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The Separation of Intestinal Invertase and Three Different Intestinal Maltases on TEAE-cellulose by Gradient Elution, Frontal Analysis, and Mutual Displacement Chromatography

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The separation of intestinal invertase and maltase activities on ion exchange columns was further studied by use of a modified ion exchange cellulose, TEAE-cellulose.

The gradient elution, frontal analysis, and mutual displacement techniques were applied to the separation of intestinal invertase and maltase activities on these columns.

The results with these separation methods supported the theory that intestinal maltase activity consists of three components, only one of which is associated with invertase activity.

The intestinal invertase activity behaved as one single enzyme. The homogeneity of intestinal invertase activity also appears from the K_s values for the invertase activity in different fractions of a chromatogram.

The method for the separation of intestinal maltase activities by partial heat inactivation was improved.

By combining the methods of heat inactivation and ion exchange chromatography, the three components of intestinal maltase activity were purified. The three maltase fractions had different K_s values, which supports the theory that they are different enzymes.

In a previous paper the separation of intestinal maltase activity in one heat-stable component (amounting to 1/3 of the total maltase activity) and one heat-labile component, was described [1]. The heat-stable maltase was without invertase activity. The heat-labile maltase, too, was partially separated from the invertase activity. In a preparation containing heat-labile maltase and invertase activity with a maltase/invertase activity quotient of 0.6, these activities could not, however, be further separated. In this preparation the invertase and maltase activities may therefore possibly be caused by the same enzyme.

As a working hypothesis, the possible existence of three different maltases in intestinal mucosa was therefore assumed. For the further study of these enzymes, however, improved separation methods were required.

In the present paper further separation experiments with intestinal invertase and maltase activities are described. Use was made of a modified ion exchange cellulose (TEAE-cellulose) and, in addition to *gradient elution*, two other techniques for chromatography, namely *frontal analysis* and *mutual displacement chromatography*. The separation by partial heat inactivation was improved, too. The enzyme fractions obtained were characterized by their K_s values.

MATERIALS AND METHODS

Solubilized glycosidases were prepared from pig intestinal mucosa as previously described [2].

Substrates. Sucrose of analytical grade purity was obtained from J. T. Baker Co. Maltose of analytical grade purity was obtained from Merck A. G. (Germany) and Pfanstiehl Chemical Co. The maltose preparations were found to contain oligosaccharides with a lower mobility than maltose on the paper chromatogram. For maltase determinations the preparations were used without further purification, but for K_s determinations chromatographically pure maltose was used, which was obtained by carbon column chromatography [3].

Determination of invertase and maltase activity was performed under the same conditions as used earlier, *i. e.* at 0.1388 M substrate concentration, in 0.05 M maleate buffer pH 6.5 and with toluene as preservative [1,2]. One unit of glycosidase activity produces 1 mg of monohexoses (*i. e.* 1 % of hydrolysis in 2.0 ml of reaction mixture) in 1 h at 37°C under these conditions. When the degree of hydrolysis did not exceed 10 %, the amount of monohexoses produced was proportional to the amount of enzyme present.

Determination of invertase activity. 1.0 ml of substrate solution (9.5 % sucrose *, 0.1 M maleate buffer pH 6.5 and a few ml of toluene per lit.) and 1.0 ml of enzyme solution with 0.5 – 3 units of invertase were mixed in a test-tube in a water-bath at 37°C. After 60 min the reaction was interrupted by the addition of 2.0 ml of the 3,5-dinitrosalicylic acid reagent of Sumner [4]. A blank was prepared with the same composition, in which, however, the 3,5-dinitrosalicylic acid reagent was added *before* the enzyme solution. The samples were heated in a boiling water-bath for 10 min, chilled with tap water, and diluted with 20.0 ml of distilled water. After mixing, the red colour was determined with a Beckman B spectrophotometer at 530 mμ. The colour was stable for at least 20 h. The amount of monohexoses produced was calculated from a standard curve with known amounts of glucose. Glucose and fructose have the same extinction coefficient with the 3,5-dinitrosalicylic acid reagent.

