. Hydrolyses</td>
<td>3.2.1.65</td>
<td>levanase</td>
<td>2,6-β-α-fructan fructohydrolase</td>
<td>levanopyrase = levan-6-fructohydrolase</td>
<td>endo</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>exo</td>
<td>levan</td>
</tr>
</tbody>
</table>

with predominantly β(2-6)-linkages branched at C₁ (18,19). Furthermore, levan resembles inulin in that it also possesses a terminal glucose moiety.

Microbial degradation of fructans can be accomplished by two types of enzymes: transferases and hydrolyses (19,20). In Table 2 a survey is given of the characteristics of the microbial fructan-degrading enzymes. Transferases produce difructose anhydrides via intra-molecular transfructosylation. Hydrolyases (inulinase and levanase) may split fructans either endo-wise or exo-wise, producing a series of oligofructans or only fructose, respectively. The disaccharide sucrose can be regarded as the smallest inulin molecule, with a degree of polymerization of two. As some microbial inulinases show a high activity with sucrose, they strongly resemble the classical enzyme invertase (19,42,51). However, invertases show a weak activity or none at all with the fructose polymers (23,24,42,51). To distinguish between invertase and inulinase, the so-called S/I ratio (relative activity with sucrose and inulin) is used. A low S/I ratio (< 50) is indicative for an inulinase (42,51). Originally, the classification of inulinase and levanase was based on the occurrence of specific enzymes in plants, molds, and bacteria (10,17,51). These enzymes rarely show any
activity with sucrose. In contrast, yeasts produce non-specific inulinases, capable of hydrolyzing both fructose polymers as well as sucrose and raffinose (43). These inulinases act exo-wise; and no endo-wise-acting inulinases, levanases, or transferases have been encountered in yeast so far (19,20,38). Thus, the inulinase produced by yeast always hydrolyzes both sucrose and inulin. As a consequence, yeasts that are able to grow on inulin can also grow on sucrose.

Regulation of invertase and inulinase synthesis in yeasts. Regulation of invertase synthesis in S. cerevisiae has been extensively studied. Research in the physiology and in the molecular biology of invertase production made clear that synthesis of extracellular invertase is only subject to catabolite repression (11,22,31). No specific inducer is required for the production of extracellular invertase. The yeast S. cerevisiae also produces a cytoplasmic, non-glycosylated invertase, that is not sensitive to glucose repression but constitutively synthesized in very low amounts (21,31). Under non-repressive conditions, the cytoplasmic invertase is about 1% of the total invertase activity. Both the cytoplasmic invertase and the extracellular invertase are derived from the same SUC gene. However, their mRNA's differ in the 5'-region that, in the case of extracellular invertase, encodes for the signal sequence (6,7,8).

No reports are available on the molecular biology of inulinase synthesis in yeasts. The formation of yeast inulinases has been investigated mainly by determination of enzyme levels in batch cultures. Highest yields of inulinase were obtained when the yeasts were grown in media with either inulin or fructose, and to a lesser extent sucrose, as the carbon source. Growth on glucose or lactose did not support the production of inulinase (24,42,51). GrootWassink and Hewitt (24) also used chemostat cultivation of K. marxianus in complex media to establish the mode of regulation of inulinase production. In media with sucrose or fructose, the enzyme activity showed an optimum at an intermediate dilution rate and then decreased with increasing dilution rate. From these results on inulinase production in batch and continuous cultures, it was concluded that inulinase is inducible by fructose and subject to catabolite repression. Other factors that appeared to influence the inulinase production by yeasts include the culture pH and the growth temperature. As long as culture pH and temperature support maximum cell yields, the inulinase yield is not affected (23).
CHAPTER 1

Deviation from the optimal pH and temperature of growth leads to decrease in inulinase yield.

Distribution of invertase and inulinase. Limited information is available on the factors that influence the distribution of invertase over cell wall and culture liquid. However, it is generally assumed that, independent of culture conditions, the extracellular invertase of S. cerevisiae is almost completely localized within in the cell wall and that only a small portion (about 5%) is released into the culture fluid (13, 21, 25, 31, 49). This has led to the remarkable fact that in most studies on invertase production only cell wall-associated invertase and not the amount of invertase present in the culture liquid is determined. Even in the production of S. cerevisiae invertase by Pichia pastoris, it is taken for granted that all invertase is retained in the cell wall of P. pastoris (50).

Esmon and co-workers (13) reported on a relation between oligomeric structure of invertase and its location. They found the cell wall-associated enzyme to be octameric, whereas the enzyme released into the culture liquid was a dimer. Obviously, in S. cerevisiae not only binding of invertase to cell wall components, but also the impermeability of the cell wall to larger molecules plays a role in retention of invertase. In contrast to the invertase of S. cerevisiae, a much higher level of inulinase is reported to be excreted into the culture liquid by K. marxianus ATCC 12424. In the stationary phase of batch cultures, up to 75% of the inulinase can be found in the culture liquid (24). When the yeast is grown in continuous culture under carbon limitation, it releases about 50% of the inulinase into the culture liquid, irrespective of the dilution rate (30). Whether K. marxianus also produces an intracellular inulinase and whether the other inulinase-producing yeast species show the same pattern of inulinase distribution is not known.

OUTLINE OF THIS STUDY

In this thesis a physiological study on the production and localization of inulinase in Kluyveromyces yeasts, and especially in K. marxianus var. marxianus CBS 6556, is presented. It includes a biochemical study into the nature of the glycoprotein inulinase. As outlined above, the glycoprotein inulinase is in part associated with the cell wall of K. marxianus and partially released into the culture liquid. So far, no systematic studies on
CHAPTER 1

the relationship between growth conditions and glycoprotein localization have been performed. In this thesis, extracellular inulinase at three different locations are distinguished:

1. inulinase present in the culture liquid (supernatant inulinase),
2. inulinase that can be released from the cell wall by 2-mercaptoethanol (cell wall inulinase),
3. inulinase that can only be released from the yeast by disruption of the cells (cell-bound inulinase).

If *K. marxianus* produces an intracellular inulinase, then the cell-bound fraction might contain both cell wall-associated inulinase and intracellular inulinase.

The experimental work was focused on answering two questions:

I. Should inulinase be regarded as a special kind of invertase or as a different enzyme?

II. Which factors influence the production and localization of inulinase?

The first question arises in view of the fact that in yeast both invertase and inulinase may accomplish the extracellular hydrolysis of β-fructosides like sucrose and raffinose. Because of the similarity in mode of enzyme action, some authors doubt the validity of distinguishing inulinase from invertase. They regard inulinase as a special type of invertase (1,27).

In chapter 2 an inventory is made of the general physiological characteristics of *K. marxianus* var. *marxianus* CBS 6556 in batch cultures. Substrate specificity, maximal growth rate, temperature optimum of growth and pH optimum of growth are determined.

In order to get more insight into the culture conditions that regulate production and localization of inulinase, *K. marxianus* CBS 6556 was grown in aerobic chemostat cultures. By determination of the relationships between inulinase production and dilution rate, and between inulinase production and carbon-limiting substrate, conclusions could be drawn concerning inulinase regulation in *K. marxianus*. The distribution of inulinase over supernatant, cell wall, and cell-bound fractions was determined in relation to growth-limiting substrate, temperature and pH (chapter 2). From determination of the kinetic parameters of the crude enzyme preparations, it was concluded that the enzymes present in supernatant, cell wall, and cell-bound fraction all represent the same inulinase (chapter 2).

The distribution of inulinase over the three enzyme fractions in steady-state sucrose-limited chemostat cultures of *K. marxianus* CBS 6556 as given
in chapter 2, showed considerable variation with time. It was hypothesized that this variation was caused by a variation in inulinase release from the cell wall, due to synchronized budding of part of the cell population. A convenient technique for establishing the relationship between enzyme localization and budding cycle is the use of oscillating continuous cultures. However, such cultures could not be obtained with K. marxianus. Therefore, the production and localization of invertase in oscillating and steady-state continuous cultures of S. cerevisiae CBS 8066 were examined, and compared to the production and distribution of inulinase in steady-state continuous cultures of K. marxianus CBS 6556 (chapter 3).

Esmen and co-workers (13) suggested that retention of invertase in the cell wall is mainly caused by the oligomeric structure of the enzyme. Since a comparable situation could exist with respect to inulinase of K. marxianus CBS 6556, both the supernatant inulinase and the cell wall inulinase of K. marxianus CBS 6556 were purified and their molecular weight, degree of glycosylation, and kinetic parameters determined. Of the purified inulinsases the amino acid sequence of the amino-terminal end was determined, and the results are compared to those of the invertase of S. cerevisiae (chapter 4).

During purification of the inulinsases, difficulties arose with the estimation of protein concentrations. Colorimetric methods for protein determination such as the Lowry and Bradford methods, gave non-linear results when inulinase preparations were serially diluted. Based on the invariable carbon content in proteins and carbohydrates, a fast and accurate method for the determination of protein concentration was developed. This method is described in chapter 5.

According to current yeast taxonomy, K. marxianus is the only representative of the genus capable of assimilating the polysaccharide inulin (3,28). However, it was observed that some other Kluyveromyces species obtain the ability to grow on inulin under culture conditions different from those applied in yeast taxonomy. Chapter 6 deals with the conditions necessary for growth on inulin of these species, and with the role of the cell wall in the ability of yeasts to use polysaccharides.

Martinus Willem Beijerinck, the first professor of microbiology of the Delft University of Technology, The Netherlands, published his discovery of lactase in the yeast Candida kefyr (renamed K. marxianus var. marxianus) in 1889 (5). We found a flask, containing lactase prepared by Beijerinck in 1899. This lactase preparation was still active and also contained
detectable inulinase activity. In chapter 7 a reappraisal of Beijerinck's discovery of lactase is given.
CHAPTER 1

LITERATURE CITED


CHAPTER 1


CHAPTER 1


Chapter 2

Production, Distribution and Kinetic Properties of Inulinase in Continuous Cultures of *Kluyveromyces marxianus* CBS 6556.

R.J. Rouwenhorst, L.E. Visser, A.A. van der Baan, W.A. Scheffers, and J.P. van Dijken.

CHAPTER 2

SUMMARY

From a screening of several Kluyveromyces strains, the yeast Kluyveromyces marxianus CBS 6556 was selected for a study of the parameters relevant to the commercial production of inulinase (EC 3.2.1.7). This yeast exhibited superior properties with respect to growth at elevated temperatures (40 to 45°C), substrate specificity, and inulinase production. In sucrose-limited chemostat cultures growing on mineral medium, the amount of enzyme decreased from 52 U mg of cell dry weight⁻¹ at a dilution rate of 0.1 h⁻¹ to 2 U mg of cell dry weight⁻¹ at D=0.8 h⁻¹. Experiments with nitrogen-limited cultures further confirmed that synthesis of the enzyme is negatively controlled by the residual sugar concentration in the culture. High enzyme activities were observed during growth on non-sugar substrates, indicating that synthesis of the enzyme is a result of a derepression/repression mechanism. A substantial part of the inulinase produced by K. marxianus was associated with the cell wall. The enzyme could be released from the cell wall via a simple chemical treatment of cells. Results are presented on the effect of cultivation conditions on the distribution of the enzyme. Inulinase was active with sucrose, raffinose, stachyose and inulin as substrates and exhibited an S/I ratio (relative activities with sucrose and inulin) of 15 under standard assay conditions. The enzyme activity decreased with increasing chain length of the substrate.

INTRODUCTION

Representatives of the genus Kluyveromyces are well-known for their ability to grow on fructans such as inulin. Inulin is a storage polysaccharide of plant origin and consists of a linear chain of β(2-1) linked D-fructofuranose molecules terminated at the reducing end by a D-glucose residue. In yeasts the enzyme responsible for the degradation of inulin is a non-specific β-fructosidase (inulinase: 2,1-D-fructanfructohydrolase, EC 3.2.1.7) that liberates fructose molecules from sugars with β(2-1)-linked fructose units at the terminal, non-reducing end (6,19). In spite of similarity in enzyme action and of correspondence in
CHAPTER 2

affinity for sucrose, yeast inulinase is distinguished from another well-known β-fructosidase: invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26). The latter enzyme shows a low activity with higher molecular-weight substrates such as inulin. The so-called S/I ratio (relative activities with sucrose and inulin) is now commonly used to discriminate between inulinase and invertase (24). This parameter, however, is strongly dependent upon the method employed to determine enzyme activities (18,26).

The inulinase of yeasts is an extracellular enzyme partially associated with the cell wall and partially excreted into the culture fluid. Regulation of inulin synthesis in yeasts has been studied in batch and continuous cultures, mainly with complex media (1,9,16,18). From these studies, it was concluded that the enzyme is inducible and subject to catabolite repression. Highest enzyme production so far was obtained with constitutive, derepressed mutants of Kluuyveromycetes fragilis in chemostat cultures (9,11).

In our studies on the optimization of inulinase production by yeasts, K. marxianus CBS 6556 was found to exhibit many properties which compare favourably with those reported for other Kluuyveromycetes strains. These include fast growth on a wide range of substrates at temperatures above 40 °C and high enzyme productivity. We present the results of a continuous-culture study on inulinase production by this strain.

MATERIALS AND METHODS

Microorganism and culture conditions. Kluuyveromycetes marxianus var. marxianus CBS 6556 was obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, and maintained on YEPD agar slopes. YEPD contained the following per liter of distilled water: yeast extract (Difco Laboratories, Detroit, Mich.), 10 g; Bacto Peptone (Difco), 10 g; glucose, 20 g). The organism was grown at 40°C in a laboratory fermenter (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter. Dissolved oxygen was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland) and controlled at 50-70% of air saturation. The pH was maintained at pH 4.5 by automatic addition of 1 M KOH or 0.5 M H₂SO₄.

For carbon- and energy-limited growth, mineral medium contained the following, per liter: (NH₄)₂SO₄, 5 g; K₂HPO₄, 3 g; MgSO₄·7H₂O, 0.5 g; EDTA,
CHAPTER 2

15 mg; ZnSO₄·7H₂O, 0.45 mg; FeSO₄·7H₂O, 3 mg; CuSO₄·5H₂O, 0.3 mg;
CaCl₂·2H₂O, 0.45 mg; MnCl₂·4H₂O, 1 mg; CoCl₂·6H₂O, 0.3 mg; NaMoO₄·2H₂O, 0.04
mg; H₃BO₃, 1 mg; KI, 0.1 mg; 0.025 ml silicon antifoaming agent (BDH
Chemicals, Poole, Dorset, England), calcium pantothenate (1 mg) and
nicotinic acid (1 mg). The medium was sterilized at 120°C. For nitrogen-
limited growth the concentration of (NH₄)₂SO₄ was lowered to 200 mg liter⁻¹.
Carbon sources (glucose, fructose, lactose, sucrose, inulin and glycerol)
were heat-sterilized separately at 110 °C, except for ethanol and fructose
which were filter-sterilized. Carbon sources were added to give final
concentrations of 2.5 g liter⁻¹, unless mentioned otherwise.

Fractionation of cultures for inulinase assays. Both cells and culture
supernatants were routinely assayed for inulinase activity. The fraction of
cell-associated enzyme that could be released by treatment with sulfhydryl
reagents will be referred to as cell wall enzyme. The activity that could
only be solubilized by means of sonication will be referred to as cell-bound
enzyme. The fractionation of cultures into supernatant enzyme, cell wall
enzyme and cell-bound enzyme is described below.

(i) Supernatant enzyme. Samples (100 ml) of steady-state cultures
(containing approximately 0.1 g [dry weight] of cells) were harvested by
centrifugation at 4°C (10 min, 4,000xg). The supernatant was used as a
source of extracellular enzyme.

(ii) Cell wall enzyme. The liberation of cell wall-associated enzyme was
induced by suspension of the cells in 10 ml of enzyme release buffer (50 mM
potassium phosphate, pH 7, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol and
2 mM MgSO₄) and incubation for 1 h at 30°C. The suspension was then
centrifuged at 4°C and washed twice, first with 5 ml of enzyme release
buffer and subsequently with 5 ml of sonication buffer (50 mM potassium
phosphate, pH 7, 10 mM MgSO₄). Enzyme activities present in the supernatant
after incubation of the cells in enzyme release buffer and in the
supernatants obtained after washing the cells, are designated as
preparations of extracellular enzyme formerly trapped in the cell wall. The
amount of enzyme released by washing accounted for <1% of the total enzyme
produced.

(iii) Cell-bound enzyme. After release of cell wall enzyme and washing,
cells were suspended in 5 ml of sonication buffer and sonicated at 4°C with
an MSE 150W ultrasonic disintegrator (MSE Ltd., London, England) for 5 min
with intermittent periods of cooling. Cell debris was removed by

-32-
centrifugation at 4°C (15 min, 30,000 x g) and suspended in 5 ml of sonication buffer. Enzyme activities present in cell-free extract and resuspended debris were taken as preparations of cell-bound enzyme. The amount of enzyme present in the debris always accounted for <1% of the total enzyme produced.

Occurrence of cell lysis after treatment with enzyme release buffer was routinely checked by following the activity of the constitutive intracellular β-glucosidase using ortho-nitrophenyl-β-D-glucopyranoside (2-NPG) as a substrate. For the assay, enzyme preparation was added to a pre-warmed (37°C) solution of 0.1 M potassium phosphate, pH 7, 10 mM KCl, 1 mM MgCl₂, and 4 mg of 2-NPG ml⁻¹. The hydrolysis of 2-NPG was followed at 420 nm in an LKB Ultrospec II spectrophotometer (LKB-Produkter, Bromma, Sweden). In no cases could β-glucosidase activity be detected in the preparations of cell wall enzyme. In cell-free extracts, however, irrespective of the growth substrate or dilution rate, high activities of β-glucosidase (0.1 to 1.87 μmol 2-NPG hydrolysed min⁻¹ mg cell dry weight⁻¹) were present.

Analytical methods. Biomass concentrations were measured by drying to constant weight at 70°C after membrane filtration (0.45 μm, Schleicher & Schull, Dassel, FRG) of samples from steady-state cultures.

Glucose and fructose were determined enzymatically using hexokinase, 6-phosphogluconate dehydrogenase and phosphoglucone isomerase (Glucose/Fructose Test combination, Boehringer, Mannheim, FRG). Sucrose and lactose were determined by the same method after addition of 10 U of either invertase β-galactosidase (Boehringer) ml⁻¹. Inulin was determined by measuring the amount of fructose and glucose released after acid hydrolysis in sulfuric acid at 100°C as well as after total enzymatic hydrolysis by a preparation of cell wall enzyme from K. marxianus. The average chain length of the inulin used was 30 units of fructose plus one unit of glucose. The molar weight of the inulin therefore was assumed to be 5,040.

Protein was measured by a modified Bradford method (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as a standard, according to the instructions of the manufacturer.

Inulinase activity was measured by essentially the same method as described by GrootWassink & Hewitt (9), determining the rate of appearance of fructose and glucose with the Boehringer glucose/fructose Testcombination, in the presence of 2% sucrose or 2% inulin in a 0.1 M sodium acetate buffer, pH 4.5, at 50°C. In all cases enzyme activity was
proportional to the amount of enzyme when diluted in 0.1 M sodium acetate, pH 4.5. One unit of inulinase activity is defined as the amount of enzyme catalyzing the liberation of 1 μmol of fructose min⁻¹ under the conditions mentioned above. Specific enzyme activities of cultures are given as the sum of the activities from the various fractions and are expressed per milligram cell dry weight.

Chemicals. Fructose, glucose, lactose, raffinose, sucrose and 2-mercaptoethanol were from Baker Chemicals BV, Deventer, The Netherlands. Dithiothreitol, inulin (chicory root), stachyose and 2-NPG were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Choice of organism and cultivation conditions. All strains of K. marxianus var. lactis, K. marxianus var. marxianus and K. vanudenii available from the Centraalbureau voor Schimmelcultures (CBS) were compared with respect to growth on agar containing either mineral or complex medium and a wide variety of carbon sources. K. marxianus var. marxianus CBS 6556 was selected for further studies in view of its rapid growth at elevated temperatures on inulin and other sugars. Growth rates of this strain in shake-flask cultures are summarized in Table 1. Except for ethanol, the optimal growth temperature was in the range of 37 to 42°C. Growth in a mineral medium supplemented with pantothenic acid and nicotinic acid was almost as fast as in complex medium. The growth rates of the organism on sugars are the highest reported so far for yeasts.

The response of the organism towards pH was tested in glucose-limited chemostat cultures at a dilution rate of 0.15 h⁻¹. Stable steady states could be obtained between pH 2.6 and 7.5. At the extreme pH values, however, uncoupling of growth and sugar utilization occurred (Fig. 1).

In view of the above results, cultivation was routinely performed at pH 4.5 and 40°C. A mineral medium rather than yeast extract as a source of minerals and vitamins (9,24) was chosen since this allowed the study of effects of various growth limitations. Moreover, the use of mineral media allows cultivation at high cell densities as required in a commercial process since complex media lead to excessive foaming.
TABLE 1. Effect of temperature on maximal growth rate of *K. marxianus* CBS 6556<sup>T</sup>.

<table>
<thead>
<tr>
<th>Medium and Carbon substrate (2%)</th>
<th>Temp (°C)</th>
<th>Growth rate (h&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEPD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>33</td>
<td>0.87</td>
</tr>
<tr>
<td>Glucose</td>
<td>37</td>
<td>0.89</td>
</tr>
<tr>
<td>Glucose</td>
<td>42</td>
<td>0.89</td>
</tr>
<tr>
<td>Glucose</td>
<td>45</td>
<td>0.72</td>
</tr>
<tr>
<td>Mineral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>33</td>
<td>0.89</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>0.86</td>
</tr>
<tr>
<td>Glucose</td>
<td>42</td>
<td>0.83</td>
</tr>
<tr>
<td>Glucose</td>
<td>45</td>
<td>0.53</td>
</tr>
<tr>
<td>Inulin</td>
<td>37</td>
<td>0.45</td>
</tr>
<tr>
<td>Inulin</td>
<td>42</td>
<td>0.43</td>
</tr>
<tr>
<td>Ethanol</td>
<td>33</td>
<td>0.30</td>
</tr>
<tr>
<td>Ethanol</td>
<td>40</td>
<td>0.25</td>
</tr>
<tr>
<td>Ethanol</td>
<td>45</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Cells were grown in shake flask cultures on the given medium.*

Release of cell wall-associated inulinase. For the release of the cell wall enzyme the methods used by Kidby and Davies (12) and Sommer and Lewis (20) were modified. The effects of various relevant parameters such as buffer composition, ionic strength of the buffer, buffer pH and concentration of the sulfhydryl compounds 2-mercaptoethanol and dithiothreitol, were studied. The highest enzyme release was obtained with a 50 mM potassium phosphate buffer, pH 7, containing 10 mM 2-mercaptoethanol and 10 mM dithiothreitol (enzyme release buffer). The enzyme appeared to be
FIG. 1. Effect of culture pH on growth yield of *K. marxianus* CBS 6556 in glucose-limited chemostat cultures. The organism was grown at a dilution rate of 0.15 h⁻¹.

completely stable under these conditions. Prolonged incubation of cells in enzyme release buffer for more than one hour did not increase the amount of enzyme released. Incubation of cells in a 50 mM potassium phosphate buffer, pH 7, or in a 100 mM sodium acetate buffer, pH 4.5, released 40 to 70% and 30 to 40%, respectively, of the cell wall enzyme within 1 h. These amounts increased during prolonged incubation periods. For this reason, washing of cells before incubation in the enzyme release buffer was omitted. Buffer solutions with other sulfhydryl compounds such as cysteine (13) yielded 60 to 80% of the amount of solubilized enzyme released by the combined activity of 2-mercaptoethanol and dithiothreitol.

The release of cell wall enzyme resulted in an inulinase preparation of considerable purity. It has been reported that a purified inulinase preparation of *K. fragilis*, that yielded one band on isoelectric focusing, had a specific activity of 2,552 U mg of protein⁻¹ (25). Cell wall enzyme obtained from our cultures grown on sucrose at D=0.2 h⁻¹ (29 U mg of cell
CHAPTER 2

dry weight$^{-1}$), had an activity of 1,739 U mg of protein$^{-1}$ whereas the enzyme excreted into the culture fluid had a specific activity of 1,310 U mg of protein$^{-1}$. The activity of inulinase that remained cell-bound after incubation in enzyme release buffer could be solubilized by sonication. The specific activity of the enzyme in this fraction was relatively low: 40 U mg of protein$^{-1}$.

