Purification and Characterization of an α-Glucosidase from *Saccharomyces carlsbergensis*†

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ABSTRACT: α-Glucosidase (EC 3.2.1.20) was purified to homogeneity from logarithmically growing cells of *Saccharomyces carlsbergensis*. The purification involved the following steps: (a) ammonium sulfate fractionation; (b) Sephadex G-100 chromatography; (c) DEAE-cellulose chromatography; and (d) hydroxylapatite chromatography. This procedure gave a preparation judged to be greater than 98% pure by Na-DodSO$_4$–polyacrylamide gel electrophoresis. The enzyme was shown to be a monomer of 63 000 daltons by gel filtration on Sephacryl S-200 under native conditions and by polyacrylamide gel electrophoresis under denaturing conditions. The K_m values of the enzyme for the substrates maltose and *p*-nitrophenyl α-D-glucoside were found to be 1.66×10^{-2} and 3.1×10^{-4} M, respectively. The corresponding V_{max} value for maltose was 44.8×10^{-6} mol min^{-1} mg^{-1} and that for *p*-nitrophenyl α-D-glucoside was 134×10^{-6} mol min^{-1} mg^{-1}. The pH optimum for the purified enzyme was found to be between pH 6.7 and 6.8. The enzyme has an absolute anomeric specificity for α-glycosidic linkages and appears to recognize a glucosyl residue in α linkage on the nonreducing end of its substrate. For the strain used in this study, which carries the MAL 6 locus, only a single form of the enzyme was detected.

An inducible enzyme α-glucosidase or maltase (EC 3.2.1.20), which is present in some yeast species, catalyzes the intracellular hydrolysis of maltose (De la Fuente & Sols, 1962). Investigations on its purification, characterization, and regulation have been the subject of numerous reports (ten Berge et al., 1973; Khan & Eaton, 1967; Halvorson & Ellias, 1958). In spite of this interest, there is uncertainty regarding the size of enzyme and number of enzyme species in yeast cells that have related specificities. Molecular weight determinations of yeast maltase preparations from four independent investigations have yielded values ranging from 40 000 to 85 000 (Legler & Lotz, 1973; McDermott, 1968; Khan & Eaton, 1967; Halvorson & Ellias, 1958).

The presence of any one of seven unlinked MAL genes is necessary for maltose fermentation in yeast (Mortimer & Hawthorne, 1969; Oeser & Windisch, 1964). Initially, it was suggested that each of these loci defined a maltase structural gene; however, more recent data indicates that at least several of these loci play a regulatory role (ten Berge et al., 1973). Halvorson et al. (1963) and later Needleman (1975) have shown that maltase isolated from strains carrying different MAL genes has similar physical and enzymatic characteristics. The interpretation of these results is complicated by the recent suggestion of three distinct forms of maltase in a strain with a single MAL gene (Eaton & Zimmerman, 1976).

Recent technical advances, particularly the ability to clone structural genes from a variety of sources in the bacterium *Escherichia coli*, have stimulated interest in and have provided new methods to study the organization, expression, and regulation of both eukaryotic and prokaryotic genes. These recent developments, together with the availability of yeast mutants affecting the expression of maltase and the inducible character of the enzyme, make the system attractive for further investigation. To facilitate such an investigation, specific information regarding the size and number of forms of maltase is required. This report describes the isolation and purification of maltase from *S. carlsbergensis*.

Materials and Methods

Strains. S. carlsbergensis strain CB 11 (α MAL 6 *ade* 1) was obtained from A.M.A. ten Berge.

Materials. The chemicals used in this study were maltose (Difco or Sigma), ammonium sulfate (Schwarz/Mann, enzyme grade), Sephadex G-100 medium, Sephacryl S-200 (Pharmacia), DEAE-cellulose (Whatman), hydroxylapatite (Bio-Rad; DNA grade), *p*-nitrophenyl α-D-glucoside, *p*-NPG[1] (Sigma), sodium dodecyl sulfate (Pierce), urea (Schwarz/Mann; UltraPure), acrylamide (Sigma; recrystallized from ethyl acetate), *N'*,*N'*-methylenebis(acrylamide) (Sigma; recrystallized from acetone), ampholines (LKB), NP-40 (Particle Data Labs). All other chemicals used were of reagent grade.

