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Purification of Rat Intestinal Maltase/Glucoamylase and its Anomalous Dissociation either by Heat or by Low pH

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Maltase/glucoamylase, a microvillous membrane ectoenzyme, was solubilized from rat intestinal mucosa by digestion with papain and subsequently purified to homogeneity with an overall yield of 10–20%. An antibody to the purified enzyme formed a single precipitin line in immunodiffusion experiments with an intestinal homogenate. The enzyme was shown to be an acidic glycoprotein (20% sugar by weight) which contained low amounts of cysteine and no sialic acid. At pH 3–6, maltase activity was slowly lost, but the enzyme was re-activated by re-adjustment of the pH to neutrality. However, in the presence of sodium dodecyl sulphate, acid pH values inactivated maltase irreversibly, and at the same time converted the enzyme (mol.wt. 500000 approx.) into five new species with apparent molecular weights ranging from 134000 to 480000 as judged by polyacrylamide-gel electrophoresis. The same five fragments were also formed by boiling the enzyme for brief periods in the presence of sodium dodecyl sulphate or urea either with or without reducing agents. The dissociated species were stable on re-electrophoresis, and amino acid analysis showed them to be very similar to each other and to the original enzyme. The bands migrated anomalously on polyacrylamide gels of different concentration, thereby preventing the assignment of precise molecular weights. It is possible that the five species may represent stable aggregates of a common monomer of the enzyme.

Maltase (α-D-glucoside glucohydrolase, EC 3.2.1.20)/glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is one of a group of oligo- and disaccharidases which are constituents of the intestinal brush-border membrane (Miller & Crane, 1961; Dahlqvist & Thomson, 1963; Eichholz & Crane, 1965; Forstner *et al.*, 1968; Alpers & Solin, 1970). These carbohydrases are easily solubilized from the membrane in good yield by papain digestion (Auricchio *et al.*, 1963; Eichholz, 1968; Forstner, 1971) and thus their purification is greatly facilitated (Cogoli *et al.*, 1972). Two of the dissacharidases, sucrase (EC 3.2.1.48) and isomaltase (EC 3.2.1.10), have been intensively studied. These enzymes represent distinct subunits in a dimeric protein complex which has been isolated from rabbit (Cogoli *et al.*, 1972) and from human intestine (Conklin *et al.*, 1975). Both subunits are glycoproteins (Cogoli *et al.*, 1973) and have almost equal molecular weights of between 110000 and 140000 (Cogoli *et al.*, 1973; Mosimann *et al.*, 1973; Conklin *et al.*, 1975).

In contrast, maltase/glucoamylase is much less well characterized. Intestinal glucoamylase activity has been distinguished from pancreatic amylase activity (Dahlqvist & Thomson, 1963; Alpers & Solin, 1970) and has been shown to be identical with one of the maltase activities of the intestine (Alpers & Solin, 1970). The enzyme sediments faster (Eggermont & Hers, 1969) and is more heat-stable (Auricchio *et al.*, 1965) than the other disaccharidases. The enzyme has been partially purified from the intestine of the monkey (Seetharam *et al.*, 1970), suckling rat (Schlegel-Haueter *et al.*, 1972), adult rat (Kolinska & Kraml, 1972), human (Kelly & Alpers, 1973) and rabbit (Sivakami & Radhakrishnan, 1973), and kinetic studies have been performed by Kelly & Alpers (1973) and by Sivakami & Radhakrishnan (1976). It is a glycoprotein (Forstner, 1971; Kelly & Alpers, 1973; Forstner & Galand, 1976) and appears to be the largest component of the brush-border membrane from rat (Galand & Forstner, 1974*b*; Forstner & Galand, 1976), hamster (Critchley *et al.*, 1975) and human (Maestracci *et al.*, 1975) intestine. Published values of the molecular weight by a number of techniques have varied from approx. 500000 (Forstner, 1971; Maestracci *et al.*, 1973, 1975; Galand & Forstner, 1974*a*) to about half that value (Kelly & Alpers, 1973). It seems probable that a molecule of such size must consist of more than a single polypeptide chain, and the present study was undertaken to investigate this possibility. Preliminary reports of the present work have been presented (Flanagan & Forstner, 1976*a*,*b*).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Chemicals

Mercuripapain, cysteine, bovine serum albumin, phosphorylase, *p*-nitrophenyl α-D-glucoside and lactose were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and dithiothreitol and maltose were from Calbiochem, La Jolla, CA, U.S.A. β-Galactosidase and catalase were products of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Soluble starch and ampholytes were obtained from Fisher Scientific Co., Don Mills, Ontario, Canada. Sepharose 4B was purchased from Pharmacia (Canada) Ltd., Dorval, Quebec, Canada, and DEAE-cellulose (DE32) was a Whatman product supplied by Mandel Scientific Co. Ltd., Ville St. Pierre, Quebec, Canada. Reagents for polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Canada.

Animals

Male white rats of the Wistar strain were used throughout the study. The animals weighed 150–250g and their food supply was removed 12h before death.

Buffers

Electrophoresis buffers were prepared simply by mixing the constituents. All other buffers were prepared by adjustment to a particular pH at 24°C. For example, 0.1M-EDTA/0.2M-potassium phosphate, pH7, was prepared by adjusting a solution of approx. 0.2M-EDTA/0.4M-KH_2PO_4 to pH7 with 5M-NaOH and then diluting it to the final concentration. Similarly the buffers used in the pH studies were prepared by adjustment of a solution of approx. 50mM-maleic acid/50mM-acetic acid/50mM-Tris to the appropriate pH value with NaOH, followed by dilution to 25mM for each component.

Polyacrylamide-gel electrophoresis

The nomenclature of Hjerten (1962) was used to describe the gel composition. Total concentration (%T) = (g of acrylamide + g of methylenebisacrylamide)/100ml of solution; cross-linking concentration (%C) = 100 × (g of methylenebisacrylamide/100ml of solution)/%T.