Determination of total maltase activity. Equal volumes of substrate solution (10.0 % maltose monohydrate, 0.1 M maleate buffer pH 6.5 and a few ml of toluene per lit.) and enzyme solution with 5 – 10 units of maltase per ml were mixed in a test-tube in a water-bath at 37°C. At zero time and after 60 min, 0.10 ml samples were withdrawn from the reaction mixture and immediately precipitated [5] with 1.0 ml of 5 % $ZnSO_4$ and 1.0 ml of 0.3 N $Ba(OH)_2$, diluted with water to 4.0 ml and centrifugated. 2.0 ml of the supernatant was transferred to a Folin's tube for sugar determination, 2.0 ml of the acid copper reagent of Tauber and Kleiner [6] was added, and the tube was boiled for 8 min in a water-bath. After chilling with tap water for 2 min, 2.0 ml of Benedicts molybdate reagent [6] was added, and after mixing the solution was diluted to the 25 ml mark with distilled water. After 3 – 30 min the blue colour was determined with a Beckman B spectrophotometer at 600 mμ. With each set of determinations, a series of tubes were boiled containing known amounts of maltose and glucose in mixture, corresponding to 0, 5, 10, and 15 % hydrolysis.

* Per cent means g of solute per 100 ml of solution.

Determination of heat-stable maltase activity. To the enzyme solution was added water or phosphate buffer (depending on the buffer content of the enzyme solution) until reaching a final concentration of 0.01 M phosphate buffer pH 6.0. The solution was then heated in a water-bath at 60°C for 45 min, which completely inactivated the heat-labile maltase activity, while the heat-stable maltase was unaffected [1]. The heat-stable maltase activity was determined with the procedure described above.

The heat-labile maltase activity was calculated as the difference between the total maltase activity and the heat-stable maltase activity.

K_s *for invertase activity* was determined as described earlier [7].

K_s *for maltase activity* was determined with 0.030—0.002 M maltose solutions. The degree of hydrolysis was not allowed to exceed 20 %. With these dilute substrate solutions it was necessary to use a larger volume of the reaction mixture for the quantitative determination of the glucose produced. Since amounts greater than 5 μmoles of the maleate buffer interfered with the reagents of Tauber and Kleiner, the modified reagents described by Caputto, Leloir and Trucco [8] were used for these determinations. Amounts as large as 50 μmoles of maleate buffer pH 6.5 did not interfere with these reagents.

Protein was determined with the method of Lowry *et al.*[9] by use of human serum albumin (kindly supplied by A.B. Kabi) as a standard.

Conductivity measurements (for determining the concentration of phosphate buffer in chromatography fractions) were made with a Philips conductometer, type PR 9500.

Diethylaminoethyl cellulose (DEAE-cellulose) was prepared from Solkafloc cellulose powder SW 40 A with the method of Peterson and Sober [10]. The product contained 0.75—0.85 mequiv. of titrable hydroxyl groups per g dry weight. The titrations were performed in 0.5 M NaCl [10].

Triethylaminoethyl cellulose (TEAE-cellulose) was prepared from DEAE-cellulose by ethylation as described by Porath [11]. The ethylation process increased the pK value of the ion exchanger from 9.5 (DEAE-cellulose) to 10.0 (TEAE-cellulose). Since the free base of TEAE-cellulose was found unstable even at room temperature, TEAE-cellulose was stored and used in its bromide form.

Before use, the TEAE-cellulose was stirred for 30—60 min with the buffer to be used as starting buffer in the chromatography. After packing the column, it was washed with several column volumes of the same buffer.

RESULTS AND DISCUSSION

Gradient elution from TEAE-cellulose. During gradient elution chromatography on DEAE-cellulose, intestinal maltase and invertase activties formed one single peak, which, however, was not homogeneous [2]. Porath [11] has reported the preparation of a modified anion exchange cellulose, TEAE-cellulose, with somewhat different adsorption properties. The chromatography of the intestinal glycosidases on TEAE-cellulose was therefore studied.

To a 1 × 11 cm column of TEAE-cellulose, which had been equilibrated with 0.010 M phosphate buffer pH 6.0, a solubilized intestinal glycosidase preparation containing 12.2 mg of protein, 180 units of invertase and 360 units of maltase in 0.010 M phosphate buffer pH 6.0 was applied. The enzymes were eluted with phosphate buffer pH 6.0 with continuously increasing concentration (Fig. 1). The experiment was performed at room temperature with a flow rate of 0.5 ml/min. Each fraction collected had a volume of 3.0 ml.

