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PURIFICATION AND CHARACTERIZATION OF MALTASE AND α-METHYL GLUCOSIDASE FROM YEAST

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SUMMARY

A method for the simultaneous purification of maltase and α-methyl glucosidase from a strain of *Saccharomyces cerevisiae* carrying the MA_3 gene has been described.

The two enzymes have been characterized with respect to substrate specificities, pH optima, molecular weights, heat inactivation and behavior on ion-exchange columns.

The structural relationship of these two enzymes and the problem of their simultaneous induction have been discussed.

INTRODUCTION

SPIEGELMAN, SUSSMAN AND TAYLOR[1] reported the formation of two inducible enzymes in response to maltose in yeast. One enzyme was specific for maltose as a substrate and the other for α-methyl glucoside. Simultaneous induction of maltase and α-methyl glucosidase (isomaltase)[2,3] by maltose or α-methyl glucoside (N. A. KHAN AND N. R. EATON, unpublished results), poses interesting questions regarding the genetic control mechanisms for the synthesis of these two enzymes.

In order to investigate the nature of this genetic control, it was found essential, first to purify these two enzymes for qualitative and quantitative comparisons. Maltase has been purified by HALVORSON AND ELLIAS[2] from *Saccharomyces italicus*. The low yield of enzyme, however, made it impossible to establish the purity by standard criteria. Another attempt to purify maltase from yeast was made by PHILIPS[4]. The final yield in this case was also low and no extensive characterization was made. Both of these authors reported hydrolysis of α-methyl glucoside by maltase in their pure preparations.

In this report a method for simultaneous purification of these two enzymes and their partial characterization will be described.

Abbreviation: PNPG, *p*-nitrophenyl-α-D-glucopyranoside.

MATERIALS AND METHODS

Saccharomyces cerevisiae, strain 1412-4D, carrying the MA_3 gene, was used throughout these studies. The yeast was grown in 1% yeast extract and 2% Bacto-peptone containing either 2% maltose or 2% α-methyl glucoside as a carbon source. 1 ml of freshly-grown culture was inoculated per l of media in Fernbach flasks and was incubated for 24 h in a rotary shaker at a constant temperature of 30°.

Activities of both the enzymes were estimated by following the continuous release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside[2] (PNPG). The assay mixture contained enzyme, 0.02 mmole of phosphate buffer (pH 7.2) 0.02 mmole 2-mercaptoethanol, and 0.3 μmole of PNPG in a total volume of 1 ml. The adsorption of *p*-nitrophenol was determined at 400 mμ using a Beckman DU-Gilford spectrophotometer.

The rates of hydrolysis of other glucosides were determined by estimating the glucose released with a coupled glucose oxidase and peroxidase system (Glucostat special, Worthington Biochemical Co.).

Protein was determined by the microbiuret method at 310 mμ. The microbiuret reagent was prepared as described by ZAMENHOFF[5].

Sephadex G-100 and DEAE–Sephadex A-50 were obtained from Pharmacia and prepared according to the manufacturer's instructions.

An estimate of purity was obtained by polyacrylamide-gel electrophoresis with 7.5% 'Standard gel' according to the manual supplied by the Canal Industrial Corporation (Bethesda, Md.). All the samples were run at 5° with a constant current of 3 mA per tube.

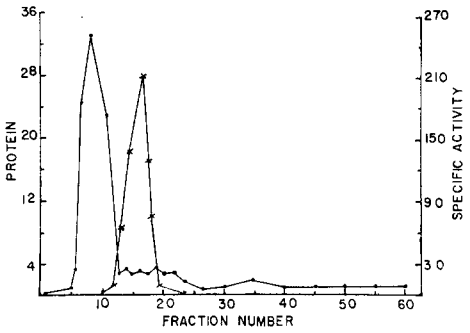
The molecular weights of maltase and α-methyl glucosidase were determined on Sephadex G-100 columns as described by ANDREW[6,7], with minor modifications. The pure proteins, α-chymotrypsinogen (6 times crystallized beef pancreas, Mann Research Laboratories), crystalline aldolase (Worthington Biochemical Co.), hemoglobin (human, 2 times crystallized, Mann Research Laboratories), and bacterial alkaline phosphatase (Worthington Biochemical Co.) were used as standards for the estimation of molecular weights. The column of Sephadex G-100 (2.5 cm × 34 cm) was equilibrated with phosphate buffer (pH 7.2) containing 2.5% $(NH_4)_2SO_4$. All samples were run separately at 5°. After each run the column was rewashed overnight with the same buffer.