Production and distribution of inulinase of *K. marxianus* in continuous cultures. In carbon- and energy-limited continuous cultures of *K. marxianus* CBS 6556, the highest inulinase yields were obtained with either sucrose or inulin as the limiting substrate (Table 2). Fructose, which is believed to be the primary inducer of inulinase (9), gave an enzyme production half of that observed with sucrose. Growth on glucose or lactose gave very low

TABLE 2. Total inulinase activities and distribution in carbon-limited continuous cultures of *K. marxianus* CBS 6556 in mineral medium with 0.25% of various carbon substrates

<table>
<thead>
<tr>
<th>Carbon substrate</th>
<th>Dilution rate (h$^{-1}$)</th>
<th>Total inulinase activity (U mg of cell dry wt$^{-1}$)$^{a}$</th>
<th>% of Inulinase in supernatant</th>
<th>% of Inulinase in cell wall</th>
<th>% of Inulinase in cell-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>0.05</td>
<td>58</td>
<td>65</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.15</td>
<td>25</td>
<td>60</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.10</td>
<td>52</td>
<td>48</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.10</td>
<td>29</td>
<td>51</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.10</td>
<td>3.9</td>
<td>87</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.13</td>
<td>2.8</td>
<td>97</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.10</td>
<td>3.4</td>
<td>43</td>
<td>37</td>
<td>20</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.10</td>
<td>26</td>
<td>57</td>
<td>31</td>
<td>12</td>
</tr>
</tbody>
</table>

$^{a}$ Enzyme activities were measured with sucrose as substrate.
yields of inulinase. However, considerable amounts of enzyme were produced during growth on the non-fermentable substrate glycerol or ethanol (Table 2).

The levels of inulinase in sucrose-limited chemostat cultures were strongly dependent on the dilution rate. The enzyme levels (sum of cell-bound enzyme, cell wall enzyme and supernatant enzyme) decreased from a maximum of 52 U mg of cell dry weight\(^{-1}\) at \(D = 0.1\ h^{-1}\) to 2 U mg of cell dry weight\(^{-1}\) at \(D = 0.8\ h^{-1}\) (Fig. 2).

![Graph showing the effect of dilution rate on total inulinase activity](image)

**FIG. 2.** Effect of dilution rate on total inulinase activity in sucrose-limited chemostat cultures of *K. marxianus* CBS 6556. Enzyme activities were measured with sucrose as substrate.

The profile of inulinase synthesis in carbon-limited chemostat cultures suggests that the enzyme is regulated by the residual sugar concentration in the culture (2,3). To substantiate this hypothesis, the effect of nitrogen limitation on cells growing at a fixed rate was studied. Nitrogen-limited growth, obtained with an ammonium sulfate concentration of 200 mg liter\(^{-1}\) and a reservoir sucrose concentration of 2.5 g liter\(^{-1}\), resulted in an enzyme activity of 18 U mg of cell dry weight\(^{-1}\). A higher concentration of sucrose in the medium reservoir resulted in a further increase in the
TABLE 3. Effect of medium composition on residual sugar concentration and inulinase activity in chemostat cultures of \textit{K. marxianus} CBS 6556.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution rate (h(^{-1}))</th>
<th>Influent sucrose (g liter(^{-1}))</th>
<th>Limitation</th>
<th>Residual substrate (mg liter(^{-1}))(^a)</th>
<th>Total inulinase activity (U mg cell dry wt(^{-1}))(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral</td>
<td>0.1</td>
<td>2.5</td>
<td>Carbon</td>
<td>&lt;10</td>
<td>52</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.8</td>
<td>2.5</td>
<td>Carbon</td>
<td>110</td>
<td>2.0</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.1</td>
<td>2.5</td>
<td>Nitrogen</td>
<td>70</td>
<td>18</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.1</td>
<td>5.0</td>
<td>Nitrogen</td>
<td>430</td>
<td>2.8</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.1</td>
<td>7.5</td>
<td>Nitrogen</td>
<td>2620</td>
<td>2.1</td>
</tr>
<tr>
<td>Complex</td>
<td>0.1</td>
<td>2.5</td>
<td>Carbon(^e)</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>Complex</td>
<td>0.1</td>
<td>5.0</td>
<td>?</td>
<td>90</td>
<td>26</td>
</tr>
<tr>
<td>Complex</td>
<td>0.1</td>
<td>7.5</td>
<td>Nitrogen ?</td>
<td>690</td>
<td>5.5</td>
</tr>
</tbody>
</table>

\(^a\) Residual substrate was determined after centrifugation of culture samples and is the sum of residual glucose, fructose and sucrose.

\(^b\) Enzyme activities were measured with sucrose as substrate.

\(^c\) Nitrogen limitation with mineral medium was obtained by lowering the ammonium sulfate concentration to 200 mg liter\(^{-1}\).

\(^d\) The complex medium contained 2.5 g of yeast extract liter\(^{-1}\) as a source of minerals and vitamins.

\(^e\) limitation unknown.

residual sugar concentration. This was accompanied by a decrease in inulinase activity to a level comparable to the activity found during growth under carbon limitation at high dilution rates (Table 3).

So far, no studies have been published on the synthesis of inulinase by yeasts growing in chemostat cultures on mineral media. GrootWassink and coworkers (8, 9, 11, 13) used a complex medium for chemostat cultivation of \textit{K. fragilis}. The medium was composed of yeast extract and a sugar in a 1 : 2 ratio. Growth of \textit{K. marxianus} CBS 6556 at a low dilution rate on this medium was, however, not carbon-limited as indicated by the residual sugar concentration (Table 3). Probably, this culture was nitrogen limited because addition of (NH\(_4\))\(_2\)SO\(_4\) led to a decrease in the residual sugar concentration.
(results not shown). There still was, however, incomplete consumption of the sugar, indicating the presence of another limiting nutrient or the presence of a growth-inhibiting factor. The enzyme yields of cultures grown on media with a yeast extract/sucrose ratio of 1:2 or 1:3, were lower than the enzyme yield of a culture grown on a medium composed of yeast extract and sucrose in a ratio of 1:1. Growth on this latter medium led to an inulinase yield (37 U mg of cell dry weight⁻¹) which still was lower than that obtained during carbon-limited growth on the defined mineral medium (52 U mg of cell dry weight⁻¹). Since the residual sugar concentrations in the various cultivation conditions were determined after centrifugation of culture samples, the values listed in Table 3 are an underestimation of the real concentrations, as a consequence of sugar consumption during sample processing. Nevertheless, the data clearly show that the inulinase activity of cultures is negatively correlated with the residual sugar concentration in both mineral and complex media. The results also demonstrate that enzyme production in complex medium is inferior to that in mineral media due to unnoticed limitation even at low yeast extract/sucrose ratios.

The distribution of enzyme activity over supernatant, cell wall and cell-bound fractions was dependent on the nature of the growth-limiting carbon substrate. Especially the cell-bound enzyme was present at very low levels when lactose or glucose were used as carbon source (Table 2). Fructose, glycerol and ethanol gave roughly the same distribution as sucrose and inulin. The distribution of inulinase activity over the fractions was determined at various dilution rates (Fig. 3). Although the amounts of enzyme detected in the supernatant and in the cell wall showed a rather broad variation, the overall picture was that the relative amount of the supernatant enzyme remained constant at about 50%. The amount of cell-wall enzyme exhibited a slight increase with increasing growth rates up to a dilution rate of 0.6 h⁻¹. The increase in the amount of cell-wall enzyme was paralleled by a decrease in the amount of cell-bound enzyme. The cell-bound fraction became nil above a dilution rate of about 0.6 h⁻¹. In contrast to carbon-limited growth on a mineral medium, growth under nitrogen limitation or growth on a complex medium resulted in relatively higher inulinase levels in the supernatant (60 to 80%) and lower levels (6 to 9% of total inulinase activity) in the cell-bound fraction.
CHAPTER 2

The ratio of the activities of the enzyme with sucrose and inulin, determined with 2% substrate solutions at pH 4.5 and 50°C, was 15 ± 3 irrespective of the growth substrate, growth rate and medium composition.

FIG. 3. Effect of dilution rate on the distribution of inulinase activity in (■) supernatant, (□) cell wall and (■■) cell-bound fractions in sucrose-limited chemostat cultures of K. marxianus CBS 6556. Vertical bars represent the standard deviations.

Effect of temperature and pH on the activity and distribution of inulinase. Both production and distribution of the inulinase of K. marxianus were affected by the growth temperature. The highest enzyme production was encountered at temperatures between 37 and 42°C (Table 4). Apparently, the temperature range of optimal enzyme production corresponded to the optimal temperature of growth of K. marxianus CBS 6556. The enzyme location also varied with the growth temperature. Temperatures below the optimal temperature range of growth and inulinase production gave rise to a larger fraction of enzyme present in the supernatant and a concomitant reduction in the amount of cell wall enzyme (Table 4). The reverse was found at temperatures higher than the optimal growth temperature.
CHAPTER 2

TABLE 4. Effect of growth temperature on total inulinase activity and its distribution in sucrose-limited cultures of K. marxianus CBS 6556

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Total inulinase * activity (U mg of cell dry wt⁻¹)*</th>
<th>Super-</th>
<th>Cell</th>
<th>Cell-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>26</td>
<td>62</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>35</td>
<td>39</td>
<td>68</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>37</td>
<td>45</td>
<td>61</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>40</td>
<td>52</td>
<td>48</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>42</td>
<td>36</td>
<td>46</td>
<td>33</td>
<td>21</td>
</tr>
<tr>
<td>46</td>
<td>12</td>
<td>25</td>
<td>42</td>
<td>33</td>
</tr>
</tbody>
</table>

*a Enzyme activities were measured with sucrose as substrate.

Since a rise in buffer pH stimulated the release of inulinase from the cell wall it was anticipated that cultivation of the organism at higher pH values would similarly increase the relative amount of enzyme excreted into the culture fluid. When the organism was grown on mineral medium with sucrose at a dilution rate of 0.1 h⁻¹ and pH 6.7, the total inulinase activity decreased to approximately half of that at pH 4.5. Surprisingly, however, the distribution of the enzyme over the various fractions was the same during growth at pH 6.7 and 4.5.

Effect of temperature and pH on enzyme activity. The activities of inulinase with sucrose and with inulin were tested at different temperatures using a 2% (wt/vol) substrate solution in 0.1 M sodium acetate buffer, pH 4.5. Irrespective of the enzyme location or of the dilution rate at which the cells were grown, the inulinase showed different temperature optima with sucrose (70° C) and inulin (50° C) (Fig. 4). Thus, the S/I-ratio was fairly
Fig. 4. Effect of temperature on activity of inulinase with sucrose and inulin as a substrate. Activities were determined at various temperatures using 2% (wt/vol) solutions of substrate in 0.1 M sodium acetate, pH 4.5.

Fig. 5. Effect of pH on activity of inulinase with sucrose and inulin as substrate. Activities were determined at 50 °C using 2% (wt/vol) solutions of substrate in 0.1 M sodium acetate (pH 3 to 5.5) or 0.1 M potassium phosphate (pH 5 to 9).
constant up to about 50°C (almost parallel lines), but greatly increased with higher temperatures. Above 70°C, no activity with inulin was measurable, whereas the activity with sucrose was still high (Fig. 4).

No loss in activity of inulinase was observed when inulinase preparations were incubated for 8 hours at temperatures up to 50°C. Incubation at higher temperatures gave rise to heat inactivation of inulinase measured either with sucrose or inulin. At 60°C the half-life was about 30 min.

The effect of pH on the activity of inulinase was tested in standard assay conditions. Irrespective of its origin (cell-bound, cell wall or supernatant) it exhibited a lower pH optimum with sucrose than with inulin as a substrate (Fig. 5). The S/I ratio was dependent on the pH of the assay. The ratio decreased with increasing pH up to 6.5 and again increased at higher pH values. The pH activity profiles were independent of enzyme location or growth rate of the cells.

Kinetic constants of inulinase. Substrates hydrolyzed by yeast β-fructosidases include the oligosaccharides raffinose and stachyose, with chain lengths of three and four sugar moieties, respectively (18). Hydrolysis of sucrose, raffinose and stachyose followed Michaelis-Menten kinetics. Values for the apparent affinity constant (Km) and maximal velocity (Vmax) with these oligosaccharides (Table 5) were calculated from

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (mM)</th>
<th>Vmax (μmol g−1 min−1)</th>
<th>Vmax/Km</th>
<th>(Kcat[E0]/Km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>15.7</td>
<td>122</td>
<td>7.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Raffinose</td>
<td>8.2</td>
<td>38</td>
<td>4.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Stachyose</td>
<td>9.7</td>
<td>32</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Inulinase was isolated from cells grown in a sucrose-limited continuous culture at D=0.2 h−1.

-44-
plots by the method of Hanes (10). With the chicory inulin preparation (mean chain length, 31 fructose units), however, a separate estimation of $K_m'$ and $V'$ is impossible since a linear relation between the enzyme activity and the substrate concentration was observed up to the saturation concentration of this substrate. Assuming that hydrolysis of inulin also exhibits Michaelis-Menten kinetics, it follows that the maximal chicory inulin concentration that can be achieved is well below the apparent affinity constant. Under these conditions, the Michaelis-Menten equation can be represented by: $V = \frac{K_{cat}[E] \cdot S}{K_m}$, where [E] represents the concentration of free enzyme. At substrate concentrations well below $K_m$, the concentration of free enzyme approximates $[E_0]$, the total enzyme concentration. Therefore the behaviour of the enzyme towards the different substrates can be compared using the slopes of the plots of $V$ versus $[S]$ at equal total enzyme concentrations. The slopes represent $K_{cat}[E_0]/K_m$ which, under these conditions, should be equal to the calculated $V'/K_{m}'$. With this method, it appears that sucrose is a better substrate than inulin for inulinase and that the enzyme specificity ($K_{cat}/K_m$) decreases with increasing chain length (Table 5). The apparent affinity constant $K_m'$, however, was lower for the oligosaccharides raffinose and stachyose than for sucrose. The kinetics of the enzyme were independent of its origin. No difference in $V'/K_{m}'$ values was observed when supernatant, cell wall and cell-bound enzyme were compared.
CHAPTER 2

DISCUSSION

Regulation of enzyme synthesis. In previous studies on the formation of inulinase by Kluyveromyces spp. in batch (17,18) and continuous cultures (8,9), it was concluded that the enzyme is regulated by induction and repression. However, in various cases high enzyme levels were encountered in the absence of inducer (Table 6). Results from batch cultures (Table 6) should be interpreted cautiously, since in these cultures enzyme levels depend on the time of harvesting and thus are subject to large variations.

The use of chemostat cultivation permits a more precise analysis of the regulation of inulinase synthesis, provided that defined media are used and that the limiting nutrient is known. Inulinase production by K. marxianus CBS 6556 decreased with increasing dilution rate (Fig. 2). The same relationship between dilution rate and inulinase production was reported for K. fragilis ATCC 12424, a yeast now classified as K. marxianus var. marxianus. This was, however, determined in complex medium over a very small range of dilution rates (18). The decrease in inulinase activity with increasing dilution rates is primarily caused by the increase in residual sugar concentration at higher dilution rates. This was also evident from an analysis of enzyme production in cultures grown under different degrees of nitrogen limitation at a fixed dilution rate. The presence of higher concentrations of residual substrate led to lower enzyme activities (Table 2). These observations indicate that the enzyme is regulated by catabolite repression (2,3). The finding that ethanol and, to a lesser extent, glycerol also gave rise to fairly high levels of inulinase (Table 1) is in contradiction to the suggested inducible nature of the enzyme (9). Rather, derepression of enzyme synthesis is likely to occur when cells are grown on these substrates.

Regulation of another yeast β-fructosidase, invertase, has been more extensively studied. In Saccharomyces cerevisiae, synthesis of this enzyme is only subject to carbon catabolite repression and does not require a specific inducer (7,14,15). This conclusion was based on invertase levels in cultures of wild type S. cerevisiae, enzyme levels in cultures of regulatory mutants and also on messenger RNA contents of cells under repressive and derepressive growth conditions (4,15).
CHAPTER 2

TABLE 6. Published data on inulinase production by yeast during growth on various carbon sources.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>S. fragilis</th>
<th>K. fragilis</th>
<th>K. marxianus</th>
<th>K. marxianus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>351, batch</td>
<td>ATCC 12424</td>
<td>UCD 55-82</td>
<td>CBS 6556</td>
</tr>
<tr>
<td>Inulin</td>
<td>1000</td>
<td>500</td>
<td>nd</td>
<td>294</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fructose</td>
<td>340</td>
<td>100</td>
<td>111</td>
<td>88</td>
</tr>
<tr>
<td>Glucose</td>
<td>170</td>
<td>100</td>
<td>33</td>
<td>79</td>
</tr>
<tr>
<td>Lactose</td>
<td>nd</td>
<td>50</td>
<td>&lt;1</td>
<td>nd</td>
</tr>
<tr>
<td>Galactose</td>
<td>170</td>
<td>50</td>
<td>1</td>
<td>nd</td>
</tr>
<tr>
<td>Ethanol</td>
<td>nd</td>
<td>100</td>
<td>&lt;1</td>
<td>nd</td>
</tr>
<tr>
<td>Glycerol</td>
<td>nd</td>
<td>150</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

For comparison, enzyme levels are presented as percentages of the activities of cells grown on sucrose as carbon source. ND, not determined.

Snyder and Phaff (18); complex medium with 2% substrate.

GrootWessink and Hewitt (9); complex medium with 1% substrate.

Parekh and Margaritis (17); complex medium with 1% substrate.

This study; carbon-limited cultures on mineral medium with 0.25% substrate.

The yeast K. marxianus CBS 6556 had a maximum inulinase yield of about 50 U mg of cell dry weight⁻¹ when grown in sucrose-limited continuous cultures at low dilution rates (Table 2; Fig. 2). This inulinase yield is among the highest reported so far. Most research on inulinase formation and regulation, reviewed by Vandamme and Derycke (24), has been performed using cultivation on complex media. In our experience a complex medium is less favourable to enzyme formation than a defined mineral medium (Table 3).
CHAPTER 2

Probably this was caused by the absence of true carbon limitation when yeast extract was used as source of nitrogen and vitamins.

In our studies with mineral medium only the effects of carbon-limited growth and nitrogen-limited growth on inulinase production were determined. It is possible, however, that other limitations may have effects on inulinase production. In this respect, the work of Toda and co-workers is of importance. They reported a pronounced positive effect of phosphate limitation on the production of invertase by the yeast S. carlsbergensis, which overruled the repressive effect of high residual sugar concentration (22,23).

Distribution of inulinase. The distribution of inulinase over supernatant, cell wall and cell-bound fractions was dependent on the (i) nature of the carbon-limiting substrate, (ii) dilution rate, (iii) medium composition (mineral or complex) and (iv) growth temperature. As already observed by Lam and GrootWassink (13) for K. fragilis, irrespective of the dilution rate, about half of the total amount of enzyme produced in sucrose-limited continuous cultures of K. marxianus CBS 6556 was present in the culture supernatant. The distribution of the other half of the enzyme over cell wall and cell-bound fractions, however, differed with the dilution rate. The cell-bound fraction decreased with increasing dilution rates (Fig. 3). Carbon-limited growth on glucose or lactose or nitrogen-limited growth (both in mineral and in complex media) with sucrose resulted in high percentages of inulinase present in the culture supernatant and low amounts of cell wall-and cell-bound enzyme (Table 2). Retention of inulinase in the cell wall thus seems to be less pronounced when cells are grown under conditions that result in a low level of enzyme. It remains to be elucidated whether this is a consequence of differences in cell wall composition or to differences in enzyme structure.

As yet it is not clear whether the cell-bound enzyme represents a tightly bound enzyme located outside the cytoplasmic membrane or, comparable to the invertase of Saccharomyces spp. (14), an intracellular enzyme. In this respect it is relevant to mention the findings of Esmen et al. (5) and Tammi et al. (21) on the multimeric structure and excretion of invertase in Saccharomyces cerevisiae. These authors demonstrated that invertase is an octameric complex of four invertase dimers throughout the secretory process and that this complex appears to play a role in the retention of invertase within the cell wall. Both invertase released into the culture fluid and the
fraction of cell wall invertase that can be released after treatment of the cells with sulphydryl compounds are composed of invertase dimers with the same kinetic properties as the octamer. Conversion of octamer to dimer is promoted by several treatments (e.g., sonication) but not by treatment with 2-mercaptoethanol. Even when intact cells are treated with 2-mercaptoethanol the octamer is preferentially retained in the cell wall and release requires cell wall disruption (21). It is thus possible that the distribution of inulinase in K. marxianus, like that of invertase in Saccharomyces spp., may depend on the subunit composition of the enzyme.

Kinetic properties of inulinase. In standard assay conditions the S/I ratio of the β-fructosidase of K. marxianus CBS 6556 was 15, which would make it a true inulinase (24). Temperature and pH optima (Fig.4; Fig.5) differed with sucrose or inulin as substrate. This phenomenon has also been observed for β-fructosidases from other Kluyveromyces strains (18,25) and from other yeasts (1). As a consequence S/I ratios cannot be compared without prior knowledge of the dependence of enzyme activity on pH and temperature. Moreover, the value of the S/I ratio is influenced by the substrate concentration and by the origin of the inulin preparation used.

Contrary to bacteria and molds, all yeast β-fructosidases show a restricted mode of action. They all remove fructose moieties exo-wise and all have the capability of hydrolyzing both sucrose and inulin (6). The question remains as to whether differences in values such as S/I ratios and apparent kinetic constants for substrates with undefined molecular weights, provide sufficient evidence for the classification of yeast β-fructosidases into two different enzymes: inulinase and invertase.

LITERATURE CITED


Chapter 3

Production and Localization of $\beta$-Fructosidase in Synchronous and Asynchronous Chemostat Cultures of Yeasts.

R.J. Rouwenhorst, A.A. van der Baan, W.A. Scheffers, and J.P. van Dijken.

Submitted to Applied Environmental Microbiology.
CHAPTER 3

SUMMARY

In synchronized continuous cultures of *Saccharomyces cerevisiae* CBS 8066, the production of the extracellular invertase (EC. 3.2.1.26) showed a cyclic behavior that coincided with the budding cycle. The invertase activity increased during bud development and ceased at bud maturation and cell scission. The cyclic changes in invertase production resulted in cyclic changes in the amount of invertase localized in the cell wall. However, the amount of invertase present in the culture liquid remained constant throughout the budding cycle.

Also in asynchronous continuous cultures of *S. cerevisiae*, the production and localization of invertase showed significant fluctuation. The overall invertase production in an asynchronous culture was 2-3 times higher than in synchronous cultures. This could be due to more severe invertase-repressive conditions in a synchronous chemostat culture. In synchronous chemostat cultures both the intracellular glucose-6-phosphate concentration and the residual glucose concentration were significantly higher than in asynchronous chemostat cultures.

In asynchronous and synchronous continuous cultures of *S. cerevisiae* about 40% of the invertase was released into the culture liquid. Thus far, it was generally believed that *S. cerevisiae* releases only about 5% of its invertase.

In contrast to invertase production and localization in chemostat cultures of *S. cerevisiae*, no significant changes in inulinase (EC. 3.2.1.7) production and localization were observed in chemostat cultures of *K. marxianus* CBS 6556. In cultures of *K. marxianus* about 50% of the inulinase was present in the culture liquid.

INTRODUCTION

In yeasts, enzymes that hydrolyze oligosaccharides are located in the cell wall. Depending on culture conditions and yeast species, variable amounts of enzyme may be released in the culture medium (9,13). Two mechanisms for the retention of these enzymes in the cell wall have been
proposed. One is based on the assumption that these glycoproteins are associated with phospho-mannan components of the outer cell wall region by either covalent or non-covalent linkages (9). The alternative proposal is that the outer layers of the cell wall function as a permeability barrier (7). The barrier hypothesis was affirmed by the finding that the localization of the two \(\beta\)-fructosidases known in yeasts, namely invertase (EC 3.2.1.26) produced by \textit{Saccharomyces cerevisiae} and inulinase (EC 3.2.1.7) produced by \textit{Kluveromyces marxianus} (13,16), was dependent on the degree of oligomerization of the enzymes. Both enzymes are excreted into the culture liquid as a dimer. The enzyme retained in the cell wall is a tetramer in the case of inulinase, and an octamer in the case of invertase (4,14).

The secretion of glucanase, acid phosphatase, and invertase follows the pattern of cell-surface growth in \textit{S. cerevisiae} (2,5,17). During budding an increased amount of transport vesicles containing newly synthesized cell wall material and extracellular enzymes are localized at the developing bud. As a result of exocytosis, newly secreted enzymes mainly reside in the cell wall surrounding the developing bud. The secretion continues until growth of the daughter cell is completed, suggesting a close relationship between cell division cycle and enzyme secretion (5). The developing wall of the bud is less rigid than that of the mother cell (15). Therefore, the developing wall could temporarily contain larger pores and be more permeable to the glycoproteins. The release of the glycoproteins into the culture liquid could thus be highest at the stage of bud development. However, the possibility that release of extracellular enzymes into the culture medium might be related to the budding cycle, has not been tested.

A convenient method for the study of a relation between enzyme production and budding cycle of yeasts is the use of oscillating continuous cultures. In these cultures part of the cell population exhibits synchronized budding. This method has proved useful for the study of the synthesis of intracellular enzymes in relation to the budding cycle (1,6,8,20). In this paper, using two well-known \(\beta\)-fructosidases namely invertase and inulinase as model enzymes, the production and release of extracellular enzymes in synchronous and asynchronous continuous cultures of yeasts are investigated.
CHAPTER 3

MATERIALS AND METHODS

Yeast strains and growth conditions. *Kluyveromyces marxianus* var. *marxianus* CBS 6556 and *Saccharomyces cerevisiae* CBS 8066 were obtained from the Yeast Division of the Centraal Bureau voor Schimmelcultures (CBS), Delft, The Netherlands. Yeasts were maintained on YEPD agar slopes (Yeast Extract, 10 g; Bacto-Peptone, 10 g; glucose, 20 g; per liter of demineralized water). The organisms were grown in a laboratory fermenter with a working volume of 1 liter (Applikon, Schiedam, The Netherlands). The pH was maintained at the desired value by automatic addition of 1 M KOH and 0.5 M H₂SO₄. *S. cerevisiae* was cultivated at D=0.18 h⁻¹, pH 5.0, and 30°C, *K. marxianus* was cultivated at D=0.1 h⁻¹ or D=0.18 h⁻¹, pH 4.5, and 40°C. For carbon-and energy-limited growth a mineral medium according to Van Urk et al. (18) was used. For carbon- and energy-limited growth sucrose was added to the mineral salts medium to a final concentration of 5 g l⁻¹.