Also with TEAE-cellulose the invertase and maltase activities formed one common peak. The resolution within this peak seemed better, however, than that with DEAE-cellulose (*cf.* Fig. 1 in this publication with Fig. 3 in Ref.[2]). The first half of the invertase activity peak had a low maltase/invertase activity quotient (0.6) and is apparently identical with the fraction obtained earlier [1], which had the same maltase/invertase activity quotient, and in which the invertase

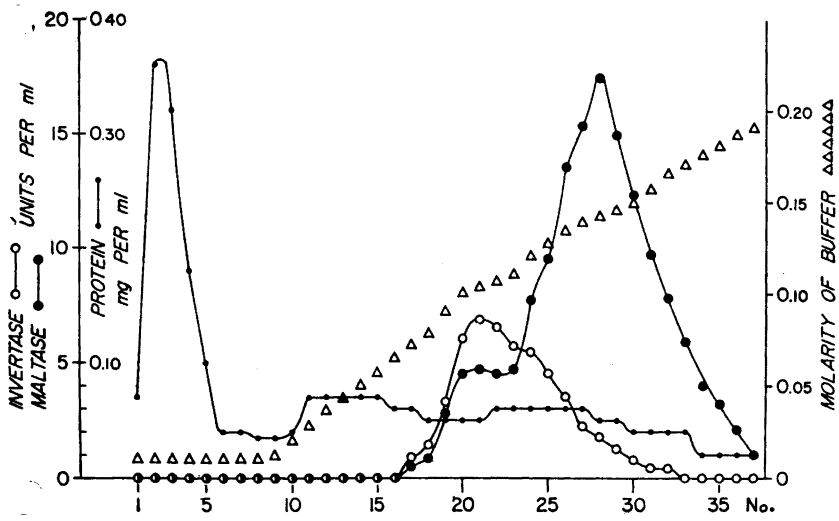

Fig. 1. Gradient elution of intestinal glycosidases on TEAE-cellulose at pH 6.0. For experimental conditions, see text. The molarity of the buffer in the effluent was determined by conductometry.

and maltase activities could not be further separated by rechromatography or by heat inactivation at varying pH. With TEAE-cellulose, too, the heat-stable maltase activity was the main component of the descending branch of the maltase peak.

Attempts to improve the separation by use of longer columns, lower flow rate, or step-wise elution, were not successful.

Frontal analysis. In frontal analysis the column is first washed with pure solvent. Then a relatively large volume of a solution of the substance to be analyzed is filtered through the column. Since the substance is adsorbed to the column, the filtrate is at first pure solvent. While more solution is filtered through the column, however, an increasing part of the column becomes saturated with the substance, and at last the substance appears in the effluent. If the flow rate is not too high, the concentration of the substance in the effluent rises rapidly from zero to the same concentration as in the influent. If there are more than one substance present in the solution, these substances may from separate fronts, depending on their concentration and the capacity of the column for each substance, and also on the competition of the different substances for the adsorbent (mutual displacement).

The frontal analysis principle was introduced by Tiselius [12], and its application to the separation of sugars and fatty acids on charcoal columns has been thoroughly studied by Claesson [13]. The frontal analysis principle has also been applied to the separation of inorganic ions on ion exchange column, which has been studied by Samuelsson [14]. As far as the present author is aware, however, frontal analysis has not been applied to the separation of proteins.

