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PURIFICATION BY AFFINITY CHROMATOGRAPHY AND CHARACTERIZATION OF A NEUTRAL α-GLUCOSIDASE FROM HORSE KIDNEY

J. GIUDICELLI, R. EMILIOZZI, C. VANNIER, G. DE BURLET and P. SUDAKA

Laboratoire de Biochimie, U.E.R. de Médicine, Chemin de Vallombrose, 06034 Nice Cedex (France)

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Summary

A horse kidney neutral α-D-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) was purified about 580-fold with a yield of 33% by an affinity chromatography technique using the *p*-aminophenyl-β-D-maltoside, a substrate derivative, as ligand. The purified enzyme, homogeneous in polyacrylamide gel electrophoresis, was a glycoprotein with a molecular weight of 280 000 as calculated by gel filtration and its isoelectric focusing points was found to be pH 4.1.

The purified enzyme was able to hydrolyze various substrates having (α-1,2), (α-1,3), (α-1,4) and (α-1,6) glucosidic linkages. The V/K_m ratio shows that the (α-1,4) linkages are the best substrates.

The pK_m of the purified enzyme determined at different pH values indicated that two ionizable groups with pK values 5.2 and 6.9 could be essential in the active site. Enzyme modification with cardodiimide abolished the maltase activity. The turanose, a substrate analogue, protected the enzyme against this inactivation.

Introduction

The brush border membrane of kidney proximal tubule cells is known to possess many hydrolases [1—4] considered as markers for this organelle. Some of these enzymes may be involved in reabsorption phenomena [5,6]. A kinetic

Abbreviation: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

stud of the glucose active transport by horse kidney brush border vesicles has been previously described [7]. An approach to the understanding of the molecular aspect of this transport needs a structural knowledge of the membrane and of some of its well represented markers.

One of these markers, neutral maltase was partially purified by conventional procedures from rat [8] and rabbit [9]; it was purified to homogeneity from human kidney in our laboratory [10]. The present paper describes a purification method of the horse kidney neutral maltase including an affinity chromatography on a Sepharose column substituted with a maltose derivative. Some physicochemical and catalytic properties of this purified neutral maltase were established.

Materials and Methods

Pig heart fumarase, bovine liver catalase and trypsin inhibitor (beef lung aprotinin) were obtained from Boehringer; trypsin from Worthington; horse serum cholinesterase from Sigma Chemical Co; pig kidney aminopeptidase was purified by the technique described by Maroux et al. [11] applied to the renal enzyme [12]. CH-Sepharose 4B and DEAE-Sepharose 6B-CL were from Pharmacia; maltosaccharides were prepared and purified as previously reported [13]. [U-^{14}C]Maltose (10 Ci/mol) was purchased from Amersham. Other chemical products were of the best available grade.

Preparation of p-aminophenyl-β-D-maltoside-CH-Sepharose 4B. The heptaacetyl *p*-nitrophenyl-β-D-maltoside prepared according to Babers and Goebel [14], was deacetylated in methanol saturated with ammonia to give the *p*-nitrophenyl-β-D-maltoside; this compound was reduced, under hydrogen atmosphere, with palladium-activated charcoal (10% Pd) as catalyst in tetrahydrofuran to give the *p*-aminophenyl-β-D-maltoside. This ligand was bound to CH-Sepharose 4B as described by Junowicz and Paris [15]. Its amino group reacts with the carboxyl group ending the six carbon long spacer arm of the CH-Sepharose 4B gel. Using *p*-aminophenyl-β-D-[U-^{14}C]maltoside prepared from [U-^{14}C]maltose we found 7 μmol of ligand bound per ml of gel.

Enzymatic determinations. Maltase and glucoamylase activities were measured as previously reported [13]. One unit of maltase activity is the amount of enzyme required to hydrolyze 1 μmol of maltose per min in the assay conditions. Catalase activity was assayed by the method of Bergmeyer [16], fumarase by the procedure of Kanarek and Hill [17], serum cholinesterase by the technique of Knedel and Böttger [18] and β-galactosidase by the two-step method of Dahlqvist [19] with lactose as substrate. Aminopeptidase M activity was determined with L-alanine *p*-nitroanilide as substrate [20].