RESULTS

Purification of enzymes

6 l of cells were harvested by centrifugation and washed twice with distilled water. The washed cells were crushed as described previously[8], suspended in approx. 75 ml of 0.1 M phosphate buffer, and centrifuged at 27 000 × *g* for 15 min to remove cellular debris. The crude extract was then centrifuged at 105 000 × *g* in a Spinco model L ultracentrifuge. The supernatant fluid was treated with $(NH_4)_2SO_4$ (enzyme grade, Fisher Scientific Co.) and the precipitate obtained between 30% and 75% satn. was retained for further purification. The increase in specific activity accomplished by this step was approx. 3–4 fold.

The $(NH_4)_2SO_4$ precipitate was dissolved in 20–25 ml phosphate buffer and



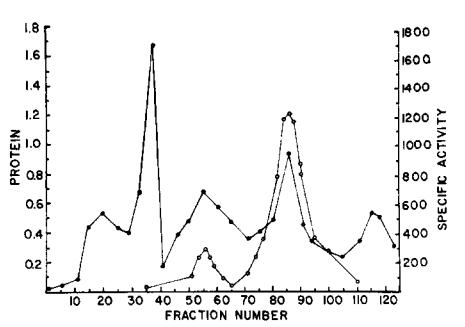


Fig. 1. Gel filtration on Sephadex G-100. Approx. 350 mg of protein in 10-ml vol. were layered on (2.5 cm × 34 cm) column. Fractions of 5 ml were collected. ●—●, absorbance at 280 mμ; ×—×, specific activity of enzymes (Δ absorbance per min per mg protein).

Fig. 2. Chromatography of maltase and α-methyl glucosidase on DEAE-Sephadex A-50. Approx. 200 mg of protein were adsorbed to the (2.5 cm × 30 cm) column. Fractions of 4 ml were collected. ●—●, absorbance at 280 mμ; ○—○, specific activity (Δ absorbance per min per mg protein) of maltase (Fractions 70–100) and α-methyl glucosidase (Fractions 50–65).

the pH was adjusted to 7.0. The resulting solution was dialyzed 6–8 h against 25 mM phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol and 5% $(NH_4)_2SO_4$, which appeared to stabilize the protein during purification. The small amount of insoluble material that separated during dialysis was removed by centrifugation. Aliquots of the clear dialyzed solution, containing approx. 300–400 mg of protein were then layered on the columns of Sephadex G-100 (2.5 cm × 34 cm), which had been previously equilibrated with the same buffer as that used for dialysis. Fig. 1 illustrates a typical elution pattern. The main peak of the activity emerges from the column shortly after the turbid front fractions. The enzyme-containing fractions, characterized at this stage by clear yellowish color, were checked qualitatively for the presence of the enzymes by PNPG. The gel filtration generally resulted in an increase of specific activity of 3–4 fold over the $(NH_4)_2SO_4$ precipitate.

The fractions containing enzyme were pooled after gel filtration, concentrated by 65% $(NH_4)_2SO_4$ satn., and dialyzed against 50 mM phosphate buffer for 4–6 h.