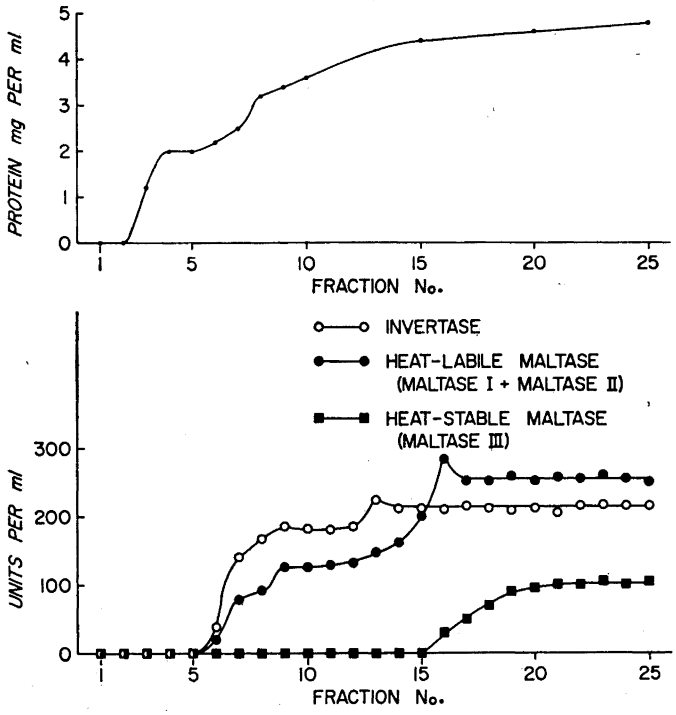

Fig. 2. Frontal analysis of intestinal glycosidases on TEAE-cellulose. 0.070 M phosphate buffer pH 6.0 was used as solvent. For experimental conditions, see text.

The frontal analysis of a solubilized intestinal glycosidase preparation on TEAE-cellulose was studied by use of phosphate buffer pH 6.0, and with varying molarity, as solvent. The experiments were performed on 1 × 6 cm columns containing 0.5 g (dry weight) of TEAE-cellulose, previously equilibrated with the same buffer as was used as solvent for the glycosidase preparation.

It was found necessary to use a relatively low flow rate, 2—3 drops (< 0.2 ml) per min, because otherwise very flat fronts were obtained. The experiments were always performed at room temperature (at lower temperature, a still lower flow rate would have been required).

The buffer concentration was found essential for the results. At low buffer concentration (0.010 M) all the glycosidases appeared as one common front. At higher concentrations (0.060—0.080 M) separate fronts were obtained. At still higher buffer concentration the glycosidases were not adsorbed to the column, but appeared in the effluent together with the solvent front. Since an important factor in frontal analysis seems to be the mutual displacement effect, these observations on the importance of the buffer concentration are in accordance with the findings of Boman [15,16].

A frontal analysis experiment is demonstrated in Fig. 2. The enzyme solution used contained 220 units/ml of invertase, 360 units/ml of maltase

and 4.8 mg/ml of protein in 0.070 M phosphate buffer pH 6.0. The flow rate was 3 drops/min and each fraction collected had a volume of 1.8 ml. The first glycosidase front was formed by invertase activity, accompanied by a heat-labile maltase activity with maltase/invertase activity quotient 0.6. This front corresponds to the maltase fraction I (invertase fraction) postulated earlier [1]. The appearance of these activities as one single, homogeneous front, strongly supports the theory that these activities are caused by one single enzyme. The fact that a second front of heat-labile maltase later appeared, is also in favour of the theory that two different heat-labile intestinal maltases exist.

The second front of heat-labile maltase activity (maltase II [1]) and the front of the heat-stable maltase activity (maltase III [1]) appeared in the same tubes. This front is, however, not homogeneous, since the increase in heat-labile maltase activity is more rapid than the increase in heat-stable maltase activity. This indicates an incomplete mutual displacement of these two activities, which was also found in mutual displacement technique chromatography.

Mutual displacement chromatography. In mutual displacement cromatography the substances to be separated are adsorbed to the upper end of an adsorbent column. The substances are then eluted by the addition of another substance to the influent, which is adsorbed to the column with greater force than any of the substances to be separated. The developer should have a sufficiently high concentration to elute the substances to be separated completely. These substances therefore travel down the column as one band, just in front of the developer. During the passage down the column, the different components of the band compete with each other for the adsorbent, which makes them form separate zones of increasing adsorbability. If sufficiently small fractions are collected, the components may therefore be obtained in different fractions.

This principle for chromatography was introduced by Tiselius [17], and was first applied to the separation of sugars on charcoal columns. It has also been applied to the separation of proteins, but only in a few cases and not always under clear-cut experimental conditions [16]. That one protein may displace another from an ion exchange column has been demonstrated by Boman [15,16]. He found, however, that protein-protein displacement is ineffective at low buffer concentration, *i.e.* when the protein to be displaced is strongly adsorbed.