Protein determinations. Protein concentrations were determined according to the method of Lowry et al. [21] modified by Eggstein and Kreutz [22] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. 7.5% acrylamide gels were run using an asparagine-imidazole buffer at pH 7.4 [23]. Glycoproteins were stained by the technique of Zacharius et al. [24]. In order to measure the enzymatic activity, the gel was sliced into 2-mm sections, the slices were eluted overnight in distilled water at 4° C and an aliquot was assayed for maltase activity.

Molecular properties. Isoelectric focusing was carried out in a 110 ml column (L.K.B. 8100.1) at 4°C with a linear 0—60% glycerol gradient. The carrier ampholyte (Ampholine L.K.B.) concentration was 0.8% and the pH range was 3.5—5.0. Gel filtration for molecular weight determination was carried out according to the method of Andrews [25]. The Ultrogel AcA 2/2 (L.K.B.) column, equilibrated with 0.1 M NaCl/0.05 M sodium phosphate buffer, pH 7.5, was calibrated with pig heart fumarase (194 000), beef liver catalase (232 000), pig kidney aminopeptidase M (280 000) and horse serum cholinesterase (315 000). The determination of the sedimentation coefficient was carried out according to the method of Martin and Ames [27] as previously reported [26].

Enzyme chemical modification studies. *N*-Ethylmaleimide (25 mM) was allowed to react with neutral maltase (1 U) at 37°C for 60 min in 0.1 M sodium citrate buffer, pH 6.2. Then maltase activity was tested on aliquots diluted in 0.1 M sodium citrate buffer (1 : 100) and compared with the control activity. Reaction with diethylpyrocarbonate was realized as reported by Paus [28] with a 200 : 1 molar ratio of reagent to enzyme in 0.1 M sodium phosphate buffer, pH 6.1, for 60 min. Reaction with 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide was realized according to Hoare and Koshland [29]. To 2 μM solution of neutral maltase in 1 M NaCl/0.1 M MES buffer, pH 5.4, was first added glycine ethyl ester (2 mM) and then carbodiimide to a final concentration of 0.2 mM.

Results

Purification of neutral maltase

Fresh horse kidneys were obtained at the slaughterhouse immediately after killing. All experiments were carried out at 4°C.

Solubilization

One part of horse kidney cortex was homogenized in three parts (w/v) of 0.01 M sodium phosphate buffer, pH 6.5, with a blade Omnimixer (Virtis Macro 45) for three periods of 30 s and filtered through gauze. This homogenate, used as reference for the determination of yield and purification factor, was centrifuged for 60 min at 40 000 × *g*. The pellet was resuspended in 0.02 M sodium phosphate buffer, pH 6.5, and the detergent Emulphogen BC 720 (GAF, France) was added with stirring to a 2% (v/v) final concentration. The solution was stirred for 30 min at room temperature and then for 16 h at 4°C. After centrifugation for 60 min at 40 000 × *g* the pellet was discarded.

Ammonium sulphate fractionation

Solid $(NH_4)_2SO_4$ was added to supernatant to give a 30% saturated solution; after 15 min the precipitate was removed by centrifugation for 20 min at 20 000 × *g*. The supernatant was then adjusted to 60% saturation in $(NH_4)_2SO_4$ and centrifuged for 20 min at 20 000 × *g*. The pellet thus obtained was dissolved in 0.05 M Tris-HCl buffer, pH 8.0, containing 1% Emulphogen BC 720 and dialyzed overnight against the same buffer. The solution was then centrifuged and the supernatant was used for the proteolysis.

Trypsin proteolysis

The supernatant was treated by trypsin (0.5 mg/ml) for 4 h at 4°C according to Maroux et al. [11]. Then proteolysis was inhibited by addition of aprotinin to a 0.20 mg/ml final concentration. The hydrolyzate was brought to 60% saturation with solid $(NH_4)_2SO_4$. After centrifugation, maltase and aminopeptidase M activities were recovered in the pellet. This contrasts with small intestine and renal aminopeptidases M of pig [11,12] which up to this step were prepared by the same method but were recovered in the supernatant after trypsin treatment and $(NH_4)_2SO_4$ fractionation. The pellet was dissolved in 0.05 M NaCl/0.02 M sodium phosphate buffer, pH 6.5, and dialyzed against the same buffer. After centrifugation the supernatant was collected for the following steps.