TABLE I

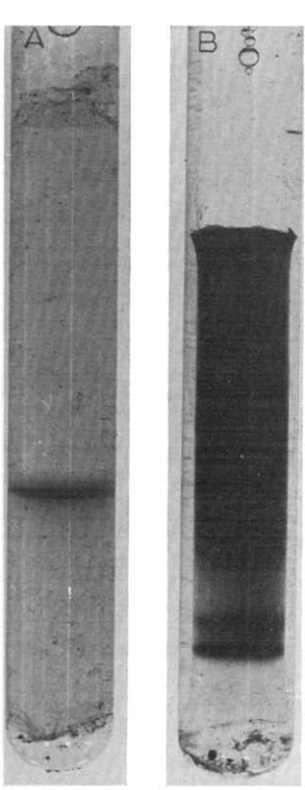
PURIFICATION OF MALTASE AND α-METHYL GLUCOSIDASE

Fraction	*Total vol. (ml)*	*Protein (mg/ml)*	*Units/mg*	*Total units ($\times 10^{-6}$)*	*Recovery from preceding step (%)*	*Purification*
Crude	65	62	12 800	51.5	—	—
105 000 × *g* supernatant	55	47	18 700	48.3	93.7	1.2
30% $(NH_4)_2SO_4$	65	28.7	29 000	54.0	111.9	2.2
75% $(NH_4)_2SO_4$	46	30.0	39 000	53.8	99.4	3.0
Sephadex G-100	97	2.5	164 000	39.7	73.8	12.8
DEAE-Sephadex A-50						
Maltase	28	0.35	3 040 000	29.7	74.8	237.5
α-Methyl glucosidase	32	0.38	270 000	3.3	8.2	21.1

The final step of the purification procedure involved chromatography on DEAE-Sephadex A-50. The column (2.5 cm × 30 cm) was equilibrated with 50 mM phosphate buffer containing 1 mM mercaptoethanol. The dialyzed material from Sephadex G-100 was adsorbed to the column and eluted with a linear gradient of 0 to 0.5 M NaCl in buffer. Typical results are given in Fig. 2. Both enzymes obtained after this step were dialyzed immediately against 50 mM phosphate buffer to remove NaCl, and 5% $(NH_4)_2SO_4$ was added to stabilize the enzymes. A summary of the purification procedure is given in Table I.

Criteria of purity

The purity of the enzymes was checked by electrophoresis on polyacrylamide gel and by rechromatography on Sephadex G-100 columns. Maltase has a single



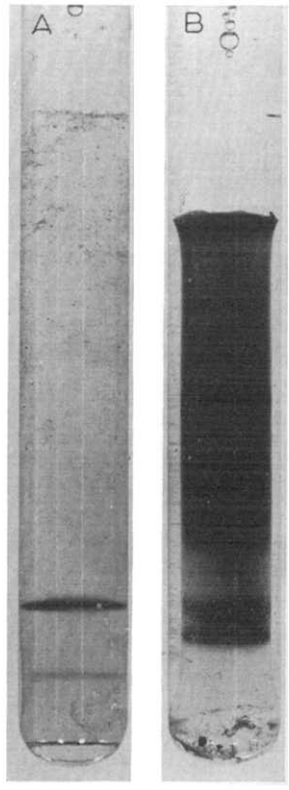


Fig. 3. Polyacrylamide-gel electrophoresis patterns of crude extract (B) and purified maltase (A).

Fig. 4. Polyacrylamide-gel electrophoresis of crude extract (B) and purified α-methyl glucosidase (A).

band on the gel as shown in Fig. 3. Preparations of α-methyl glucosidase, on the other hand, contained 2 bands (Fig. 4), the heavier of which probably represents the enzyme. Rechromatography of the purified maltase on Sephadex G-100 under conditions identical to those described above revealed no protein contaminant.

TABLE II

RECHROMATOGRAPHY OF PURIFIED MALTASE ON SEPHADEX G-100 COLUMN

Fraction number	*Protein (mg/ml)*	*Specific activity*
14	0.04	920
15	0.07	1004
16	0.085	1215
17	0.090	1205
18	0.065	1157
19	0.045	1116
20	0.021	1096
21	0.015	845

The specific activities were similar in all the fractions except in very dilute ones. (Table II). It has consistently been observed that maltase is relatively unstable in dilute solution. Recovery of no other protein peak on Sephadex G-100 except maltase and the similarity of specific activity in all fractions suggest that the maltase preparation is free from any significant protein contamination.

