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Kinetic Studies on Glucoamylase of Rabbit Small Intestine

By SUBRAMANYAM SIVAKAMI AND AMURTUR N. RADHAKRISHNAN
Wellcome Research Unit, Christian Medical College Hospital, Vellore-632004, Tamil Nadu, India

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The kinetic properties of a maltase–glucoamylase complex with a neutral pH optimum, purified to homogeneity from the brush borders of the rabbit small intestine, are described. It has a broad range of substrate specificity, hydrolysing di- and polysaccharides with α-1,4 and α-1,6 linkages. The K_m and $V_{max.}$ values of the enzyme for the various substrates were determined. Starch and maltose were its best substrates. The kinetics of hydrolysis of two synthetic linear maltosaccharides, namely maltotriose and maltopentaose, were studied. Mixed-substrate incubation studies revealed the presence of at least two interacting sites on the enzyme, and the data were further analysed by the use of a number of non-substrate inhibitors.

Glucoamylase (EC 3.2.1.3) is an exo-enzyme that splits glucose residues directly from starch. It has a broad range of substrate specificity, acting on disaccharides, oligosaccharides and polysaccharides with α-1,4 and α-1,6 linkages. The glucoamylase of the small intestine, which is of brush-border origin, has been purified from the monkey (Seetharam *et al.*, 1970), rat (Schlegel-Haueter *et al.*, 1972), human (Kelly & Alpers, 1973) and the rabbit (Sivakami & Radhakrishnan, 1973). An α-glucosidase of lysosomal origin with an acid pH optimum has been purified and characterized from a number of tissues. Thus the properties of the acid α-glucosidases from bovine spleen (Fujimori *et al.*, 1968), bovine liver (Bruni *et al.*, 1969, 1970), rat liver (Lejeune *et al.*, 1963), rabbit liver (Belenki & Rosenfeld, 1972), rabbit muscle (Palmer, 1971*a*,*b*) and human placenta (De Barsy *et al.*, 1972) have been studied in detail. The acid α-glucosidases in general possess more than one catalytic site, as shown by kinetic studies with the enzyme from rabbit muscle (Palmer, 1971*a*,*b*) and rat liver (Jeffrey *et al.*, 1970*a*,*b*). Among the intestinal disaccharides, the maltase–sucrase–isomaltase complex (Kolinska & Semenza, 1967; Swaminathan & Radhakrishnan, 1970) and the lactase–phlorrhizin hydrolase complex (Birkenmeier & Alpers, 1974; Ramaswamy & Radhakrishnan, 1973, 1975) have been shown to possess more than one catalytic site. However, the kinetic data on the human intestinal glucoamylase (Kelly & Alpers, 1973) indicate the presence of only one binding site.

A simple procedure based on the affinity of the enzyme for Sephadex G-200 for the isolation of rabbit intestinal glucoamylase in a homogeneous form was described previously (Sivakami & Radhakrishnan, 1973). Enzyme preparations with a specific activity ranging from 15 to 20 units with starch as substrate were used in the present work. In the present paper we give some of the kinetic data on the enzyme, which provide evidence for the presence of multiple substrate- and inhibitor-binding sites.

Materials and Methods

Chemicals

The following chemicals were bought commercially as indicated: starch (E. Merck A.-G., Darmstadt, Germany); maltose, glucose oxidase (type II, purified), horseradish peroxidase (type VI), *o*-dianisidine, Tris, isomaltose, palatinose, trehalose, D-cellobiose, lactose hydrate, bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.); turanose, glycogen, D-(+)-raffinose pentahydrate, α-D-(+)-melibiose monohydrate (Mann Research Laboratories, New York, N.Y., U.S.A.); pentaerythritol (J. and H. Berge, Plainfield, N.J., U.S.A.); maltotriose, maltopentaose (Koch–Light Laboratories, Colnbrook, Bucks., U.K.). Triton X-100 was a gift from Rohm and Haas Co., Philadelphia, Pa., U.S.A. All other chemicals were of analytical grade.