DEAE-Sepharose 6B-CL chromatography

The supernatant was fractionated on DEAE-Sepharose 6B-CL. The fractions containing maltase activity were pooled, dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and submitted to affinity chromatography. Although not essential in the enzyme purification, the DEAE-Sepharose chromatography was used here because this step allows us to increase the number of utilizations of the affinity chromatography. The experimental conditions and elution patterns are shown in Fig. 1.

Affinity chromatography on p-aminophenyl-β-D-maltoside Sepharose 4B

The column was equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The enzyme solution was applied on the column at 5 ml/h. After washing the gel with the equilibration buffer, up to the disappearance of the absorption at 280 nm, the enzyme was eluted at 10 ml/h by a linear 0.25—0.5 M NaCl gradient in 0.1 M sodium phosphate buffer, pH 7.0. Fractions containing maltase activity were pooled (Fig. 2), dialyzed against distilled water and lyophylized. The results of a typical purification are summarized in Table I.

Purity of enzyme preparation

Purity of the final step product was checked by polyacrylamide gel electro-

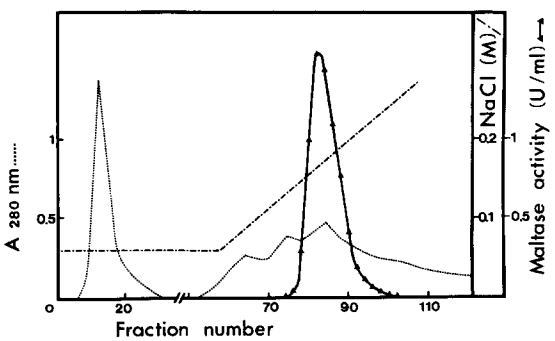


Fig. 1. Chromatography on DEAE-Sepharose 6B-CL. The column (2.1 × 5.5 cm) was equilibrated with 0.05 M NaCl/0.02 M sodium phosphate buffer, pH 6.5. Elution was performed using a linear 0.05—0.3 M NaCl concentration gradient in the buffer (total volume 400 ml). Fraction 8.5 ml; flow rate 20 ml/h.

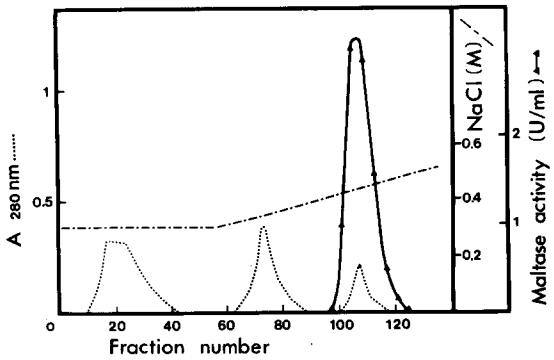


Fig. 2. Affinity chromatography on *p*-aminophenyl-β-D-maltoside. The enzyme was applied to the affinity column (2.1 × 4.5 cm) preequilibrated with 0.1 M sodium phosphate buffer, pH 7.0. Elution was performed with a linear 0.25—0.5 M NaCl gradient (total volume, 300 ml). 2-ml fractions were collected and assayed for maltase activity.

phoresis. As shown in Fig. 3, the protein or glycoprotein bands present the same R_F value as the maltase activity band.

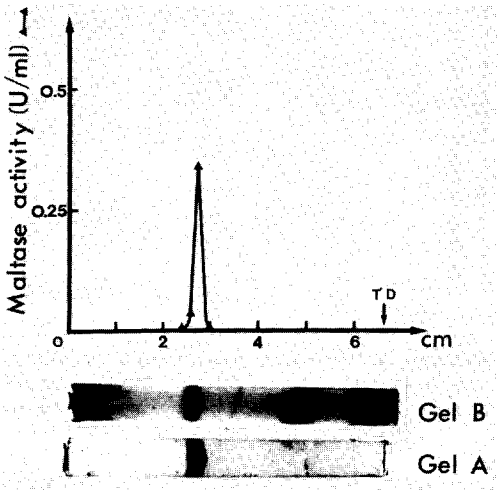
Behavioural study of the enzyme towards affinity gel chromatography