Partial characterization

Comparisons of the two enzymes were made with respect to their substrate specificities, pH optima, molecular weights, and heat inactivation.

Substrate specificities were tested with maltose, α-methyl glucoside, sucrose and isomaltose. Sucrose and PNPG were hydrolyzed by both the enzymes. However the rates of reaction in both cases were slower with α-methyl glucosidase. Maltase hydrolyzed maltose but could not hydrolyze α-methyl glucoside and isomaltose. α-Methyl glucosidase, on the other hand, hydrolyzed α-methyl glucoside and isomaltose, but was unable to use maltose as a substrate. These results are given in Table III.

The specific activities of the two enzymes were determined at different pH

TABLE III

SUBSTRATE SPECIFICITIES OF MALTASE AND α-METHYL GLUCOSIDASE

Assay mixture contained: 1–5 μmoles of substrate, 0.045 mmole of phosphate buffer (pH 7.0) and enzyme 0.05 ml (approx. 3000 units of maltase and 2700 units of α-methyl glucosidase) in a total volume of 1 ml. The mixtures were incubated at 30°, and samples were withdrawn at 30-min intervals for the determination of glucose as described (see MATERIALS AND METHODS).

Enzyme	*Substrate*	*μmoles of glucose/h*
Maltase	Maltose	0.90
	Isomaltose	0.00
	α-Methyl glucoside	0.00
	Sucrose	0.60
α-Methyl glucosidase	Maltose	0.00
	Isomaltose	0.92
	α-Methyl glucoside	0.66
	Sucrose	0.48

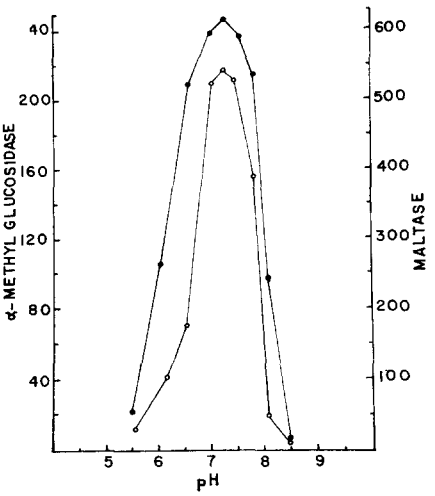
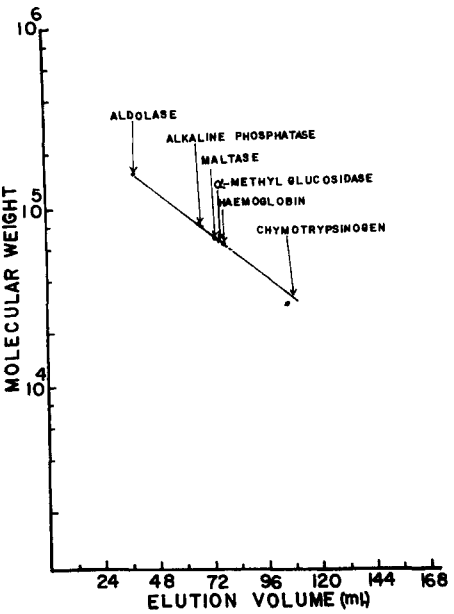


Fig. 5. pH optima of maltase and α-methyl glucosidase. Each point represents an average of 6–9 readings. ●—●, α-methyl glucosidase; ○—○, maltase. Ordinates are Δ absorbance per min. per mg protein using PNPG as the substrate.