Electrophoresis was carried out either in glass tubes or in a Lucite [poly(methyl methacrylate)] vertical slab-gel apparatus. Two electrophoresis systems were used.

In the non-dissociating system, the gel contained 50mM-Tris/7mM-glycine, pH9.5, 0.025% (v/v) *NNN'N'*-tetramethylethylenediamine and 1.5mg of ammonium persulphate/ml. The gel concentration was 3.32%T and the cross-linking concentration was 3.61%C. The electrode solutions were 50mM-Tris/7mM-glycine, pH9.5.

In the dissociating system, the SDS method of Fairbanks *et al.* (1971) as modified by Steck & Yu (1973) was used throughout. Except where indicated, the gel concentrations used were identical with those in the non-dissociating system.

Proteins (10–20μg) were dissolved in electrophoresis buffer (10–20μl), except where stated otherwise, then water and/or other solutions (SDS, β-mercaptoethanol), as appropriate, were added to adjust the volume to 100μl. Finally 10μl of 50% (w/v) sucrose prepared in 0.01% Bromophenol Blue was added and the mixture was electrophoresed at a current of 2mA/tube (non-dissociating) and 8mA/tube (dissociating). The apparatus was turned off after the Bromophenol Blue had migrated 8.5cm and the dye position was marked by stabbing the gel with a needle dipped in black ink.

Coomassie Blue staining and destaining procedures for the detection of protein on the gels were described previously (Flanagan & Zbarsky, 1977). The periodic acid–Schiff stain for the detection of carbohydrate was carried out as described by Forstner (1971).

Density-gradient ultracentrifugation

This was carried out by the method of Martin & Ames (1961) as described previously (Galand & Forstner, 1974*a*) by using 5–20% (w/v) mannitol or sucrose gradients, except that the buffer used was 0.1M-Tris/HCl, pH7.4.

Boundary-sedimentation-velocity studies

A Beckman model E analytical ultracentrifuge was used at 20°C with single-sector cells and a speed of 52000rev./min. Samples were dialysed overnight against 0.1M-potassium phosphate buffer, pH7.0, and sedimentation rates were followed by using u.v. absorption at 280nm. The sedimentation coefficients were calculated as described previously (Jabbal *et al.*, 1976).

Isoelectric focusing

Isoelectric focusing was performed in a LKB Uniphor 7900 column electrophoresis system (LKB-Producter AB, S-161 25 Bromma 1, Sweden) equipped with a 35ml column. The column was loaded with a 5–50% (w/v) sucrose density gradient containing 1% (v/v) Ampholine carrier ampholytes pH3–6 and 50μg of pure maltase/glucoamylase. The initial voltage was 400V and was gradually increased to 600V at a maximum current of 2mA over 2h and maintained at 600V for 24h: 1000V was then used for 10h.

Immunodiffusion

Immunodiffusion was performed by the double-diffusion technique of Ouchterlony as previously described (Forstner *et al.*, 1973). Anti-maltase antibody was raised in rabbits by subcutaneous injection of 20μg of purified enzyme in complete Freund's adjuvant with 0.1vol. of pertussis vaccine, followed by two additional injections with incomplete adjuvant at monthly intervals. Rabbit antiserum was prepared 3–4 months after the initial injection.

Determinations

Maltase activity was measured by the method of Dahlqvist (1968), and glucoamylase activity, with soluble starch as substrate, by the method of Schlegel-Haueter *et al.* (1972). α-Glucosidase activity was determined by the method of Kolinska & Kraml (1972) with *p*-nitrophenyl α-D-glucoside as substrate. β-Galactosidase activity was measured with lactose as substrate (Dalhqvist, 1968) and catalase activity as described in the Worthington enzyme manual. Glucoamylase activity was expressed as μmol of glucose produced/min, whereas other enzyme units were equivalent to 1μmol of substrate hydrolysed/min. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and total hexose by the anthrone reaction (Spiro, 1966). Amino acid analyses were performed on a Beckman–Spinco automatic amino acid analyser, model 120C, after hydrolysis of samples under vacuum in 6M-HCl for 12–36h at 105°C. Sugar analysis was performed by g.l.c. after methanolysis (Chambers & Clamp, 1971).

Purification of maltase/glucoamylase

Step 1. Rats were killed by cervical dislocation and the small intestine was quickly removed, thoroughly washed with ice-cold 0.9% NaCl and slit open to expose the mucosa, which was then scraped gently with a microscope slide. All further operations were carried out at 4°C. Mucosal scrapings from five to ten rats were combined for each experiment, and homogenized in 50vol. (ml/g of tissue) of 5mM-EDTA, pH7.4, for 10s at top speed in a Waring blender.

Step 2. The homogenate was centrifuged at 35000$g_{max.}$ for 20min in the SS34 rotor of a Sorvall RC2-B centrifuge and the supernatant solution was discarded. The sediment was resuspended in 90ml of ice-cold water.

Step 3. To the suspension 5ml of 0.1M-EDTA/0.2M-potassium phosphate, pH7, and 5ml of 0.1M-cysteine were added, and the mixture was then incubated at 37°C for 30min. The sediment was again collected by centrifugation at 35000$g_{max.}$ for 20min and the supernatant solution discarded. The washed sediment was then resuspended in 50ml of water and the protein concentration determined. The volume to which the suspension required diluting to give a protein concentration of 10mg/ml was calculated (V). Papain was used at 0.1mg/ml and the required amount of mercuripapain was activated by the addition of 0.05 V of 0.1M-EDTA/0.2M-potassium phosphate, pH7, and 0.05 V of 0.1M-cysteine to the papain in a test tube followed by a brief (2min) incubation at 37°C. This activated papain solution was then added to the intestinal suspension and water was added to adjust the volume to V. Thus the papain digestion was carried out in 5mM-EDTA/10mM-potassium phosphate/5mM-cysteine, pH7, with 10μg of papain/mg of protein for 30min at 37°C, with occasional swirling. After this, the suspension was cooled quickly and centrifuged for 1h at 105000$g_{max.}$ in a Spinco no. 30 rotor. The sediment was discarded and the supernatant solution, after dialysis over a 24h period against three changes of water (40vol. each time), was freeze-dried. The residue was redissolved in 4ml of 10mM-potassium phosphate, pH7.