Assay of enzyme activities

Maltase and glucoamylase activities were determined by the measurement of the glucose formed by the glucose oxidase–peroxidase procedure of Dahlqvist (1964) as described earlier (Seetharam *et al.*, 1970) in a total volume of 0.5ml, with potassium phosphate buffer, pH7.0 (0.1M final concentration). For assays with maltotriose and maltopentaose as substrates the total volume of the assay system was scaled down to 0.05ml.

Enzyme units

One unit of glucoamylase activity was defined as the amount of enzyme required to produce 1 μmol

of glucose/min at 37°C with starch as substrate. One unit of maltase activity was the amount of enzyme required to hydrolyse 1 μmol of substrate/min at 37°C. Specific activity was expressed as units per mg of protein. Substrate concentrations have been expressed as mol of glucosidic bonds/litre (=g of substrate/litre per mol of glucose anhydride) (Van Dyk & Caldwell, 1956).

Protein estimation

Protein was determined by the procedure of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Results

Substrate specificity

The enzyme catalyses the hydrolysis of α-1,4 as well as α-1,6 linkages. It hydrolyses the polysaccharides starch and glycogen and, among the disaccharides, maltose, isomaltose, turanose and palatinose. It is completely free of sucrase activity. Table 1 gives the K_m and $V_{max.}$ values for the hydrolysis of the various substrates; starch and maltose are the best substrates, especially if the ratios $V_{max.}/K_m$ are considered.

Effect of pH

The pH/activity determinations were performed with different buffers under assay conditions described above: citrate/phosphate buffer (pH4.0–7.0) and sodium phosphate buffer (pH7.0–8.0); sodium acetate buffer (pH4.0–5.6) and sodium phosphate buffer (pH5.4–8.0). Similar activity profiles were obtained in both the buffer systems with each of the substrates (Fig. 1). The hydrolysis of maltose is maximum at pH7.0, with a shoulder on the graph at pH6.2. The hydrolysis of starch is maximum at pH7.0, though an almost constant rate of hydrolysis occurs from pH6.2 to 6.8.

K_m and $V_{max.}$ values for maltose and starch were determined at various pH values between 3.5 and 8.0 by using citrate–phosphate buffer (Fig. 2). The plot of pK_m of starch with pH shows a break at pH4.3, whereas a plot of $V_{max.}$ with pH shows a break at pH5.7. The plot of both pK_m and $V_{max.}$ of maltose with pH shows a single break at 5.7. The group with the pK of 5.7–6.0 may possibly be imidazole.

The effect of pH on activity in the range pH3.0–5.0 has been checked with sodium acetate as well as citrate–phosphate buffers. No significant differences in activity were noticed, indicating the absence of organic acid stimulation of the type observed for the monkey intestinal phlorrhizin hydrolase (Ramaswamy & Radhakrishnan, 1973).

Neither maltotriose nor maltopentaose showed a pH optimum for hydrolysis in the pH range (4.0–8.0) investigated. Possibly no ionizable groups are involved in their hydrolysis.

Action of glucoamylase on maltosaccharides

Two of the maltosaccharides, namely maltotriose and maltopentaose, were tested as substrates for the

Table 1. *Substrate specificity: K_m and $V_{max.}$ values of the various substrates*

$V_{max.}$ values are given as μmol of glucose formed/min per mg of protein. For further details, see the text.

Substrate	K_m (mM)	$V_{max.}$
Maltose	1.43	25
Starch	2.0	16
Glycogen	11.3	5
Turanose	20.7	1.5
Palatinose	16.66	0.83
Isomaltose	50.0	1.66
Maltotriose	25.0	15.6
Maltopentaose	20.0	25.0