The mutual displacement chromatography of intestinal glycosidases was performed on TEAE-cellulose columns, which had been equilibrated with phosphate buffer pH 6.0 with varying molarity. After adsorption of the glycosidase solution to the column, this was first eluted with the starting buffer to remove some inactive protein which was not adsorbed to the column [2]. The molarity of the influent buffer then at once was increased to 0.200 M, which eluted all the glycosidases. The enzymes appeared in the effluent as one single protein peak, which was, however, composed of zones with different enzymatic activity (Fig. 3).

The experiments were always performed at room temperature. To obtain good separation, it was necessary to use so low a flow rate as 1 drop/min (0.04 ml). In accordance with the experiences of Boman, the molarity of the starting buffer was of great importance for the separation. With 0.010 M

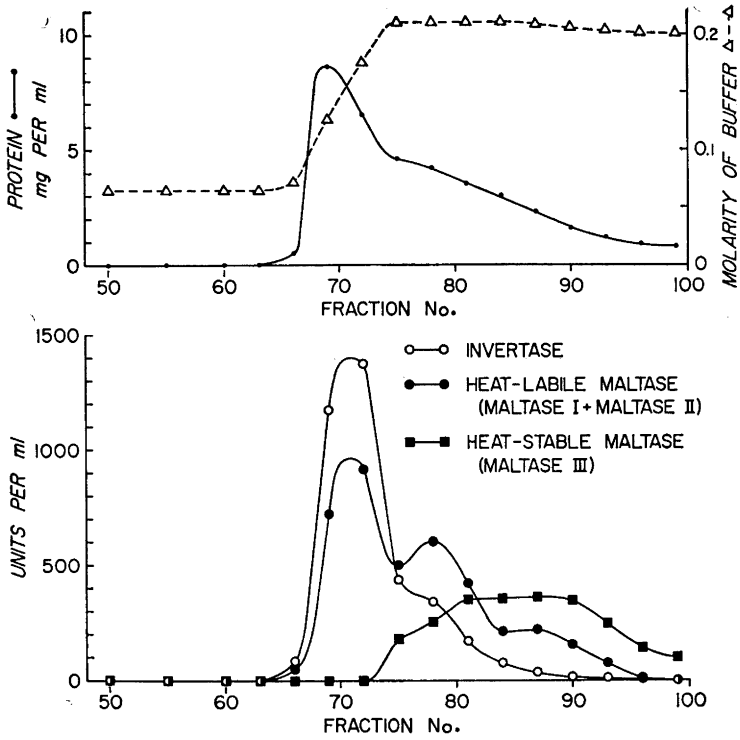

Fig. 3. Mutual displacement chromatography of intestinal glycosidases on TEAE-cellulose with 0.065 M phosphate buffer pH 6.0 as starting buffer. For experimental conditions, see text.

buffer practically no separation was obtained. The most effective separation was found with 0.065—0.080 M buffer. With 0.090 M buffer the separation was again poor, although the glycosidases were adsorbed to the column with this buffer.

The length of the column also had an influence on the separation, since better separation was obtained with a 1 × 100 cm column than with a 1 × 50 cm column. This is in contrast to the gradient elution technique, in which the length of the column was found to be of practically no importance.

These facts seem to prove that the separation obtained is really caused by mutual displacement, and that the experiments cannot be described as a gradient elution with a very steep gradient [16].

One mutual displacement chromatography of intestinal glycosidases is demonstrated in Fig. 3. A 1 × 100 cm column containing 9 g (dry weight) of TEAE-cellulose was equilibrated with 0.065 M phosphate buffer pH 6.0. 48.5 ml of an intestinal glycosidase preparation containing 313 units/ml of invertase, 430 units/ml of maltase, and 5.75 mg/ml of protein in 0.065 M phosphate buffer pH 6.0 was applied to the column with a flow rate of 3 drops

(0.12 ml)/min. The column was first eluted with 230 ml of 0.065 M phosphate buffer pH 6.0, which eluted 37 % of the protein applied to the column, but no invertase or maltase activity. The influent buffer was then shifted to 0.200 M phosphate pH 6.0, and at the same time the flow rate was lowered to 1 drop (0.04 ml)/min. Each fraction collected contained 20 drops (0.8 ml). The number of the fractions in Fig. 3 refers to the number of fractions collected from the moment the influent buffer was shifted to 0.200 M.

