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PROPERTIES OF HUMAN INTESTINAL GLUCOAMYLASE

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SUMMARY

1. Human intestinal glucoamylase has been purified almost to homogeneity by polyacrylamide disc electrophoresis. At least two isoenzymes were found with identical catalytic properties.

2. Linear oligosaccharides ($N = 9$) containing glucose residues linked α (1 → 4) have the greatest affinity for the active site. Both decreasing and increasing the polysaccharide chain length increased the K_m.

3. The purified enzyme hydrolyzed both α (1 → 4) linkages and α (1 → 6) linkages. Linear glucose polymers linked α (1 → 4) were hydrolyzed more rapidly than polymers containing α (1 → 6) cross linkages. Activity of the purified enzyme against both linkages was identically heat inactivated, and no sucrase activity was detectable. Thus, hydrolysis of α (1 → 6) bands was not due to contamination by intestinal sucrase–isomaltase.

4. Intestinal glucoamylase contained 32–38% carbohydrate by weight, and had a partial specific volume of 0.684.

5. Molecular weight of the purified enzyme was 210 000 by equilibrium sedimentation.

6. The purified enzyme contained neither sialic acid nor phosphate, both membrane components. It is not clear yet whether a portion of glycocalyx might be removed with the enzyme during purification to account for the high carbohydrate content.

INTRODUCTION

Amylases are enzymes which hydrolyze starch and are classified according to the location of the α (1 → 4) glucose bonds which are hydrolyzed. The intestinal brush border of the rat contains only a single amylase activity[1] which shows characteristics of a glucoamylase, in that it removes successive glucose units from non reducing ends of glucose polymers and produces glucose as the end product. By contrast, the familiar pancreatic amylase is an example of an α-amylase, in that it hydrolyzes α (1 → 4) glucosidic bonds in a random fashion with maltose as the final

product. Human mucosa has been reported to contain a variety of amylase activities[2]. Since the work in the rat and in human biopsy specimens[3] suggests that amylase in the intestine is glucoamylase, we focused our attention on this enzymatic activity when purifying human intestinal amylase.

Other intestinal disaccharidases have been intensively studied as examples of membrane bound enzymes. Sucrase–isomaltase has been purified and characterized[4,5] as has been lactase–phlorizin hydrolase[6–8]. However, maltase has not been purified to homogeneity, although partial purification has been reported previously[1,9]. Schlegel-Hauter *et al.*[7] reported approximately a 500-fold purification of rat intestinal glucoamylase, but the preparation seemed heterogeneous on disc gel electrophoresis.

Both sucrase–isomaltase[5] and lactase–phlorizin hydrolase[10] have been found to exist either as an enzyme complex, or as a single enzyme with multiple substrate binding sites. No such data are available for intestinal glucoamylase. Data from monkey suggest that intestinal glucoamylase corresponds to Maltase II of Dahlqvist[28]. Finally, some data suggest that intestinal disaccharidases are glycoproteins[6,12]. We report here the purification of human intestinal glucoamylase with a description of some of its enzymatic and chemical properties.

MATERIAL

Soluble starch was obtained from Merck and Company, Inc. (Rahway, N.J.). Isomaltose was obtained from Miles Laboratories, Inc. (Kankakee, Ill.) and rechromatographed on paper in butanol–pyridine–water (6:4:3, v/v/v) to remove contaminants. The isomaltose was eluted from the paper and then used for enzyme assays. Sephadex G-200 was a product of Pharmacia (Uppsala, Sweden), DEAE-cellulose (DE52) was purchased from Reeve-Angel (Clifton, N.J.). Papain and glucose oxidase were obtained from Sigma Chemical Co., St. Louis, Mo. Hexasaccharide standard was generously provided by Dr Barbara Illingworth Brown, Washington University, St. Louis, Mo. Human intestine was obtained at autopsy within 6 h of death. Intestines were obtained from patient N.S., age 36 (myocardial infarction), W.S., age 41 (cerebrovascular hemorrhage), H.S., age 67 (myocardial infarction), and M.M., age 57 (cardiac arrest). No patient was malnourished or had been on intravenous therapy at time of death. The small intestine appeared normal in all cases and was removed from the ligament of Treitz to the mid-small intestine, opened longitudinally, and washed very gently with cold normal saline. Mucosa was obtained by scraping with glass slides and frozen at −70 °F until needed. All samples were used within four months.