The study was done on material obtained after proteolytic treatment by trypsin. When an aliquot was layered on a control column filled with CH-Sepharose 4B saturated by ethanolamine rather than by *p*-aminophenyl-β-D-maltoside, the enzyme was desorbed by a linear 0.25—0.5 M NaCl gradient, at an ionic strength (0.37 M) close to that which eluted other proteins (0.34 M) (Fig. 4a). In this case, the desorbed enzyme appears impure by polyacrylamide gel electrophoresis. If the same aliquot was layered on a column filled with *p*-aminophenyl-β-D-maltoside-Sepharose 4B, the enzyme could be eluted in two ways: (1) if the elution was achieved with the *p*-aminophenyl-β-D-maltoside at 0.1 M concentration, a sharp peak of maltase activity emerged from the column (Fig. 4b); (2) eluted by a linear 0.25—0.5 M NaCl gradient, the maltase activity was recovered at 0.45 M NaCl concentration in a larger peak (Fig. 4c). In both cases no glucose was detected in the eluate, indicating that the ligand was not hydrolyzed, and the enzyme appeared pure by polyacrylamide gel electro-

TABLE I

PURIFICATION OF HORSE KIDNEY NEUTRAL MALTASE

	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Recovery of activity (%)	Purification factor
Homogenization	242	4760	0.051	100	1
Solubilization	228	1395	0.164	94	3.2
Ammonium sulphate fractionation	151	600	0.273	62	5.4
Trypsin treatment	131	359	0.366	54	7.2
DEAE-Sepharose 6B-CL chromatography	96	60	1.575	40	31
Affinity chromatography	79	2.7	29.300	33	580



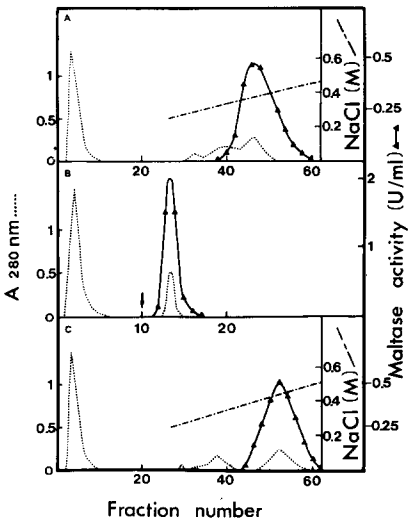


Fig. 3. Polyacrylamide gel electrophoresis of neutral maltase. The electrophoresis was carried out at 5 mA/tube with gels of 8 × 0.5 cm (30 μg of purified protein). A, Coomassie brillant blue R 250 staining; B, periodic acid-Schiff staining; TD, tracking dye.

Fig. 4. Elution pattern of the neutral maltase on the control column (A) and on affinity column (B, C). The columns (1 × 2.5 cm) were equilibrated and washed with 0.1 M sodium phosphate buffer, pH 7.0. Elution of adsorbed proteins was carried out with a linear 0.25—0.5 M NaCl concentration gradient (A, C) or with 0.1 M *p*-aminophenyl-β-D-maltoside (B). Flow rate 10 ml/h; fractions 2.5 ml (A, C), 2 ml (B). The arrow indicates the point at which the elution by ligand (100 mM) was started.

phoresis. The other proteins were eluted at the same concentration (0.34 M) as described above. The maltose did not desorb the enzyme even at high concentration (0.15 M); interestingly again, no glucose was detected in the eluate, showing that the adsorbed enzyme do not exhibit maltase activity.

Molecular properties

The purified enzyme gave a single peak with p*I* = 4.1 when subjected to isoelectric focusing (Fig. 5). The molecular weight of the enzyme was estimated by filtration on Ultrogel AcA 2/2. A value of 280 000 was found. The sedimentation coefficient of the enzyme determined by sucrose density centrifugation was found to be 10 S.

Kinetic properties

The enzyme was shown to be able to catalyze the hydrolysis of disaccharides with various α-linkages: kojibiose (α-1,2), nigerose (α-1,3), maltose (α-1,4) and isomaltose (α-1,6). However, it had no action on trehalose (α-1,1). The enzyme was also completely devoid of cellobiase (β-1,4) and sucrase activities. Moreover the enzyme was able to hydrolyze oligo- and polysaccharides as (α-1,4) maltooligosaccharides and starch. The V/K_m ratio shows that maltotetraose is the best substrate. Synthetic substrate as *p*-nitrophenyl-α-D-glucopyranoside was also hydrolyzed by the enzyme. The kinetic constants for the above substrates are summarized in Table II.