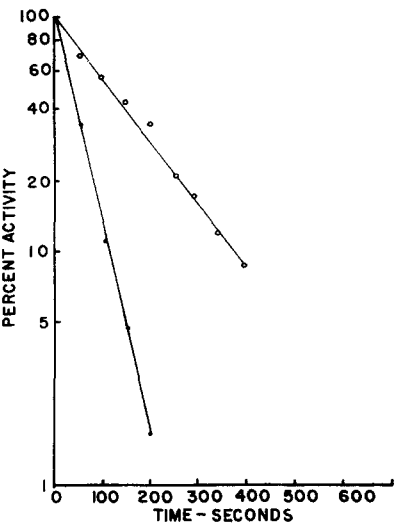


Fig. 6. Plot of log mol. wts. against elution volume of various proteins on Sephadex G-100.

Fig. 7. Heat inactivation of purified maltase and α-methyl glucosidase . Samples were incubated at 51° in 75 mM phosphate buffer (pH 7.2) and 2.5% $(NH_4)_2SO_4$. ●—●, maltase; ○···○, α-methyl glucosidase.

values, using PNPG as a substrate. As illustrated by Fig. 5, both enzymes have pH optima between 7.0 and 7.5.

The behavior of maltase and α-methyl glucosidase during purification on Sephadex G-100 indicated similarity in size of both the molecules. However, the estimates of the molecular weights of two isolated enzymes on Sephadex G-100 according to the method of ANDREW[6,7], revealed slight but reproducible differences in size. The molecular weight of maltase is approx. 68 500 ± 1200 (95% confidence interval), and α-methyl glucosidase 64 700 ± 2870 (95% confidence interval). These results are illustrated in Fig. 6.

The stability of maltase and α-methyl glucosidase towards heat was compared at 51°. The kinetics of inactivation for both enzymes are essentially first order, but α-methyl glucosidase is more stable towards heat than maltase as shown in Fig. 7.

DISCUSSION

The purification procedure described in this communication permits the preparation of maltase and α-methyl glucosidase in relatively few steps with a high yield. The final product is virtually homogeneous in the case of maltase as judged by electrophoresis and by rechromatography on Sephadex G-100. A significant improvement over the previously described methods results from the use of Sephadex-gel filtration and DEAE-Sephadex chromatography.

This method has also been used successfully in this laboratory to purify maltase from a constitutive strain of yeast. The procedure described should be equally applicable for the ready preparation of enzyme from strains carrying single alleles of any of the six maltose genes[9,10].

In the earlier attempts to purify maltase, we found that the enzyme was unstable after 10–12 fold purification and was inactivated after a few days at −15°, even in the presence of mercaptoethanol. $(NH_4)_2SO_4$ in 2.5–5% concn. has been found to stabilize the enzyme and apparently activates it also (Table I, Step 3). Both maltase and α-methyl glucosidase show increases in specific activities after the addition of 5% $(NH_4)_2SO_4$ following the last step of purification, although the mechanism of this activation is not clear. When stored in presence of $(NH_4)_2SO_4$ at −15°, 70–80% of the activities of both the enzymes can be recovered after 30 days.

Purification and characterization of α-methyl glucosidase of yeast has not been previously reported in detail. This enzyme has been distinguished in the present study from maltose on the basis of substrate specificity, molecular weight, heat inactivation, and its behavior on DEAE-Sephadex ion-exchange column. The similarity in size, pH optimum, and behavior in all the steps of purification, except on DEAE-Sephadex column, indicates a very close structural similarity between the two proteins, and this may account for the reports[2,4] of α-methyl glucosidase activity in maltase preparation.

The simultaneous induction of maltase and α-methyl glucosidase by either maltose or α-methyl glucoside poses interesting questions concerning control of the formation of the two enzymes. Genetic studies in our laboratory (N. A. KHAN AND N. R. EATON, unpublished) and by MORTIMER AND HAWTHORNE[11], have shown that the MA_3 gene is closely linked to an α-methyl glucoside gene (MG_2). The close genetic linkage of these two genes suggested that they might be under the control of

a common operator[12]. However, a preliminary study (N. A. KHAN AND N. R. EATON, unpublished), using various conditions of induction, has shown that the two enzymes are not produced in a constant ratio. These studies suggest that the formation of maltase and α-methyl glucosidase are not controlled by a common operator.

Further investigation of these problems is currently in progress.

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