Step 4. The enzyme sample was applied to a 2.5cm × 90cm column of Sepharose 4B which had been equilibrated in 10mM-potassium phosphate, pH7, containing 0.2% NaN_3, and elution was continued with this solution at a flow rate of 15ml/h. The eluted fractions (20ml) containing maltase activity were combined.

Step 5. A column (1.2cm×20cm) of DEAE-cellulose was equilibrated in 10mM-potassium phosphate, pH7, and the active fractions from step 4 were applied to the column, which was then washed with 100ml of the equilibration buffer. Adsorbed proteins were eluted with a linear 10–70mM-potassium phosphate, pH7, gradient (total volume 500ml). Two peaks of maltase activity were eluted from the column, one at 25mM-potassium phosphate and the other at about 40mM.

Step 6. The second peak of maltase activity was dialysed against water, freeze-dried and resuspended in 1ml of 1mM-Tris/HCl, pH7.5. This was mixed with some Bromophenol Blue and placed in a vertical preparative electrophoresis apparatus on a slab of 3.3%T polyacrylamide gel (9cm×8cm high×0.4cm thick; non-dissociating system prepared as described under 'Polyacrylamide-gel electrophoresis', above). Electrophoresis was carried out of a current of 50mA for about 90min, and after removal of the gel slab a central vertical slice was cut from it and stained for protein with Coomassie Blue. The major protein band was shown, in preliminary experiments, to co-electrophorese with the maltase activity on the gel. Based on the migration of this band, the appropriate maltase-rich regions were cut from the remainder of the slab and homogenized thoroughly

in a Potter–Elvehjem apparatus in 10ml of 1M-Tris/HCl, pH7.5. The homogenate was centrifuged at 105000 $g_{max.}$ for 1h and the supernatant solution removed, dialysed against water and freeze-dried. The residue was resuspended in 1mM-Tris/HCl, pH7.5, at a concentration of 1mg of protein/ml.

Results

Purification of maltase/glucoamylase

A summary of an experiment appears in Table 1, along with the range of specific activities obtained in a series of such experiments. Routinely the enzyme was purified about 300-fold in a yield of 10–20%. The first maltase peak from the DEAE-cellulose column (DEAE I) contained the sucrase/isomaltase (Kolinska & Kraml, 1972). The second peak (DEAE II) was due to the maltase/glucoamylase. No sucrase activity was detected in the latter fraction. Electrophoresis of the fractions in a non-dissociating system (Fig. 1*a*) showed that material from the second peak was composed principally of a slowly migrating band (mobility, *m*, relative to Bromophenol Blue, 0.12), which corresponded to all the maltase activity in a similarly run gel which was sliced and analysed. Several contaminating proteins were present, the major one being a protein (*m*, 0.34) which electrophoresed in a position close to sucrase (*m*, 0.27), but which contained no sucrase activity (Fig. 1*a*). Sucrase, maltase and the contaminating species were also visualized by using the periodic acid–Schiff stain. Maltase was conveniently separated from the contaminants by preparative electrophoresis, and the final preparations of the enzyme migrated as a single band on re-electrophoresis (Fig. 1*a*). Chromatography on Sephadex G-200, a procedure used by Kolinska & Kraml (1972), Schlegel-Haueter *et al.* (1972), Sivakami & Radhakrishnan (1973) and Kelly & Alpers (1973), was not successful in removing the contaminating proteins.

The enzyme was pure by the following criteria: gel electrophoresis of the enzyme in the presence of SDS showed a single protein band (Fig. 1*b*): it sedimented as a single species in sedimentation-velocity experiments (see below); an antibody to the purified enzyme gave a single precipitin line in immunodiffusion experiments with an intestinal homogenate and a crude papain digest as well as with the antigen (Fig. 2). Interestingly, the antibody also reacted with a naturally soluble 'heavy' maltase, which Galand & Forstner (1974*a*) showed was present in young rats. No precipitate was formed with sucrase/isomaltase (Fig. 2).

Some properties of the enzyme

The pure enzyme exhibited a K_m for maltose of 1.08mM and a maximum velocity of 71 μmol of maltose hydrolysed/min per mg of protein, both values being obtained at 36°C. In a second experiment the maltase, glucoamylase and α-glucosidase activities of the enzyme, expressed as μmol of glucose released/min per mg of protein, were found to be 152, 1.4 and 1.5 respectively. The energy of activation of maltose hydrolysis by the enzyme was 39kJ/mol. Maltase activity was relatively thermostable; it was unaffected by heating at 55°C for 10min but was completely abolished by exposure to 70°C for a similar period. In electrofocusing experiments a single maltase peak was found with pI 4.2. The effects of pH on the enzyme are described below.

Composition of the enzyme

The amino acid and sugar analyses of maltase/glucoamylase are given in Table 2. The polarity of the enzyme, calculated from the amino acid composition by the method of Capaldi & Vanderkooi (1972), was 46.1, which is similar to the average value of 47.0 obtained from 205 soluble proteins by these authors. The sugar content of the enzyme was about 20% by weight and was mainly composed of mannose and *N*-acetylglucosamine. The acidic isoelectric point of the enzyme agrees well with the high amounts of glutamate and aspartate residues

Table 1. *Purification of rat intestinal maltase/glucoamylase*

The procedures were carried out as described in the Experimental section. The experiment shown was performed with scrapings from five rats. Other experiments used up to ten rats, and the mean specific activity ±S.D. with the number of experiments in parentheses is shown for these in the last column.