Growth of Cells. Strain CB 11 was grown to late logarithmic phase (density of 1.1×10^8 cells/mL) in a medium containing 1% Difco yeast extract, 2% Difco peptone, 2% maltose (YPM) supplemented with 20 μg/mL adenine sulfate in a 14-L New Brunswick fermentor at 30 °C with moderate aeration. Cells were harvested using a CEPA continuous flow centrifuge. The yield of cells under these conditions was approximately 12 g wet weight/L. Cells could be frozen at −20 °C for up to 2 months without significant loss of maltase activity.

Enzyme Assays. The chromogenic substrate, *p*-NPG, was routinely used for assay of maltase activity. Assay mixtures contained 50 mM potassium phosphate, pH 6.8, 1 mM β-mercaptoethanol, 1 mM EDTA, and a final concentration of 0.3 mg/mL of *p*-NPG. Reactions were stopped after 15 s by the addition of 1 volume of 1 M sodium carbonate and absorbance at 400 nm was measured. Specific activities were

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Na-DodSO$_4$, sodium dodecyl sulfate; PhCH$_2$SO$_2$F, phenylmethanesulfonyl fluoride; *p*-NPG, *p*-nitrophenyl α-D-glucoside.

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TABLE I: Purification of Maltase from *S. carlsbergensis*.

purification step	vol (mL)	protein (mg/mL)	total act. (units × 10^{-6})	sp act. (units/mg)	recovery (%)
fraction 1: high speed centrifugation	61	25	4.0	2 662	100
fraction 2: ammonium sulfate fractionation	12	56.5	3.4	4 960	85
fraction 3: Sephadex G-100 chromatography	70	2.0	2.1	15 080	53
fraction 4: DEAE-cellulose chromatography	13	1.5	1.3	62 070	33
fraction 5: hydroxylapatite chromatography	22	0.5	1.3	131 313	33

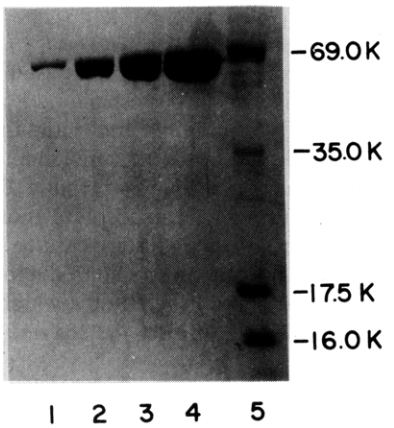

FIGURE 1: The purity of fraction V was evaluated by 12.5% Na-DodSO$_4$-poly(acrylamide) gel electrophoresis according to the method of Laemmli (1970). Samples were boiled in 1% NaDodSO$_4$ and 20 mM dithiothreitol for 5 minutes before loading. Lanes 1, 2, 3, and 4 represent the Coomassie blue stain patterns of increasing amounts of fraction V. The corresponding protein loads for these lanes were 1, 5, 10, and 20 μg, respectively. Lane 5 represents the stained pattern of the molecular weight reference markers bovine serum albumin (mol wt = 69 000), rabbit muscle lactate dehydrogenase (subunit mol wt = 35 000), bovine β-lactoglobulin (subunit mol wt = 17 500), and bovine hemoglobin (subunit mol wt = 16 000).

expressed as nmol of *p*-nitrophenol (*p*-NP) released per min per mg of protein. One unit of activity was defined as the amount of enzyme that liberated 1 nmol of *p*-NP/min at 23 °C. Protein determinations were carried out by the method of Lowry et al. (1951).

Substrate specificity assays using compounds which release glucose were determined by the two-step glucose oxidase method of Lloyd & Whelan (1969). The cleavage of substrates containing umbelliferone was assayed fluorometrically (Robinson, 1956).

Results

Preparation of the Crude Extract. Washed yeast cells suspended in an equal volume of 50 mM potassium phosphate, pH 6.8, containing 1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), were disrupted in a Braun homogenizer with glass beads (0.45–0.50 mm diameter; 1:2 v/v beads:cell suspension) for 2 min with cooling. The broken cells were centrifuged at 12 000*g* for 10 min; the supernatant was decanted and then recentrifuged at 110 000*g* for 1.5 h. The high speed supernatant was taken as the crude extract (fraction I). All manipulations were performed at 4 °C unless otherwise indicated.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was slowly added to fraction I to 35% saturation over a 30-min period. A pH of 6.8 was maintained throughout by the addition of 1 M KOH. Centrifugation at 7700*g* for 20 min yielded a supernatant containing maltase activity. The ammonium sulfate concentration of the supernatant was then adjusted to 85% saturation and centrifuged at 7700*g* for 20 min. The pellet was suspended in approximately 5 volumes of 50 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, 1 mM $PhCH_2SO_2F$, and 2% (v/v) glycerol (fraction II).