METHODS

Enzyme purification

Intestinal mucosa was homogenized with a Teflon tissue grinder in 4 vol. of 50 mM potassium phosphate buffer, pH 6.0. The homogenate was centrifuged at 1000 × *g* for 15 min to remove cell debris; then the supernatant fraction was spun at 105 000 × *g* for 60 min and the pellet resuspended in an equal volume of the original buffer. Approximately 20% of total maltase activity was discarded with the supernatant fraction. The resuspended pellet (about 10 mg protein per ml) was

TABLE I

PURIFICATION OF HUMAN INTESTINAL GLUCOAMYLASE

On DEAE chromatography, glucoamylase activity accounts for only 27% of total maltase activity. Thus, the value for the first four steps has been corrected to report only 27% of the total maltase activity. One unit = 1 μmole disaccharide hydrolysed/min.

	Total protein (mg)	*Maltase (units)*	*Yield (%)*	*Specific activity (units/mg)*
Homogenate	4672	619.4	100	0.133
Papain treatment	1513	438.5	70.8	0.29
Ethanol precipitation	1131	336	64.2	0.297
DEAE-cellulose	30.4	386	58.8	12.7
Sephadex G-200	21.07	332	53.6	15.76
Preparative acrylamide electrophoresis	1.92	70.2	11.2	36.6

treated with 0.01 mg of papain per ml, in the presence of 1 mM cysteine, for 60 min at 37 °C. The suspension was then spun at 105 000 × *g* for 60 min, and the supernatant fraction recovered.

To the supernatant fraction were added 2 vol. of ethanol (−20 °C) and the mixture was incubated at 4 °C for 30 min. All subsequent procedures were carried out at 4 °C. After centrifugation at 6000 × *g* for 15 min, the pellet was redissolved in 1/6 of the original volume with 50 mM potassium phosphate buffer, and dialyzed against the same buffer to remove the ethanol. After dialysis, the enzyme solution was again spun at 105 000 × *g* for 60 min and the supernatant fraction retained. This supernatant fraction was dialyzed against 5 mM potassium phosphate buffer and applied to a 1 cm × 30 cm column of DEAE-cellulose, equilibrated with the same buffer. The column was packed under 15 lb/inch^2. Glucoamylase activity was eluted using a 800-ml gradient of potassium phosphate, pH 6.0, from 5–100 mM, at a flow rate of 36.2 ml/h. The fractions containing glucoamylase activity were combined, concentrated by lyophilization, and placed on a column of Sephadex G-200 (1.5 cm × 90 cm), and the column developed with 50 mM potassium phosphate buffer, pH 6.0. Tubes containing glucoamylase activity were then combined, lyophilized, dialyzed against 50 mM potassium phosphate buffer, pH 6.0, and applied to a 6 mm thick preparative slab electrophoresis using an E-C vertical gel system with a discontinuous Tris–glycine buffer[13]. Electrophoresis was performed at 300 V for 6 h. One strip of the gel was cut out and stained with Coomassie Blue. A strip of remaining gel was sliced into pieces of 1 mm each, using a slicer containing razor blades fixed at 1-mm intervals. Each gel slice was homogenized into 5.0 ml of the phosphate buffer, shaken at 4 °C for 4 h, and then centrifuged at 600 × *g* for 10 min. The supernatant fraction was then assayed for maltase activity.

Oligosaccharide purification

Oligosaccharides were purified after starch hydrolysis according to the method of Gibson *et al.*[14]. 2 g of soluble starch were dissolved in 40 ml of 0.05 M H_2SO_4. After boiling 1 h, the solution was titrated to pH 7.0 with 0.5 M $Ba(OH)_2$, and the precipitate allowed to settle. The supernatant fraction was decanted and lyophilized, and resuspended in water. Aliquots were applied to Whatman 3 MM chromatography

paper, and developed in descending chromatography in butanol–pyridine–water (6:4:3, v/v/v) for three days to separate glucose polymers of 2–6 residues, and for six days to separate polymers of 6–10 residues. One strip was stained with $AgNO_3$, and appropriate areas cut, and the oligosaccharides eluted with water. Hexasaccharide was used as a standard. On rechromatography, each fraction obtained was 90–95% pure.