Step	Fraction	Maltase activity (units)	Protein (mg)	Activity recovered (%)	Specific activity (units/mg of protein)	Average specific activity (units/mg of protein)
1	Homogenate	924	4670	100.0	0.2	0.3± 0.1 (10)
2	Sediment	856	1740	92.6	0.5	0.6± 0.3 (10)
3	Papain	616	144	66.7	4.3	4.5± 0.9 (10)
4	Sepharose 4B	568	38.2	61.5	14.9	26.8± 7.8 (10)
5	DEAE peak I	124	6.3	13.4	19.7	20.6± 3.6 (9)
5	DEAE peak II	252	4.9	27.3	51.4	71.9±19.9 (9)
6	Electrophoresis	152	2.3	16.3	65.0	73.2±12.9 (9)

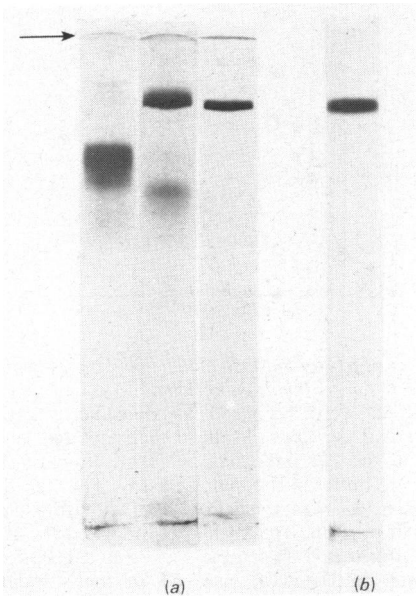

Fig. 1. *Polyacrylamide-gel electrophoresis of maltase fractions obtained during purification*
(*a*) Non-dissociating electrophoresis: the fractions depicted are, from left to right: DEAE peak I (step 5), 20μg of protein; DEAE peak II (step 5), 20μg of protein; electrophoretically purified maltase (step 6), 10μg of protein. (*b*) Dissociating electrophoresis in the presence of SDS: the final step-6 enzyme is shown (10μg of protein). Electrophoresis was performed as described in the Experimental section with the cathode at the top and the anode at the bottom of the Figure. The arrow on the left indicates the origin and the inked stab-marks at the bottom of the gels indicate the migration of the Bromophenol Blue marker.

found, although a proportion of these might be expected to amidated in the intact enzyme. Other points of interest in the composition were the low amounts of cysteine and the absence of sialic acid.

Size of the enzyme

Maltase/glucoamylase migrated as a single protein band on polyacrylamide gels, calibrated with a series of bovine serum albumin oligomers prepared by the method of Payne (1973), with an apparent mol.wt. of 500000 (Fig. 1*b*). Density gradient centrifugation in both sucrose and mannitol gradients by the method of Martin & Ames (1961) showed that the enzyme virtually co-sedimented with β-galactosidase (mol.wt. 540000; 16.6S), a result which confirms earlier studies with the impure enzyme (Galand & Forstner, 1974*a*). When catalase was used as the

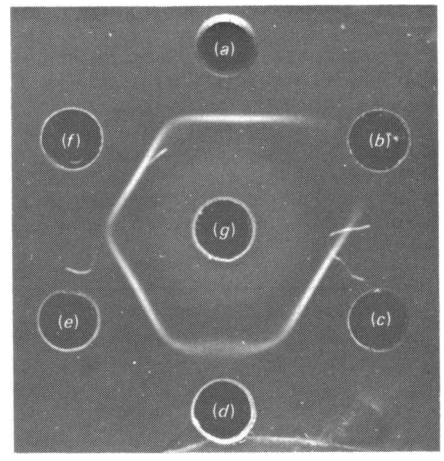

Fig. 2. *Double immunodiffusion of various maltase fractions against rabbit anti-(maltase/glucoamylase) antiserum*
Units of maltase activity are given in parentheses. The wells contains (*a*) maltase/glucoamylase, step 6 (15); (*b*) sucrase/isomaltase, step 5 peak I (15); (*c*) papain-released material, step 3 (30); (*d*) soluble maltase prepared as described by Galand & Forstner (1974*a*) (10); (*e*) homogenate, step 1 (10); (*f*) maltase/glucoamylase, step 6 (5); (*g*) rabbit antiserum prepared as described in the Experimental section.

standard, the derived sedimentation coefficient for maltase/glucoamylase was 14.9S. An $s^0_{20,w}$ value of 14.5S was determined after sedimentation-velocity runs at concentrations of 0.45 and 0.15mg of protein/ml.

Dissociation of the enzyme

Whereas it is well recognized that maltase activity persists in the presence of SDS (Maestracci *et al.*, 1973, 1975; Critchley *et al.*, 1975), our preliminary experiments showed poor recovery of activity in enzyme samples which had been treated with SDS at low pH. Several pH effects are shown in Fig. 3. The enzyme exhibited activity over a broad pH range, with maximal activity between pH 6 and 7. When the effect of pH on the stability of maltase activity was examined, the persistence of the activity over a wide pH range was again notable. However, the stability of maltase activity at pH 3–6 was decreased markedly by very brief exposure to SDS (Fig. 3). Also, the SDS-induced inactivation was irreversible; a sample of the enzyme inactivated by incubation at pH 3.2 for 48h (Fig. 3) was re-activated by dialysis for 12h against 10mM-Tris/HCl, pH 7.5, whereas this was not possible with samples to which SDS had been added.