Sephadex G-100 Chromatography. Fraction II was applied to a Sephadex G-100 column (4 × 85 cm) equilibrated with 50 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, and 2% (v/v) glycerol. The loaded column was eluted at a flow rate of 35 mL/h and 7-mL fractions were collected. Those fractions, representing a fourfold increase in specific activity, were pooled (fraction III).

DEAE-Cellulose Chromatography. Fraction III was dialyzed against two changes of buffer containing 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, and 2% (v/v) glycerol and then applied to a Whatman DE52 column (1.5 × 22 cm) equilibrated with dialysis buffer. The column was eluted with 400 mL of a 10–200 mM linear potassium phosphate gradient, pH 6.8, containing 1 mM EDTA,1 mM dithiothreitol and 2% (v/v) glycerol. Those fractions which contained the highest amount of specific activity (within 5% of the peak fraction) were pooled (fraction IV).

Hydroxylapatite Chromatography. Fraction IV was dialyzed against two changes of a buffer consisting of 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, and 2% (v/v) glycerol. The dialysate was loaded onto a hydroxylapatite column (1 × 22 cm) equilibrated in dialysis buffer. A 400-mL linear potassium phosphate gradient from 10 to 100 mM (as described in the DEAE-cellulose step) was used to elute the column. Peak fractions containing maltase activity were pooled (fraction V). Table I summarizes the relevant purification data.

Assessment of Purity. The Tris-glycine/NaDodSO$_4$-polyacrylamide gel electrophoresis system of Laemmli (1970) was used at all stages to monitor the extent of purification. Figure 1 illustrates a typical enzyme preparation displayed on a 12.5% poly(acrylamide) slab gel. Lanes 1–4 represent the stained patterns generated from using increasing amounts of fraction V. To estimate the extent of contamination of maltase by other proteins, this polyacrylamide gel was dried and the stained proteins in these lanes were scanned in a Joyce-Loebl densitometer. Lower molecular weight impurities, barely discernible to the unaided eye, were not detected by the densitometer. Evaluation of the amount of protein required to give a signal under the conditions employed for densitometry indicated that maltase impurity levels were less than 1.5%.

To examine whether there are multiple forms of maltase, two-dimensional electrophoresis was performed by the method of O'Farrell (1975). A representative two-dimensional pattern is presented in Figure 2. The single maltase spot supports the notion of one form of the enzyme. To demonstrate the comigration of maltase activity with the protein band, isoelectric focusing gels run without urea and NP-40, but including 10% glycerol, were sliced into 1-mm segments and assayed for the capacity to hydrolyze *p*-NPG. As shown in Figure 3 only a single peak of activity was observed which comigrated with stained maltase. Two-dimensional gel analysis of purified

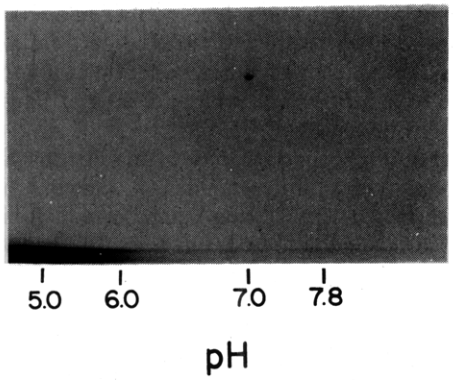

FIGURE 2: Two-dimensional gel electrophoresis was performed on fraction V by the method of O'Farrell (1975). Ten micrograms of protein was isoelectricfocused in a 4.0% poly(acrylamide) gel with pH 3.5–10 ampholines. After isoelectric equilibrium was attained the gel was layered on and electrophoresed through a 10–15% exponential $NaDodSO_4$–poly(acrylamide) gel. The pH gradient was determined from a parallel gel.