Biochemical analyses

Amylase activity was assayed as previously described[1], using soluble starch as substrate. Disaccharidase activity was determined by the two step method of Dahlqvist[15]. Amino acid analysis was performed on a Technicon NC-1 amino acid analyzer after hydrolysis of the glycoprotein in constant boiling HCl for 22 h at 105 °C in an evacuated sealed tube. Carbohydrate analysis was performed as described by Clamp *et al.*[16] on a 3% SE-30 column in an F and M Model 4000 gas–liquid chromatography analyzer. Separate analyses were performed by other established methods, as a check on the validity of the data. These methods include separation by paper chromatography, elution, and quantitation by specific enzymatic reactions for galactose, mannose, and glucose[17]. Fucose was determined by the cysteine–H_2SO_4 reaction[18], total hexosamines by a modified Elson–Morgan reaction[19], and sialic acid by the method of Warren[20]. Total phosphorus was determined by the micro method of Ames and Dubin[21]. Electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels were performed as described previously[22]. Isoelectric focusing in acrylamide gels were carried out using the method of Riley and Coleman[23]. Analytic polyacrylamide disc electrophoresis was performed as described by Davis, using a Tris–glycine system[24]. Analytical ultracentrifugation was performed by the method of Yphantis[25].

RESULTS

Purification of intestinal glucoamylase

The human enzyme was purified by steps outlined in Methods. Amylase activity could not be used to follow the degree of purification, because of the large amounts of pancreatic α amylase in the original homogenate. However, not all the maltase activity could be attributed to the glucoamylase. It was found that on DEAE-cellulose chromatography (Fig. 1), about 27% of maltase activity could be attributed to the glucoamylase (Peak B), which was eluted at a phosphate concentration of 40–50 mM. Most of the sucrase and lactase activity (Peak A) could be separated from the amylase activity. Although the small amount of maltase activity which was eluted slowly from the column (Peak C) had some properties similar to Peak B, it did not contain amylase activity and was, therefore, not included in further purification steps. Peak B was placed over Sephadex G-200 to remove the small amount of sucrase activity. Sucrase is retarded by the Sephadex[26], while maltase is eluted in the void volume. The preparation at this stage consisted largely of one protein, but had five other bands. However, when stained histochemically by the method of Jos *et al.*[27], at least two protein bands contained maltase activity. Attempts to separate these proteins by column chromatography (including hydroxylapatite, CM cellulose, and Sepharose 2B) or by isoelectric focusing were unsuccessful. However, when the eluate

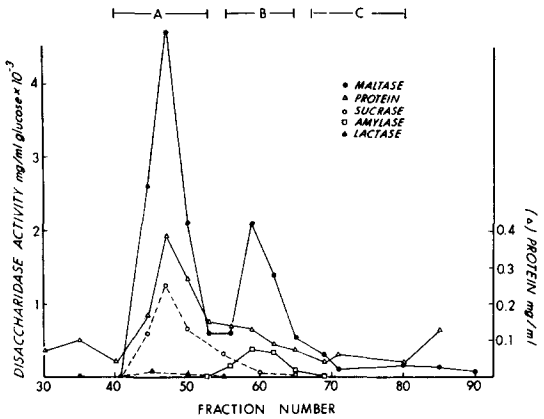


Fig. 1. DEAE-cellulose chromatography of glucoamylase 0.2 g of protein was placed on the column and chromatography carried out as described in Methods. Fractions were pooled into Groups A, B, or C as depicted at the top of the figure.

from Sephadex G-200 was electrophoresed on slab acrylamide gel electrophoresis, and the gel sliced in pieces 1 mm thick, the two proteins with maltase activity could be largely removed from the other proteins.

Fig. 2 shows the elution pattern of maltase activity obtained from preparative slab electrophoresis. The peak of activity between Fractions 37 and 45 contained maltase and amylase activity in proportions comparable to the enzyme obtained from Sephadex G-200. The fraction between Slices 45 and 63 corresponded to the major faster moving protein seen on disc gel electrophoresis, and also exhibited maltase and amylase activity. In order to obtain a single protein band, maltase activity eluted from Fractions 52–63 were combined so as to include the fastest moving component of maltase activity. When the fraction was analyzed on analytical