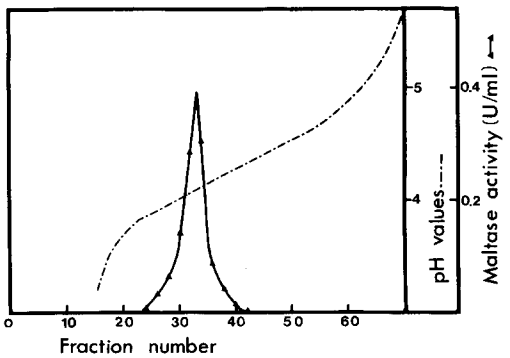


Fig. 5. Isoelectric focusing of the purified maltase. The electrofocusing run was carried out at 500 V for 60 h at 4°C. The sample contained 85 μg of purified enzyme. After separation of content of the column into 1 ml fractions, pH and maltase activity were measured.

No substrate inhibition was observed with maltose up to 50 mM concentration. In contrast, strong substrate inhibition was observed with the oligosaccharides at concentrations exceeding 0.2 mM. The K_i value determined for the maltotriose at inhibitory concentration was $7 \cdot 10^{-4}$ M. The effect of various compounds previously described as α-glucosidase inhibitors was tested on maltase and glucoamylase activities of the purified enzyme and the mode of inhibition was determined (Table III). Interestingly, the *p*-aminophenyl-β-D-maltoside, our chromatography ligand, was found to act as a mixed inhibitor of maltase activity.

Mixed-substrate studies showed that hydrolysis of maltose was competitively inhibited by starch. Maltose and starch inhibited competitively the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside. In each case the K_i values were very close to K_m values for these substrates (0.53 mM for maltose and 0.50 mg/ml for starch to compare with 0.45 mM and 0.40 mg/ml, respectively) (Table III).

TABLE II

KINETIC CONSTANTS (K_m) AND MAXIMUM VELOCITY (V) FOR VARIOUS SUBSTRATES

V is expressed as μmol linkage hydrolyzed/min per mg protein.

Substrates	K_m (mM)	V	V/K_m
Trehalose (α-1,1)	—	—	—
Kojibiose (α-1,2)	8.3	25	3
Nigerose (α-1,3)	3.3	31	9.5
Maltose (α-1,4)	0.45	66	146.5
Isomaltose (α-1,6)	13	1	0.08
p-Nitrophenyl α-D-glucopyranoside	2.7	15	5.56
Maltotriose	0.18	110	613.3
Maltotetraose	0.1	83	830
Maltopentaose	0.14	47	336
Starch	0.4 *	22.5	—

* K_m expressed as mg/ml.

TABLE III

KINETIC PARAMETERS OF INHIBITORS ON MALTASE AND GLUCOAMYLASE ACTIVITIES

Values of inhibition constants are in mM. M, mixed; C, competitive; NC, non-competitive; NI, no inhibition.

Substrates:	Maltose		Starch		*p*-Nitrophenyl-α-D-glucopyranoside
Inhibitors	Inhibition mode	K_i	Inhibition mode	K_i	
Tris	M	—	M	—	
Turanose	C	9.15	C	22.6	
Phlorizin	NC	0.5	NC	0.5	
Trehalose	NI	—	C	43	
p-Aminophenyl-β-D-maltoside	M	—	—	—	
Starch	C	0.4 *,**			C K_i = 0.5 *
Maltose					C K_i = 0.53

* K_i expressed as mg/ml.

** V expressed as the appearance of [U-^{14}C]glucose from [U-^{14}C]maltose hydrolysis vs. time.

Effect of pH on maltase and glucoamylase activities

The effect of pH on maltase and glucoamylase activities was investigated in the buffer of Teorell and Stenhagen [30]. As shown in Fig. 6a, the pH optima were 6.25 and 6.0 for maltase and glucoamylase activities, respectively. The effect of pH on enzyme stability was studied after 60 min incubation at 37°C in a 2—12 pH range. At pH 6—9, the enzyme activities were over 80% of the initial (Fig. 6b).