Oscillating continuous cultures. *Saccharomyces cerevisiae* CBS 8066 was grown batch-wise in the fermenter on the mineral salts medium containing glucose (5 g l⁻¹). After the culture entered the stationary phase, the continuous feed of medium with sucrose was started. In three out of eight trials, the transition from batch to continuous mode led to stable oscillations in carbon dioxide production rate. The oscillations persisted for a minimum period of two days and a maximum period of eight days. As a substantial part of the cultures appeared to have become synchronized in budding such cultures were labeled synchronous.

Sampling. Oscillating, synchronous chemostat cultures and non-oscillating, asynchronous continuous cultures of yeasts were sampled only after five volume changes following start-up of continuous-culture conditions. In order to maintain steady-state conditions, samples (30 ml) were taken from the outlet (sampling time 10 min). The sample was received in a cooled flask (4°C).

Yeast cells in a culture sample were counted under a light microscope (1000x) using a Burkner haemocytometer, and the percentage of budding cells in the population was estimated. All stages from the moment the bud became visible to the scission of the daughter cell were taken as a budding cell (8).

Gas analysis. Analysis of CO₂ in fermenter outlet gas was performed on line using a Beckman 864 infrared carbon dioxide analyzer (Beckman Ind.,...
CHAPTER 3

Fullerton, California), and registered with a Kipp BD 41 recorder (Kipp & Zn., Delft, The Netherlands). For calculation of carbon dioxide production ($Q_{\text{CO}_2}$) the method described by Van Urk (18) was used.

Fractionation of cultures for imulinase and invertase assays. Samples (10 ml) were fractionated into three enzyme preparations by the method described by Rouwenhorst et al. (13). Enzyme present in the culture liquid is designated as supernatant enzyme. Induced release of cell wall-associated enzyme was obtained by suspension of 7 mg of cell dry wt in 1 ml of a 50 mM potassium phosphate buffer, pH 7, containing 10 mM 2-mercaptoethanol, and 10 mM dithiothreitol, and incubation for 1 h at 30°C. The suspension was then centrifuged at 4°C and washed with 1 ml of potassium phosphate buffer (50 mM, pH 7). Enzyme activity present in the supernatant after incubation of the cells in buffer with sulfhydryl compounds is designated as cell wall enzyme. After induced release of cell wall enzyme and washing, cells were resuspended in 1 ml phosphate buffer (50 mM, pH 7) and sonicated at 4°C for 5 min with intermittent periods of cooling. Cell debris was sedimented by centrifugation at 4°C (15 min, 30,000 x g) and resuspended in 1 ml of phosphate buffer. The activity solubilized by means of sonication for 5 min at 4°C is designated as cell-bound enzyme. Enzyme activities present in resuspended cell debris always accounted for less than 1% of the total enzyme produced. No distinction is made between cell wall-retained enzyme and intracellular enzyme. Together they constitute the cell-bound fraction.

Analytical methods. Biomass concentrations were established by total organic carbon analysis. A Beckman 915B Tocamaster (Beckman Ind., Fullerton, California) was used to determine the carbon content in culture suspensions and culture supernatant, the carbon content of yeast being the difference. Yeast dry weight was calculated assuming a carbon content of 50%. As a standard a 2.137 g 1⁻¹ solution of anhydrous potassium biphthalate was used.

Glucose, glucose-6-phosphate, and fructose were determined enzymatically, using hexokinase, 6-phosphoglucone isomerase, and glucose-6-phosphate dehydrogenase (glucose/fructose test combination, Boehringer GmbH, Mannheim, Federal Republic of Germany). Sucrose was determined by the same method after addition of 10 U ml⁻¹ of invertase (Boehringer).

Residual sugar was determined after centrifugation of culture samples. Samples were collected at 4°C during 1 min from the fermenter outlet. This sampling method was required in order to preserve steady state conditions. As a result, the values for residual sugar concentration will be an.
CHAPTER 3

underestimation of the real value, due to sugar consumption during sample processing. Glucose-6-phosphate concentrations were determined in the cell-free extracts and related to the cell dry weight, assuming a 100% efficiency of cell disruption during sonication.

Sucrose-hydrolyzing activities of invertase and inulins were measured by the method described previously (13). One unit of invertase or inulins activity is defined as the amount of enzyme catalyzing the liberation of 1 μmol of fructose min⁻¹ at pH 4.5 and 50°C. Specific activities were related to cell dry weight. As sampling time, the mean time of collection was used.

Chemicals. Yeast extract and Bacto-Peptone were from Difco Laboratories, Detroit, Michigan. Sucrose and 2-mercaptoethanol were from Baker Chemicals, Deventer, The Netherlands. Dithiothreitol was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Oscillating cultures of S. cerevisiae. Oscillations in oxygen consumption and carbon dioxide production of S. cerevisiae cultures reflect the occurrence of synchronized budding in the culture. These oscillations can occur spontaneously after transition of a culture from batch to continuous cultivation (11) or can be induced by stepwise changes in either dilution rate or glucose feed rate (1,12,20). Spontaneously oscillating continuous cultures of S. cerevisiae CBS 8066 growing on sucrose were obtained after transition from stationary batch cultures to continuous mode. At a dilution rate of 0.18 h⁻¹, stable oscillations in carbon dioxide production rate with a period of 2.5 were observed (Fig. 1). During the oscillations the percentage of budding cells, the optical density at 610 nm, the intracellular glucose-6-phosphate concentration, and the residual sugar concentrations, i.e. glucose, fructose, and sucrose, were determined. The behavior of an oscillating S. cerevisiae culture is well documented (1,6,8,20). The oscillations in oxygen consumption and carbon dioxide production in fully aerobic cultures of S. cerevisiae are a result of the cyclic budding of part of the yeast population. The development of a yeast cell can be subdivided into a single cell phase and a double-cell phase (Fig. 1A). During the single-cell phase (S) the cells metabolize glucose oxidatively and accumulate reserve carbohydrates such as glycogen and
trehalose. At the initiation of budding (I) a rapid degradation of reserve carbohydrates occurs. During this period, the yeast switches over from a respiratory to a fermentative metabolism and ethanol is secreted into the medium (1, 6, 20). The production of ethanol coincides with a marked increase in carbon dioxide production, and a slight decrease in biomass in the culture (Fig. 1A). This sudden change in glucose metabolism is followed by an oxidative breakdown of the previously formed ethanol. The highest percentage of double cells is attained when the carbon dioxide production rate has dropped to nearly its original value. Of the total cell population, a maximum of 56% of budding cells was observed (Fig. 1A). Thus, a degree of synchronization of 56% was obtained in the oscillating continuous culture of *S. cerevisiae* CBS 8066. The glucose-6-phosphate concentration showed an oscillating behavior with minimal and maximal concentrations of 14 and 32 mg (g of cell dry weight)⁻¹, respectively (Fig. 1B). The residual sugar concentration in the oscillating culture was 26.6 ± 2.8 mg l⁻¹. A summary of the parameters determined in synchronous and asynchronous continuous cultures is given in Table 1.

Localization of invertase in synchronous chemostat cultures of *S. cerevisiae*. During the synchronized continuous cultivation the distribution of invertase over supernatant, cell wall, and cell-bound fraction were determined (Fig. 1C). Treatment of *S. cerevisiae* cells with sulfhydryl compounds did not result in a marked release of invertase from within the cell wall (Fig. 1C). Only about 0.3 U mg of cell dry weight⁻¹, i.e. 3% of total invertase produced, was present in this fraction (Table 1). The insensitivity of *S. cerevisiae* to treatment with sulfhydryls has long been known (3,4), and release of cell wall-retained invertase can only be achieved by complete removal of the cell wall during spheroplast formation (9). Spheroplasts still contain sucrose-hydrolyzing activity, due to an intracellular invertase which amounts to about 5% of total invertase activity (5). However, in the figures presented here, no distinction is made between cell wall-retained invertase and intracellular invertase. The cell-free extracts (cell-bound invertase) contained both these invertases. The invertase in this cell-bound fraction showed a marked oscillating behaviour in synchronously dividing cultures (Fig. 1C). The cyclic pattern of cell-bound invertase paralleled the cyclic pattern of the percentage of budding cells in the culture. The cell-bound invertase increased with increasing
CHAPTER 3

A

Optical density (610 nm)

Carbon dioxide (mmol/g)

Budding cells (%)

Time (h)

B

Carbon dioxide (mmol/g)

Glucose-6-phosphate (mg/g cells)

Time (h)

C

Carbon dioxide (mmol/g)

Invertase (U/mg of cell dry wt)

Time (h)

-60-
CHAPTER 3

FIG. 1. Synchronous growth of S. cerevisiae under sucrose limitation in an oscillating continuous culture at a dilution rate of 0.18 h\(^{-1}\) (mean generation time of 3.85 h), pH 5, and 30\(^\circ\)C. (A) Course of (◇) culture biomass, and (●) percentage of budding cells. I: initiation of budding, S: start of single-cell phase. (B) Course of (■) glucose-6-phosphate in cell-free extracts of the culture. (C) Production of invertase in the oscillating culture divided in (●) supernatant invertase, (◇) cell wall invertase, and (▲) cell-bound invertase (see text). The pattern of the carbon dioxide production rate is presented in each figure by a dashed line.

percentage of budding cells, up to a maximum of 9.6 U mg of cell dry weight\(^{-1}\) and decreased to 4.6 U mg of cell dry weight\(^{-1}\) when bud maturation and cell division took place (Fig. 1A,1C). The invertase activity correlated negatively to the glucose-6-phosphate concentration.

Irrespective of the amount of invertase present in the cells, the invertase released into the culture liquid remained constant at 5.3 ± 0.3 U mg of cell dry weight\(^{-1}\) throughout the budding cycle. During continuous cultivation a constant amount of cells and culture liquid is removed from the culture per unit of time. In the case of cessation of invertase synthesis during bud maturation and cell scission, both the cell-bound invertase and supernatant invertase are expected to decrease according to wash-out kinetics. However, as already mentioned above, the amount of supernatant invertase remained constant. This implies that there should be an increased release of invertase from the cells. The observed decrease in cell-bound invertase activity (Fig. 1C) could then be explained by the release of invertase into the culture liquid. However, if invertase synthesis is ceased, the total amount of invertase present in the culture still should follow wash-out kinetics. The decline in total invertase activity during bud maturation and the theoretical wash-out are presented in Fig. 2. The decrease in total invertase activity did not completely follow theoretical wash-out kinetics (Fig. 2). Only during the first 40 to 50 min did the decrease in total invertase appeared to follow wash-out kinetics. From 50 min onwards invertase decreased more rapidly than expected from wash-out kinetics. Apparently, from that moment on either inactivation or denaturation of invertase took place.
CHAPTER 3

FIG. 2. Decline in total invertase activity during the disappearance of budding cells, followed over three separate oscillations in a synchronous continuous culture of *S. cerevisiae* CBS 8066 at a dilution rate of 0.18 h\(^{-1}\). The dashed line represents the theoretical wash-out of invertase.

**Localization of invertase in asynchronous chemostat cultures of *S. cerevisiae*.** After several days the oscillations in carbon dioxide production rate disappeared and the culture changed over to an asynchronous cell division cycle. For comparison with the behavior of a synchronous culture, the production and localization of invertase in the asynchronous continuous culture were followed after five extra volume changes of the fermenter. As evidenced by the constancy in carbon dioxide production (4.1 ± 0.1 mmol/g h), optical density of the culture at 610 nm (7.12 ± 0.07), and residual substrate concentration (7.9 ± 0.6 mg l\(^{-1}\)), the culture then represented a steady-state continuous culture. During the steady-state the activities of supernatant invertase and cell wall invertase remained unchanged at 19.3 ± 0.7 and 0.42 ± 0.04 U mg of cell dry weight\(^{-1}\), respectively (Table 1; Fig. 3). The cell-bound invertase activity showed a remarkable variation, with activities between 20 and 28.4 U mg of cell dry weight\(^{-1}\). This variation in
cell-bound invertase activity was not due to the fractionation or assay methods. The coefficient of variation in ten independent determinations of a single measurement starting at the fractionation of a culture suspension was 5.2%.

![Graph](image)

**FIG. 3.** Course of (○) supernatant invertase, and (●) the sum of cell wall and cell-bound invertase in an asynchronous sucrose-limited continuous culture of *S. cerevisiae* CBS 8066 at pH 5, 35°C, and a dilution rate of 0.18 h⁻¹.

The total invertase activity was between 38.8 and 48.6 U mg of cell dry weight⁻¹. This is about three times the amount of invertase found in synchronous *S. cerevisiae* cultures. The variation in total invertase was only due to the variation in cell wall plus cell-bound invertase, since the amount of supernatant invertase remained unchanged.

**Localization of inulinase in asynchronous chemostat cultures of K. marxianus.** The extracellular inulinase of *K. marxianus* is strongly related to the invertase of *S. cerevisiae*. In order to determine whether the budding cycle-related variation in extracellular β-fructosidase production and localization also occurs in *K. marxianus*, we tried to obtain synchronous cultures of this yeast and determine inulinase activities. Various attempts
CHAPTER 3

to obtain an oscillating culture of *K. marxianus* CBS 6556 were unsuccessful. Only after step-wise increase in dilution rate up to 0.89 h\(^{-1}\), when the mean generation time almost equals the minimal time necessary for budding (11,20), oscillations in carbon dioxide production of a *K. marxianus* culture were observed. However, due to inulinase repression at this high dilution rate (13), the amount of inulinase was too low, viz. 0.5 - 2 U mg of cell dry weight\(^{-1}\) to obtain reproducible results with respect to

TABLE 1. Various parameters in a synchronous and an asynchronous continuous culture of *S. cerevisiae* grown at D=0.18 h\(^{-1}\), and an asynchronous continuous culture of *K. marxianus* grown at D=0.1 h\(^{-1}\). Carbon dioxide production rate, mmol/g h; glucose-6-phosphate concentration, mg/g dry wt; residual sugar concentration, mg/l, \(\beta\)-fructosidase activities (invertase in *S. cerevisiae*, inulinase in *K. marxianus*), U/mg dry wt.

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>S. cerevisiae</em></th>
<th><em>K. marxianus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CBS 8066</td>
<td>CBS 6556</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>synchronous</td>
<td>asynchronous</td>
</tr>
<tr>
<td></td>
<td>asynchronous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>asynchronous</td>
<td></td>
</tr>
<tr>
<td>(\text{CO}_2)</td>
<td>4.0 - 7.9</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>14 - 32</td>
<td>4.9 ± 1.1</td>
</tr>
<tr>
<td>Residual sugar(^1)</td>
<td>14.0 ± 1.5</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>(\beta)-Fructosidase activities:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>5.3 ± 0.2</td>
<td>19.3 ± 0.7</td>
</tr>
<tr>
<td>Cell wall(^2)</td>
<td>0.3 ± 0.07</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>Cell-bound(^3)</td>
<td>4.6 - 9.6</td>
<td>20.0 - 28.8</td>
</tr>
</tbody>
</table>

\(^1\) Residual sugar is an underestimation of the real value. Using fast sampling a residual concentration of 19.8 mg/l for *S. cerevisiae* CBS 8066 was established (18). \(^2\) Enzyme released by sulfhydryls. \(^3\) Cell wall enzyme plus intracellular enzyme in cell free extracts.

-64-
CHAPTER 3

inulinase distribution. Analogous to the invertase in asynchronous steady-state cultures of S. cerevisiae CBS 8066, the production and localization of inulinase was followed during asynchronous steady-state cultivation of K. marxi anus growing on sucrose at D=0.1 h\(^{-1}\) (Table 1). In contrast to S. cerevisiae, treatment of K. marxi anus cells with a sulfhydryl compound led to a release of cell wall-retained inulinase. The inulinase activities in cell wall fraction and cell-bound fraction were 17.6 ± 1.2 U mg of cell dry weight\(^{-1}\) and 8.4 ± 0.9 U mg of cell dry weight\(^{-1}\), respectively. In contrast to a steady-state culture of S. cerevisiae, there was no significant change in \(\beta\)-fructosidase activity in this steady-state culture of K. marxi anus. However, continuous growth of S. cerevisiae had been performed at a dilution rate of 0.18 h\(^{-1}\). Hence, also a steady-state culture of K. marxi anus growing at D=0.18 h\(^{-1}\) was followed. During 15 volume changes, the total inulinase activity in this culture also remained constant at 36.8 ± 1.4 U mg of cell dry weight\(^{-1}\). The percentage of inulinase present as supernatant inulinase was always between 46 and 57%.

DISCUSSION

Invertase production and cell division cycle. The activity of invertase in synchronous cultures of S. cerevisiae CBS 8066 showed a cyclic pattern that coincided with the budding cycle of the yeast. The synthesis of invertase was lowest during the single-cell phase. When the percentage of budding cells increased, the production of invertase increased as well (Fig. 1). This confirms the hypothesis that production of extracellular enzymes in yeasts is related to the increased amount of transport vesicles during budding (2,5,17), and that de novo synthesized and secreted invertase is mainly present at the developing bud (17).

The maximal production of invertase in a synchronous continuous culture of S. cerevisiae was about three times lower than in an asynchronous continuous culture of S. cerevisiae growing at the same dilution rate. It is of interest that the residual sugar concentration in the culture and the intracellular glucose-6-phosphate concentration of a synchronous culture, were significantly higher than in an asynchronous culture. Invertase production is subject to catabolite repression (9,10), and the lower level of invertase in a synchronous culture could be due to these higher glucose
and glucose-6-phosphate concentrations. In synchronous chemostat cultures, the residual sugar concentration did not oscillate, indicating that cyclic changes in invertase production do not result from changes in residual glucose. A candidate to regulate invertase synthesis is the intracellular glucose-6-phosphate concentration (1,20). Indeed, the glucose-6-phosphate concentration showed oscillations related to the budding cycle. After the intracellular level of glucose-6-phosphate was approximately 25 mg/g, the decay in invertase levels coincided with a supplementary increase in intracellular glucose-6-phosphate (Fig. 1B,1C). The decrease in total invertase activity during cell-scission and the single cell-phase, could not be explained only by cessation of invertase synthesis and wash-out. During the single cell phase a decay of invertase by either inactivation or denaturation of the enzyme must have occurred. Remarkably, the start of the decay stage coincided with the moment that the glucose-6-phosphate concentration had reached its maximal value.

Invertase localization and cell cycle. Only the cell-bound invertase showed cyclic changes in invertase level. It was expected that during budding more invertase would be released into the culture fluid. However, the invertase present in the culture fluid (supernatant invertase) remained constant throughout the oscillations of the culture and thus throughout the cell cycle (Fig. 1C). The cell-free extracts contained both the intracellular invertase and cell wall-retained invertase. In view of the fast solubilization of invertase during cell disruption, it is likely that these changes in invertase mainly concern cell wall-retained enzyme. Moreover, the intracellular invertase accounts for only 5% of the total invertase activity (4,9). It is apparent that during budding all the newly synthesized invertase remains in the cell wall. Only a small part is released into the culture fluid thus compensating for wash-out of supernatant enzyme. This release is a continuous process and is independent of the cell cycle. Therefore, release of the enzyme in the culture fluid is probably caused by culture conditions like ionic strength of the medium or shear force; it is not regulated by the yeast. This view is supported by the finding that supernatant invertase is a dimer and cell wall-retained invertase an octamer, and that dissociation of octamer into dimers is affected by ionic strength of the medium and by shear force (5).

In spite of a constant invertase level in the culture fluid during steady-state cultivation, the percentage of invertase present in this
fraction was between 40 and 49%. Contrary to our results, the invertase of
*S. cerevisiae* has been reported to be barely present (<10%) in the culture
fluid (4,9,10). However, these reports are mostly based on batch culture
studies using complex media. The shear force, a condition that could affect
the release of invertase from the cell wall (4), is much less in culture
flasks than in chemostats. No continuous culture studies on both production
and distribution of invertase in *S. cerevisiae* have been carried out.
McMurrough and Rose (10) established the invertase production in continuous
cultures of *S. cerevisiae* but did not determine the invertase activity in
the culture fluid.

**Inulínase production and localization in asynchronous cultures of K.
marxianus.** In contrast to the production of invertase in asynchronous
steady-state cultures of *S. cerevisiae* CBS 8066, there were no significant
changes in inulínase production during steady-state cultivation of *K.
marxianus*. Recently, we reported on the production and distribution of
inulínase in continuous cultures of *K. marxianus* CBS 6556 (13). A rather
broad variation in the distribution of inulínase over supernatant and cell
wall fraction was found between cultures grown at different dilution rates
(13). A closer look into the distribution of inulínase during steady states
at $D=0.1 \text{ h}^{-1}$ and $D=0.18 \text{ h}^{-1}$, reveals that these variations are not
significant.

**Applied aspects.** The results presented above clearly show that production
of the extracellular invertase is dependent on the budding cycle of *S.
cerevisiae*. Since this organism easily synchronizes in aerobic sugar-limited
cultures, it is clear that large variations in enzyme production may occur.
This phenomenon may also hold for production of extracellular heterologous
proteins by *S. cerevisiae*. The occurrence of oscillations seems to be
peculiar to Crabtree-positive yeasts (1,18) such as *S. cerevisiae*. The use
of Crabtree-negative yeasts for production of heterologous, glycosylated
proteins may offer the advantage of absence of oscillations and a constant
extracellular protein production level.
CHAPTER 3

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Chapter 4

Structure and Properties of the Extracellular Inulinase of *Kluyveromyces marxianus* CBS 6556.

R.J. Rouwenhorst, M. Hensing, J. Verbakel, W.A. Scheffers, and J.P. van Dijken.

Submitted to Applied Environmental Microbiology.
CHAPTER 4

SUMMARY

In the yeast *Kluyveromyces marxianus* two forms of inulinase were present namely an inulinase secreted into the culture fluid and an inulinase retained in the cell wall. Both forms were purified and analyzed by denaturing and non-denaturing gel electrophoresis. With the use of Endo-H it was established that the enzyme retained in the cell wall and the enzyme secreted into the culture fluid have similar subunits consisting of a 64 kDa polypeptide with varying amounts of carbohydrate (26-37% of the molecular mass). The two forms of inulinase differed in size, due to differences in subunit aggregation. The enzyme present in the culture fluid was a dimer and the enzyme retained in the cell wall a tetramer. The differences in oligomerization did not affect the apparent affinity constants towards the substrates sucrose and raffinose. These findings support the hypothesis that the retention of glycoproteins in the yeast cell wall may be caused by a permeability barrier towards larger glycoproteins. The amino-terminal end of inulinase was determined and compared with the amino terminus of the closely related invertase. The kinetic and structural evidence indicate that in yeasts two distinct β-fructosidases exist, viz. invertase and inulinase.

INTRODUCTION

In yeasts two extracellular glycoproteins are known to be associated with growth on sucrose: invertase (EC 3.2.1.26), e.g. in *Saccharomyces cerevisiae* and inulinase (EC 3.2.1.7), e.g. in *Kluyveromyces marxianus*. These enzymes exhibit corresponding hydrolytic activities towards sucrose, but differ in their specificities for higher molecular-weight oligosaccharides and fructans of the inulin-type (17,33). The S/I ratio (relative activities with sucrose and inulin) is generally employed to discriminate between invertase and inulinase (33,37). A low S/I ratio (high activity with inulin) is taken to indicate inulinase.

Originally, the classification of inulinases was based on the occurrence of specific enzymes in bacteria, molds and plants. These inulinases rarely show activity with sucrose and split fructans of the inulin type either endo-wise or exo-wise, producing a series of oligosaccharides or only fructose,
respectively (11,16,33,37). In contrast, yeasts produce inulinases capable of hydrolysis of inulin and levan type fructans exo-wise, as well as of sucrose hydrolysis. Differences in S/I ratios and in apparent kinetic constants are considered by some authors to be insufficient for a distinction between yeast inulinase and yeast invertase. In their view, inulinase is a special kind of invertase and should be classified as such (2,21,31).

A considerable amount of research has been devoted to the invertase of *S. cerevisiae*, as it appeared an attractive model for studies on protein synthesis and excretion of glycoproteins (6,7,13,18,24,29,30,35). Secreted invertase mainly resides in the cell wall as an octamer (1,13). The small amount of invertase present in the culture fluid, as well as the fraction that is removable from the cells by treatment with thiols, was found to be composed of dimers. It has been suggested that oligomerization helps to retain the enzyme within the cell wall (13).