When samples of maltase inactivated by low pH in the presence of SDS were electrophoresed on SDS/polyacrylamide gels, the patterns shown in Fig. 4

Table 2. *Composition of rat intestinal maltase/glucoamylase*
Enzyme (25 μg) was hydrolysed in 6M-HCl for 30h under vacuum for the amino acid determination, and the sugar analysis was carried out with 250 μg of the enzyme. By the anthrone reaction the enzyme was shown to contain 71 μg of hexose/mg of protein (range 54–80). The residues/molecule of enzyme were calculated by assuming a mol.wt. of 500000. The values for serine and threonine presumably were low because of loss owing to the extended hydrolysis time.

	Content		
	(residues/100 amino acids)	(residues/ molecule)	(% by wt.)
Asx	17.0	550	13.5
Thr	3.8	123	2.7
Ser	2.0	65	1.2
Glx	10.8	349	9.6
Pro	7.2	233	4.8
Gly	5.6	181	2.2
Ala	5.6	181	2.7
Val	6.7	217	4.6
Cys	0.4	13	0.3
Met	2.2	71	2.0
Ile	5.2	168	4.1
Leu	10.2	330	8.0
Tyr	5.2	168	5.9
Phe	5.5	178	5.7
Lys	4.7	152	4.2
His	2.6	84	2.5
Arg	5.2	168	5.6
Fuc	1.6	52	1.6
Man	4.6	149	5.1
Gal	1.6	52	1.9
GlcNAc	6.6	214	9.3
GalNAc	1.6	52	2.3
AcNeu	0	0	0

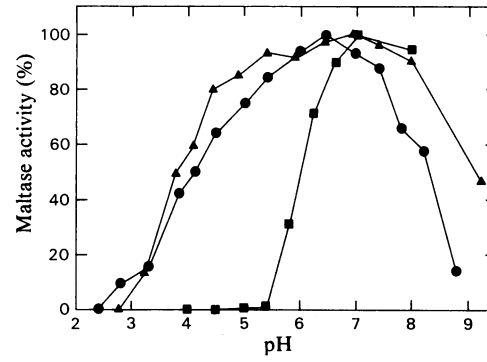

Fig. 3. *Effect of pH on maltase activity and on the stability of maltase activity*
The buffers were prepared as described in the Experimental section. Maltase activity (●) was assayed at the pH indicated by substitution of the appropriate buffer in the maltase assay. The stability of maltase (▲) was measured by incubating 5 μg samples of pure enzyme at the pH indicated for 48h at 4°C; after this each sample was diluted 1000-fold with water and quickly assayed in the standard maltase assay. The stability of maltase in SDS (■) was measured by incubating 5 μg samples of maltase at the pH values indicated; SDS, to a final concentration of 1% (w/v), was then added and after 5min at 24°C the samples were quickly diluted 1000-fold with water and assayed for residual maltase activity. Maltase activities were expressed as a percentage of the maximum value in each case. In the first two experiments these values were comparable; however, the enzyme activity at pH7 in the presence of SDS was increased by about 50% over that in its absence.

were obtained. Maltase was dissociated into five major species along with some minor components. The distribution of protein among the bands, as judged by densitometry of several gels, was: band 1, 36%; band 2, 24%; band 3, 13%; band 4, 19%; band 5, 8%. Each band also stained faintly with the periodic acid–Schiff stain.

The enzyme can be dissociated also by heat. Although it was unaffected by a 1h incubation in 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol at pH7.4 and 37°C it could readily be dissociated by boiling for a short period, as shown in Fig. 5. This procedure also totally inactivated the enzyme. Reducing agents were not required for the dissociation, which was also accomplished by boiling the enzyme in 6M-urea (Fig. 5). A longer period of heating (5min) was sometimes required for the urea treatment, since incomplete dissociation was occasionally seen after only 2min boiling (Fig. 5*e*). Boiling for periods up to 30min did not alter the pattern any further. The invariability of the dissociated pattern was also shown by parallel experiments in which the dissociation was accomplished by low pH or by heating; the patterns were indistinguishable. It is unlikely that the dissociation pattern resulted from contaminating proteinase activity since the pattern did not change when the dissociation was carried out in the presence of a large excess of albumin, ovalbumin or immunoglobulin, which should have tended to preserve the enzyme if this was the case.

Of the minor bands, all of which did not always appear, the most persistent was one which migrated (*m* 0.64) ahead of band 1, but which never amounted to more than 3% of the protein on the gel. It is not clear if this represented a contaminant or if it was derived from the enzyme.

Characterization of the dissociated species

The bands obtained by dissociation of the enzyme appeared to be quite stable entities, since on re-electrophoresis each one migrated with its appropriate

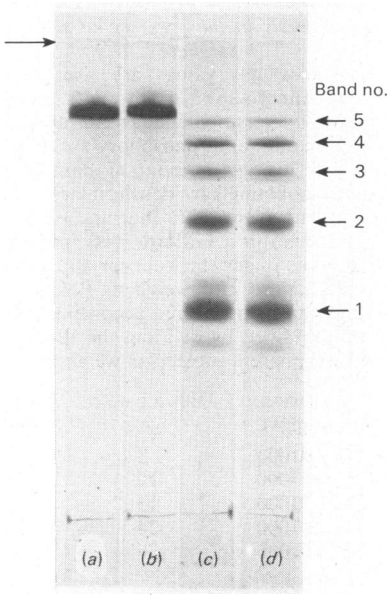

Fig. 4. *Dissociation of maltase/glucoamylase by pH*
Samples of the enzyme (15 μg each) were adjusted to the pH values given below as described in the Experimental section. Then SDS was added to a final concentration of 1% (w/v) and the samples were electrophoresed. The pH values were (*a*) 7.4, (*b*) 6.3, (*c*) 5.1 and (*d*) 4.0. The arrow on the left shows the origin and the cathode and anode were respectively towards the top and bottom of the Figure.