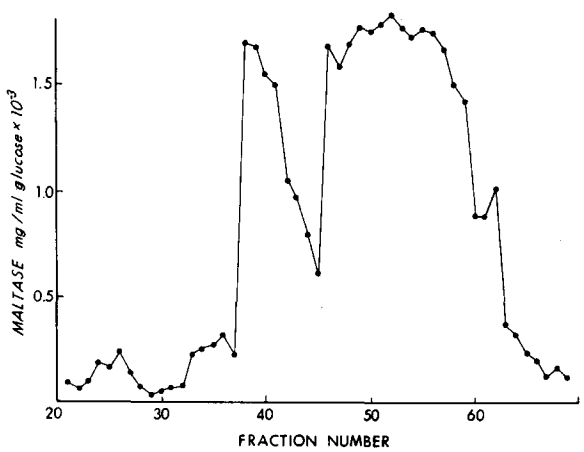
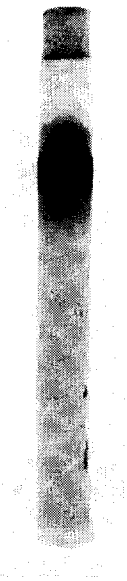


Fig. 2. Preparative slab gel polyacrylamide electrophoresis of glucoamylase. Ten μg of maltase from Sephadex G-200 was applied to a 4% slab gel acrylamide system and electrophoresed as described in Methods. For subsequent studies, Fractions 53–62 were pooled, dialyzed against 50 mM potassium phosphate buffer to remove unreacted acrylamide and Tris buffer, and concentrated by lyophilization.

disc gel electrophoresis, it seemed almost completely homogeneous (Fig. 3). Identical gel patterns were obtained using a cacodylic acid–pyridine system, pH 6.5, or a Bis–Tris–TES–cacodylic acid system, pH 7.4. The purified enzyme gave a straight line on an Yphantis plot derived from data obtained by analytical untracentrifugation. The final preparation was purified approximately 300-fold, although the yield was low, mainly due to losses incurred during preparative electrophoresis.



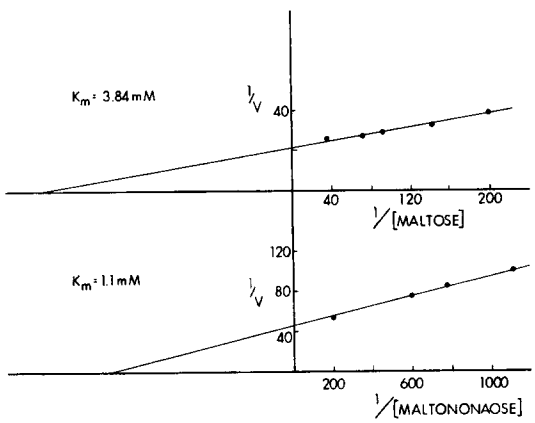


Fig. 3. Analytical disc-gel polyacrylamide electrophoresis of purified glucoamylase. 30 μg maltase obtained from slab gel electrophoresis was electrophoresed and stained as described in Fig. 2.

Fig. 4. Enzyme kinetics of glucoamylase. Incubation mixture in a volume of 40 μl included purified enzyme (1 μl) and substrate in 50 mM potassium phosphate buffer, pH 6.0. Reaction was terminated by addition of 600 μl of Tris–glucose oxidase reagent[14] and incubation for another 60 min was performed.

Both enzyme fractions (37–45 and 45–63) obtained from preparative gel electrophoresis had identical substrate specificities, maltase amylase activity ratios, molecular weights, and were equally heat sensitive. Thus, they can probably be considered as isoenzymes. The enzyme purified in Fig. 3 was most extensively tested with the results reported below. The purified enzyme had a pH optimum of 6.0. Unlike sucrase, its activity at pH 6 was stimulated by only about 10% in the presence of 50 mM Na^+. Its isoelectric point was 4.4, very similar to that reported for rat lactase[7]. Activity was stable to heat at 55 °C but destroyed after 30 min at 65 °C, similar to the properties of maltase II previously reported[28]. When a variety of linear glucose polymers were used to test activity, a consistent pattern emerged.

The K_m for all polymers tested from $N = 5$ to $N = 9$ were similar, varying between 1.0 and 1.5 mM (Fig. 4). However, the K_m increased as polymer length increased above $N = 9$ and increased again below $N = 5$. K_m for maltose hydrolysis was 3.84 mM (Fig. 4), while for starch it was 11 mM. However, V steadily decreased

as polymer length increased, so that the V for starch was only about one fifth that for maltose. The product of all these reactions, determined by paper chromatography, was glucose.