maltase revealed occasional gel artifacts related to the isoelectric focusing step. Purified maltase samples, when prepared for isoelectric focusing in the presence of urea and NP-40, would infrequently give rise to two or three bands of the same molecular weight. The bands stained unequally, the major species being the most basic. In samples prepared and run without urea and NP-40, the acidic spot(s) were not detected. To rule out the possibility that these isoelectric variants represented different primary sequences, two-dimensional peptide mapping (data not shown) was performed by a modification of the Cleveland procedure (Cleveland et al., 1977). Those isoelectric focusing gels which gave rise to multiple bands were electrophoresed into a 4.5% polyacrylamide spacer gel containing 0.1% $NaDodSO_4$ and any one of three proteolytic enzymes, trypsin, α-chymotrypsin, or *Staphylococus aureus* V8 protease. After a brief digestion period within the spacer gel, the potential was reapplied and the gels were run as described by Cleveland et al. (1977). The peptide maps generated from digestion of isoelectric variants were indistinguishable for all the proteases tested. This result indicates that isoelectric heterogeneity due to differences in primary sequence is unlikely.

Determination of Kinetic Parameters. A Lineweaver–Burk kinetic analysis was performed on fraction V with *p*-NPG as substrate (data not shown). A Michaelis constant (K_m) of 3.1×10^{-4} M and a maximal velocity (V_{max}) of 134×10^{-6} mol of *p*-NPG hydrolyzed min^{-1} mg^{-1} of protein was obtained. When maltose was used as a substrate, a K_m of 1.66×10^{-2} M and a V_{max} of 44.8×10^{-6} mol min^{-1} (mg of protein)$^{-1}$ was obtained.

A thermal inactivation experiment was performed according to the method of Eaton (Eaton & Zimmerman, 1976). In all cases monophasic heat inactivation profiles were obtained (data not shown). The decay constant for the purified enzyme (fraction V) at 48 °C is 2.42×10^{-1} min^{-1}.

Determination of pH Optimum and Substrate Specificity. The pH optimum in 100 mM potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol for the substrate *p*-NPG was between pH 6.7 and pH 6.8. Absorbance measurements of the *p*-NP moiety were all normalized to the same pH.

Substrate specificity was examined by two different methods. For those glucosides which were hydrolyzed by the enzyme, a V_{max} was determined by standard Lineweaver–Burk kinetic analysis. The second approach involved testing all glucosides for their ability to inhibit the enzymatic hydrolysis of *p*-NPG. Assuming Michaelis–Menten kinetics, a dissociation constant, K_i, was determined for those glucosides which displayed competitive inhibition with respect to *p*-NPG hydrolysis. The dissociation constant, K_i, was determined from the equation:

$$\frac{1}{V_i} = \frac{1}{V_{max}}\left(K_m + \frac{K_m[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

where V_i represents the initial velocity in the presence of inhibitor, [I] represents the inhibitor concentration, and K_i represents the dissociation constant of the enzyme–inhibitor complex. K_m, V_{max}, and [S] represent the standard kinetic parameters, Michaelis constant, maximal velocity, and substrate concentration, respectively, for the uninhibited reaction. The results of these experiments are presented in Table II.

Amino acid analysis was performed by the standard single column method as described in the Beckman amino acid manual on the Beckman amino acid analyzer, Model 120B

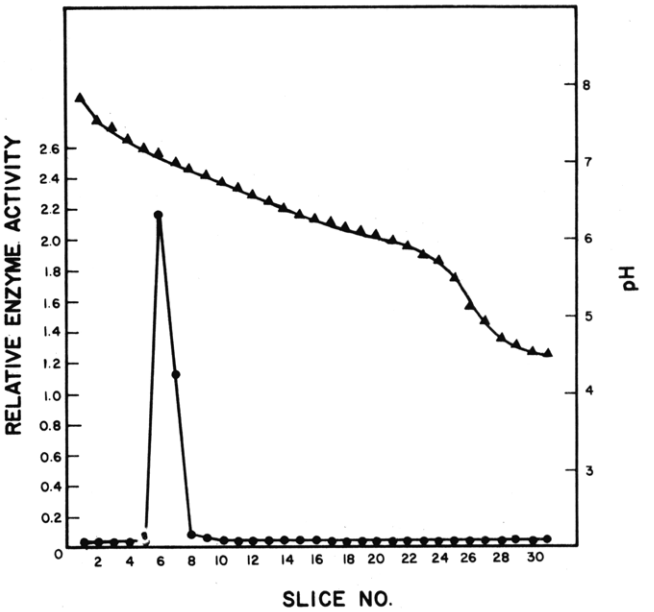

FIGURE 3: An isoelectric focusing gel of purified maltase was run with glycerol and without denaturants. The gel was sliced into 1-mm segments and each slice assayed for their capacity to hydrolyze *p*-NPG. (●) Represents the relative enzyme activity per slice expressed as the optical density at 400 nm of the liberated *p*-NP moiety. (▲) Represents the pH profile as determined from another gel run in parallel.