As is seen from the figure, the enzymes appear in the effluent as one single protein peak. The first part of this peak (fractions Nos. 66—72) contains the main part of the invertase activity and has a maltase/invertase activity quotient of 0.6. This part of the peak apparently corresponds to maltase fraction I (invertase fraction) described earlier [1]. The tailing of the invertase activity peak was reduced by the mutual displacement technique (*cf.* Fig. 1) but it was not completely abolished. K_s determinations for the invertase activity in fraction Nos. 69 and 85 gave the same values, 2.6×10^{-2}, in good accordance with the value obtained for a crude intestinal glycosidase preparation [7]. This shows that the intestinal invertase activity is caused by one single enzyme. As was found in the frontal analysis experiments, the mutual displacement between maltase II (invertase-free heat-labile maltase activity)[1] and maltase III (heat-stable maltase activity) [1] was much less effective, although a certain mutual displacement took place between these two enzymes also. The heat-stable maltase activity may be purified simply by heating the solution at 70°C [1], but the invertase-free heat-labile maltase fraction seems more difficult to separate. Rechromatography of fractions Nos. 75—84 did not appreciably increase the separation of this activity.

Heat inactivation of intestinal glycosidases. The frontal analysis experiments supported the theory that the heat-labile intestinal maltase is made up of two components. These two activities are rapidly inactivated at 60°C in 0.01 M phosphate buffer pH 6.0. At 50°C in the same buffer, the invertase activity was inactivated by 90 % in one hour. During the same time, however, the maltase activity was diminished only by 30 % (see Fig. 2 in Ref.[1]). If intestinal invertase and maltase I are supposed to be identical, this must mean that maltase II is not measurably inactivated under these conditions. Consequently, there must be a small difference in heat lability between maltases I and II as well.

At pH 7.0 this difference was found to be increased. A solubilized glycosidase preparation was used, which had been dialyzed against distilled water. The solution had a conductivity corresponding to about 0.005 M phosphate and contained 167 units/ml of invertase, 280 units/ml of maltase and 3.6 mg/ml of protein. The pH of the solution was 6.95, as measured with a glass electrode. When this solution was heated at 50°C, the invertase and maltase I activities were inactivated in 30 min, while the maltase II and III activities were not affected even after heating at 50°C for 60 min (Fig. 4). That the difference was caused by the shift in pH, was found by heating another sample of the solution in the presence of 0.010 M phosphate buffer pH 7.0 with the same results.

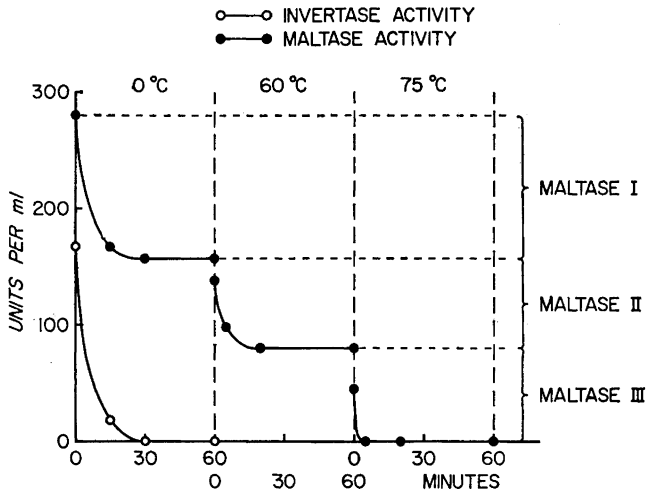

Fig. 4. Heat inactivation of intestinal maltase activity in three steps. Inactivation at 50°C was performed without buffer present (pH of the solution was 6.95). Inactivation at 60°C and 75°C was performed in 0.010 M phosphate buffer pH 6.0. The volume change by addition of the buffer is corrected for in the figure. Preheating was performed as described earlier [1]. The enzyme solution contained 167 units/ml of invertase, 280 units/ml of (total) maltase, and 3.6 mg/ml of protein.

When phosphate buffer pH 6.0 was added to a final concentration of 0.010 M (the change of volume has been corrected for in the figure) and the temperature increased to 60°C, the maltase II was rapidly inactivated while the maltase III was unaffected (Fig. 4).