Similarly, the inulinase of yeast is in part associated with the cell wall but, as compared to the invertase of *Saccharomyces*, much more of the enzyme is actually secreted into the culture fluid. When grown under conditions which derepress enzyme synthesis, the yeast *K. marxianus* secretes over 50% of its enzyme into the culture fluid, and 35% can be released from the cell wall by sulfhydryls (23,31). In contrast to the biochemistry of the invertase of *Saccharomyces*, very little is known on that of inulinase. Although inulinases of different yeasts have been partially purified (28,37,39) and characterized with respect to their kinetic properties, no information is available concerning their oligomeric structures and molecular weights. During purification of inulinase, the sucrose- and inulin-hydrolyzing activities are never separated. This has led to the conclusion that one enzyme is responsible for both activities, and that cell wall-associated activity and activity secreted into the culture fluid both represent inulinase, with identical kinetic properties (28,39). However, as to the nature of the inulinase two questions remain to be answered:

I. Do the cell wall associated inulinase and the inulinase present in the culture fluid differ in molecular weight, due to differences in oligomeric structure, like the invertase of *Saccharomyces*?

II. If *K. marxianus* produces only one enzyme, should this inulinase then be regarded as a special kind of invertase or as a hydrolase distinct from invertase but with an analogous mode of action?

-73-
CHAPTER 4

In order to answer these questions we have not only purified the inulinase associated with the cell wall (cell wall inulinase), but also that present in the culture fluid (supernatant inulinase) of K. marxianus, paying special attention to their oligomeric structures. In this report we also present the amino acid sequence of the amino-terminal end of inulinase.

MATERIALS AND METHODS

Organism and growth conditions. Kluyveromyces marxianus var. marxianus CBS 6556 was obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands, and maintained on YEPD agar slopes. YEPD contained per liter of distilled water: yeast extract (Difco Laboratories, Detroit, Mich.), 10 g; Bacto Peptone (Difco), 10 g; glucose, 20 g. The organism was grown under carbon and energy limitation in a laboratory fermenter (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter, on a mineral-salts medium supplemented with 5 g liter\(^{-1}\) sucrose and 2.5 g liter\(^{-1}\) inulin at a dilution rate of 0.15 h\(^{-1}\) at 40°C, pH 4.5, with an oxygen concentration of 50 - 70% of air saturation. Dissolved oxygen was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland) and pH was controlled by automatic addition of 1 M KOH. The mineral-salts medium was prepared according to Rouwenhorst et al. (31).

General conditions during purification. All steps were performed at room temperature, unless stated otherwise. Enzyme from each step of the purification procedure could be stored at either 4 or -20°C without any significant loss of activity (either with sucrose or inulin as the substrate). To remove any remaining whole cells, the crude inulinase preparations were membrane-filtered (0.22 μm; Schleicher & Schull, Dassel, Federal Republic of Germany) under mild pressure. Membrane filtration did not alter the enzyme activity nor the quaternary structure of the enzyme. When storage was required, the enzyme preparation was kept frozen.

Preparation of inulinase: 1. Supernatant inulinase. During steady state cultivation fifteen liters of culture effluent were collected at 4°C. Cells and culture fluid were separated by continuous-flow centrifugation (10,000 x g) with a Sorvall TZ-28 density gradient rotor (Du Pont Instruments, Biomedical Division, Newtown, Connecticut). Culture fluid was concentrated to 150 ml with a Nephross Andante hollow-fiber system (Organon Teknika, Oss,
The Netherlands) followed by removal of low molecular-weight contaminating protein with Centricon-30 microconcentrators (30 kDa cut-off; Amicon Corporation Scientific Systems Division, Danvers, Massachusetts). The concentrated preparation of extracellular inulinase from the culture fluid was taken as a source of supernatant inulinase.

Preparation of inulinase: ii. Cell wall inulinase. Release of cell wall-associated inulinase was induced by suspending the cells (0.2 g cell paste per ml) in 50 mM potassium phosphate, pH 6, containing 10 mM 2-mercaptoethanol, 10 mM dithiothreitol, and incubating for 2 h at 30°C with gentle agitation. The suspension was then centrifuged at 4°C (4,000 x g) and the inulinase containing supernatant was exhaustively dialyzed against 50 mM potassium phosphate (pH 6.5), and concentrated with Centricon-30 microconcentrators. The concentrated preparation of this inulinase formerly trapped in the cell wall will be referred to as cell wall inulinase.

To solubilize activity that was still associated with the cells after primary release of cell wall inulinase, the cells (0.2 g cell paste per ml) were suspended in 50 mM potassium phosphate (pH 6) containing 10 mM 2-mercaptoethanol, 0.1 M KCl, 0.1% Triton X-100, and incubated for 4 h at 30°C. After centrifugation (4,000 x g) at 4°C, the supernatant contained the newly released inulinase.

Preparation of inulinase: iii. Cell-bound inulinase. After removal of cell wall inulinase the cells still contained inulinase activity. This cell-bound activity could only be solubilized by complete breakage of the cells. Ultrasonic disintegration, performed as described previously (31), was used to collect this enzyme fraction.

Purification of inulinase: i. Anion-exchange chromatography. Supernatant inulinase (4 ml) and cell wall inulinase (4 ml) were loaded on a 60 ml DEAE-Sephadex A-50 column (2.5 x 50 cm) equilibrated with 20 mM potassium phosphate (pH 6.5). The column was washed with the same buffer and eluted with a 100 ml linear gradient of 0 to 500 mM sodium chloride in 20 mM potassium phosphate (pH 6.5). The flow rate throughout the experiment was 24 ml h⁻¹ and effluent was collected in fractions of 2.3 ml. The column fractions containing inulinase activity were collected as three pools, and the protein in the pools was concentrated with Centricon-30 microconcentrators.

Purification of inulinase: ii. Fast protein liquid chromatography. Further purification was performed on a small scale by means of high
performance gel filtration chromatography on a Superose 12 HR 10/30 prepacked column with a Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). Elution was carried out at room temperature with 500 mM potassium phosphate (pH 6) at a flow rate of 24 ml h⁻¹.

**Determination of molecular mass.** To estimate the molecular mass of the inulinases, the Sepharose 12 column was calibrated with standard proteins: carbonic anhydrase (Sigma Chemical Company, St. Louis, Missouri), 29 kDa; ovalbumin (Sigma), 43 kDa; phosphorylase (Pharmacia), 94 kDa; bovine albumin (Pharmacia), 67 kDa; lactate dehydrogenase (Sigma), 140 kDa; aldolase (Pharmacia), 158 kDa; catalase (Pharmacia), 232 kDa; ferritin (Pharmacia), 440 kDa; thyroglobulin (Pharmacia), 660 kDa.

**Removal of carbohydrate from inulinase.** Depletion of oligosaccharide chains from inulinase was accomplished by endo-\(N\)-acetyl-\(\beta\)-glucosaminidase H (Endo-H; EC 3.2.1.96) from *Streptococcus plicatus* (Boehringer GmbH, Mannheim, Federal Republic of Germany). Endo-H removes carbohydrate chains of the high-mannose type by cleaving the di-\(N\)-acetylchitobiose unit linked to asparagine (34). Purified inulinases (6.5 μg) were suspended in 100 μl of 50 mM sodium citrate (pH 5.5), 0.1% sodium dodecyl sulphate (SDS), 0.02% sodium azide, and heated for 5 min at 100°C. Endo-H (0.02 U) was added to the inulinases and the solutions incubated for 24 h at 30°C. Released oligosaccharides and Endo-H were removed by filtration of the reaction mixture with Centricon-30 microconcentrators (30 kDa cut-off).

**Gel electrophoresis: SDS-PAGE.** Slab gel electrophoresis on a discontinuous polyacrylamide gel (12 cm high, 12 cm wide, 1.5 mm thick) was performed according to Laemmli (22) in a 3 cm 3.25% stacking gel, and a 12.5% running gel. Samples were mixed in a 1:1 ratio (v/v) with a buffer containing 0.17 M Tris-HCl (pH 6.8), 3% SDS, 30% glycerol, and 10% 2-mercaptoethanol, and heated (10 min, 100°C) before application. Gels were stained for protein with Coomassie brilliant blue, and for carbohydrate using the periodic acid-Schiff (PAS) reagent, as described by Beeley (4). Apparent molecular masses were determined using commercially available marker proteins as standards (Pharmacia, Uppsala, Sweden).

**Gel electrophoresis: Non-denaturing PAGE.** Essentially the same method as with SDS-PAGE was used except that SDS was omitted from the running buffer and from the sample solution, and samples were not boiled before application. Inulinase activity in the gels was detected using a modification of the method described by Grossmann and Zimmermann (18). Gels
(7%) were incubated at room temperature in a solution of 0.1 M sodium acetate, pH 4.5, with 4% sucrose. After washing (three times 5 min) with distilled water, the gels were transferred to 100 ml 0.5 M NaOH, 1 mg ml\(^{-1}\) 2,3,5-triphenyl tetrazolium chloride (TTC), and heated over an open flame until coloration occurred. Staining was stopped by adding excess distilled water, followed by complete fixation of the gel in 10% acetic acid.

**Amino acid sequencing.** Purified protein was dialyzed against double-distilled water, precipitated with 9 volumes of acetone and resolubilized in water for amino acid sequencing. Sequence analysis was performed with a gas-phase sequenator (Applied Biosystems, model 470A) using 25% trifluoroacetic acid as the conversion reagent. The resulting phenylthiohydantoin (PTH) amino acids were analyzed on-line by reversed-phase high pressure liquid chromatography with a PTH C\(_{18}\) column (2.1 x 220 mm) and a PTH analyzer (Applied Biosystems, model 120A).

**Protein determination.** Protein was determined by the Lowry-Folin method (25), by the Bradford-Coomassie brilliant blue method (5), and by total carbon analysis (TOC). From 23 proteins with known amino acid composition (19) both the nitrogen content (16.12 ± 0.79) and the carbon content (53.27 ± 1.06) appeared very constant. Analogous to the representation of the protein concentration by the nitrogen content, the protein concentration of globular proteins can be obtained by determination of the carbon content. When the degree of glycosylation (G; fraction of molecular weight accounted for by carbohydrate) is known and assuming a carbon content of carbohydrates of 44%, the concentration of glycosylated protein (PG, ppm) can be calculated from the carbon content by:

\[
[\text{PG}] = \text{ppm C} \times (\frac{1}{0.53} \times (1 - G) \times \frac{1}{0.44} \times G)
\]

A Beckman Model 915B Total Organic Carbon Analyzer (Beckman Instruments, Inc., Fullerton, USA) was used to determine the carbon content of inulinase preparations. As a standard, a 2.137 g l\(^{-1}\) solution of anhydrous potassium biphthalate (1000 mg carbon liter\(^{-1}\)) was used.

**Inulinase assay.** Inulinase activity was measured as described previously (31). One unit of inulinase activity is defined as the amount of enzyme catalyzing the liberation of 1 \(\mu\)mol of fructose min\(^{-1}\) at pH 4.5 and 50° C.
CHAPTER 4

Chemicals. Fructose, sucrose and 2-mercaptoethanol were from Baker
Chemicals BV, Deventer, The Netherlands. Acrylamide, SDS, and N,N'-
methylbisacrylamide were from Merck, Darmstadt, Federal Republic of
Germany. Dithiothreitol and inulin (chicory root) were from Sigma Chemical
Co., St Louis, Missouri. Other chemicals were reagent grade.

RESULTS

Purification of supernatant inulinase and cell wall inulinase.

Preliminary experiments indicated that the inulinase of K. marxianus could
not be precipitated conveniently by ammonium sulphate. Inulinase present in
the culture fluid was therefore concentrated using a hollow-fiber device.

1. Anion exchange chromatography. In two successive runs, performed under
exactly the same conditions, 9,400 units of the crude preparation of
supernatant inulinase and 10,350 units of the crude preparation of cell wall
inulinase were loaded on a Sephadex A-50 column, equilibrated with 20 mM
potassium phosphate, pH 6.5, and eluted with a linear NaCl gradient.
Supernatant inulinase eluted at 0.2 M NaCl (Fig. 1A) but the peak coincided
with a second, contaminating protein peak. In contrast, Sephadex A-50
gradient elution of cell wall inulinase provided a rapid purification. Most
of the contaminating protein did not bind to the column and was eluted
before NaCl gradient elution was initiated. The protein peak containing
inulinase activity eluted at an NaCl molarity of 0.15 M (Fig. 1B).

The total enzyme activities recovered after Sephadex A-50 gradient
elution, both of supernatant inulinase and cell wall inulinase, were higher
(107% and 129% recovery, respectively) than the total activity originally
loaded on the column. Apparently, the anion exchange chromatography has led
to removal of constituents of either the medium or the cells that were
inhibitory to inulinase. The S/I ratios of both the crude preparations of
supernatant inulinase and cell wall inulinase were 13. Inulinase obtained
from Sephadex A-50 fractions had S/I ratios of 13 ± 2, and this value
appeared constant throughout the Sephadex A-50 inulinase peaks, although the
beginning and the end of these peaks the S/I ratios showed greater
deviations. This was probably due to a low inulinase concentration resulting
in inulin-hydrolyzing activities which were at the limits of the assay (data
not shown).

-78-
FIG. 1. Chromatography of inulinase preparations on DEAE Sephadex A-50. Crude preparations of supernatant inulinase (A) and cell wall inulinase (B), were applied to the column equilibrated with 20 mM potassium phosphate buffer (pH 6.5) and eluted with a 0 to 0.5 M linear gradient of NaCl. Fractions (2.3 ml) were collected, scanned for absorbance at 280 nm, and assayed for sucrose-hydrolyzing activity (●). Active peak fractions of supernatant inulinase and cell wall inulinase were separated into six pools as indicated.
FIG. 2. FPLC gel filtration of the pooled DEAE Sephadex A-50 chromatography fractions of supernatant inulinase (A) and cell wall inulinase (B). The pooled inulinase activities I, II, III (supernatant inulinase), and IV, V, VI (cell wall inulinase) were applied to a Superose 12 HR 10/30 gel filtration column, equilibrated with 0.5 M potassium phosphate buffer (pH 6). Chromatography was performed at a flow rate of 24 ml h⁻¹. Inulinase (●) is given as sucrose-hydrolyzing activity.
ii. Fast protein liquid chromatography. The Sephadex A-50 inulinase activities were collected as six pools, marked I, II, III (supernatant inulinase), and IV, V, VI (cell wall inulinase). These pooled activities were concentrated by Amicon-30 ultrafiltrators, and a portion of each pool was applied separately to an FPLC gel filtration system. The FPLC elution profiles of pools I, II, and III of the supernatant inulinase all showed two major protein peaks, of which only one corresponded with an inulinase activity peak (Fig. 2a). The inulinase pools IV, V, and VI of cell wall inulinase appeared to be almost pure. The FPLC elution profiles all showed one major protein peak, corresponding with inulinase activity (Fig. 2b). The inulinses purified by FPLC gel filtration were active towards sucrose as well as inulin, in a ratio of 15 ± 2 (results not shown). When compared with protein markers of known molecular weight, the supernatant inulinase eluted as a peak at approximately 180 kDa, and the cell wall inulinase eluted as a

![Graph](image-url)
peak at approximately 450 kDa (Fig. 3). However, glycoproteins tend to elute earlier from gel filtration columns than globular marker proteins, and hence these apparent molecular masses are likely to be overestimations of the real values (4).

Analytical gel electrophoresis. The isolated peak fractions from FPLC gel filtration of supernatant inulinase and cell wall inulinase were concentrated and analyzed by both SDS-PAGE and non-denaturing PAGE, before and after treatment with Endo-H. The protein in the FPLC fractions of cell wall inulinase and the protein in the FPLC fractions of supernatant inulinase both migrated on SDS-PAGE as a polydisperse band between 87 and 102 kDa (Fig. 4; lanes 1,5). No other protein bands were detected on the SDS-PAGE gels, either with Coomassie brilliant blue or with the more sensitive silver staining, indicating that the enzyme preparations only contained inulinase protein. The polydispersity of the inulinase bands is apparently caused by heterogeneity in the size of the polysaccharide chains attached to the inulinase polypeptide. To remove these carbohydrate chains, intact supernatant inulinase and cell wall inulinase were treated with Endo-H. Polyacrylamide gel electrophoresis with SDS of the Endo-H treated inulinases gave more distinct protein bands, with apparent molecular masses of about 72 kDa (Fig. 4, lanes 2,3). Deglycosylation was verified by staining the gel for carbohydrate. The Endo-H treated inulinase stained with Schiff-Pas, indicating that it still contained carbohydrate. Deglycosylation experiments with Saccharomyces inverase have shown that the susceptibility of oligosaccharides in the native glycoprotein is enhanced by its denaturation (35). Inulinase samples were therefore boiled in 0.1% SDS before addition of Endo-H. Depletion of the oligosaccharide chains after denaturation of the inulinases resulted in a sharper resolution of the inulinases. The deglycosylated protein backbones of cell wall inulinase and supernatant inulinase now migrated as one band with a uniform size of 64 kDa (Fig. 4; Lanes 4,6). Thus, the sizes of the monomeric peptide chains of supernatant- and cell wall inulinase are the same. The denatured and Endo-H treated inulinase failed to stain with Schiff-Pas. Apparently, all outer-chain carbohydrate had now been removed from the inulinases. The results of the SDS-PAGE with non-treated and Endo-H treated inulinase clearly show that intact inulinase monomer contains 26 to 37% of its mass as high-mannose type oligosaccharides linked to asparagine residues on a 64 kDa polypeptide. The
FIG. 4. SDS-Polyacrylamide gel electrophoresis of native and carbohydrate-depleted inulinases. Supernatant inulinase (128 μg ml⁻¹), cell wall inulinase (135 μg ml⁻¹), and Endo-H treated supernatant and cell wall inulinases (65 μg ml⁻¹ each) were mixed with an equal volume of sampling buffer and heated at 100°C for 5 min. Aliquots of 50 μl were then applied to a 12.5% discontinuous SDS-polyacrylamide gel system. Lanes 1,2,4; cell wall inulinase, lanes 3,5,6; supernatant inulinase. Treatment with Endo-H was performed with native (lanes 2,3) and denatured inulinases (lanes 4, 6). Molecular mass standards: phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase.

Native enzymes from culture fluid and cell wall should then represent glycosylated inulinase multimers. To establish the relationship between supernatant inulinase and cell wall inulinase, Ferguson analysis of the purified enzymes was carried out, using non-denaturing gels of different polyacrylamide concentrations and determining the migration distances of the inulinases relative to those of proteins with known molecular weight, as described by Esmen et al. (13). It appeared that the supernatant inulinase could correspond to an inulinase dimer of approximately 165 kDa, and the cell wall inulinase to a tetramer of approximately 335 kDa.

Release of cell wall inulinase by different treatments. A comparison of the initial inulinase preparations and the purified inulinases, with respect to their behavior on non-denaturing PAGE with staining for sucrose
CHAPTER 4

hydrolyzing activity, showed that the purification procedure had not altered the composition of either the supernatant inulinase or the cell wall inulinase (Fig. 5; lanes 1 and 2; lanes 3 and 4). Inulinase treated with SDS showed no sucrose-hydrolyzing activity upon native PAGE.

![Image of gel electrophoresis](image)

FIG. 5. Non-denaturing polyacrylamide gel electrophoresis of crude and purified inulinase preparations. Samples were subjected to non-denaturing PAGE on a 10% polyacrylamide gel and the gel was stained for sucrose-hydrolyzing activity. Lane 1; crude cell wall inulinase, lane 2; purified cell wall inulinase, lane 3; crude supernatant inulinase, lane 4; purified supernatant inulinase.

To substantiate that the use of sulfhydryl compounds as inulinase releasing agents did not effect the multimeric structure of the cell wall inulinase, inulinase released by incubating cells for 24 h at 4 °C in 50 mM potassium phosphate (pH 6) was purified. After Sephadex A-50 elution and subsequent FPLC gel filtration, the preparation of cell wall inulinase obtained showed profiles and molecular weights (as determined by SDS-PAGE) which did not differ from those observed for the cell wall inulinase released by thiols. Non-denaturing PAGE of crude preparations of cell wall inulinase further confirmed that the mode of enzyme release did not affect the structure of native inulinases (Fig. 6; lane 2).

The use of enzyme release buffer did not result in a complete removal of inulinase from the cells: approximately 15% of the total inulinase produced
by K. marxianus still remained attached to the cells. Incubation of these cells in a solution containing 2-mercaptoethanol, Triton X-100, and KCl, resulted in a further release of inulinase. However, after this treatment cells still contained 3% of the total inulinase produced. This residual enzyme was solubilized by sonic disintegration of the cells. On non-denaturing PAGE, the inulinase in these preparations showed the same mobility as the cell wall inulinase that was removed from the cell wall by the enzyme release buffer (Fig. 6; lanes 3,4,5). The sucrose-hydrolyzing

FIG. 6. Non-denaturing PAGE of inulinase solubilized from the cells by various, consecutive treatments. Cells were suspended in 50 mM potassium phosphate buffer (pH 6) for 24 h at 4°C (lane 2) or in enzyme release buffer for 2 h at 30°C (lane 3). Inulinase still associated with the cells after treatment with enzyme release buffer was in part solubilized by incubating the cells in buffer containing 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 0.1 M KCl for 4 h at 30°C (lane 4), and in part by sonication (lane 5) of the cells. Commercially available S. cerevisiae invertase (lane 1) and inulinases released by each treatment were applied to a 7% non-denaturing polyacrylamide gel.
activity was verified by running commercially available (Boehringer Mannheim) extracellular invertase of S. cerevisiae (molecular mass of about 800 kDa) on the same gel (Fig. 6; lane 1).

**Kinetic constants of purified supernatant- and cell wall inulinases.** The apparent affinity constants \( K'_m \) of inulinase for sucrose and raffinose appeared independent of the degree of oligomerization. Both the supernatant (dimeric) inulinase and the cell wall (tetrameric) inulinase showed \( K'_m \) values of 14.6 mM and 5.5 mM with sucrose and raffinose, respectively (Table 1). No \( K'_m \) value with chicory inulin could be calculated, as had also been observed for crude inulinase preparations (31). Because of the low solubility of this fructose polymer, the maximal inulin concentration that can be achieved is well below the apparent affinity constant.

A major problem in determining specific enzyme activities can be the establishment of protein concentrations. This is especially true in the case of chromophoric proteins and glycoproteins. As a result, large differences between observed and true protein concentrations may occur. Colorimetric protein determinations include peptide-specific reactions, as in the reduction of Folin-Ciocalteu reagent by tryptophan and tyrosine residues in the Lowry method or dye-binding properties in the Bradford method. The response of these methods depends both on the nature of the polypeptide and on the presence of either chromophoric groups or carbohydrate chains, and may vary with the amount of protein. When using these methods, standardization with proteins of comparable properties is a necessity. On the other hand, determination of protein concentrations on the basis of the carbon content is independent of peptide composition or presence of chromophoric groups, and the method need not be standardized on other proteins. To convert the measured carbon content of a purified glycoprotein preparation into the protein content, only the degree of glycosylation should be known (See Methods Section).

As expected the values obtained for the specific inulinase activities strongly depended on the method employed to determine protein concentrations in the inulinase solutions. Significant differences in the obtained specific activities were observed between the Lowry method, the Bradford method and the TOC method. The Lowry method tended to give lower values than TOC calculated activities, whereas the Bradford gave higher values. With all three methods, the specific activities of the inulinase dimer were higher than those of the inulinase tetramer. Assuming a degree of glycosylation of
TABLE 1. Apparent affinity constants and specific activities of supernatant (dimer) inulinase and cell wall (tetramer) inulinase of *K. marxianus* CS 6556 for three substrates with different degrees of polymerization. Protein concentrations were determined with the Lowry-Folin Ciocalteu method, the Bradford-Coomassie Brilliant blue method, and by measuring the carbon content in the inulinase solutions with total organic carbon analysis (TOC).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inulinase</th>
<th>$K'_m$</th>
<th>Specific inulinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lowry$^a$ Bradford$^a$ TOC$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mM) (U mg protein$^{-1}$) (U mg inulinase$^{-1}$)</td>
</tr>
<tr>
<td>Sucrose dimer</td>
<td>14.6</td>
<td>923</td>
<td>3331</td>
</tr>
<tr>
<td>Sucrose tetramer</td>
<td>14.7</td>
<td>806</td>
<td>2069</td>
</tr>
<tr>
<td>Raffinose dimer</td>
<td>5.6</td>
<td>312</td>
<td>1126</td>
</tr>
<tr>
<td>Raffinose tetramer</td>
<td>5.3</td>
<td>261</td>
<td>670</td>
</tr>
<tr>
<td>Inulin$^c$ dimer</td>
<td>-</td>
<td>59</td>
<td>212</td>
</tr>
<tr>
<td>Inulin tetramer</td>
<td>-</td>
<td>51</td>
<td>132</td>
</tr>
</tbody>
</table>

$^a$ Bovine serum albumin was used as a standard.
$^b$ For calculation of the concentrations of inulinase protein and of native glycosylated inulinase a glycosylation of 26% was used.
$^c$ Specific inulin hydrolyzing activities were measured at a substrate concentration below enzyme saturation with 1.6% (w/v) inulin.