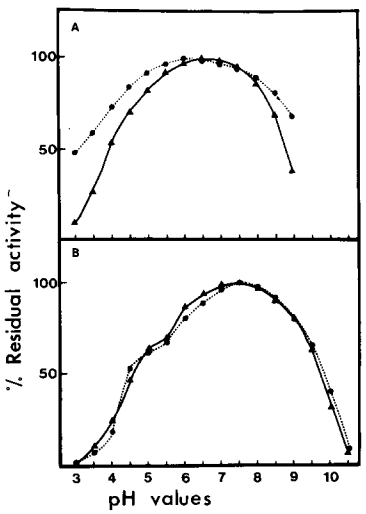


Fig. 6. pH activity (A) and stability (B) curves of maltase (▲) and glucoamylase (●) activities. In B, after 60 min at 37°C each fraction was diluted in 0.1 M sodium citrate buffer, pH 6.2 and the activities were assayed.

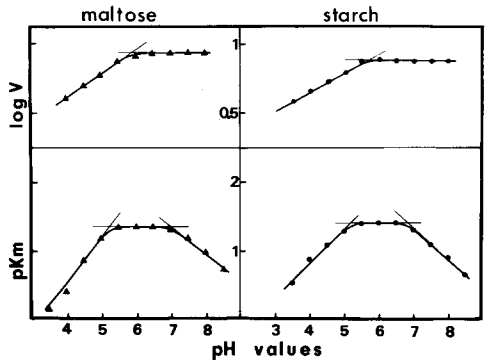


Fig. 7. Plot of K_m and V vs. pH for maltose and starch hydrolysis. V is expressed in units of enzyme activity per mg of protein.

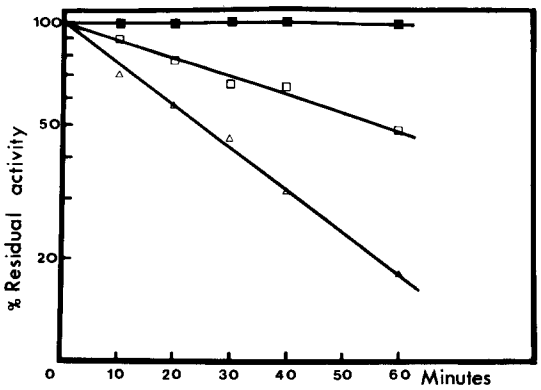


Fig. 8. Effect of carbodiimide on maltase activity in presence or absence of turanose (50 mM). (△) in presence of glycine ethyl ester and carbodiimide; (□) in presence of turanose; (■) control (no reagent added).

We have studied the pH influence on V and K_m for maltose and starch hydrolysis (Fig. 7). The plot of log V vs. pH of the two substrates shows a break at pH 5.7. The values of pK_m vs. pH for maltose and starch hydrolysis show a plateau in the pH range 5.3—7.0 and 5.2—6.8, respectively.

Since the substrate is not charged in this pH range, the bending points could be due to ionizable groups in the enzyme active site. In order to elucidate the nature of these groups, we have studied the effect of several chemical reagents capable of modifying the maltase activity. After incubation with 25 mM *N*-ethylmaleimide no inhibition occurred, indicating the absence of accessible SH groups in the active site. The involvement of the imidazole group site can be demonstrated particularly by carbethoxylation of the enzyme with diethylpyrocarbonate [28]. With this compound, an increase in absorption at 242 nm was observed, implicating the formation of *N*-carbethoxyhistine residues in the protein, but no change in the enzyme activity could be detected, indicating that no imidazole group can be involved in the active site. When the enzyme was submitted to reaction with glycine ethyl ester in presence of carbodiimide, the maltase inactivation rate followed pseudo-first-order kinetics. The inactivation rate with carbodiimide alone was very close to those observed in presence of both reagents (not shown). In presence of turanose (50 mM), a competitive inhibitor of maltase activity, the extent of inactivation was reduced, indicating that the carbodiimide acts also near or on the active site of enzyme (Fig. 8).