Since other intestinal disaccharidases can catalyze a synthetic reaction by transferring a monosaccharide to an oligosaccharide, we investigated the transglucosylation of maltose by the intestinal glucoamylase. Starting with [^{14}C]glucose and maltose (80 mM) or soluble starch (1%), incubation with enzyme was carried out for up to 60 min[29]. No evidence of incorporation of glucose into maltose, maltotriose, or starch was detected. Thus, considering substrate concentrations encountered post prandially in the intestinal lumen, transglucosylation by glucoamylase probably does not occur.

Besides substrate size, another factor which affected the rate of hydrolysis was the degree of cross linkage in the substrate. When enzyme was added to excess substrate of six glucose moieties either in linear 1–4 linkage (hexaose) or containing one 1–6 linkage (branched hexasaccharide), activity was twice as great against the linear polymer (Table II). Isomaltose itself, but not sucrose, was slowly hydrolyzed by the purified enzyme. This activity was not lost by heating the enzyme at 55 °C for 30 min. Activity against starch and amylose was lower, but not different from each other. This is not surprising, since soluble starch contains only small amounts of cross-linked amylopectin. By comparison, pancreatic amylase was nearly 300 times more active than the intestinal amylase at splitting glucosidic bonds from starch.

TABLE II

SUBSTRATE SPECIFICITY OF HUMAN INTESTINAL GLUCOAMYLASE

Purified human glucoamylase was present at a concentration of 0.24 mg/ml. Incubation was for 30 min at 37 °C. One unit = 1 μmole of glucose liberated/min. Moles of glucosidic bonds/l = (g of substrate/l)/(mol. wt of glucose anhydride) (ref. 30).

Substrate	*Concentration (moles of glucosidic bonds/l)*	*Activity (units/mg protein)*
Isomaltose	20	0.8
Sucrose	20	0
Hexaose	20	4.2
Branched hexasaccharide	20	2.0
Amylose	20	1.5
Starch	20	1.3

The purified protein was analyzed for amino acid and sugar content, with results recorded in Table III. Nothing unusual can be discerned from the pattern of amino acids. However, the carbohydrate content was striking, with large amounts of fucose, mannose, galactose, and hexosamines found. No sialic acid was found, both by gas–liquid or colorimetric determinations. Moreover, no phosphate was found in the purified enzyme. The carbohydrate composition as determined by more traditional techniques (see Methods) agreed very well with the values from gas–liquid chromatography. By paper electrophoresis and enzymatic assay, galactose was found to be 83 μg/mg protein, and mannose 14 μg/mg protein. Fucose, determined by the cysteine sulfate method, was 167 μg/mg protein. Colorimetric hexo-

TABLE III

AMINO ACID COMPOSITION OF HUMAN INTESTINAL GLUCOAMYLASE

Amino acid and sugar analyses were performed as outlined in Methods. The hexosamine content was within 10% by amino acid analysis and gas liquid chromatography, and this correction was made to compare the amino acid and sugar analysis obtained by different methods.

$$V_p = \frac{\Sigma V_i W_i}{\Sigma W_i} = 0.684$$

Amino acid or sugar residue	*μg/mg protein*	*W_i (% by wt of residue*	*V_i (Spec. vol. of residue)*	*$V_i W_i$ (% by vol. of residue)*
Aspartic acid	83.1	7.24	0.59	4.27
Glutamic acid	95.1	8.29	0.66	5.47
Glycine	84.3	7.60	0.64	4.86
Alanine	29.2	2.54	0.74	1.88
Valine	40.1	3.50	0.86	3.01
Leucine	58.2	5.07	0.90	4.56
Isoleucine	28.9	2.52	0.90	2.21
Serine	52.7	4.59	0.63	2.89
Threonine	42.2	3.68	0.70	2.58
Methionine	4.4	0.38	0.75	0.28
Proline	40.6	3.54	0.76	2.69
Phenylalanine	39.7	3.46	0.77	2.66
Tyrosine	29.8	2.60	0.71	1.85
Histidine	21.9	1.91	0.67	1.29
Lysine	37.1	3.24	0.82	2.66
Arginine	72.5	6.32	0.70	4.42
Fucose	205	17.86	0.678	10.95
Xylose	3.2	0.28	0.613	0.17
Mannose	5.9	5.14	0.613	3.15
Galactose	51.9	4.52	0.613	2.77
Glucosamine	53.6	4.67	0.666	3.11
Galactosamine	16.6	1.45	0.666	0.966
Sialic acid	—	—	—	—