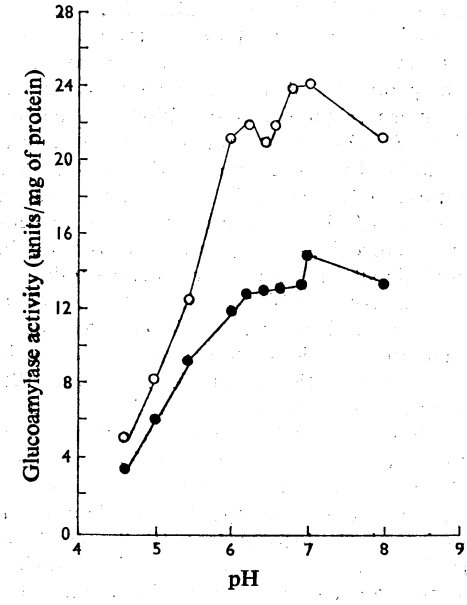

Fig. 1. *Effect of pH on the activity of glucoamylase with maltose* (○) *and starch* (●) *as substrate*

Standard assay conditions were used. Buffers used were citrate/phosphate (pH4.0–7.0), sodium phosphate (pH7.0–8.0) and sodium acetate (pH4.0–5.6), sodium phosphate (pH5.4–8.0).

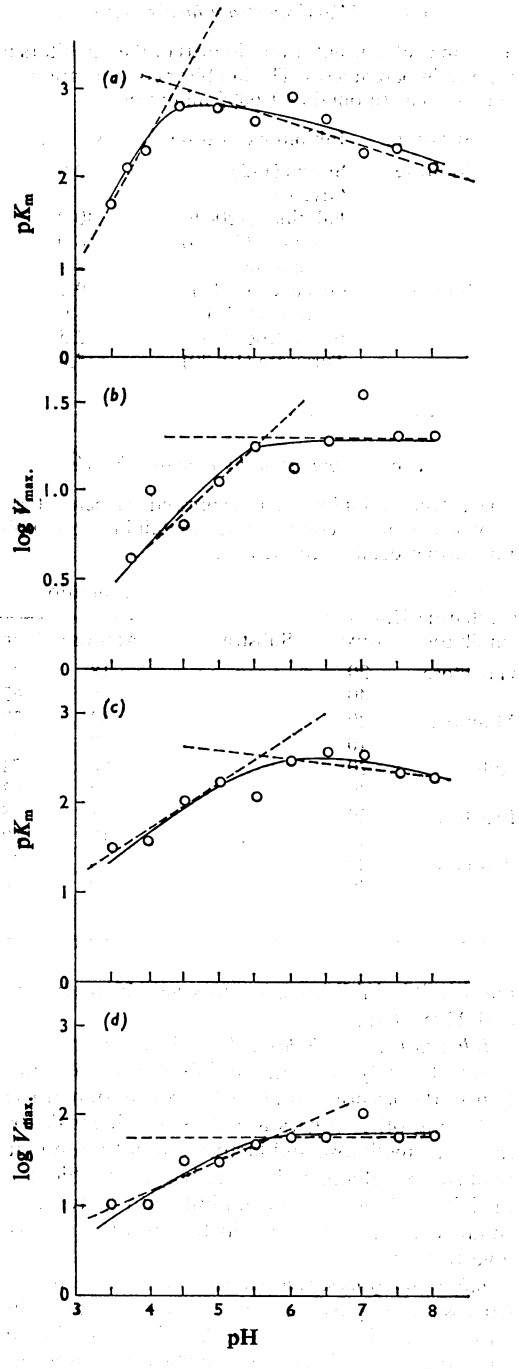

Fig. 2. *Plot of K_m and $V_{max.}$ versus pH for starch (a, b) and maltose (c, d)*

Standard assay conditions were used (see the Materials and Methods section), with maltose or starch as substrate, and citrate/phosphate buffer. $V_{max.}$ is expressed as units of enzyme activity per mg of protein.

enzyme. Both the linear maltosaccharides were hydrolysed by the enzyme at very similar rates. The reaction velocities are expressed as μmol of α-1,4 linkages hydrolysed/min per mg of protein. The rate of glucose release is taken as a measure of the rate of α-1,4-linkage hydrolysis. Pronounced substrate inhibition was observed with maltotriose at concentrations exceeding 4mM and with maltopentaose at concentrations exceeding 2mM (Fig. 3). With maltose no inhibition was observed up to a concentration of 40mM.