TABLE II: Affinities of Substrates and Inhibitors of Maltase.

	K_i [a]	V_{max} [b]
p-nitrophenyl α-D-glucopyranoside	3.1×10^{-4} [c]	134
methyl α-D-glucopyranoside	8.5×10^{-2}	4.4×10^{-2}
methyl β-D-glucopyranoside	*d*	0
saligenin β-D-glucopyranoside	1.6×10^{-2}	0
4-methylumbelliferyl β-D-glucopyranoside	2.0×10^{-3}	0
4-methylumbelliferyl α-D-glucopyranoside	9.0×10^{-5}	127
maltose	1.7×10^{-2}	45
sucrose	1.5×10^{-2}	53
turanose	3.8×10^{-3}	58
isomaltose	*d*	0
glucose	3.4×10^{-3}	0

[a] K_i expressed in mol/L. [b] V_{max} expressed as μmol per min per mg of protein. [c] This value represents the K_m for the uninhibited reaction. [d] No detectable inhibition.

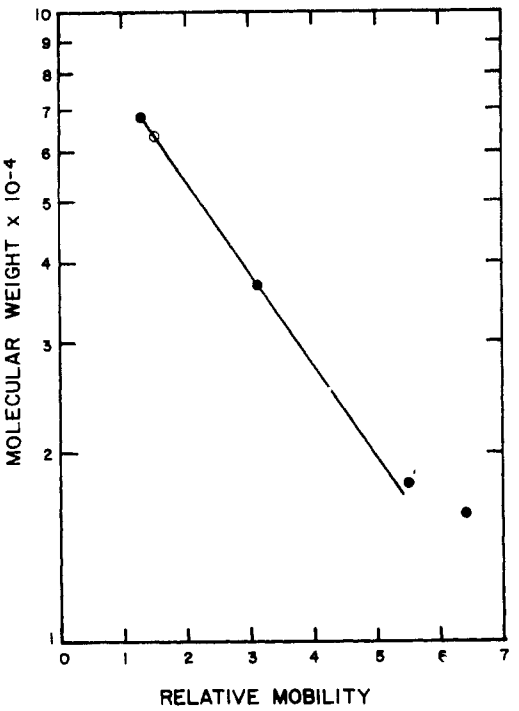

FIGURE 4: The molecular weight determination of maltase was performed by the method of Weber & Osborn (1969) using 12.5% poly(acrylamide) gels. (●) Represents the molecular weight markers bovine serum albumin (mol wt = 69 000), rabbit muscle lactate dehydrogenase (subunit mol wt = 35 000), bovine β-lactoglobulin (subunit mol wt = 17 500), bovine hemoglobin (subunit mol wt = 16 000). (○) Represents the mobility of purified maltase.

TABLE III: Amino Acid Composition.

amino acid	mole fraction	residues/63 000 daltons
Ala	0.057	40
Arg	0.044	16
Asp	0.124	59
Cys	ND[a]	
Glu	0.115	49
Gly	0.066	55
His	0.027	11
Ile	0.056	27
Leu	0.063	30
Lys	0.082	35
Met	0.014	6
Phe	0.076	29
Pro	0.055	30
Ser	0.069	41
Thr	0.063	33
Trp	ND	
Tyr	0.043	15
Val	0.046	25

[a] Not determined.

fitted with 6-mm (i.d.) columns. The results of the analysis are presented in Table III.

Molecular Weight Determination and Subunit Composition. Molecular weight analysis on the purified enzyme was performed by NaDodSO$_4$-poly(acrylamide) gel electrophoresis and gel filtration chromatography on Sephacryl S-200. The molecular weight estimate by poly(acrylamide) gel electrophoresis under fully dissociating conditions was performed on fraction V by the method of Weber & Osborn (1969) on 12.5% NaDodSO$_4$-polyacrylamide slab gels. Between 2 and 10 μg of each sample or reference marker was boiled for 5 min in a buffer containing 1% NaDodSO$_4$ and 20 mM dithiothreitol to ensure complete dissociation. Maltase, which migrated slightly faster than bovine serum albumin, was estimated to have a molecular weight of 63 000. This result is presented in Figure 4.