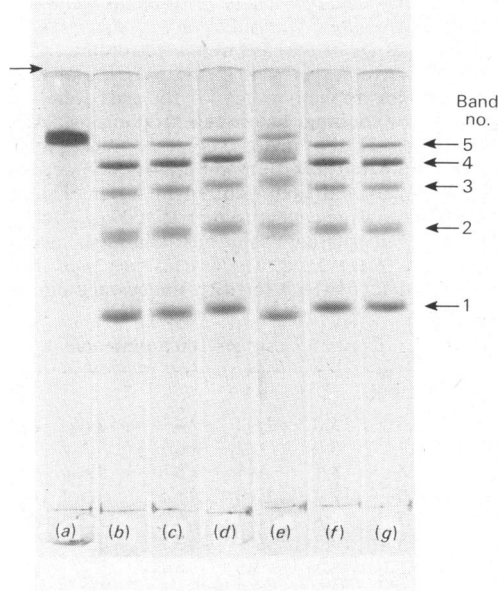

Fig. 5. *Dissociation of maltase/glucoamylase by heat*
The enzyme (10 μg) was incubated as indicated below with 1% (w/v) SDS/6M-urea/1% (v/v) β-mercaptoethanol/0.1M-dithiothreitol in 1mM-Tris/HCl, pH7.4, either at 37°C (1h) or 100°C (2min). After cooling at 24°C the samples were electrophoresed. The treatments were: (*a*) SDS and β-mercaptoethanol at 37°C; (*b*) SDS and β-mercaptoethanol at 100°C; (*c*) SDS and dithiothreitol at 100°C; (*d*) SDS alone at 100°C; (*e*) urea alone at 100°C; (*f*) urea and β-mercaptoethanol at 100°C; (*g*) urea and dithiothreitol at 100°C. The arrow on the left indicates the origin and the cathode and anode were respectively towards the top and bottom of the figure.

mobility and showed no tendency towards further breakdown.

In addition, amino acid analysis was performed on protein extracted from the appropriate regions of ten identically run gels (Table 3). Although some differences were found, the general impression was that the compositions of the bands were very similar to each other and to that of the original enzyme.

Because of the apparent high molecular weight of the dissociated species somewhat unusual marker proteins were used to calibrate the SDS/polyacrylamide gels used. The standards were synthetic oligomers of bovine serum albumin (Payne, 1973) and natural oligomers of phosphorylase (Weber & Osborn, 1969). Apparent molecular weights of the bands are given in Table 4, which shows that the molecular weight of the smallest band was greater than 140000 whereas that of band 5 was of the same order as that of the undissociated enzyme. In this connection, it may be noted that this band was not just an undissociated portion of the original maltase but actually arose from smaller components of the enzyme during dissociation (P. R. Flanagan & G. G. Forstner, unpublished work), suggesting, perhaps, that it represented an aggregate form. Aggregation was also suggested as an explanation for the very large apparent sizes of some of the bands by the following considerations. It is possible to calculate the relative molar amounts of the species from the data in Table 4, provided that (*a*) the bands represent single polypeptide chains and (*b*) the amount of Coomassie Blue staining is proportional to the mass of each band. When this was done it was found that band 1 was present in a 16-fold molar excess over band 5, and a minimum molecular weight for the composite enzyme, calculated from the apparent sizes and molar ratios of all the bands (Table 4), was 6×10^6. Even if the enzyme was taken to be composed of one of each of the subspecies, the calculated molecular weight would be more than three times the experimentally determined value.

Table 3. *Amino acid analysis of the original maltase band and the five bands obtained from it by dissociation*

Samples (20 μg) of maltase were electrophoresed on each of twelve 3.3%T gels in the presence of SDS. The samples applied to ten of the gels were dissociated by boiling before electrophoresis. After electrophoresis the regions corresponding to the bands (numbered according to Figs. 4 and 5) were cut from the gels and homogenized in water. The gel homogenates were centrifuged at 105000$g_{max.}$ for 1 h and the supernatant solutions dialysed against water and freeze-dried. The dry residues were then hydrolysed in 6M-HCl for 12h under vacuum and analysed.

	Content (residues/100 amino acids)					
	Original	5	4	3	2	1
Asx	15.5	15.0	15.2	14.9	16.6	14.7
Thr	6.1	5.9	6.2	6.1	6.5	6.7
Ser	7.6	8.1	9.1	8.8	8.6	9.5
Glx	10.0	11.4	11.0	11.6	10.9	12.2
Pro	5.7	4.8	6.2	6.7	5.7	5.3
Gly	8.3	10.5	9.1	10.0	9.3	9.8
Ala	6.1	6.1	5.5	6.1	5.9	6.7
Val	6.8	6.1	6.2	5.8	6.0	4.8
Ile	5.0	5.2	4.9	4.6	4.8	3.8
Leu	8.8	7.7	7.9	7.6	7.3	7.2
Thr	3.9	2.6	3.6	1.2	3.1	4.1
Phe	5.6	4.4	4.3	4.3	4.3	4.8
Lys	3.9	6.8	3.8	6.7	5.1	4.8
His	1.2	0.8	1.2	0.6	1.0	0.9
Arg	5.4	4.7	5.7	5.2	5.1	4.5

Table 4. *Apparent molecular weights of maltase subspecies by gel electrophoresis in the presence of sodium dodecyl sulphate*

The molecular-weight values are average values obtained by interpolation from plots of electrophoretic mobility versus the logarithm of molecular weight of albumin and phosphorylase oligomeric standards. The average amount of stain bound by the bands was obtained by densitometry. Five gels were stained with Coomassie Blue and subsequently scanned at 550nm in a Gilford 2400 spectrophotometer. The areas under the appropriate peaks were cut from the charts and weighed. Relative molar ratios of the bands (numbered according to Figs. 4 and 5) were obtained by dividing the 'Bound stain' values by the apparent molecular weight.