Discussion

We have purified to homogeneity the horse kidney neutral maltase by affinity column chromatography on immobilized *p*-aminophenyl-β-D-maltoside, a substrate derivative of the enzyme. This choice was made after unsuccessful attempts with ligands known as α-glucosidase inhibitors (Tris, Tricine, glucose). With our substrate derivative ligand no hydrolysis by maltase was observed during affinity chromatography in accordance with similar observations previously described for affinity chromatographies of other glycosidases [15,31]. The neutral maltase adsorption on the affinity column was due to specific

ligand-enzyme interactions as demonstrated by the elution experiments: (1) maltase was the unique protein desorbed when the enzyme was eluted with ligand; (2) when maltase was eluted by an ionic strength gradient, its activity was desorbed at an higher ionic strength in the affinity column as compared to the control column. In contrast unspecifically-bound proteins were eluted at similar and lower ionic strength in both experiments (Fig. 3). Elution by salt gradient was used instead of elution by ligand since the *p*-aminophenyl-β-D-maltoside can be synthesized in limited amounts and since after enzyme elution the ligand elimination by dialysis proved to be very difficult. Moreover, the facts that: (1) no glucose could be detected in the eluate after the enzyme fixation; (2) the bound enzyme exhibits no more maltase activity; (3) the enzyme cannot be desorbed at high maltose concentration, would be consistent with the hypothesis that the ligand-enzyme interactions were located in an area distinct from but near to the enzyme active site. These ligand-enzyme interactions, external to those which involve the substrate recognition site, have been already observed for glycosidases [31—33].

The purified enzyme is a glycoprotein with apparent M_r 280 000 and sedimentation coefficient 10S. The p*I* of the neutral maltase was 4.1 as determined by isoelectric focusing and no charged isomer is found. The molecular properties of horse kidney neutral maltase appear not very different from the corresponding enzyme of pig serum [34].

The enzyme is a glucosidase exhibiting a narrow specificity towards α-linkages; although it is able to hydrolyze (α-1,2) and (α-1,3) linkages, the V/K_m ratio shows that it is mainly active towards (α-1,4) linkages. It presents a great affinity and an important hydrolytic activity towards maltooligosaccharides among which maltotetraose is the best substrate. It also hydrolyzes polysaccharides such as starch. With maltooligosaccharides but not maltose, we observed a potent inhibition brought about by substrates at concentrations (0.2 mM and above) lower than those required for other α-glucosidases [13,35,36].

Several observations are in favour of a unique catalytic site for maltose and starch hydrolysis; the variation of log *V* vs. pH showed that an ionic group of p*K* 5.7 is involved in starch and in maltose hydrolysis. Such a group has already been described for the hydrolysis of these substrates in the case of glucoamylase from rabbit intestine [35]. Maltase and glucoamylase activities are inhibited by phlorizin in a non-competitive manner with identical dissociation constants; the neutral α-glucosidase from human kidney also exhibits such a property [13].

The competition observed between maltose and starch substrates could be related either to close proximity and consequently to interactions between the recognition sites of substrates or to the identity of these recognition sites. The intervention of two ionizable groups with the same p*K* for the fixation of maltose and starch agrees well with the postulated existence of a common fixation site for both substrates. However, the different action of trehalose on maltase and glucoamylase activities suggests the existence of several subsites. The starch fixation requires one or several subsite(s) implicated in the maltose and trehalose fixation, but the maltose fixation subsite(s) is(are) independent of the trehalose site(s). Similar results were obtained for neutral α-glucosidase from human kidney [13] and glucoamylase from rabbit intestine [35].

Plots of pK_m vs. pH give information on the ionizable groups participating in the active site [37]. These plots curved near pH 5.2 and 6.9. In order to elucidate the nature of these ionizable groups, several chemical modifications on the enzyme were performed. Diethylpyrocarbonate and *N*-ethylmaleimide treatments demonstrate the absence of imidazole and sulphydryl groups in the active site, respectively. Enzyme treatment with carbodiimide leads to a decrease of its maltase activity; this inactivation was lowered when a competitive inhibitor such as turanose was added before reaction with carbodiimide. Therefore one carboxyl group is probably located in the active site; a p*K* value of 5.2 could be assigned to this group. Since no imidazole or sulphydryl group can be detected in the enzyme active site, the p*K* value at 6.9 could correspond to a second carboxyl group with an unusually high p*K* value. Such a p*K* has been already described for a lysozyme [38], α-mannosidase from *Phaseolus vulgaris* [28], sweet almond β-glucosidase [39] and has been postulated for the rabbit small intestine sucrase isomaltase [40] which present similar p*K* values.

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