In this way it was found possible to inactivate the invertase and maltase I activities with preservation of the maltase II and III activities. An attempt

Table 1. Change in adsorbability of the protein denaturated at 50°C at pH 7.0. The table shows the distribution of the protein in two corresponding mutual displacement chromatograms on TEAE-cellulose of the same enzyme solution, before and after heating to 50°C at pH 7.0 for 1 h. The amount of protein *not* eluted from the column was calculated from the amount of protein applied to the column.

	Protein as per cent of total	
	Enzyme solution not heated	Enzyme solution heated at 50°C at pH 7.0 for 1 h
Eluted with 0.080 M phosphate buffer pH 6.0	38	39
Eluted with 0.200 M phosphate buffer pH 6.0	32	18
Not eluted from the column	30	43

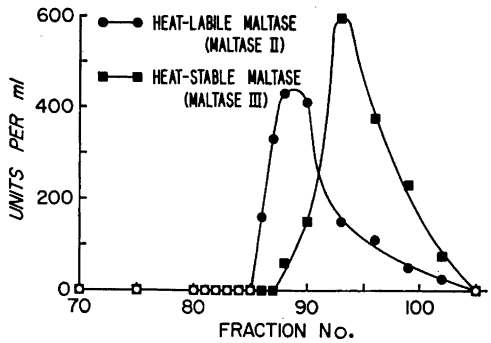

Fig. 5. Mutual displacement chromatography of intestinal maltase activities II and III on TEAE-cellulose with 0.080 M phosphate buffer pH 6.0 as starting buffer. For experimental conditions, see text.

was made to remove the inactivated protein by centrifugation, but it could not be sedimented even at 50 000 *g* (in a Spinco preparative ultracentrifuge). The adsorption properties of the inactivated protein were, however, found to be changed. The distribution of the protein in two corresponding chromatograms of the same glycosidase solution, one before and the other after heating at 50°C for 1 h, is shown in Table 1. It is seen that the heat-inactivated protein is not eluted from TEAE-cellulose by 0.200 M phosphate pH 6.0.

Mutual displacement chromatography of maltase II and III. A 1 × 100 cm column of TEAE-cellulose was equilibrated with 0.080 M phosphate buffer pH 6.0. 50 ml of an intestinal glycosidase preparation, in which the invertase and maltase I activities had been inactivated by heating for 1 h at 50°C, was used for the experiment. The solution contained 160 units/ml of maltase (II + III) and 3.6 mg/ml of protein. After addition of phosphate buffer pH 6.0 to a final concentration of 0.080 M, the solution was applied to the column, which was first eluted with 100 ml of 0.080 M phosphate buffer pH 6.0, and then with 0.200 M phosphate buffer of the same pH. The flow rate was 1 drop (0.04 ml)/min and each fraction collected contained 20 drops (0.8 ml).

The results of the chromatography is seen in Fig. 5. The mutual displacement between maltase II and maltase III was very incomplete. Only the two first fractions (Nos. 86 and 87) of the enzyme peak were free from heat-stable maltase activity.

Table 2. K_s values for three different intestinal maltases.

	K_s
Maltase I (invertase fraction)	1.1×10^{-2}
Maltase II (invertase-free heat-labile maltase activity)	2.2×10^{-3}
Maltase III (heat-stable maltase activity)	4.2×10^{-3}

K_s-values for the different maltase activities. For K_s determinations the following fractions were used:

Maltase I: Heat-labile maltase activity with maltase/invertase activity quotient 0.6, obtained by mutual displacement chromatography (fractions Nos. 66—72 of the chromatogram shown in Fig. 3).

Maltase II: Heat-labile maltase activity without invertase activity, obtained by mutual displacement chromatography of enzyme solutions which has been heated at 50°C at pH 7.0 for 1 h (fractions Nos. 86—87 of the chromatogram shown in Fig. 5 and corresponding fractions from similar chromatograms).

Maltase III: Heat-stable maltase activity, obtained from fractions Nos. 86—99 of the chromatogram shown in Fig. 3, fractions Nos. 93—102 of the chromatogram shown in Fig. 5, and the corresponding fractions from similar chromatograms. Traces of heat-labile maltase and invertase activity were inactivated by heating at 60°C for 1 h.

The K_s values for the different maltase activities are shown in Table 2. The figures reported are the mean of several separate determinations, with good agreement between them. The divergent K_s values demonstrate that the different maltase fractions are really different enzymes.

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