26%, the specific sucrose-hydrolyzing activities of dimeric and tetrameric inulinase were approximately 1840 and 610 U mg of inulinase protein$^{-1}$, or 1370 and 460 U mg of glycosylated protein$^{-1}$, respectively (Table 1). The maximal concentration of chicory inulin that could conveniently be used for measuring the velocity of inulin hydrolysis was 2% (w/v). At this concentration, the activities of dimeric inulinase and tetrameric inulinase
were 119 and 55 U mg of inulinase protein⁻¹, or 88 and 40 U mg inulinase⁻¹, respectively. The ratio of the activities of pure inulinase with sucrose and chicory inulin, determined at pH 4.5 and 50°C, at a saturating sucrose concentration and at 2% inulin, respectively, was about 16. This value for the S/I ratio is consistent with that of crude inulinase preparations (31).

The inulinases had an absorption maximum at 280 nm. Specific absorption coefficients were calculated from the TOC-analyzed inulinase concentrations and the absorption of inulinase dilutions at 280 nm. These coefficients for solutions of 1 mg/ml (A₂₈₀₀.₁%) were 1.03 ± 0.01 and 1.00 ± 0.01 for the inulinase dimer and tetramer, respectively.

N-terminal amino acid sequence of inulinase. In order to clarify the relationship among the inulinase of K. marxianus CBS 6556 and the invertase of S. cerevisiae, the amino acid sequence of the amino-terminal end of inulinase was analyzed, and compared with the amino-terminal sequence of invertase as obtained by Carlson et al. (7) (Fig. 7). Apart from the serine residue at position 1, of the twenty amino acids determined no two amino acids aligned between the amino-terminal sequence of inulinase and invertase. Thus, there was no homology in amino acid sequence of the amino-terminal ends of inulinase and invertase. Partial amino-terminal sequence analysis of both the purified supernatant-inulinase and the purified cell

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<tr>
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<th>1</th>
<th>20</th>
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<tbody>
<tr>
<td></td>
<td>Ser - Gly - Asp - Ser - Lys - Ala - Ile - Thr - ? - Thr -</td>
<td>Thr - Phe - ? - Leu - Aan - Arg - Pro - Ser - Val - Tyr -</td>
</tr>
<tr>
<td></td>
<td>Ser - Met - Thr - Aan - Glu - Thr - Ser - Aas - Arg - Pro -</td>
<td>Leu - Val - His - Phe - Thr - Pro - Aan - Lys - Gly - Trp -</td>
</tr>
</tbody>
</table>

FIG. 7. Amino acid sequences of the amino-terminal ends of the inulinase polypeptide of K. marxianus CBS 6556 and the invertase polypeptide of S. cerevisiae. The amino terminal sequence of invertase was obtained from Carlson et al. (7).
CHAPTER 4

Wall inulinase was conducted. The amino-acid sequence of the first twenty amino acid residues of supernatant-inulinase and cell wall inulinase were identical.

DISCUSSION

Structure of the extracellular inulinase of K. marxianus. In the present paper we have presented evidence showing that the inulinase secreted into the culture fluid and the inulinase retained in the cell wall of K. marxianus CBS 6556 have similar subunits, consisting of a 64 kDa polypeptide that contains 26-37% of the molecular mass as carbohydrate. The two inulinase forms differ in size due to differences in subunit aggregation.

Inulinase monomer may contain varying amounts of carbohydrate, as suggested by the diffuse protein bands that ranged from 87-102 kDa on SDS-PAGE (Fig. 4). This outer-chain carbohydrate can be removed by treatment of denatured inulinase with Endo-H, indicating that it is present as high-mannose oligosaccharide chains linked to asparagine residues of the polypeptide chain. The polydisperse behavior of glycoproteins on SDS-PAGE has also been observed with yeast invertase (9,34). This dispersity appeared to be an effect of different lengths of the carbohydrate chains, and not of differences in number of carbohydrate chains attached to the invertase polypeptide (9).

During the first step of purification of the intact inulinases with anion exchange chromatography, it was obvious that the supernatant inulinase and the cell wall inulinase differed (Fig. 1). Cell wall inulinase eluted from the column at a lower sodium chloride concentration than supernatant inulinase. Such chromatographic behavior can be caused by a difference in the amount of carbohydrate attached, or by a difference in the quaternary structure that masks the DEAE-binding properties of the polypeptide moieties (35). Non-denaturing PAGE and FPLC gel filtration demonstrated that the inulinases differ in size, but the anomalous behavior of glycoproteins in electrophoresis and in gel filtration makes it difficult to obtain accurate molecular weights. Ferguson analysis and analytical gel filtration gave estimated values of 165 - 180 KDa and 335 - 450 KDa for the supernatant and cell wall inulinase, respectively (Fig. 3). These values coincide with a dimer structure of the purified supernatant enzyme and a tetrameric
structure of the purified cell wall enzyme. During purification the structure of the inulinases had not altered. Neither did the use of 2-mercaptoethanol, in the initial step of inulinase release from the cell wall, influence the oligomeric structure of cell wall inulinase. Release of cell wall inulinase by potassium phosphate alone led to solubilization of tetrameric inulinase. Moreover, inulinase that could only be solubilized by incubating the cells in a buffer containing KCl, Triton X-100 and 2-mercaptoethanol or by cell disruption, showed the same electrophoretic behavior on non-denaturing PAGE as purified cell wall enzyme (Fig. 5 and 6). Apparently, the inulinase tetramer is the largest inulinase aggregate present in K. marxianus CBS 6556. The absence of extracellular inulinase monomer as well as activity with any peak smaller than a dimer, as determined by gel filtration, precludes the recognition of activity with the monomeric form of inulinase. Indeed, during purification of supernatant inulinase with FPLC gel filtration we observed a contaminating protein with an apparent molecular weight of 72 kDa. Although no activity could be measured, this protein could represent inulinase monomer formed by dissociation of multimer inulinase due to shear force and heat production during concentration with the hollow fiber device.

Among the different yeast inulinases reported, only a few have been purified and no information is available on their subunit structure. Workman and Day (39) purified cell wall inulinase of K. fragilis ATCC 12424 and stated that this enzyme was a glycoprotein containing 66% carbohydrate, a percentage exceeding by far the value reported here. Unfortunately, Workman and Day (39) did not mention the molecular weight of their purified inulinase in which they estimated the percentage of carbohydrate. By Uhm et al. (36), a molecular weight of 250 KDa was cursorily mentioned for K. marxianus inulinase. More information is available on the non-specific exo-inulinases of fungi. The molecular weights of inulinase subunits determined with SDS-PAGE were found to be 74 kDa for Aspergillus ficiuum (14), 81 KDa for Aspergillus spec. (3), 85 kDa for A. niger (36), 70 and 84 kDa for Chrysosporium parvum (40), 60 and 80 kDa for Penicillium sp.1 (27), and all contained 20-40% carbohydrate. Moreover, native inulinase of A. niger appeared to be a tetramer with a molecular weight of approximately 315 kDa (36), as determined by analytical gel filtration. The molecular weights and the degrees of glycosylation of these fungal inulinases are in the same range as those of the extracellular inulinases of K. marxianus.
CHAPTER 4

Kinetic properties of the extracellular inulinases of *K. marxianus*. Inulinase dimer and tetramer had identical apparent affinity constants and specific absorption coefficients at 280 nm, but the specific enzyme activities were different. Recently, we reported on the kinetic parameters of non-purified inulinase preparations (31). The apparent affinity constants ($K'_m$) of inulinase dimer and inulinase tetramer with sucrose (both 14.6 mM) had not altered during purification. However, a $K'_m$ of 5.6 mM with raffinose of the purified inulinase differs from the 8.2 mM observed for non-purified inulinase. The reason for these slightly different $K'_m$ values is not known (31).

The specific enzyme activity of inulinase dimer was consistently higher than the specific enzyme activity of inulinase tetramer. Apparently, aggregation leads to negative cooperativity for enzyme activity. For the *S. cerevisiae* invertase no such difference between specific enzyme activity of invertase dimer and specific enzyme activity of invertase octamer had been observed (13). The specific enzyme activities of purified inulinase were markedly lower than those reported for crude inulinases (31). The latter activities were calculated with protein concentrations determined with the Bradford protein assay. As compared to the TOC-carbon content method, the Bradford protein assay underestimates the actual protein content 2.5 to 3-fold. As a result, the specific inulinase activities will be too high.

Invertase vs inulinase. Apart from the S/I ratio and the apparent affinity constants, further differences between yeast invertase and yeast inulinase are encountered when enzyme structures are compared: (1). A 62 KDa polypeptide backbone as observed for Endo-H treated extracellular invertase (9,35) is slightly smaller than that of the inulinase reported here. (2). The carbohydrate attached to the polypeptide of invertase accounts for 50% of the molecular mass of invertase (35), while in inulinase the carbohydrate content is only around 30%. This difference could reflect a smaller number of glycosylation sites in the inulinase polypeptide, and thus a dissimilarity in polypeptide structures of the two proteins. Another finding that suggests different polypeptide structures is the low homology in amino acid sequences at the amino-terminal ends of inulinase and invertase.

Preliminary experiments using oligonucleotides derived from the amino acid sequence of the amino-terminal end of *K. marxianus* CBS 6556 inulinase, did not result in any detectable hybridization with the genome of various *Saccharomyces* yeasts (J. Verbakel, Unilever Research, Vlaardingen; personal

-91-
CHAPTER 4

communication). The SUC gene family has been reported to be a highly conserved sequence in *Saccharomyces* yeast (6). Strong homology has been demonstrated even between the SUC2 gene of *S. cerevisiae* and the invertase genes of the mold * Neurospora crassa* (8) and the bacterium *Bacillus subtilis* (26). Remarkably, the *S. cerevisiae* SUC2 sequence hardly hybridized with the genome of the yeast *S. kluveri* UCD 51-242 (6). Further research into the nature of the sucrose hydrolyzing activity of *S. kluveri* showed that this yeast possesses an inulinase, as defined by S/I ratio and behavior on native gel electrophoresis (32). The sequence of the inulinase gene should eventually confirm or renounce the conclusion that inulinase is not a specific kind of invertase, but a second kind of β-fructosidase with a similar mode of action as invertase.

Retention of glycoproteins within the periplasmic space of the cell wall of yeasts is thought to be caused by a permeability barrier in the outer regions of the cell wall (13,20). Oligomerization of glycoproteins may then play a role in this cell wall retention. Almost all of the invertase produced by *S. cerevisiae* is ultimately retained in the cell wall as an octameric complex of four invertase dimers. Invertase that is completely secreted into the culture fluid and invertase that can be released by use of sulfhydryl compounds are invertase dimers (13). In contrast to the *S. cerevisiae* invertase, both the cell wall-retained inulinase and the inulinase released from the cell wall of *K. marxianus* by sulfhydryl-treatment are tetramers. This is probably a consequence of differences between the two yeasts in susceptibility of their cell walls to sulfhydryls. The cell wall of *K. marxianus* may be less rigid than the cell wall of *S. cerevisiae* and hence treatment with sulfhydryl compounds might result in a more pronounced elimination of the permeability barrier (10, 38).

Various treatments such as shear force, heat, low-ionic strength buffer, and sonic disintegration of the cells, have been reported to cause disintegration of the *S. cerevisiae* invertase octamer, resulting in appearance of dimeric invertase without any change in kinetic parameters (9, 13). We did not observe a decay of inulinase tetramer into dimers during purification. On the contrary, we could induce tetramer decay only by the use of enzyme-denaturing agents such as sodium dodecyl sulphate or guanidine hydrochloride (results not shown). The tetramer inulinase thus appears to be a very stable form. However, we can not exclude the possibility that
supernatant inulinase is actually a tetramer that is dissociated during concentration of culture fluid by the use of a hollow fiber device.

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Chapter 5

Protein Determination by Total Organic Carbon Analysis.

R.J. Rouwenhorst, J. Frank, Jzn, W.A. Scheffers, and J.P. van Dijken.

Submitted to Analytical Biochemistry.
CHAPTER 5

SUMMARY

Determination of the carbon concentration in protein solutions by total organic carbon analysis was found to be a sensitive and reliable method for the estimation of protein concentrations. Using a carbon content of 0.53 g/g in protein and of 0.44 g/g in carbohydrate, the concentrations of normal proteins, proteins containing chromophoric groups, and proteins containing carbohydrate could be established. The method appeared to be independent of the nature of the protein and showed complete linearity (25 to 1,000 mg/l) when protein was serially diluted. Determination of specific absorption coefficients by measuring the absorbance of protein solutions at 280 nm and measuring their carbon concentration gave values that, on the average, coincided within 12% with values reported in the literature. The method may have special applicability in protein purification studies, since it does not require knowledge of molar extinction coefficients beforehand, and it monitors the disappearance of carbon compounds other than protein.

INTRODUCTION

Determination of protein concentrations in purification schemes should be accomplished in a fast, and simple manner without loss of large amounts of protein. As most purification procedures end up with small amounts of protein, concentrations are usually determined by spectrophotometric, chromogenic, dye-binding or fluorometric methods (1,2). Chromogenic methods, like the Folin phenol of Lowry et al. (3) or the Coomassie dye-binding (4) and fluorometric techniques (5), are suitable in view of the low amount of protein required. However, these methods show a wide variation in their response to different protein species and may give unacceptable errors whenever calibration is performed with a structurally unrelated, and thus inappropriate, standard protein.

A most accurate and sensitive method for estimation of protein concentration is determination of nitrogen content by the Kjeldahl method (6), since proteins contain a relatively constant percentage of nitrogen. However, this method is time consuming and needs technical skill and experience (1,6).
CHAPTER 5

We recently purified two forms of the glycoprotein inulinase, a dimer and a tetramer, of the yeast Kluyveromyces marxianus and we were unable to obtain consistent values for their protein concentrations and the molar absorption coefficient by chromogenic or dye-binding methods. The average carbon content of amino acids (0.46 g/g) compares favorably with the average nitrogen content (0.15 g/g) and, more importantly, the coefficient of variation is lower (28% vs 42%). Total organic carbon analysis by rapid combustion of organic matter and measurement of the carbon dioxide formed by infrared analysis, has proven to be a helpful tool in the measurement of water pollution (7,8) and in the determination of microbial cell concentrations (9). However, as far as we are aware, it has not been applied to the determination of protein. Since total organic carbon analysis is simple, rapid, and sensitive, we have explored the potential of this method as an attractive alternative to the Kjeldahl nitrogen determination.

MATERIALS AND METHODS

Materials. Proteins were from the highest grade available and tested for purity with a fast protein liquid chromatography system and a Superose 12 gel permeation column (Pharmacia, Uppsala, Sweden). Albumin (bovine serum), aldolase (rabbit muscle), catalase (bovine liver), and ferritin (horse spleen) were from Sigma Chemical Co., St. Louis, Missouri, invertase (yeast), and glucose oxidase (Aspergillus niger) were from Boehringer GmbH, Mannheim, Federal Republic of Germany. Purified glucose dehydrogenase of Acinetobacter calcoaceticus was a kind gift of B. Groen, Department of Microbiology and Enzymology, Delft University of Technology, The Netherlands. Inulinase dimer and inulinase tetramer of the yeast Kluyveromyces marxianus were recently purified in our laboratory (unpublished result). Potassium biphthalate and 2-mercaptoethanol were purchased from Merck, Darmstadt, Federal Republic of Germany.

Preparation of protein solutions. Commercially obtained proteins were dissolved in 0.025 M potassium phosphate, pH 4.5, and exhaustively dialyzed against the same buffer to remove carbon-containing low molecular-weight contaminants. Glucose dehydrogenase and inulinase were obtained, after purification, as solutions of protein in 0.05 M potassium phosphate, pH 6.5.
CHAPTER 5

Resulting protein solutions were serially diluted with 0.025 M potassium phosphate, pH 4.5. Dilution factors were determined by weighing.

Total organic carbon analysis (TOC). A combustion type total organic carbon computational system (Tocamaster model 915B, Beckman Industrial, Fullerton, California) was used for measurement of the carbon content in the protein solutions. As the standard, a 2.137 g/l solution of anhydrous potassium biphthalate (1,000 mg C/l) in carbon-free water was used. Carbon-free water was obtained by extensively boiling de-ionized water that had been acidified (pH < 2) by addition of phosphoric acid. Analysis of carbon contents with the Beckman 15B TOC analyzer can conveniently be performed in the range of 2-4,000 mg C/l (4-7,500 mg protein/l). The determination requires 10-50 μl of sample and takes about two minutes. To establish the carbon contents in protein solutions, a calibration curve for 0-500 mg C/l was used, in order to determine the performance of TOC with protein concentrations up to 1 mg/ml. Of serial dilutions of carbon standards (range 0-500 mg C/l) and proteins, 20 μl were applied in quadruplicate to the combustion column with a Hamilton CR-700-50 pressure syringe (Hamilton Co., Reno, Nevada). Presence of inorganic carbon in the samples was checked by acidifying part of the sample (25 μl phosphoric acid per ml) and sparging with carrier gas (21% O₂, 79% N₂, free from CO₂). The resulting carbon dioxide-free sample was analyzed and compared with the non-acidified samples. Use of phosphate buffer, pH 4.5, to dilute protein solutions, appeared sufficient to remove all dissolved carbon dioxide.

Standard protein assays. The Coomassie Brilliant Blue protein micro-assay was as described by Bradford (4). The Folin phenol protein method was as described by Lowry et al. (3) with the absorbance measured at 660 nm. In both assays, 50 μl of protein dilutions were used to construct the standard curve for each protein. Absorbances were measured with a Vitalab 20 spectrophotometer (Vital Scientific, Dieren, The Netherlands).

Spectrophotometric measurements at 280 nm were made on a Hitachi model 100-60 double beam spectrophotometer (Hitachi Ltd., Tokyo, Japan). Readings were made in 1 cm light path quartz cuvettes of 1 ml capacity. Specific absorption coefficients (A0.1%) are expressed as the absorption of a protein solution of 1 mg/ml in a 1 cm light path at 280 nm.
CHAPTER 5

RESULTS AND DISCUSSION

Carbon content of proteins and measurement with TOC. Determination of protein concentrations by Kjeldahl nitrogen is based on the more or less constant nitrogen content in proteins. To see whether the amount of carbon

TABLE 1. Carbon and nitrogen contents of 20 proteins. Contents were calculated from the amino acid compositions in ref. (12).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular wt</th>
<th>C content</th>
<th>N content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/g</td>
<td>g/g</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>65,238</td>
<td>0.528</td>
<td>0.163</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>30,310</td>
<td>0.540</td>
<td>0.169</td>
</tr>
<tr>
<td>Casein</td>
<td>23,791</td>
<td>0.553</td>
<td>0.161</td>
</tr>
<tr>
<td>Chymotrypsinogen B</td>
<td>26,216</td>
<td>0.537</td>
<td>0.157</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>79,714</td>
<td>0.531</td>
<td>0.175</td>
</tr>
<tr>
<td>Deoxyribonuclease A</td>
<td>29,830</td>
<td>0.536</td>
<td>0.153</td>
</tr>
<tr>
<td>Enolase</td>
<td>105,767</td>
<td>0.527</td>
<td>0.161</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>89,499</td>
<td>0.497</td>
<td>0.145</td>
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<tr>
<td>β-Galactosidase</td>
<td>131,477</td>
<td>0.533</td>
<td>0.162</td>
</tr>
<tr>
<td>GAP-3-P-dehydrogenase</td>
<td>137,309</td>
<td>0.531</td>
<td>0.161</td>
</tr>
<tr>
<td>Glycerol-3-P-dehydrogenase</td>
<td>30,133</td>
<td>0.536</td>
<td>0.157</td>
</tr>
<tr>
<td>Invertase (dimer)</td>
<td>173,943</td>
<td>0.535</td>
<td>0.154</td>
</tr>
<tr>
<td>Methionyl-tRNA synthetase</td>
<td>173,967</td>
<td>0.538</td>
<td>0.159</td>
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<tr>
<td>Penicillinase</td>
<td>30,310</td>
<td>0.534</td>
<td>0.171</td>
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<td>Pro lactin</td>
<td>22,574</td>
<td>0.527</td>
<td>0.160</td>
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<td>Rhodopsin</td>
<td>27,729</td>
<td>0.542</td>
<td>0.146</td>
</tr>
<tr>
<td>D-Serine dehydratase</td>
<td>45,668</td>
<td>0.533</td>
<td>0.159</td>
</tr>
<tr>
<td>Thrombin</td>
<td>38,482</td>
<td>0.537</td>
<td>0.168</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>582,478</td>
<td>0.528</td>
<td>0.171</td>
</tr>
<tr>
<td>Urease</td>
<td>75,463</td>
<td>0.528</td>
<td>0.171</td>
</tr>
</tbody>
</table>

Mean value 0.532 0.161
Standard deviation ±0.010 ±0.008
Coefficient of variation 1.879 4.968
in proteins is equally invariable, the carbon and nitrogen contents of 20, randomly chosen, proteins with known amino acid constitution were determined (Table 1). The mean values were 0.53 g C and 0.16 g N per gram of protein. The carbon content of these 20 proteins appeared to be less variable than the nitrogen content, as predicted by the smaller variation in carbon content of the individual amino acids. The relative coefficients of variation of carbon and nitrogen were 2 and 5%, respectively (Table 1). Given this very constant amount of carbon in proteins, it was expected that the use of TOC analysis of protein solutions could be a possible way of establishing protein concentrations.

FIG. 1. Relation between BSA concentration and TOC-analyzed carbon content. Protein was dissolved in either (□) carbon-free potassium phosphate, pH 4.5, (R = 0.9996, a₀ = 0.69, a₁ = 0.529), or (●) potassium phosphate, pH 4.5, with 2-mercaptoethanol (R = 0.999, a₀ = 237, a₁ = 0.51). Each protein dilution was analyzed in quadruplicate and deviations in carbon contents are visualized by heights of the markers.

Use of a carbon-containing buffer to solubilize or stabilize protein could affect the precision of the method. Dissolving BSA in a 10 mM solution
of 2-mercaptoethanol in phosphate buffer and measuring the carbon content, also gave a straight line with a slope of 0.51 g C/g protein (Fig. 1), which deviates less than 3% from the theoretical value. The line is shifted upwards over 237 mg C/l (=19.7 mM buffer carbon), in agreement with the expected value of 240 mg C/l due to the presence of 2-mercaptoethanol. Therefore, in the presence of 19.7 mM buffer carbon a protein concentration as low as 46 mg/l (24.4 mg C/l) could reliably be determined. In general, the protein-carbon concentration should exceed the variation in TOC measurements of 1-2%. Measurement of protein-carbon in buffers that are frequently employed in protein purifications, like acetate or citrate (50 mM equals 1,200 and 2,400 mg C/l, respectively), thus require higher, but still moderate, protein contents, as well as the use of more extended calibration curves.

Carbon content of proteins containing carbohydrates. The carbon content of 0.53 g/g found only holds when it concerns proteins that do not contain carbohydrate residues. Carbohydrate carbon contributes to the total carbon of glycosylated proteins. The amount of carbon in carbohydrates increases from 0.4 g/g for monosaccharides, to 0.435 g/g for pentasaccharides, and 0.445 g/g for a polymer with a chain length of 500 monosaccharide residues. As most carbohydrate chains of glycoproteins contain at least 5 residues (10), a mean value of 0.44 g/g carbon for carbohydrate residues in glycosylated proteins was used. When the degree of glycosylation (G; fraction of molecular weight accounted for by carbohydrate) is known, the TOC-measured carbon concentration \( \text{TOC}_C \) can be converted into the concentration of glycosylated protein (PG) by:

\[
\text{PG} = \text{TOC}_C \times \left[ \frac{1 - G}{0.53} + \frac{G}{0.44} \right]
\]

The degree of glycosylation of glycoproteins can be determined by SDS polyacrylamide gel electrophoresis of both native and carbohydrate-depleted protein (10). When, however, no correction for carbohydrate-carbon is made and a maximum degree of glycosylation of 50% is assumed, the error in the protein concentration determined by TOC is only about 10%, which is still acceptable in view of the large errors occurring when the Bradford or Lowry methods are used (2).

Determination of protein concentration and specific absorption coefficients. A requirement for all methods of protein determination is that

-103-
a number of reproducible dilutions of the sample can be made. However, large deviations frequently occur when results from different dilutions are observed. A glucose oxidase solution was serially diluted and the protein concentration in the non-diluted solution estimated by TOC analysis, the Lowry method, and the Bradford method (Fig. 2). The Lowry method and the

FIG. 2. Relation between glucose oxidase concentration and calculated protein concentrations. Protein concentrations in serial dilutions of glucose oxidase were established by (○) the Lowry method, (●) the Bradford method, and (■) TOC analysis, and calculated for the original non-diluted solution. For TOC analysis a degree of glycosylation of 14% (10) was used.