Band no.	Apparent mol.wt.	Bound stain (%)	Relative molar ratio
5	480000	8	1.0
4	410000	19	2.8
3	350000	13	2.2
2	245000	24	5.9
1	134000	36	16.1

Evidently, then, the bands do not represent conventional subunits, i.e. single polypeptide chains.

An alternate explanation for the anomaly is that the molecular weights of the bands were overestimated. Because it is known that glycoproteins may exhibit non-ideal behaviour during electrophoresis in the presence of SDS (Bretscher, 1971; Segrest *et al.*, 1971) an attempt was made to detect such anomalies by plotting the electrophoretic mobilities of the bands in gels of different concentration according to the relationship proposed by Ferguson (1964). Such 'Ferguson plots' have proved useful for detecting anomalously migrating proteins in various polyacrylamide-gel-electrophoresis systems (Rodbard & Chrambach, 1971; Neville, 1971; Banker & Cotman, 1972). A 'Ferguson plot' of the oligomeric standards and the maltase bands is shown in Fig. 6, and it is clear that the enzyme bands behaved quite differently from those of the standards. The bovine serum albumin and phosphorylase markers together formed a group whose plots intersected at 2%T. Rodbard & Chrambach (1971) have suggested that the extent of cross-linking of the gel shifts the intersection point of the plots away from the ideal value of 0%T; the gels in the present study were cross-linked at 3.6%C. The legitimacy of the standards was further corroborated by the fact that the behaviour of the phosphorylase dimer (mol.wt. 190000) was almost indistinguishable from that of the albumin trimer (mol.wt. 201000) on the gels.

On the other hand, the maltase bands clearly behaved anomalously (Fig. 6). For example, band 1 migrated more slowly than the albumin dimer at a gel concentration of 3.3%T, whereas at higher gel concentrations it moved ahead of the dimer. Band 3 migrated similarly relative to the phosphorylase trimer. In addition, the 'free electrophoretic mobilities' (for explanation see legend to Fig. 6) of the maltase bands did not increase with apparent molecular weight as did those of the standards and, in fact, bands 2 and 4 displayed identical values (Fig. 6). As a group, the enzyme bands exhibited lower 'free electrophoretic mobilities' and 'retardation coefficients' (see legend to Fig. 6) than might be expected from the apparent molecular weights determined at a single gel concentration (Table 4), and so molecular-weight estimates obtained from plots of 'free electrophoretic mobility' versus molecular weight and 'retardation coefficient' versus molecular weight (Banker & Cotman, 1972) gave values of 80000–100000 for band 1 and 150000–280000 for band 5. These values were not sufficiently different from those shown in Table 4 to alter the suggestion that at least some of the bands represented aggregated forms (see above).

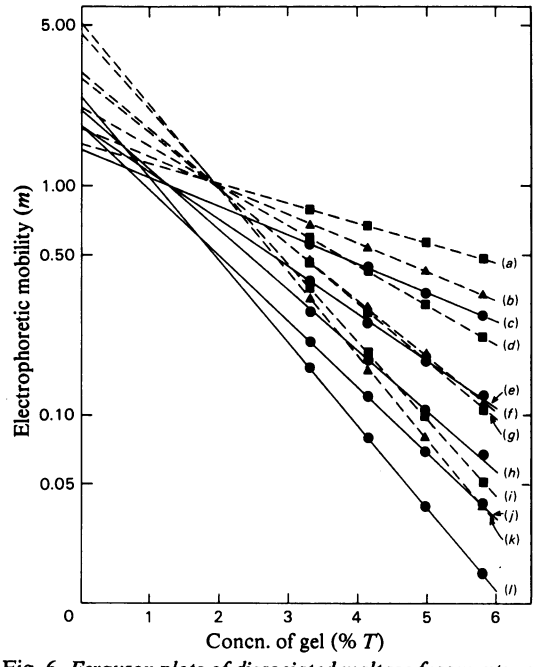

Fig. 6. *Ferguson plots of dissociated maltase fragments and of albumin and phosphorylase oligomeric standards*

The electrophoretic mobilities (m) of the various proteins were measured in SDS/polyacrylamide gels of different concentration (3.32, 4.15, 4.98 and 5.81%T). For clarity the data for the albumin (■) and phosphorylase (▲) standards are shown by the broken lines, whereas data for the maltase bands (●), numbered according to Figs. 4 and 5, are shown by the continuous lines. The individual curves are for: (*a*) albumin monomer; (*b*) phosphorylase monomer; (*c*) maltase band 1; (*d*) albumin dimer; (*e*) maltase band 2; (*f*) phosphorylase dimer; (*g*) albumin trimer; (*h*) maltase band 3; (*i*) albumin tetramer; (*j*) maltase band 4; (*k*) phosphorylase trimer; (*l*) maltase band 5. The relationship proposed by Ferguson (1964) to describe electrophoretic behaviour is: $\log m = \log M_0 - K_r T$, where m is the experimentally determined mobility of the protein in a gel of concentration T, M_0 is the 'free electrophoretic mobility' and K_r is the 'retardation coefficient'.

Discussion

The present preparation of maltase/glucoamylase was demonstrably pure by several criteria and therefore the specific activity of the enzyme was higher than that reported by Schlegel-Haueter *et al.* (1972), Kolinska & Kraml (1972) and Kelly & Alpers (1973). However, we have found that pure maltase specific activities were quite variable, and on occasion we have measured values as high as 100 μmol of maltose hydrolysed/min per mg of protein.