Gel filtration chromatography of maltase on Sephacryl S-200 was performed under nondenaturing conditions. The column was loaded with fraction V and bovine serum albumin in a relative ratio of 1 to 100 and eluted with a potassium phosphate buffer as described in the Sephadex G-100 step above. Maltase elution was followed by its activity on *p*-NPG and bovine serum albumin elution was monitored by absorbance at 280 nm. Sephacryl, a mixed matrix of polyacrylamide and agarose, was chosen in order to minimize the potential affinity of maltase for the glucosyl α-1→6 linkages prevalent in Sephadex. Under these conditions, maltase eluted from the column just after the bovine serum albumin monomer (data not shown). These results are in agreement with the NaDodSO$_4$-poly(acrylamide) gel molecular weight estimates and therefore support the assignment of a value of 63 000 as the monomeric molecular weight of maltase.

Discussion

A procedure for the purification of an α-glucosidase or maltase from *S. carlsbergensis* has been described which allows the preparation of large quantities of enzyme. The enzyme is stable in its purified form, but is subject to proteolysis and inactivation in crude extracts. The serine protease inhibitor, $PhCH_2SO_2F$, was found to be essential for the reproducible preparation of maltase.

Based on a comparison of specific activities of the crude extract (fraction I) and the purified enzyme (fraction V), the relative abundance of maltase as a cytosolic constituent can be estimated. In the yeast strain used, grown on maltose to the logarithmic phase, maltase represents approximately 2% of the total soluble protein. If it is assumed that polypeptide abundance reflects messenger RNA abundance, it can be inferred that maltase gene transcription is a very active process.

Some physical characteristics of maltase have been presented. The molecular weight determinations by NaDodSO$_4$-poly(acrylamide) gel electrophoresis, under fully dissociating conditions, and by gel filtration chromatography on Sephacryl S-200, under native conditions, are in good agreement and indicate that maltase is a 63 000 dalton monomeric enzyme. This result is similar to the conclusion of Kahn & Eaton (1967).

The kinetic data, pH optimum, and substrate specificity further define the enzymatic characteristics of maltase. The kinetic constants, K_m and V_{max}, as well as the pH optimum are consistent with values obtained by others (Legler & Lotz, 1973). However, the temperature inactivation data obtained is in conflict with the report of Eaton & Zimmerman (1976). Using their experimental conditions, multiphasic thermal decay kinetics were not observed with purified maltase. One possible explanation is that the multiphasic decay kinetics, which Eaton & Zimmerman observed in crude extracts, reflected changes due to the proteolytic degradation of maltase.

The substrate specificity data indicate the enzyme's absolute requirement for an α-D-glycosidic linkage. The ability of maltase to split sucrose, turanose, and *p*-NPG further suggests that its primary specificity is directed toward the glucosyl residue in the nonreducing position in α linkage to another moiety. Amino acid composition data was presented in order to provide a basis for comparison to other purified yeast α-

glucosidases. The data of McDermott (1968) and Legler & Lotz (1973), when expressed in residues per 63 000 dalton protein, are in agreement with our findings.

The strain used in this study, CB 11, which carries the MAL 6 gene contains only a single form of maltase. This conclusion is supported by the observed homogeneity of enzyme preparations when subjected to isoelectric focusing under both denaturing and nondenaturing conditions. The multiple banding pattern sometimes observed when samples for isoelectric focusing were run in the presence of urea and NP-40 does not result from inherent protein heterogeneity. This interpretation is supported by its infrequent occurrence and urea dependence. One-dimensional peptide maps failed to reveal major differences in the primary structure of these isoelectric variants. The explanation for the heterogeneity may require an understanding of the chemical modification reactions occurring in samples containing urea and NP-40. Isoelectric focusing artifacts due to either carbamoylation or deamidation have been extensively reported (Righetti & Drysdale, 1973). Alternative explanations for multimodal isoelectric focusing banding patterns from apparently homogeneous proteins have been suggested (Cann & Stimpson, 1977; Stimpson & Cann, 1977).

The purification and characterization of yeast maltase are the first parts of a program designed to explore the expression and regulation of this enzyme. The development of a maltase sequence probe, by the application of recently developed recombinant DNA technology, will enable one to analyze the molecular events ensuing after the induction of the enzyme.

Acknowledgments

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