Bradford method were standardized with bovine serum albumin and each dilution was measured in triplicate. For estimation of glucose oxidase concentration by TOC analysis, a degree of glycosylation of 14% was used (11). The glucose oxidase concentration calculated from TOC-analyzed carbon content corresponded to the weighed glucose oxidase concentration, was linear over all dilutions tested and showed a coefficient of variation of 2.8% (Fig. 2). Protein concentrations established with the Bradford method were lower than the weighed concentration and appeared linear, but the
CHAPTER 5

coefficient of deviation was relatively high (13%). No linearity between glucose oxidase dilutions and calculated protein contents was observed when the Lowry method was applied. The Lowry method was particularly unreliable at low glucose oxidase concentrations. The same experiment was performed with a 'normal' protein (aldolase), or proteins containing a chromophoric group (catalase, ferritin, glucose dehydrogenase), or proteins containing carbohydrate residues (invertase, inulinase dimer, inulinase tetramer). With these proteins linearity between dilution and established protein concentration was also limited when the Lowry or Bradford methods were used. Moreover, large differences between protein concentrations obtained with the Bradford method and protein concentrations obtained with the Lowry method occurred, as was already observed in (2). Irrespective of the nature of the protein, all TOC values lower than 500 mg C/l were linear with protein dilutions. These values were used to estimate protein concentrations of the non-diluted solutions and compared to protein values estimated with UV-absorbance at 280 nm (Table 2). For the glycoproteins, a degree of glycosylation of 47% for invertase (11), and 26% for the two inulinase forms (unpublished results) was used. Protein concentrations obtained from absorption at 280 nm were calculated for the original non-diluted solutions from at least four near-linear results of serial dilutions, using the mean value of published specific absorption coefficients. Table 2 shows that the coefficient of variation of TOC-estimated protein concentrations was not related to the presence of chromophoric groups or glycosyl chains.

Specific absorption coefficients were calculated from the TOC analyzed protein concentration and the absorption of the protein dilutions at 280 nm. The resulting mean value of the specific absorption coefficients are compared to literature values (Table 2). Good agreement in specific absorption coefficients with literature values were found for the 'normal' proteins albumin and aldolase, the chromophoric proteins catalase and glucose dehydrogenase, and the glycoprotein glucose oxidase. However, large deviations from literature values were observed with ferritin and invertase. A considerable error occurred in the estimation of the specific absorption coefficient for ferritin. This was due to low linearity of ferritin solutions with absorbance at 280 nm. Only three out of eight dilutions used to calculate the specific absorption coefficient showed a near-linear relation between protein concentration and absorbance at 280 nm. The reason for the observed difference in TOC-calculated absorption coefficient and literature
TABLE 2. Protein concentrations and specific absorption coefficients determined by total organic carbon analysis and measurement of absorbance at 280 nm.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein concentration (mg/l)</th>
<th>Specific absorption coefficient (A\textsubscript{280} \textsuperscript{0.1%})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOC</td>
<td>A\textsubscript{280}</td>
</tr>
<tr>
<td>Albumin</td>
<td>912 ± 29</td>
<td>961 ± 14</td>
</tr>
<tr>
<td>Aldolase</td>
<td>653 ± 23</td>
<td>634 ± 21</td>
</tr>
<tr>
<td>Catalase</td>
<td>1,934 ± 13</td>
<td>2,324 ± 65</td>
</tr>
<tr>
<td>Ferritin</td>
<td>437 ± 26</td>
<td>645 ± 23</td>
</tr>
<tr>
<td>Glucose dehydrogenase</td>
<td>484 ± 10</td>
<td>478 ± 1</td>
</tr>
</tbody>
</table>
| Glucose oxidase
d      | 1,033 ± 29                   | 1,010 ± 52                                                             | 1.66 ± 0.08 | 1.67 |
| Invertase\textsuperscript{d}      | 4,180 ± 58                   | 672 ± 45                                                               | 0.37 ± 0.03 | 2.30 |
| Inulinase\textsuperscript{d}                     |                               |                                                                       |     |                      |
| (dimer)         | 2,918 ± 21                   | -                                                                     | 1.03 ± 0.01 | -                      |
| (tetramer)      | 1,447 ± 18                   | -                                                                     | 1.00 ± 0.01 | -                      |

Mean coefficient of variation (%) 2.4  3.3  5.1

\textsuperscript{a} For determination of protein concentrations, mean literature values of the specific absorbance at 280 nm were used; \textsuperscript{b} Values from ref. (13); \textsuperscript{c} Value from ref. (14); \textsuperscript{d} Glycoproteins (see Text): glucose oxidase, G=14%; invertase G=47%; inulinase, G=26%.

value of invertase, is unclear. The invertase concentration determined by TOC (4,180 ± 58 mg/l) was consistent with the concentration weighed before dialysis (4,300 mg/l). Though improbable, it could be that the commercial invertase contained contaminating carbon which had not been removed by dialysis.
CHAPTER 5

The inulinase dimer and inulinase tetramer showed specific absorption coefficients of 1.03 ± 0.01 and 1.00 ± 0.01, respectively. Dissociation of the dimer by sheer force or 4 M Guanidine-HCl, gave a monomer with a specific absorption coefficient of 1.06 ± 0.01. Apparently, formation of multimers leads to a slight decrease in the specific absorption coefficient. Inulinase is a glycosylated enzyme and it could be envisaged that a higher degree of aggregation would lead to a different conformational structure and to a different grouping of the carbohydrate attached. These changes might then lead to more light scattering and less absorption.

Use of TOC analysis during purification of proteins. Establishment of concentrations of an unknown protein in chromatography fractions can quickly be performed by TOC analysis. During the purification of two forms of inulinase from the yeast Kluyveromyces marxianus CBS 6556, inulinase dimer and an inulinase tetramer, the carbon content in the eluted fractions was not parallel with the 280 nm-measured protein. However, the carbon content appeared informative with regard to specific inulinase activities and purity of the 280 nm measured protein peaks, since impurities other than protein are measured by their carbon (unpublished results). However, a possible source of error in the determination of protein concentration in the eluted fractions could be the presence of carbon originating from column material. TOC analysis of fractions from columns eluted with carbon-free potassium phosphate buffer, revealed that during elution the column materials DEAE-Sephadex A-50 (Pharmacia), CM-Sepharose Cl.-6B (Pharmacia), and Superose 12/16 (Pharmacia) released very low amounts of carbon (6.0 ± 2.1 mg C/l). This amount of column-carbon remained unaltered when gradient elution with DEAE-Sephadex or CM-Sepharose was performed.

The method of establishing protein concentrations by measuring the carbon concentration with TOC analysis, was originally intended for fast and sensitive estimation of protein contents during protein purification studies. However, it appeared reliable and can generally be used to determine protein concentrations and specific absorption coefficients. The variations observed in protein concentrations calculated from carbon concentrations were lower than those found with the classical colorimetric methods of Lowry and Bradford.

TOC analysis of protein solutions achieves the primary objectives of simplicity, independence of amino acid composition, independence of serial protein dilutions, independence of chromophoric groups or carbohydrate
residues, does not need standardization by other proteins, and protein concentrations can be calculated without prior knowledge of specific absorption coefficients. Moreover, TOC analysis requires very low amounts of protein and represents a valuable alternative to the direct spectrophotometric determination (14). It should be noted that the method cannot be used for the direct determination of protein concentrations when the solution to be studied contains carbon other than protein- and buffer-carbon. However, during purification of protein from cell-free extracts or blood serum, it can be feasible to use TOC analysis when specific activities are defined as activity/mg C. This value increases upon removal of lipids, nucleic acids, and carbohydrates, and is a measure for increase in purity. This is not the case with protein values measured by chromogenic, dye-binding or spectrophotometric methods.

REFERENCES

CHAPTER 5


Chapter 6

Localization of Invertase and Inulinase in *Kluyveromyces* species.

R.J. Rouwenhorst, W.A. Ritmeester, W.A. Scheffers, and J.P. van Dijken.

Submitted to Applied Environmental Microbiology.
CHAPTER 6

SUMMARY

In vivo hydrolysis of inulin and sucrose was examined in selected yeasts of the genus *Kluyveromyces*. Cells, grown in sucrose-limited chemostat cultures, were subjected to treatments for removal of inulinase, the enzyme responsible for both inulin and sucrose hydrolysis. The effects of these treatments were studied by measurement of inulin-dependent and sucrose-dependent oxygen consumption by cell suspensions.

In *K. marxianus* var. *marxianus*, inulinase was partially secreted into the culture fluid. Removal of culture fluid inulinase by washing had no effect on sucrose-dependent oxygen consumption by this yeast. However, this treatment drastically reduced inulin-dependent oxygen consumption. Treatment of washed cells with sulfhydryls removed part of the cell wall-retained inulinase and reduced inulin-dependent oxygen consumption by another 80%. Sucrose-dependent oxygen consumption was less affected, decreasing by 40%.

Cell suspensions of *K. marxianus* var. *drosophilum*, *K. marxianus* var. *vanudenii*, and *Saccharomyces kluyveri* rapidly utilized sucrose but not inulin. This is in accordance with the classification of these yeasts as inulin-negative. Supernatants of cultures grown at pH 5.5 did not catalyze the hydrolysis of inulin and sucrose. This suggested that these yeasts contained a strictly cell-bound invertase, an enzyme not capable of inulin hydrolysis. However, upon washing, cells became able to utilize inulin. The inulin-dependent oxygen consumption further increased after treatment of cells with sulfhydryl compounds. These treatments did not affect the sucrose-dependent oxygen consumption of the cells. Apparently, these treatments removed a permeability barrier for inulin that does not exist for sucrose. Non-denaturing PAGE and determination of the S/I ratio (relative activity with sucrose and inulin) of enzyme preparations proved that in these yeasts, as in *K. marxianus* var. *marxianus*, hydrolysis of sucrose and inulin is catalyzed by the same enzyme, namely inulinase. This 'cryptic' inulinase activity is not a physiological artefact. When instead of the standard cultivation conditions used in yeast taxonomy (pH 5.6, 25 °C) cells were inoculated in media of pH 4.5 and incubated at 35 °C, rapid growth on inulin occurred. Under these conditions, both inulin- and sucrose hydrolyzing activities could be detected in culture supernatants of these
CHAPTER 6

yeasts. Physiological, ecological, and taxonomical aspects of the occurrence and localization of inulinase in *Kluyveromyces* strains are discussed.

INTRODUCTION

Snyder & Phaff (22) first described the production of inulinase by *Saccharomyces fragilis*, a yeast now known as *Kluyveromyces marxianus* var. *marxianus*. The hydrolysis of inulin, a fructose polymer, can also be catalyzed, albeit very slowly, by invertase (EC 3.2.1.26). The separate classification of both enzymes has been disputed (2,15). The main question is whether inulinase should be regarded as a special type of invertase, or as a different enzyme with an analogous mode of action.

We recently studied the biochemistry of the inulinase of *K. marxianus*. From this study it appeared that, apart from substrate specificity, also considerable structural differences exist with the invertase of *Saccharomyces* (20). Inulinase secreted into the culture fluid has a molecular weight of about 165 kDa, consists of two protein subunits, and contains 34% of its mass as carbohydrate. The inulinase associated with the cell wall has the same carbohydrate content, but is a tetramer with an average size of 350 kDa. In *S. cerevisiae* invertase is secreted in the culture fluid as a dimer with a molecular weight of 270 kDa, whereas the enzyme retained in the cell wall is an octamer of about 800 kDa. Both invertase forms contain up to 50% carbohydrate (10). Virtually no homology was found in the amino acid sequences of the amino-terminal ends of invertase and inulinase (20). In view of the above mentioned structural differences, and the low activity of invertase with inulin as a substrate (11,23,27), the separate classification of inulinase and invertase in yeast seems justified.

Secreted invertase of *Saccharomyces* mainly resides in the cell wall, where it performs its physiological function: the cleavage of sucrose which diffuses into the cell wall (1,5). From an ecological point of view the retention of invertase in the cell wall may be beneficial. In this way an efficient scavenging of the hydrolysis products can be accomplished. Similarly, the retention of inulinase in the cell wall of *K. marxianus* may be advantageous for sucrose utilization by this yeast. However, this does
not hold for inulin utilization, since inulin cannot penetrate the cell wall (18,21) and must therefore be hydrolyzed outside the cell wall. The aim of the present study was to compare Klyuyveromyces and Saccharomyces strains with respect to the localization of sucrose-hydrolyzing activities and to study to what extent inulin consumption depends on the presence of inulinase in the culture fluid.

MATERIALS AND METHODS

Yeast strains and growth conditions. Klyuyveromyces marxianus var. drosophilaram CBS 2103; K. marxianus var. marxianus CBS 6397, and CBS 6556; K. marxianus var. lactis CBS 683, CBS 739, CBS 1067, CBS 2359, and CBS 8043; K. marxianus var. vanudenii CBS 5669; K. loddereae CBS 2758; K. waitii CBS 6430, and Saccharomyces cerevisiae CBS 8066 were obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands, S. klyuyveri UCD 51-242 was a gift from Dr. H.J. Phaff, University of California, Davis, U.S.A. Yeasts were maintained on YEPD (yeast extract, 10 g; Bacto-Peptone, 10 g; glucose, 20 g; per liter of demineralized water) agar slopes. Batch cultivation was done at various pH values and temperatures in 50 ml shake flasks containing either Yeast Nitrogen Base (5 g liter\(^{-1}\)) or mineral-salts medium (7) as source of vitamins and minerals, supplemented with 10 g liter\(^{-1}\) of carbon substrate (sucrose, raffinose, maltose, inulin).

Chemostat cultivation was performed in laboratory fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter at a dilution rate of 0.1 h\(^{-1}\) at 33\(^\circ\)C, and an oxygen concentration of 50-70% of air saturation. Dissolved oxygen was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland) and pH was controlled by automatic addition of 1 M KOH at pH 5.5, unless mentioned otherwise. A mineral medium according to Bruinenberg et al. (7) was used, except for a tenfold increase of NaMoO\(_4\).2H\(_2\)O concentration. For carbon- and energy-limited growth, sucrose was added to the mineral salts medium to give a final concentration of 10 g liter\(^{-1}\).

Measurement of substrate-dependent oxygen consumption. Inulin, sucrose, fructose and glucose metabolism by suspensions of intact cells was assayed by following the rate of oxygen consumption with a Clark-type oxygen
electrode (Yellow Springs Instr. Inc., Yellow Springs, Ohio) in a reaction volume of 4 ml, with a final cell concentration of 0.5 mg of cell dry weight ml\(^{-1}\) at 33°C. Cell suspensions from sucrose-limited continuous cultures were assayed: 1: directly after diluting with mineral medium pH 5.5; 2: after removal of culture fluid by centrifugation (4,000 \(\times\) g), washing of the cells with mineral medium and suspension of the cells in mineral medium; 3: after treatment of the cells with enzyme release buffer (50 mM potassium phosphate, pH 7, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol, 10 mM MgSO\(_4\)), washing, and suspension of the cells in mineral medium. The reaction was started by addition of glucose, fructose or sucrose, to a final concentration of 2 mM. Inulin dependent oxygen consumption was assayed with 0.2% inulin. In the case of sucrose and inulin, the oxygen consumption rate increased with reaction time and became constant after 10 min. These final values, corrected for endogenous respiration, were used to calculate the rate of sugar-dependent oxygen consumption.

Fractionation of cultures for enzyme assays. Cells and culture supernatants were assayed for inulinase, invertase and \(\alpha\)-glucosidase activities. For the fractionation of cultures into three enzyme preparations, the method described by Rouwenhorst et al. (19) was used. Enzyme activity present in the culture fluid is referred to as supernatant enzyme. Enzyme released from the cell wall by incubation of the cells in enzyme release buffer and incubation for 1.5 h at 30°C is referred to as cell wall enzyme. The activity only solubilized by means of sonication is referred to as cell-bound enzyme.

Enzyme assays. Sucrose- and inulin-hydrolyzing activities were measured by following the rate of appearance of monosaccharides with the glucose/fructose Test Combination of Boehringer (Boehringer GmbH, Mannheim, Federal Republic of Germany) in the presence of 2% sucrose or 2% inulin in a 0.1 M sodium acetate buffer (pH 4.5) at 50°C.

\(\alpha\)-Glucosidase activity was determined with \(\alpha\)-nitrophenyl-\(\alpha\)-D-glycopyranoside (2-NP\(\alpha\)G; Boehringer) as a substrate. Enzyme preparations were added to a prewarmed (33°C) solution of 0.1 M sodium phosphate (pH 7), 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM MgSO\(_4\), and 4 mg ml\(^{-1}\) of 2-NP\(\alpha\)G. The hydrolysis of 2-NP\(\alpha\)G into 2-nitrophenol and D-glucose was followed on-line at 420 nm in a Vitalab 20 spectrophotometer (Vital Scientific, Dieren, The Netherlands).
CHAPTER 6

Analytical methods. Biomass concentrations were measured by drying culture samples to constant weight at 70°C after membrane filtration (0.45 μm; Schleicher & Schull, Dassel, Federal Republic of Germany) and washing.

Non-denaturing polyacrylamide gel electrophoresis and detection of sucrose-hydrolyzing activity in the gels, was done according to Rouwenhorst et al. (20).

Chemicals. Yeast Nitrogen Base and Bacto-Peptone were from Difco Laboratories, Detroit, Michigan. Fructose, glucose, sucrose and 2-mercaptoethanol were purchased from Baker Chemicals BV, Deventer, The Netherlands. Dithiothreitol and inulin (chicory root) were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Sucrose- and inulin-dependent oxygen consumption by cell suspensions of \textit{K. marxianus} \textit{var. marxianus}. Sucrose and inulin can be hydrolyzed into the monosaccharides glucose and fructose. These monosaccharides are subsequently catabolized by the cells at the expense of oxygen. Thus, measurement of oxygen consumption after addition of sucrose or inulin to yeast cells is a convenient way for the determination of \textit{in vivo} invertase and inulinase activities. The cell wall of yeasts may act as a permeability barrier to the polymer inulin, but not to the disaccharide sucrose (21). Therefore, it was of interest to investigate whether treatments that lead to removal of inulinase have the same effect on oxygen consumption with sucrose as with inulin. Washing of cells results in removal of enzyme activity present in the culture fluid (designated as supernatant enzyme). Incubation of the cells for 1.5 h at 30°C in potassium phosphate buffer (50 mM, pH 7) or in potassium phosphate buffer containing sulphydryl compounds (enzyme release buffer) and subsequent washing, may give further depletion of enzyme activity, due to solubilization of part of the enzyme retained in the cell wall (19).

Washing or treatment with sulphydryl compounds of \textit{K. marxianus} \textit{var. marxianus} cells had no effect on fructose and glucose oxidation. The oxygen consumption rate after addition of monosaccharide remained 7.3 ± 0.2 mmol h^{-1} g of cell dry weight^{-1}. The oxygen consumption rate of untreated \textit{K. marxianus} \textit{var. marxianus} cell suspensions with sucrose was equal to that
observed with the monosaccharides glucose and fructose (Fig. 1). Washing of K. marxianus var. marxianus cells had no effect on the sucrose-dependent oxygen consumption rates, indicating that the inulinase activity present in the cell wall is high enough to saturate monosaccharide catabolism. Removal of part of the cell wall inulinase by incubation of cells in either 50 mM potassium phosphate buffer (pH 7) or enzyme release buffer resulted in

![Diagram showing oxygen consumption rates]

**FIG. 1.** Effect of inulinase removal on the oxygen consumption by suspensions of K. marxianus var. marxianus CBS 6556. The oxygen consumption rates of (■■■) fresh chemostat cultivated cell suspension; (□□□) washed cells; (■■■) cells treated with phosphate buffer (pH 7); (■■■) cells treated with enzyme release buffer, (■■■) and after addition of supernatant, were determined after four-fold dilution with mineral medium and addition of 2 mM sucrose or 0.2% inulin. Cells were obtained from a steady-state chemostat culture grown on sucrose at pH 5.5, and 33°C. A 100% oxygen consumption equals an oxygen consumption rate of 7.3 mmol h\(^{-1}\)g of cell dry weight\(^{-1}\) as observed with glucose and fructose.
decrease of sucrose-dependent oxygen consumption rates by 20 and 40%, respectively. Apparently, after these treatments hydrolysis had become the rate-limiting step in sucrose catabolism. By addition of the original amount of culture supernatant, the oxygen consumption rate could be completely restored to the level observed with untreated cell suspension.

Removal of supernatant inulinase by washing of the cells with mineral medium led to a 50% decrease of the inulin-dependent oxygen consumption rate. An even larger effect was observed when the cells were resuspended and incubated in potassium phosphate buffer or in enzyme release buffer. In these cases, the oxygen consumption rates decreased by 70% and 82%, respectively. Addition of culture supernatant to washed and sulfhydryl-treated cells resulted in an equal restoration of the oxygen consumption rates by approximately 50% of the original rates (Fig. 1). When excessive supernatant was added the original rate of inulin-dependent oxygen consumption was fully restored (data not shown).

To obtain insight into the factor(s) that affect the inulin-hydrolyzing activity, the oxygen consumption rates of untreated, washed, and sulfhydryl-treated cells of *K. marxianus* var. *marxianus* were determined in relation to inulin concentration (Fig. 2). From the curves it is obvious that the oxygen consumption rate changed when cells were subjected to treatments that remove inulinase. A threshold level of inulin was required for the detection of inulin-dependent oxygen consumption. This threshold concentration increased when more inulinase was removed from the cells (Fig. 2). By extrapolation of Lineweaver-Burk plots, derived from the data in Fig. 2, and disregarding very low oxygen consumption rates, $K_s$ and $V_{O_2}^{max}$ values could be calculated. Untreated cell suspension of *K. marxianus* var. *marxianus* gave the highest $V_{O_2}^{max}$ and the lowest $K_s$ value (7.1 mmol O$_2$ h$^{-1}$ g of cell dry weight$^{-1}$ and 0.3 g inulin liter$^{-1}$, respectively). Removal of inulinase present in the culture fluid by washing with mineral medium resulted in an overall decrease in oxygen consumption rate (maximal 5.6 mmol O$_2$ h$^{-1}$ g of cell dry weight$^{-1}$) and an increase in $K_s$ value (1.3 g inulin liter$^{-1}$). A similar effect was observed when cells were treated with enzyme release buffer: The maximal oxygen consumption rate and $K_s$ became 2.2 mmol O$_2$ h$^{-1}$ g of cell dry weight$^{-1}$ and 2.5 g inulin liter$^{-1}$, respectively. By removal of inulinase from the system, hydrolysis of inulin became rate-limiting and thus oxygen consumption rates decreased. The reason for the observed
increase in affinity constant of whole cells towards inulin is unknown but it may, at least in part, be due to a decrease in accessibility of the inulinase to the inulin molecules.

![Graph showing the relationship between inulin concentration and oxygen consumption rates](image)

**FIG. 2.** Relationship between inulin concentration and oxygen consumption rates for (●) fresh culture suspension; (○) washed cells; and (□) sulfhydryl-treated cells of *K. marxianus* var. *marxianus* CBS 6556. Cells were obtained from a steady-state chemostat culture grown on sucrose at pH 5.5 and 33°C.

Sucrose- and inulin-dependent oxygen consumption in cell suspensions of *K. marxianus* var. *drosophilaram*. The yeast *K. marxianus* var. *drosophilaram* was originally included in our study as a representative of a sucrose-utilizing *Kluyveromyces* strain that is unable to grow on inulin. It was anticipated that growth of this yeast on sucrose would involve a genuine invertase, not capable of inulin hydrolysis.

*K. marxianus* var. *drosophilaram* was grown in a sucrose-limited chemostat culture at pH 5.5 and 33°C. Cells from these cultures showed a sucrose-dependent oxygen consumption rate that was not influenced by washing or by
treatment with enzyme release buffer (Fig. 3a). The oxygen consumption rate remained constant and was equal to the oxygen consumption rate with glucose or fructose (4.2 mmol O₂ h⁻¹ g of cell dry weight⁻¹). As expected, no oxygen consumption was observed when untreated *K. marxianus* var. *drosophilarum* cells were given inulin as substrate. Surprisingly, however, washing of these cells resulted in an inulin-dependent oxygen consumption. A further increase of the oxygen consumption rate was observed when the cells were treated with enzyme release buffer (Fig. 3a).

**FIG. 3.** Effect of enzyme depletion on the oxygen consumption rate of *K. marxianus* var. *drosophilarum* CBS 2103. The oxygen consumption rates of (■) cell suspension; (□) washed cells; and (▲) cells treated with enzyme release buffer, were determined after four-fold dilution with mineral medium and addition of 2 mM sucrose or 0.2% inulin. Cells were obtained from (A) a steady-state chemostat culture grown on sucrose at pH 5.5 and 30°C, or (B) from a steady-state chemostat culture grown on sucrose at pH 4.5 and 33°C.

These results indicate that *K. marxianus* var. *drosophilarum* contains a 'cryptic' inulinase activity that becomes functional after washing or treatment of the cells with sulphydryl compounds. Indeed, enzyme assays showed that, in contrast to the situation in *K. marxianus* var. *marxianus*, culture supernatants of *K. marxianus* var. *drosophilarum* did not contain sucrose-or inulin-hydrolyzing activity. Apparently, the inulinase in this
strain is strictly cell wall-bound and only becomes accessible to inulin after special treatment of the cells. The localization of inulinase in this strain was strongly affected by the culture conditions: When *K. marxianus* var. *drosophilarum* was grown on sucrose at pH 4.5 instead of at pH 5.5, untreated cell suspensions of *K. marxianus* var. *drosophilarum* were not only able to utilize sucrose, but also inulin. Similar results as observed for *K. marxianus* var. *marxianus* were now obtained (Fig. 3b). Supernatants of cultures grown at pH 4.5 contained sucrose- and inulin-hydrolyzing activities with an S/I ratio (relative activity with sucrose and inulin) of 22.