samine determination was 56 μg/mg protein. These carbohydrate residues amounted to 32–38% of the protein by weight in various determinations. The resulting partial specific volume of 0.684 differs considerably from that of ordinary proteins, and should be taken into consideration when molecular weight determinations are carried out. The glucoamylase had an apparent molecular weight of 312 000 by sodium dodecyl sulfate–acrylamide gel electrophoresis, and by equilibrium sedimentation, the value was 210 000. Thus, due to the large carbohydrate content, its apparent molecular weight on sodium dodecyl sulfate acrylamide electrophoresis was not accurate.

DISCUSSION

Partial purification of intestinal glucoamylase has been reported by a number of different groups in the past[1,7–9]. This report demonstrates the most highly purified glucoamylase to date. In fact, by polyacrylamide electrophoresis, the preparation seems almost homogeneous.

Using heat inactivation, Dahlqvist has earlier described four maltases: Ia (sucrase), Ib (isomaltase), II, and III. Human intestinal maltase, like the monkey gluco-

amylase[8], seems to be similar to the maltase II of Dahlqvist[28] or 1 and 2 of Semenza[31]. It is heat-stable at 55 °C (whereas sucrase is not) but not at 65 °C, it contains no sucrase activity, and it is eluted on DEAE-cellulose chromatography in the position of maltase II (ref. 32). Purified human glucoamylase contains isomaltase activity, as did maltase II and III when studied by Semenza *et al.*[31] and by Kerry and Messer[33]. Thus, it is unlikely that sucrase–isomaltase is the enzyme responsible for glucoamylase activity. Maltase II has been reported to account for 25–30% of total maltase activity in the intestine[33] so that glucoamylase is probably not a minor component of the brush border membrane.

Two facts in the literature are inconsistent with our results. Kerry and Messer[33] found by heat inactivation that maltase II contained little if any activity against maltotriose, and presumably against longer polymers. However, crude mucosal extracts were used in these experiments, and heat inactivation may not accurately reflect the contribution of the various maltases to oligosaccharide hydrolysis. Moreover, Eggermont and Hers[3] found that in sucrase-(maltase Ia)-deficient patients, amylase activity decreased by 65%. Eggermont postulated that the loss of glucoamylase activity might be associated with the change in tertiary structure observed on sucrose gradients.

The most striking finding concerning human glucoamylase was its high carbohydrate content. Many glycoproteins are relatively heat stable and are excreted outside the cell[34]. Intestinal glucoamylase fits these generalities, but differs from most glycoproteins in its high carbohydrate content. Most glycoproteins contain $< 30\%$ carbohydrate[34]. Recently, Cogoli *et al.*[12] have found that purified rabbit sucrase–isomaltase contained 15% carbohydrate. The relative proportion of sugars differed from human maltase in that fucose was only found as 0.84% of the purified enzyme. Membrane glycopeptides usually contain sialic acid[35], and mannose is usually absent from mucous glycoproteins[34]. Of all glycoproteins with $> 30\%$ carbohydrate, the only ones lacking sialic acid are the blood group substances. In fact, data from our laboratory confirm the identity of blood group antigens in purified human disaccharidases[36].

The kinetic data suggest that there may be only one catalytic site on intestinal glycoamylase. The affinity for starch and oligosaccharides differs only moderately. In fact, the enzyme might more properly be considered an oligosaccharidase. Finally, maltase and amylase activity are identically heat inactivated. Unlike glucoamylase, each of the other disaccharidases from intestine contains more than one binding site, (*e.g.* sucrase–isomaltase[5] and lactase–phlorizin hydrolase[10]) Cogoli and coworkers have recently separated two catalytically different subunits of the sucrase–isomaltase complex[37]. It is possible that glucoamylase, which has a molecular weight similar to the sucrase-isomaltase complex, might be composed of two identical subunits.

The high carbohydrate content and the presence of isoenzymes of identical molecular weight suggest that as the enzyme is solubilized, a piece of membrane or glycocalyx is removed along with the catalytic site. This postulate might explain why the disaccharidases have behaved similarly on column chromatography and electrophoresis, and why most preparations contain more than one protein band on electrophoresis[7]. However, the purified enzyme contained neither sialic acid nor phosphate, both important membrane components. Whether or not the disaccharidases form an important portion of the glycocalyx remains to be determined.