The composition of rat maltase/glucoamylase appears to be quite different from that of the human enzyme (Kelly & Alpers, 1973), particularly in the sugar distributions. Whereas the major carbohydrate of the human enzyme was found to be fucose (18% by weight) by Kelly & Alpers (1973), this sugar was only a minor component of the rat enzyme (1.6% by weight). The major sugar of the rat maltase was glucosamine (9.3%) and this was also the case for rabbit intestinal sucrase/isomaltase (Cogoli *et al.*, 1972). In common with alkaline phosphatase (Engstrom, 1961), sucrase/isomaltase (Cogoli *et al.*, 1972) and human maltase/glucoamylase (Kelly & Alpers, 1973), sialic acid was absent. The fact that the polarity of the enzyme which was released from the membrane by papain digestion was comparable with that of average globular proteins (Capaldi & Vanderkooi, 1972) is in keeping with present ideas about its topology in the brush-border membrane. Maroux & Louvard (1976) have proposed that maltase is an amphipathic molecule comprising a large, enzymically active, polar 'head' which is coupled via a papain-sensitive region to a much smaller, relatively hydrophobic, 'tail' which anchors the enzyme in the membrane.

Although dual forms of human intestinal (Kelly & Alpers, 1973) and human kidney (de Burlet & Sudaka, 1976) maltases have been reported, we did not find this for the rat intestinal enzyme. Two other intestinal membrane enzymes, alkaline phosphatase (Saini & Done, 1972) and aminopeptidase (Kim & Brophy, 1976), have been shown to exist in several forms, the most likely explanation being heterogeneity of carbohydrate composition, although limited proteolysis (Malik & Butterworth, 1976) is a possibility.

Previous studies by Critchley *et al.* (1975), Maestracci *et al.* (1973, 1975) and Alpers (1972) have demonstrated the considerable stability of maltase and other brush-border enzymes, which enables them to be localized on polyacrylamide gels after electrophoresis in SDS. The present work, however, shows that this stability is markedly dependent on pH. At acidic pH values a rapid inactivation of maltase occurred in SDS solutions which was not seen in the absence of the detergent. Concomitantly, there was a considerable disruption of the structure of the enzyme, as shown by the formation of at least five new glycoprotein bands. The change was also accomplished by heating the enzyme in SDS or in urea. Reducing agents were not necessary.

It is unlikely that proteolysis was responsible for the phenomenon for four reasons: first, boiling in SDS, a procedure known to restrict proteolysis severely or eliminate it altogether (Pringle, 1970; Weber *et al.*, 1972), consistently gave a dissociation pattern identical with that obtained by lowering the pH; secondly, even prolonged incubations at 37°C

in SDS produced no low-molecular-weight species that might have indicated proteolysis (Pringle, 1970; Fairbanks *et al.*, 1971; Kobylka *et al.*, 1972); thirdly, when maltase was dissociated in the presence of an excess of other proteins, which might be expected to act as alternative substrates for a putative proteinase and thus exert a sparing effect on maltase, no decrease in the intensities of the maltase bands was observed; and fourthly, dissociation of a sample of maltase which had been solubilized by Triton X-100 extraction of the brush-border membrane (P. R. Flanagan & G. G. Forstner, unpublished work) gave a gel pattern very similar to that obtained with the papain-solubilized enzyme, indicating that the bands were not papain-induced.

The new species were formed in constant proportions whether dissociation was carried out by heat or by low pH and no evidence of a dissociation equilibrium between them was obtained, i.e. upon re-electrophoresis each band did not again redistribute itself.

The behaviour of the fragments on 'Ferguson plots' suggests that they did not fulfil the criteria necessary for valid measurements of molecular weight. These conditions are, first, that the species should bind the same amount of SDS, gram for gram, as the standards, and secondly that the relationship between hydrodynamic size (Stokes radius) and molecular weight should be identical for both standards and unknown.

Since the bands are glycoproteins, errors may result from non-fulfilment of the first condition (Pitt-Rivers & Impiombato, 1968; Weber *et al.*, 1972; Grefrath & Reynolds, 1974). This artifact has been most clearly demonstrated for glycophorin (Segrest *et al.*, 1971; Javaid & Winzler, 1974), although it should be noted that this glycoprotein contains a very high amount of carbohydrate (60%). Reasonable estimates of the molecular weights of several intestinal glycoproteins with low sugar contents have been obtained by SDS/polyacrylamide-gel electrophoresis (Maroux *et al.*, 1973; Kim & Brophy, 1976; Malik & Butterworth, 1976).

Non-fulfilment of the second condition also may be responsible for the anomaly. Intact maltase has also been characterized as a very large molecule by gel-filtration experiments (Forstner, 1971) which rely on the relationship between Stokes radius and molecular weight. Maltase and its dissociated fragments may therefore be asymmetric molecules which exhibit anomalous hydrodynamics leading to exaggerated estimates of their sizes.

In view of the similar amino acid compositions of the fragments (Table 3), their large apparent sizes (Table 4) and their inappropriate molar ratios (Table 4), it is possible that they represent aggregates of a common monomer. Alternatively, the bands may represent common protein backbones containing different amounts of carbohydrate. Periodic acid–Schiff staining, however, suggested that this was not the case.

Aggregation of glycoproteins under dissociating conditions has indeed been reported for glycophorin (Marton & Garvin, 1973; Tuech & Morrison, 1974; Slutzky & Ji, 1974; Potempa & Garvin, 1976), the anion-transport glycoprotein of erythrocytes (Fairbanks *et al.*, 1971; Yu & Steck, 1975) and a glycoprotein recently isolated from kidney basement membrane by Ohno *et al.* (1975).

In summary, maltase/glucoamylase can be dissociated either by high temperatures or by low pH in SDS. The resultant fragments are probably stable macromolecular aggregates of an unknown monomer. If band 1 is the monomer then the molecular-weight estimates, though inaccurate, suggest that the intact enzyme may be a trimer or tetramer.

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