The rather unexpected presence of an inulinase in *K. marxianus* var. *drosophilarum*, the localization of which is dependent on cultivation conditions, led us to reinvestigate the ability of this and other *Kluyveromyces* strains to assimilate inulin. *S. cerevisiae* was included as the reference organism, since the inability of this yeast to utilize inulin is well established (22). The yeast *S. kluyveri* UCD 51-242 was included, since it has been reported to contain an invertase, although no homology was found between its genome and the *SUC2* gene of *S. cerevisiae* (8).

Sucrose and inulin utilization by *Kluyveromyces* and *Saccharomyces* species. In yeast systematics, physiological properties such as the aerobic and anaerobic utilization of saccharides, are used to describe and identify yeasts. These physiological characteristics are often dependent on the culture conditions employed. Reproducibility of the taxonomic tests has been achieved by international standardization. Aerobic utilization of saccharides is generally tested in reagent tubes with 5 ml of medium containing Yeast Nitrogen Base (pH 5.6) supplemented with 50 mM of sugar. These tubes are then incubated with gentle shaking at 25°C for 3 to 21 days. Fermentative utilization is tested in Durham tubes with the same medium and incubation at 25°C for 7 days (6,28).

The growth characteristics of 13 *Kluyveromyces* and *Saccharomyces* strains with the oligosaccharides maltose, sucrose, and raffinose, and with the polysaccharide inulin are listed in Table 1. All of these yeasts are able to assimilate sucrose aerobically. The nature of the sucrose-hydrolyzing enzyme in the various strains can be predicted when growth on other saccharides is taken into account (4,5). Growth on both sucrose and inulin indicates the production of an inulinase (i.e. *K. marxianus* var. *marxianus* CBS 6397, CBS 6556). Yeast strains that are able to utilize raffinose but not inulin,
CHAPTER 6

possess an extracellular invertase (i.e. K. marxianus var. drosophilum CBS 2103, K. marxianus var. vanudenii CBS 5669, K. lodderi CBS 2758, K. waltii

TABLE 1. Aerobic and anaerobic utilization of some sugars by Kluyveromyces species according to the taxonomic tests of the Yeast Division of the Centraal bureau voor Schimmelcultures, Delft. Sugar utilization was tested at pH 5.6 and 25 °C.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Sucrose</th>
<th>Raffinose</th>
<th>Maltose</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. marxianus var. drosophilum CBS 2103</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K. marxianus var. lactis CBS 663</td>
<td>+</td>
<td>+</td>
<td>+k</td>
<td>-</td>
</tr>
<tr>
<td>CBS 739</td>
<td>+</td>
<td>+</td>
<td>+k</td>
<td>-</td>
</tr>
<tr>
<td>CBS 1067</td>
<td>+</td>
<td>+</td>
<td>+k</td>
<td>-</td>
</tr>
<tr>
<td>CBS 2359</td>
<td>+</td>
<td>+</td>
<td>+k</td>
<td>-</td>
</tr>
<tr>
<td>CBS 8043</td>
<td>+k</td>
<td>-</td>
<td>+k</td>
<td>-</td>
</tr>
<tr>
<td>K. marxianus var. marxianus CBS 397</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CBS 6556</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>K. marxianus var. vanudenii CBS 5669</td>
<td>+</td>
<td>+k</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K. lodderi CBS 2758</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K. waltii CBS 6430</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. cerevisiae CBS 8066</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. kluveri UCD 51-242</td>
<td>+</td>
<td>+</td>
<td>+k</td>
<td>-</td>
</tr>
</tbody>
</table>

* Nomenclature according to van der Walt & Johanssen (28).
+: growth; -: no growth; k: Kluyver effect with oligosaccharide.
CHAPTER 6


It is known that a number of yeast which are able to utilize disaccharides aerobically cannot do so anaerobically (4). This feature is referred to as Kluver effect and it only occurs with oligosaccharides that are hydrolyzed intracellularly. The effect is probably caused by the inability of the yeast to transport the oligosaccharides across the plasma membrane under anaerobic conditions (4, 5). Yeasts that show the Kluver effect with both sucrose and maltose are supposed to metabolize the sucrose only via an intracellular α-glucosidase (i.e. *K. marxianus* var. *lactis* CBS 8043). The coincidence of the occurrence of the Kluver effect with both maltose (an α-glucoside) and sucrose in certain yeast species is not surprising, since sucrose can be regarded both as an α-glucoside and as a β-fructoside.

**TABLE 2. Growth of yeast species on mineral medium with 2% (w/v) inulin at different pH values and temperatures in batch cultures.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH 4.5</th>
<th>pH 5.2-5.5</th>
<th>pH 6.0-6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T (°C)</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td><em>K. marxianus</em> var.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>drosophilium</em> CBS 2103</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>K. marxianus</em> var.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>vanudenii</em> CBS 5569</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. kluveri</em> UCD 51-242</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

++: growth within 2 days; +: growth within 4 days; *: growth within 8 days, -: no growth; v: variable growth.
CHAPTER 6

Growth conditions different from those commonly used in yeast systematics did not result in changes in growth characteristics with disaccharides of the yeasts listed in Table 1. However, three out of thirteen yeast strains behaved anomalously (Table 2). The use of shake flasks instead of tubes, a synthetic mineral medium instead of Yeast Nitrogen Base, and increased growth temperature changed the growth characteristics of *K. marxianus* var. *drosophilum* CBS 2103, and *K. marxianus* var. *vanudii* CBS 5669. When, moreover, the culture pH was changed to pH 4.5, both of the above *Kluyveromyces* strains and *S. kluveri* UCD 51-242 were capable of rapid utilization of inulin. Use of higher pH values of 6.0-6.5 did not result in rapid growth on inulin. Only at the highest temperature tested (35°C), weak growth on inulin of the *Kluyveromyces* species at pH 5.5 was observed. Apparently, utilization of inulin by some yeasts is primarily dependent on culture pH, and to a lesser extent on the incubation temperature. Possible effects of aeration, which is much better in shake flasks than in the shake tubes as used in yeast taxonomy, were not investigated.

The above results explain the absence of in vivo inulinase activity in *K. marxianus* var. *drosophilum* pregrown at pH 5.5 on sucrose. When grown at this pH the inulinase is retained in the cell wall and not accessible to inulin, unless the cells are washed or treated with thiols. When grown at pH 4.5, the inulinase is partially secreted into the culture liquid, as in *K. marxianus* var. *marxianus*. In this yeast the localization of inulinase is independent of culture pH (19). The two other yeast species that only showed growth on inulin at the lower pH value, *K. marxianus* var. *vanudii* and *S. kluveri*, were also tested for sucrose-and inulin-dependent oxygen consumption. Oxygen consumption patterns after various treatments were similar to those found with *K. marxianus* var. *drosophilum* when these yeasts were grown in chemostat culture on sucrose at pH 4.5 instead of at pH 5.5 (data not shown).

Sucrose-dependent oxygen consumption by yeasts that do not contain an inulinase. Treatment of *S. cerevisiae* cells with sulfhydryl compounds has hardly any effect on the solubilization of invertase (10,29). Indeed, the sucrose-dependent oxygen consumption rate in *S. cerevisiae* was not affected by either washing of the cells or treatment of the cells with sulfhydryl compounds. Oxygen consumption rates with sucrose were the same as those observed with glucose or fructose (results not shown). Similar observations were made with 7 invertase-producing *K. marxianus* strains of the varieties
lactis, lodderii, and waltii; with the exception being K. marxianus var. lactis CBS 739 (Table 1). This latter yeast showed a far lower oxygen consumption rate with fructose (60%) as compared to the rates with glucose and sucrose. As mentioned above, K. marxianus var. lactis CBS 8043 probably does not produce an inulinase or invertase. Hydrolysis of sucrose in this organism may occur via an intracellular α-glucosidase. Irrespective of treatment of the cells, this yeast showed a sucrose-dependent oxygen consumption rate that was 70% of the rate observed with glucose. Obviously, monosaccharide catabolism is not saturated when sucrose is the substrate. The rate-limiting process in this case may be either transport of sucrose into the cell, or hydrolysis of sucrose by internal α-glucosidase. None of the K. marxianus strains of the varieties lactis, lodderii, and waltii listed in Table 1 showed inulin-dependent oxygen consumption, either as untreated cell suspension or after washing with thiols. This is in line with their inability to grow on inulin in a variety of culture conditions.

Characterization of enzyme activities. Snyder and Phaff (22) introduced the S/I ratio, the ratio of the activities with sucrose and inulin as substrates, to discriminate between invertases and inulinases. Inulinases are characterized by S/I ratios lower than 50 (27). In cell-free extracts of chemostat-grown yeasts, hydrolase activities with sucrose, maltose and inulin were determined. The yeasts K. marxianus var. drosophilarum, K. marxianus var. vanudenii and S. kluveri showed a ratio of sucrose- and inulin-hydrolyzing activities of 22, 9, and 18, respectively. These S/I ratios are comparable with the value of 15 found for the K. marxianus var. marxianus inulinase (19). The enzyme assays confirmed that the yeast strains that did not produce an inulinase contained an invertase (S/I ratios > 1200) and/or an α-glucosidase, as was predicted by the growth characteristics (Table 1) and sucrose-dependent oxygen consumption patterns. The strain that showed the Kluyver effect with both sucrose and maltose, K. marxianus var. lactis CBS 8043, probably possessed an α-glucosidase. The sucrose-hydrolyzing activity in cell-free extracts of this strain exhibited optimal activity at pH 7, whereas under optimal conditions for invertase (pH 5) activity was greatly reduced.

Cell-free extracts of K. marxianus var. marxianus CBS 6556, S. cerevisiae, K. marxianus var. lactis CBS 739, K. marxianus var. drosophilarum, and K. waltii CBS 6430, were subjected to non-denaturing PAGE and the gel stained for sucrose-hydrolyzing activity. The invertases of K.

-I25-
waltii, \textit{K. marxianus} var. \textit{lactis}, and \textit{S. cerevisiae} (Fig. 4; lanes 1,3,5) showed similar migration distances. The inulinases of \textit{K. marxianus} var. \textit{drosophilurum} and \textit{K. marxianus} var. \textit{marxianus} (Fig. 4; lanes 2,4) showed lower activity bands. However, these two inulinases did not migrate in the same way. The sucrose activity band of the inulinase of \textit{K. marxianus} var. \textit{drosophilurum} migrated between those of the \textit{K. marxianus} var. \textit{marxianus} inulinase (Mr. 400 kDa) and of the \textit{S. cerevisiae} invertase (Mr. 800 kDa).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig4.png}
\caption{Sucrose-hydrolyzing activities after non-denaturing PAGE of invertases and inulinases in cell-free extracts of different yeast species. Invertase of \textit{K. waltii} CBS 6430 (lane 1), \textit{K. marxianus} var. \textit{lactis} CBS 739 (lane 3), \textit{S. cerevisiae} CBS 8066 (lane 5), inulinase of \textit{K. marxianus} var. \textit{drosophilurum} CBS 2103 (lane 2), and \textit{K. marxianus} var. \textit{marxianus} CBS 6556 (lane 4), were applied on a 7% polyacrylamide gel and the gel was stained for sucrose-hydrolyzing activity.}
\end{figure}
CHAPTER 6

DISCUSSION

Occurrence of inulin utilization among yeasts. According to systematic studies, K. marxianus var. marxianus, which includes the former species K. fragilis and K. bulgaricus, is the only representative of the genus capable of inulin utilization (6,28). However, it appears that utilization of inulin is much more widespread among Kluyveromyces species (Table 2). It may well be that also other yeast genera in which inulin utilization has been established (e.g. Debaromyces, Hansenula, and Candida) may contain more species capable of inulin utilization than those recognized to date.

Especially interesting was the finding that the yeast S. kluveri UCD 51-242 could grow on inulin when culture conditions differed from those generally employed in yeast systematics (Table 2). Carlson and Botstein (8) reported that this yeast produced an invertase different from that of S. cerevisiae. These authors investigated the physical structure of the invertase-coding (SUC) gene family by using cloned SUC2 DNA probes to detect homologous sequences in 14 S. cerevisiae strains and in 10 closely related Saccharomyces species. They found that the SUC DNA sequence was highly conserved within the genus Saccharomyces. Only DNA from S. kluveri UCD 51-242 failed to hybridize with the SUC2 DNA probe. Carlson and Botstein (8) concluded that this species was more distantly related to S. cerevisiae than the other species tested and that during evolution its SUC gene had diverged sufficiently to leave no detectable homology. The growth of S. kluveri on inulin, reported in this paper, and the S/I ratio of the enzyme indicates that this organism does not produce an invertase but an inulinase, which is a completely different enzyme (20).

The unrecognized ability of some yeasts to grow on inulin is mainly due to the fact that in yeast systematics, for reasons of standardization and convenience, growth on sugars is tested under special cultivation conditions (4,6,28). As with some of the strains tested above, utilization of inulin strongly depended on growth conditions (Tables 1,2), one should be careful in interpreting the growth characteristics derived from taxonomic tests. It is conceivable that not only growth on inulin, but on polymers in general is strongly afflicted by growth conditions.

Localization of inulinase and invertase in yeasts. The localization of inulinase and invertase in Kluyveromyces and Saccharomyces strains depends on the yeast strain and on the cultivation conditions (5,25). In S.
cerevisiae almost all invertase produced is retained in the cell wall (10). A large part of the inulinase of K. marxianus var. marxianus is secreted into the culture fluid (16,19). Removal of the supernatant enzyme of K. marxianus var. marxianus had no effect on sucrose consumption, but treatment of the cells with sulfhydryl compounds decreased the sucrose-dependent oxygen consumption rate and thus limited sucrose catabolism (Fig. 1). Contrary to invertase, inulinase is able to hydrolyze fructans like inulin and levan (22,23). These polysaccharides do not enter the cell wall of yeasts (21) and hydrolysis must occur outside the cell wall. Measurements of inulin-dependent oxygen consumption of K. marxianus var. marxianus cells revealed that both inulin concentration and inulinase localization determine the rate of inulin hydrolysis (Figs. 1,2).

Cultivation of K. marxianus var. drosophilum, K. marxianus var. vanudenii, and S. kluvyeri at lower pH values, and at higher growth temperatures than those normally used in yeast systematics allowed growth of these strains on inulin (Table 2). During growth in sucrose-limited continuous cultures at pH 5.5, a pH value that does not support growth on inulin, these yeasts produced an inulinase that is located in the cell wall. Cell suspensions were not able to utilize inulin but washing or treatment with sulfhydryl compounds restored this ability (Fig. 3A). Obviously, these cells acquired their ability to hydrolyze inulin due to removal of a permeability barrier in the outer regions of the cell wall. This either enables inulin to enter the cell wall, or inulinase to diffuse out of the cell wall. Washing of yeast cells has been reported to affect the lipid, protein, and carbohydrate contents of the yeast cell wall (25). Due to this treatment, the carbohydrate content increases whereas the protein and lipid content decreases. The decrease in lipid content could be of particular importance, as it has been suggested that the lipid plays a role in maintaining the ordered structure of the wall (25). Treatment of yeast cells with sulfhydryl compounds like β-mercaptoethanol and dithiothreitol increase the permeability of the cell wall by reducing the disulfide linkages in the outer layers of the cell wall, thus giving rise to the occurrence of pores (9,14).

Alteration of growth conditions promoted secretion of inulinase into the culture liquid by cells of K. marxianus var. drosophilum, K. marxianus var. vanudenii, and S. kluvyeri. Apparently, molecular sieving by the cell wall was less efficient than during cultivation conditions employed in yeast.
systematics. Unfortunately, reports available on pH- and temperature-induced morphological changes in yeast cell walls are limited. The available information mainly concerns the chemical composition of cell walls but not their structure (13,25). Inability of yeast cells to grow on inulin in spite of the presence of an inulinase has also been reported for a respiratory-deficient mutant of K. marxianus var. marxianus by Guiraud et al (12). These authors observed that the mutant could still grow on sucrose but that the ability to grow on inulin was lost. Hydrolysis of sucrose appeared to be catalyzed by inulinase that, as a side-effect of the mutation, was completely retained in the cell wall.

The reason for the difference between S. cerevisiae and K. marxianus with respect to the retention of their hydrolyzing enzymes in the cell wall remains unclear. Both the molecular mass of the native enzyme (invertase is a much larger aggregate than inulinase; Fig. 4) and the cell wall composition may be of importance. Compared to the cell wall of S. cerevisiae, the wall of K. marxianus var. marxianus is much more sensitive to changes in ionic strength of the surrounding medium (29), changes in pH (9,29), and treatment with thiols (9,14,29). The latter may indicate that the cell wall of K. marxianus var. marxianus is less rigid than that of S. cerevisiae.

The physiological role of inulinase. Apart from structural differences and differences in enzyme kinetics (10,11,19,22,27), invertase and inulinase also differ in physiological function. The physiological role of invertase mainly concerns the hydrolysis of sucrose within the cell wall. The main physiological role of inulinase is the breakdown of fructans outside the cell wall. Retention of invertase within the cell wall results in an enzyme concentration and a possible way to outcompete non-invertase producing micro-organisms (1,4). That excretion of invertase into the culture liquid may be regarded as disadvantageous is exemplified in bakers' yeast production. The small amounts of the invertase released by this yeast are sufficient to promote growth of infections like Candida krusei (24) a yeast unable to grow on sucrose. On the other hand, with inulin as a substrate, excretion of inulinase into the culture medium is a necessity since this polymer cannot penetrate the cell wall. The cell wall-retained inulinase does contribute to inulin metabolism, but probably only when small oligofructosides are present.
CHAPTER 6

In *K. marxianus* var. *drosophilaram* and *K. marxianus* var. *vanudenii*, the localization of inulinase strongly depended on environmental pH. Only at low pH values these organisms were able to consume inulin. If this pH-dependent localization of inulinase also exists under natural conditions, it follows that inulin degradation by these yeasts is only carried out in acidic environments. In this respect it is relevant that these yeasts can be found in root exudates of many types of trees and in the alimentary tracts of fruitflies (6,17). These environments are acidic and therefore may allow inulinase to become functional.

**Applied aspects of inulinase-producing yeasts.** The observed decrease in inulin-dependent oxygen consumption by washing of the cells could be of importance in possible applications of *K. marxianus* var. *marxianus*. Production of D-fructose or ethanol from inulin by immobilized *K. marxianus*, in a continuous process is in most cases lower than expected (3,26). This might result from a limited inulin-metabolizing capacity due to low inulinase concentrations: Cells are washed before immobilization, and high flow rates are used during the process. Both process steps may lead to loss of inulinase activity.

Studies into the localization of extracellular enzymes can be of interest for the expression of heterologous genes in yeasts. In particular, the downstream processing of heterologous gene products depends on their localization. By adapting cultivation conditions, all the protein may be retained in the cell wall. Subsequently, it can then be easily released by changing culture conditions or by chemical treatment of the cells, without a significant loss of cell viability. This opens the opportunity to concentrate the desired protein without cell disruption and may allow recycling of the cells.

**LITERATURE CITED**


-130-


CHAPTER 6

Yeasts: Metabolism and regulation of cellular processes, ENSAM-CRAM, Montpellier, France.


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Chapter 7

The Discovery of β-Galactosidase.

R.J. Rouwenhorst, J.T. Pronk, and J.P. van Dijken.

Reflections on Biochemistry

The discovery of β-galactosidase

R. J. Rouwenhorst, J. T. Pronk and J. P. van Dijken

The enzyme β-galactosidase was first mentioned in the literature by Beijerinck exactly a hundred years ago. The Department of Microbiology and Enzymology of the Delft University of Technology keeps the memory of Beijerinck, its first professor, alive by maintaining a 'Beijerinck-room' in the attic of the building. In addition to manuscripts and laboratory notebooks, this room contains some of his chemicals and biological preparations, and it was here that we recently found a 90-year-old lactase preparation. Even after storage under suboptimal conditions, the preparation still exhibited measurable enzyme activities.

Lactase

β-Galactosidase (lactase; EC 3.2.1.23) is a well known and extensively studied enzyme which catalyses the hydrolysis of milk sugar (lactose) into the monosaccharides D-galactose and D-glucose. Lactase is produced by a wide variety of organisms including bacteria, yeasts and fungi. Lactases of yeasts and filamentous fungi are of industrial importance in the saccharification of whey permeate, allowing the subsequent alcoholic fermentation with Saccharomyces cerevisiae. The enzyme is also applied in the treatment of skinned milk to allow its consumption in developing countries where the incidence of lactose intolerance is high.

The lactase operon in Escherichia coli, first described in 1961 by Jacob and Monod, has had an enormous impact on modern molecular genetics. At present, the lacZ gene and lac-Z fusions are widely used as indicators of gene integration and promoter activity. Lactase, encoded by the lacZ gene, can easily be detected by using the artificial substrate o-nitrophenyl-β-D-galactopyranoside (ONPG).

Lactase was among the first hydrolases to be discovered. In the 1880s and 1890s, many enzymes were described. In most cases yeasts (the word enzyme literally means 'in yeast') were used as a source of these proteins (e.g. invertase, maltase and trehalase). The first report that yeast cells may split lactose enzymatically into its hexose constituents was published by Beijerinck in 1889 (Fig. 1).

Beijerinck as a microbiologist

Martinus Willem Beijerinck started his scientific career as a teacher of botany in 1873 at the Agricultural School of Wageningen, The Netherlands. After 12 years he became an industrial microbiologist at the Dutch yeast factory in Delft. In 1895 he returned to the academic world as professor of Bacteriology at the Polytechnical School in Delft, a position he held until his retirement in 1921.

During his scientific career, Beijerinck published over 100 articles dealing with a great variety of subjects in the fields of botany, microbiology and virology. His scientific achievements include fundamental papers on the physiology of luminescent bacteria, the root nodules of Leguminosae and bacterial nitrogen fixation. Beijerinck successfully applied microbiological methods to the study of unicellular green algae, zoochlorellae and lichen gonidia, thereby achieving for the first time pure cultures of these organisms. Beijerinck's work on tobacco mosaic disease may be considered to mark the beginning of modern virus research. He established properties which later appeared characteristic of all viruses: multiplication in dividing tissue cells, transfer of infection by virus-containing fluid, inactivation by heating, and viability after drying or ethanol precipitation. Further topics of research include the discovery of yeast Schizosaccharomyces octosporus, studies on the butyl alcohol fermentation, investigations into the microorganisms of milk and other dairy products, and systematic studies on acetate acid bacteria and sulphate reducers. Beijerinck also made extensive investigations on the nutritional requirements of microorganisms and developed new techniques for studies in this field (the auxanographic method). A very important contribution to general microbiology was the development of the enrichment principle. Beijerinck and Winogradsky were the first to apply the idea that culture conditions such as medium composition lead to selective enrichment from natural samples of those microbes that are optimally adapted to these conditions.

β-galactosidase 100 years ago

The fermentation of the milk sugar lactose was a subject which attracted Beijerinck's interest while working at the Dutch yeast factory in Delft. He isolated two yeast species, Saccharomyces kefyr and Saccharomyces tyrocola, that were able to ferment lactose (S. kefyr has been renamed Kluyveromyces marxianus var. marxianus; S. tyrocola has not yet been classified). For the detection of disaccharide-splitting enzymes in microorganisms, Beijerinck developed a very elegant bioassay based on the ability of Photobacterium phosphoreum (renamed as Photobacterium phosphoreum) to emit light when hexoses are available as a source of carbon. However, the bacterium is unable to use disaccharides directly. When plating a suspension of the photobacteria on gelatine slants supplemented with lactose, Beijerinck

Die Lactase, ein neues Enzym.

M. W. Beijerinck

in Delft.

Mit 8 Figuren.

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* A factory then called 'Nederlandse Gist-en Spiritusfabriek' and at present known as 'Kowinklijke Gist-broodjes NV Delft'.

Fig. 1. Title page of Beijerinck's paper on the discovery of lactase in the yeasts S. kefyr and S. tyrocola.
did not observe growth or luminosencce. However, when cells of S. kefir or S. tyrocola were plated together with bacteria, light emission could be observed around the yeast colonies. From these 'luminescent plates', Beijerinck concluded that lactose fermentation by the yeast is preceded by enzyme-catalysed hydrolysis of the disaccharide and, furthermore, that the responsible enzyme activity (which he named lactase) was secreted by the yeast cells into the environment. In the original paper, there is also a brief description of the procedure by which the lactase preparations were obtained. In essence, an S. kefir culture was filtrated, and then the enzyme activity was precipitated with 85% ethanol. Using this bioassay but with cane sugar instead of milk sugar as a carbon source, Beijerinck demonstrated that these crude lactase preparations also possessed sucrose-inverting activity. He concluded that lactase was capable of hydrolysing both lactose and sucrose.

Peculiarly, the lactose-hydrolysing activity of the ethanol precipitate was not mentioned explicitly; only the sucrose-hydrolysing activity of the preparation was described.