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REFERENCES

1 Alpers, D. H. and Solin, M. (1970) *Gastroenterology* 58, 833
2 Dahlqvist, A. and Thomson, D. L. (1963) *Biochem. J.* 89, 272
3 Eggermont, E. and Hers, H. G. (1969) *Eur. J. Biochem.* 9, 488
4 Takesue, Y. (1969) *J. Biochem. Tokyo* 65, 545
5 Kolinska, J. and Semenza, G. (1972) *Biochim. Biophys. Acta* 258, 506
6 Wallenfels, K. and Fischer, J. (1960) *Hoppe Seyler's Z. Physiol. Chem.* 321, 223
7 Schlegel-Haueter, S., Hore, P., Kerry, K. R. and Semenza, G. (1972) *Biochim. Biophys. Acta* 258, 506
8 Swaminathan, N. and Radhakrishnan, A. N. (1969) *Indian J. Biochem.* 6, 101
9 Seetharam, B., Swaminathan, N. and Radhakrishnan, A. N. (1970) *Biochem. J.* 117, 939
10 Kraml, J., Kolinska, J., Ellederova, D. and Hirsova, D. (1972) *Biochim. Biophys. Acta* 258, 520
11 Forstner, G. G. (1971) *Biochem. J.* 121, 781
12 Cogoli, A., Mosimann, H., Vock, C., Balthazar, A. K. and Semenza, G. (1972) *Eur. J. Biochem.* 30, 7
13 E.C. Bulletin (1966) *Electrophoresis and Countercurrent,* Vol. 11, No. 1, E. C. Apparatus Corp., Philadelphia, Pa.
14 Gibson, W. B., Brown, B. I. and Brown, D. H. (1971) *Biochemistry* 10, 4253
15 Dahlqvist, A. (1968) *Anal. Biochem.* 22, 99
16 Clamp, J. R., Bhatti, T. and Chambers, R. E. (1971) *Method. Biochem. Anal.* 19, 229
17 Kornfeld, R., Kelley, J., Baenziger, J. and Kornfeld, S. (1971) *J. Biol. Chem.* 246, 3259
18 Dische, Z. and Shettles, L. B. (1948) *J. Biol. Chem.* 175, 595
19 Boas, N. F. (1953) *J. Biol. Chem.* 204, 553
20 Warren, L. (1959) *J. Biol. Chem.* 234, 1971
21 Ames, B. N. and Dubin, D. T. (1960) *J. Biol. Chem.* 235, 769
22 Dunker, A. R. and Reuckert, R. R. (1969) *J. Biol. Chem.* 244, 5074
23 Riley, R. F. and Coleman, M. K. (1968) *J. Lab. Clin. Med.* 72, 714
24 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404
25 Yphantis, D. A. (1964) *Biochemistry* 3, 297
26 Takesue, Y. and Kashiwagi, T. (1969) *J. Biochem. Tokyo* 65, 421
27 Jos, J., Frezal, J., Rey, J., Lamy, M. and Wegmann, R. (1967) *Ann. Histochim.* 12, 53
28 Dahlqvist, A. (1962) *J. Clin. Invest.* 41, 463
29 Alpers, D. H. and Gerber, J. E. (1971) *J. Lab. Clin. Med.* 78, 265
30 Van Dyck, J. W. and Caldwell, M. L. (1956) *J. Am. Chem. Soc.* 78, 3345
31 Semenza, G., Auricchio, S. and Rubino, A. (1965) *Biochim. Biophys. Acta* 96, 487
32 Dahlqvist, A. and Telenius, V. (1969) *Biochem. J.* 111, 139
33 Messer, M. and Kerry, K. R. (1967) *Biochim. Biophys. Acta* 132, 432
34 Eylar, E. H. (1965) *J. Theor. Biol.* 10, 89
35 Glick, M. C., Comstock, C. and Warren, L. (1970) *Biochim. Biophys. Acta* 219, 290
36 Alpers, D. H. and Kelly, J. J. (1972) *J. Clin. Invest.* 51, 3a
37 Cogoli, A., Eberle, A., Sigrist, H., Joss, C., Robinson, E., Mosimann, H. and Semenza, G. (1973) *Eur. J. Biochem.*, 33, 40