In the years following these observations, considerable doubt was expressed in the literature as to the validity of Beijerinck's conclusions. These doubts were caused by the ambiguous results that may be obtained with Beijerinck's bioassay. Using a different (chemical) method for the detection of monosaccharides, Schuurmans Stekhoven could not detect any lactose-hydrolysing activity in culture fluids of S. kefir, nor could he solubilize any such activity from fresh or dried cells by treatment with water at 30°C. This author therefore concluded that the luminescence observed in Beijerinck's experiments with lactose could not be based on the metabolism of monosaccharides by the photobacteria. Instead, he suggested that the photobacterium used glycerol, a product of lactose fermentation by the yeast. Moreover, Schuurmans Stekhoven pointed out that the dual activity of Beijerinck's lactase was a misinterpretation. He found that kefir yeast produces an extracellular enzyme, distinct from lactase, capable of inverting sucrose. A few years later Fischer 'rediscovered' lactase. The enzyme could be solubilized from S. kefir cells by treatment with glass beads or toluene and its activity detected by chemical methods. These observations demonstrated the intracellular localization of lactase in yeasts, a fact now generally accepted. Fischer concluded that Beijerinck's observations had not provided conclusive evidence for the presence of lactase in S. kefir.

The bottle in the attic

Among other preparations from Beijerinck's time, we recently found a small stoppered flask dated 4 December 1899 (see Fig. 2). According to the label, the stoppered flask contained a dried lactase preparation. Indeed, from his hand-written laboratory notebooks it can be concluded that ten years after his discovery of lactase, Beijerinck returned to his studies of lactose utilization by microorganisms. The conditions under which the preparation was stored in Beijerinck's laboratory is not known. However, over the last 30 years it has been kept in the attic of the current laboratory building and exposed to widely fluctuating temperatures (between approximately -10°C and 40°C).

The lactase preparation consists of a dry powder of brownish colour. By using a very sensitive enzymatic alcohol assay, we were able to demonstrate the presence of traces of ethanol in this preparation. This probably reflects the use of ethanol for the precipitation of lactase, a procedure mentioned in Beijerinck's first description of the enzyme. Phase contrast microscopy revealed that the lactase preparation was not cell-free: large numbers of yeast cells could be seen (Fig. 3).

Old enzymes never die

Surprisingly, after 90 years of storage under sub-optimal conditions, the lactase preparation still exhibited measurable hydrolytic activities. When assayed with ONPG as a substrate, the preparation showed a low but signifi-
cantly lactase activity, linear both with time and enzyme concentration. Lactase activity increased when the preparation was first treated with toluene (Fig. 4), indicating that an additional amount of enzyme could be released from the yeast cells present in the preparation. Enzyme activities measured with lactose as a substrate were somewhat lower than those with ONPG (0.41 vs. 0.93 nmol of substrate hydrolysed per minute per milligram of the lactase preparation).

In his paper, Beijerinck reported the occurrence of sucrose-hydrolysing activity in lactase preparations. The S. kefyr strain originally isolated by Beijerinck has since been renamed Kluyveromyces marxianus. It is well known that K. marxianus produces an extracellular inulinase (EC 3.2.1.7) that is highly active towards sucrose. It was to be expected that, if the lactase preparation had actually been obtained from Beijerinck’s original S. kefyr culture, the preparation still might possess inulinase activity. Indeed, both sucrose- and inulin-hydrolysing activities could be measured in the lactase preparation (Fig. 5). The ratio of sucrose- to inulin-hydrolysing activity was about 16. This is entirely consistent with ratios observed with fresh K. marxianus inulinase preparations.

Wild-type K. marxianus produces a maximum inulinase activity of approximately 0.2 U mg⁻¹ cells when grown in batch cultures on lactose. The 90-year-old, dried lactase preparation contained approximately 0.005 U mg⁻¹. Thus, over a period of 90 years during which the preparation has been subjected to widely fluctuating temperatures, the inulinase activity probably had declined to a few percent of its original activity.

**Blessed are those who start now**

In summary, our experiments indicate that the lactase prepared 90 years ago in Beijerinck’s laboratory originated from a yeast. This yeast most probably was S. kefyr, the only yeast used by Beijerinck for research of lactase fermentation in that period. This is confirmed by the presence of both β-galactosidase and inulinase activity in the preparation. If the enzyme preparation described by Beijerinck in 1889 was prepared in the same way as the 1899 preparation described here, his crude enzyme preparations indeed contained lactase activity. However, Beijerinck’s paper did not provide conclusive evidence for enzyme-catalysed lactose hydrolysis. Fischer can be considered the real discoverer of lactase, since he was the first to prove beyond doubt that lactose hydrolysis can be catalysed by an enzyme. However, from Beijerinck’s paper it is clear that he was the first to realize that an enzyme activity is involved in lactose hydrolysis. This perception represents just one of the many original ideas of a great scientist, whose work laid the foundation of modern microbiology.

After his retirement, Beijerinck once made the remark ‘Gelukkig zij, die nu beginnen’ (‘Blessed are those who start now’). The spectacular advances that have been made since and the fascinating problems still ahead demonstrate the relevance of this motto for those working in microbiology today.

**Acknowledgement**

The authors wish to acknowledge Dr John R. Woodward, University of Leeds, for his encouragement. His curiosity led to the discovery of the bottle in the attic.

**References**

Chapter 8

Summary

Samenvatting
CHAPTER 8

SUMMARY

In yeasts, extracellular glycoproteins can be found both in the cell wall and in the culture liquid. The distribution of glycoprotein over these locations depends on the yeast species, or even yeast strain, and on the culture conditions. In this thesis, an effort was made to determine the factors that influence the production and localization of the glycoprotein inulinase (EC 3.2.1.17) in Kluyveromyces species. As inulinase strongly resembles the well-studied invertase (EC 3.2.1.26) of S. cerevisiae, both the nature of the two enzymes and their localization are compared. For establishing the distribution of the extracellular enzymes, enzyme activities were determined in three fractions: 1. enzyme present in the culture liquid (supernatant enzyme); 2. enzyme that can be released from the cell wall by chemical treatment (cell wall enzyme); 3. enzyme that can only be solubilized by breakage of the cells (cell-bound enzyme). This distinction of enzyme activities into three fractions allowed a survey of the factors that influence protein localization.

Production and localization of inulinase were studied in carbon- and energy-limited chemostat cultures of K. marxianus var. marxianus CBS 6556 (Chapter 2). Highest enzyme yields were encountered in cultures grown on a mineral medium with either sucrose or inulin as the limiting substrate. In sucrose-limited continuous cultures, the amount of inulinase decreased from 52 U (mg cell dry weight)\(^{-1}\) at D=0.1 h\(^{-1}\) to 2 U (mg cell dry weight)\(^{-1}\) at D=0.8 h\(^{-1}\). Since at increasing dilution rates, the residual substrate concentration in the culture gradually increases, the decrease in inulinase level can be caused by either the growth rate or the residual sugar concentration. Experiments with nitrogen-limited cultures confirmed that the synthesis of inulinase is negatively controlled by the residual substrate concentration. The presence of high inulinase activities during growth of K. marxianus CBS 6556 on non-sugar carbon sources, further indicated that the synthesis of inulinase is a result of a derepression/repression mechanism. The distribution of inulinase over supernatant, cell wall, and cell-bound fractions depended on carbon source, dilution rate, and growth temperature. During growth under derepressed conditions, about 50% of the enzyme was present as supernatant inulinase and about 30% as cell wall inulinase. When inulinase synthesis was repressed, less inulinase was present as cell-bound.
enzyme, and more was released into the culture liquid when inulinase-repressive conditions became more severe. The growth temperature negatively influenced inulinase production when it deviated from the optimal temperature of growth of *K. marxianus* CBS 6556 (37-42°C). At lower temperatures, the decrease in inulinase yield was mainly reflected in a decrease in inulinase released into the culture liquid.

Independent of its localization, the inulinase of *K. marxianus* CBS 6556 had an S/I ratio (ratio between relative activities with sucrose and inulin) of 15±3. The apparent affinity constants of inulinase for the β-fructosides raffinose (trisaccharide) and stachyose (tetrasaccharide) were lower than for sucrose (disaccharide). However, the enzyme specificity decreased with increasing chain length of the substrate; just as for the invertase of *S. cerevisiae*, sucrose is a better substrate than raffinose, stachyose, or inulin (Chapter 2).

In Chapter 3 the localization of invertase was studied in oscillating and steady-state chemostat cultures of *S. cerevisiae* CBS 8066, and compared to the inulinase localization in steady-state chemostat cultures of *K. marxianus* CBS 6556. Oscillations in the rates of carbon dioxide production and oxygen consumption reflect a synchronized budding of part of the culture which is initiated by cyclic production and consumption of ethanol. The latter is related to the specific metabolic regulation of *S. cerevisiae* as a Crabtree-positive yeast. The production of invertase was restricted to the budding phase; invertase synthesis terminated when cell separation took place. The newly produced invertase remained in the cell wall, and did not provoke a more pronounced release of invertase in the culture liquid. Indeed, the supernatant invertase remained constant during the budding cycle. It is hypothesized that the release of invertase from the cell wall into the culture liquid is only affected by the shear force. In a synchronized budding continuous culture, due to higher concentrations of residual glucose or higher intracellular concentrations of glucose metabolites, the invertase production was two to three times lower than in a steady-state continuous culture. However, even in steady-state cultures of *S. cerevisiae*, variations in invertase production and distribution were observed and these are probably caused by the synchronized division of a small part of the cell population. No significant deviations in inulinase
production and localization occurred in steady-state continuous cultures of the Crabtree-negative yeast *K. marxianus* CBS 6556.

In Chapter 4, factors that might influence the localization of inulinase in *K. marxianus* CBS 6556 were investigated via a biochemical approach. The supernatant inulinase and the cell wall inulinase were purified and analyzed by denaturing and non-denaturing polyacrylamide gel electrophoresis. Both inulinases had similar subunits, consisting of a 62 kDa polypeptide with varying amounts of carbohydrate (26-37% of the molecular mass). The inulinases differed in size, due to differences in subunit aggregation. The supernatant inulinase was a dimer and the cell wall inulinase a tetramer. The cell-bound inulinase also appeared to be tetrameric. These findings support the hypothesis that retention of glycoproteins in the yeast cell wall may be caused by a permeability barrier. The differences in oligomerization did not affect the apparent affinity constants of these inulinases. However, the specific activity of the dimeric inulinase was higher than that of the tetrameric form, indicating that there is negative co-operativity.

No homology was found between the amino acid compositions of the amino terminal ends of *K. marxianus* CBS 6556 inulinase and *S. cerevisiae* invertase. The kinetic and structural evidence indicates that in yeasts two distinct β-fructosidases exist, viz. invertase and inulinase (Chapter 4).

During the purification of the two *K. marxianus* CBS 6556 inulinases, problems arose concerning the assessment of protein concentrations. The presence of carbohydrate groups in inulinase disturbed the linearity of the classic chromogenic methods for protein determination. A new sensitive and reliable method for the determination of protein concentrations by its carbon content is described in Chapter 5. Using a carbon content of 0.53 g/g in protein and of 0.44 g/g in carbohydrate, the concentrations of normal proteins, proteins containing chromophoric groups and proteins containing carbohydrate could be established. Determination of specific absorption coefficients by measuring the absorbance of protein solutions at 280 nm and measuring their carbon concentrations gave values that agreed with values reported in the literature. The method may have special applicability in protein purification studies since it does not require prior knowledge of molar extinction coefficients and since it monitors the disappearance of carbon compounds other than proteins.
CHAPTER 8

In Chapter 6 it is reported that the capacity for utilization of inulin is much more widespread among yeast species than is known from yeast taxonomy. The formerly unrecognized ability of K. marxianus var. drosophilareum, K. marxianus var. vanudenii, and S. kluveri to grow on inulin, is mainly due to the fact that in taxonomy assimilation tests are performed under special, standardized cultivation conditions. Alteration of culture conditions, like lowering the culture pH and increasing the growth temperature, resulted in rapid growth on inulin of these yeasts. It is hypothesized that altered culture conditions such as these, lead to removal of a permeability barrier in the outer region of the cell wall. This would either enable inulin to diffuse into the cell wall or inulinase to diffuse out of the cell wall. Measurement of inulin-dependent oxygen consumption by cell suspensions of K. marxianus revealed that both inulin concentration and inulinase localization determined the rate of inulin hydrolysis (Chapter 6). The physiological, ecological, and taxonomical aspects of the occurrence and localization of inulinase in Kluyveromyces species are discussed in this chapter.

A 90-year old preparation of lactase (β-galacosidase; EC. 3.2.1.23) from Candida kefiri, stored under suboptimal conditions, still exhibited measurable enzyme-activities (Chapter 7). The lactase was prepared by Beijerinck, who, 10 years earlier, had reported his discovery of lactose-hydrolyzing enzymes in yeasts. A closer look into the experimental methods used by Beijerinck and the older literature indicated that Beijerinck was unlikely to be the discoverer of lactase. Apart from lactose-hydrolyzing activity, the preparation contained sucrose- and inulin-hydrolyzing activity in a S/I ratio of 16, indicating that an inulinase was present too.
CHAPTER 8

SAMENVATTING

De extracellulaire, geglycosyleerde eiwitten van gisten kunnen worden aangetroffen in de celwand van de gist of in de kweekvloeistof. De verdeling van geglycosyleerde eiwitten over deze twee lokaties blijkt zowel afhankelijk te zijn van de gistsoort, of zelfs de giststam, als van de kweekcondities. In dit proefschrift wordt nagegaan welke factoren van invloed zijn op de produktie en lokalisatie van het geglycosyleerde eiwit inulinase (EC. 3.2.1.7) in gistsoorten uit het geslacht Kluyveromyces. Het inulinase katalyseert de hydrolyse van het fructosepolymer inuline en van het disaccharide saccharose. Daar dit inulinase sterk overeenkomende karakteristieken vertoont met het invertease (EC. 3.2.1.26) van de gist S. cerevisiae, worden aard en lokalisatie van deze enzymen vergeleken. Daarbij is onderscheid gemaakt tussen drie cultuurfracties waarin enzymactiviteit aanwezig kan zijn:

1. activiteit in de kweekvloeistof (supernatant-enzym)
2. activiteit die vrijkomt uit de celwand na behandeling van de gist met β-mercaptoethanol (celwand-enzym),
3. activiteit die alleen na openbreken van de gistcellen vrij in oplossing te krijgen is (celgebonden enzym).

De produktie en lokalisatie van het inulinase werden bestudeerd in koolstof- en energie-gelimiteerde continu-culturen van de giststam K. marxianus var. marxianus CBS 6556 (Hoofdstuk 2). De hoogste opbrengst aan inulinase werd gevonden in culturen die gekweekt waren op een mineraal basismedium met saccharose of inuline als limiterende koolstofbron. In saccharose-gelimiteerde culturen nam de totale hoeveelheid inulinase af van 52 naar 2 U (mg drooggewicht)⁻¹ wanneer de verdunningsnelheid van de cultuur werd verhoogd van 0.1 naar 0.8 h⁻¹. Een verhoging van de verdunningsnelheid ging gepaard met een verhoging van de residuele suikerconcentratie in de fermentor. Experimenten met stikstof-gelimiteerde culturen waarbij de residuele suikerconcentratie hoog was, bevestigden dat de produktie van het inulinase onder een negatieve controle staat van de residuele suikerconcentratie. Dit betekent dat de synthese van het inulinase onderworpen is aan kataboliet-repressie. De vorming van vrij hoge inulinase niveaus gedurende de groei van K. marxianus CBS 6556 op andere koolstofbronnen dan suikers, gaf aan dat de synthese van het inulinase
CHAPTER 8

gereguleerd wordt door een repressie/derepressie mechanisme. De verdeling van het inulinase over supernatant, celwand, en celgebonden fractie bleek afhankelijk van de koolstofbron, de verdunningsnelheid en de kweektemperatuur. Gedurende de groei van de gist onder inulinase-derepresserende condities was ongeveer 50% van het enzym aanwezig als supernatant-enzym en ongeveer 30% als celwand-enzym. Deze verdeling veranderde onder inulinase-represserende condities. De algemene tendens was dat wanneer de represserende condities strenger worden er steeds meer enzym vrijkomt in de kweekvloeistof en dat het celgebonden enzym steeds sterker afneemt. De kweektemperatuur oefende een negatieve invloed uit op de inulinaseproduktie als deze afweek van de optimale groeitemperatuur van K. marxianus CBS 6556 (37-42°C). Bij te lage kweektemperaturen nam de opbrengst aan inulinase af en dit ging gepaard met een afname in het percentage inulinase dat vrijkwam in de kweekvloeistof.

Onafhankelijk van de lokatie van het inulinase was de S/I ratio (relatieve enzymactiviteit met saccharose en inuline als substraat) van het enzym 15±3. Een dergelijke waarde voor de S/I ratio wordt algemeen als kenmerkend voor een inulinase beschouwd. Dit in tegenstelling tot invertases die een S/I ratio >1000 hebben en dus bijna of geen activiteit met inuline vertonen. De schijnbare affiniteitsconstantes ($K_m'$) van het inulinase voor de β-fructosiden raffinose (een trisaccharide) en stachyose (een tetrasaccharide) waren lager dan de $K_m$ voor saccharose. De waarde voor de enzymspecificiteit ($K_{kat}/K_m'$) nam echter toe met toenemende lengte van het te hydrolyseren substraat. Dit laatste betekent dat saccharose een beter substraat is voor het enzym dan raffinose, stachyose of inuline, en dat het inulinase in dit opzicht niet verschilt van het invertase van S. cerevisiae (Hoofdstuk 2).

In Hoofdstuk 3 wordt de lokalisatie van invertase bestudeerd in oscillerende en steady-state continu-culturen van S. cerevisiae CBS 8066. De resultaten hiervan worden vergeleken met de inulinase-lokalisatie in steady-state continu-culturen van K. marxianus CBS 6556. Oscillaties in kooldioxide-productiesnelheid en zuurstof-consumptiesnelheid weerspiegelen de synchrone deling van een deel van de celpopulatie in de cultuur. Deze synchrone deling wordt waarschijnlijk veroorzaakt door een plotselinge vorming van ethanol door de gist, samenhangend met de specifieke regulering van het metabolisme van de gist S. cerevisiae. Deze kan namelijk onder
volledig aerobe condities overschakelen van een oxydatieve naar een fermentatieve (ethanol vormend) stofwisseling als glucose in overmat aanwezig is (Crabtree-effect). De productie van invertase bleek sterk afhankelijk van de delingsfase van de gist. De invertase werd geproduceerd tijdens de knopvorming en de produktie stopte tijdens voltooiing van de celdeling. Het tijdens de knopvorming gevormde invertase bleef voornamelijk in de celwand van de gist gelokaliseerd, m.a.w. het leidde niet tot een sterker vrijkomen van invertase in de kweekvloeistof. Integendeel, de absolute hoeveelheid enzym in de kweekvloeistof bleef constant tijdens de gehele celcyclus. Het idee wordt geopperd dat het vrijkomen van invertase in de kweekvloeistof veroorzaakt wordt door de afschuifkracht in de cultuur.

Als gevolg van de hogere residuele glucoseconcentratie en de hogere intracellulaire concentratie van glucosemetabolieten was de invertase productie in een oscillerende cultuur twee tot drie keer lager dan in een steady-state cultuur. Echter, zelfs in een steady-state cultuur van S. cerevisiae werden variaties in invertaseproductie en in verdeling over celwand en kweekvloeistof waargenomen. Dit werd naar alle waarschijnlijkheid veroorzaakt doordat fracties van de celpopulation gelijktijdig deelden. In steady-state continu-culturen van de Crabtree-negatieve K. marxianus CBS 6556 werden dergelijke significante variaties in inulinaseproductie en lokalisatie niet waargenomen.

CHAPTER 8

Dit was wel het geval met de specifieke enzymactiviteit: het dimer inulinase had met zowel saccharose, raffinose als inuline een veel hogere specifieke activiteit dan het tetramer. Blijkbaar is er bij inulinase negatieve cooperativiteit in het spel.

Er bestaat weinig of geen homologie in de aminozuurvolgorde van het N-terminale einde van het inulinase en dat van invertase. De kinetische en structurele verschillen geven aan dat er in gisten twee soorten β-fructosidases voorkomen, nl. inulinase en invertase (Hoofdstuk 4).

Tijdens het opzuiveren van de twee inulinases van K. marxianus CBS 6556 bleek het bepalen van de eiwitconcentraties problemen te geven. De aanwezigheid van suikerketens in de inulinases verstoord de lineairiteit van de klassieke, op specifieke kleuringen gebaseerde, methodes voor het bepalen van eiwitgehaltes. In Hoofdstuk 5 wordt een nieuwe, gevoelige en betrouwbare methode beschreven voor het bepalen van eiwitconcentraties, waarbij het koolstof gehalte in eiwitpreparaten gemeten wordt. Uitgaande van een koolstofgehalte van 0.53 g/g in eiwit en een koolstofgehalte van 0.44 g/g in suikerketens kan de concentratie bepaald worden van globulaire eiwitten, eiwitten met chromofore groepen, en eiwitten met suikerketens. Na bepaling van zowel de absorptie bij 280 nm als het koolstofgehalte van deze enzympreparaten konden specifieke absorptiecoefficienten worden berekend. De waarden hiervan bleken goed overeen te komen met die in de literatuur. De methode kan speciaal toepassing vinden tijdens het zuiveren van eiwitten. Het meten van het koolstofgehalte vereist geen kennis op voorhand omtrent de specifieke absorptiecoefficient van het te zuiveren eiwit en geeft de mogelijkheid tot het volgen van de verdwijning van koolstof die niet afkomstig is van eiwit maar van andere componenten, zoals lipiden en DNA.

In Hoofdstuk 6 wordt vastgesteld dat het gebruik van inuline als koolstofbron vaker kan voorkomen onder gistsoorten dan bekend is in de gisttaxonomie. Van gisten als K. marxianus var. drosophilareum, K. marxianus var. vanudinii en S. kluveri was het voorheen onopgemerkt gebleven dat ze in staat zijn te groeien op inuline. Dit wordt voornamelijk veroorzaakt doordat in de gisttaxonomie gebruik wordt gemaakt van gestandaardiseerde kweekcondities om de groei op suikers te testen. Wijziging van de kweekcondities, zoals toepassing van een lagere pH of een hogere groeitemperatuur, resulteerde bij deze drie gisten in een snelle groei op inuline. Dit leidde tot de hypothese dat deze veranderingen van de
kweekcondities aanleiding geeft tot een vermindering of verwijdering van een diffusiebarrière in de buitenste regionen van de celwand van de gisten. Hierdoor wordt het mogelijk dat het inuline de celwand in diffundeert, of dat het inulinase uit de celwand diffundeert en de kweekvloeistof vrijkomt. Aan de hand van de meting van inuline-afhankelijke zuurstofconsumpties door celsuspensies van *K. marxianus* CBS 6556 werd duidelijk dat zowel de inuline-concentratie als de inulinase-localisatie de in vivo hydrolysesnelheid van inuline bepalen. De fysiologische, ecologische en taxonomische aspecten die met de aanwezigheid en localisatie van inulinase in *Kluveromyces*-soorten gepaard gaan, worden tevens in Hoofdstuk 6 besproken.

Een 90-jaar oud lactase (**β**-galactosidase)-preparaat verkregen uit de gist *Candida kefir*, vertoonde, ondanks dat het al die tijd was bewaard onder suboptimale omstandigheden, nog steeds meetbare enzymactiviteiten (Hoofdstuk 7). Het lactase was geïsoleerd door Beijerinck die tien jaar eerder zijn 'ontdekking' van het lactose-hydrolyserende enzym in gisten had gepubliceerd. Na het bestuderen van de door Beijerinck gebruikte methodieken en het bestuderen van de oude literatuur bleek een echte ontdekking van lactase door Beijerinck twijfachtig te zijn. Behalve de lactose-hydrolyserende activiteit bevatte het preparaat ook saccharose- en inuline-hydrolyserende activiteit in een S/I ratio van 16. Dit betekent dat er in het preparaat ook een inulinase aanwezig is.
DANKWOORD.

IEDEREEN DIE OP WELKE WIJZE DAN OOK BIJGEDRAGEN HEeft TOT DE TOTSTANDKOMING VAN DIT PROEFSCHRIFT: HARTELIJK BEDANKT!

Bovenstaande zin is de snelste en meest eenvoudige manier voor het geven van een dankwoord waarbij je zeker weet dat je niemand vergeten hebt. De laatste jaren is het echter 'fashion' geworden om in het proefschrift een wat meer persoonlijke noot aan het dankwoord te breien m.a.w. je moet NAMEN noemen. Uit vrees dat ik iemand vergeet zal ik die trent niet navolgen. Bovendien vind ik dat iedereen die op het laboratorium werkt/werkte (of die op het laboratorium aanwezig was) in de tijd dat ik er vertoefde, door zijn/haar aanwezigheid een bijdrage geleverd heeft. Zelfs de anonieme student die op een sombere winteravond de deur van het lab voor mij open deed (de sleutel vergeten!). Anders had ik die avond toch mooi niet mijn kluyver-beestjes kunnen koesteren en had het proefschrift misschien uit zeven hoofdstukken bestaan.

Gedurende totaal vijf jaar heb ik bijna elke ochtend de trip naar het laboratorium gemaakt. Die jaren zijn erg snel voorbij gegaan omdat ik het er gewoon erg naar m'n zin had. Misschien wel te snel. Het werken was er interessant en leuk en de omgang met iedereen erg plezierig. Allen nogmaals bedankt. Plink! (-klein traantje).