By plotting the reciprocal of velocity against inhibitory concentration of substrate, the K_i values for the substrate itself acting as inhibitor for the enzyme were determined (Fig. 4). The K_i values were 5mM (maltotriose) and 25mM (maltopentaose).

Mixed-substrate incubation studies

Mixed-substrate incubation studies were performed with various concentrations of maltose and starch, by using fixed concentrations of each of the other substrates as inhibitor (Table 2). The hydrolysis of starch is inhibited competitively by all the other substrates. In the hydrolysis of maltose, turanose and palatinose compete with maltose, whereas glycogen and isomaltose do not inhibit maltose hydrolysis at all. These results show that there may be more than one catalytic site on the enzyme.

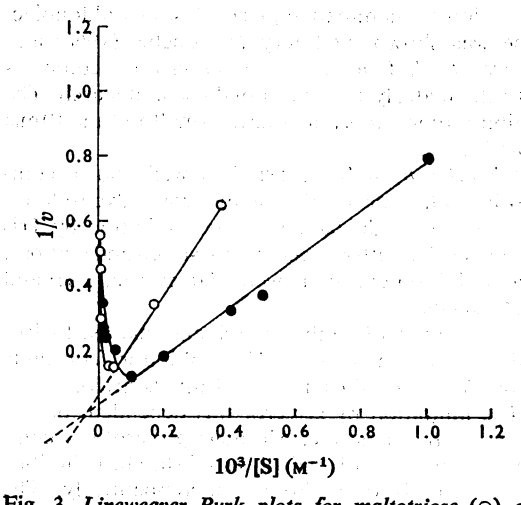

Fig. 3. *Lineweaver–Burk plots for maltotriose (○) and maltopentaose (●), showing substrate inhibition*

The reaction velocities are expressed as μmol of α-1,4 linkages hydrolysed/min per mg of protein. For the conditions of assay, see the Materials and Methods section.

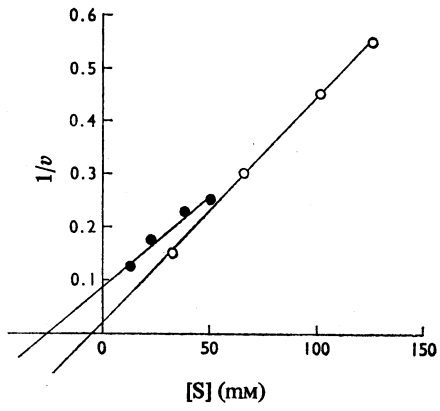

Fig. 4. *Dixon plots of substrate inhibition by maltotriose* (○) *and maltopentaose* (●)

The reciprocals of the initial velocities are plotted against substrate concentration at inhibitory concentrations of each substrate. The reaction velocities are expressed as μmol of α-1,4 linkages hydrolysed/min per mg of protein. For the conditions of assay see the Materials and Methods section.

Effect of other inhibitors

To elucidate further the number and nature of the catalytic sites involved, a number of substances were tried as inhibitors for the hydrolysis of starch and maltose. The extent and type of inhibition further support the presence of multiple sites on the enzyme.

Inhibition by glucono-δ-lactone. The competitive inhibition of mammalian glycosidases by aldonolactone was shown by Levvy & Conchie (1966) and Levvy *et al.* (1964). Glucono-δ-lactone competes with the hydrolysis of both maltose and starch. The inhibition constants were 13.5 mM (maltose) and 10 mM (starch).

Inhibition by polyols. Tris is a well known competitive inhibitor of the mammalian glycosidases. Similarly with glucoamylase, the inhibition by Tris is competitive with respect to both starch and maltose. The inhibition constants were 100 mM (maltose) and 33.4 mM (starch).

Pentaerythritol inhibits starch and maltose hydrolysis non-competitively. The inhibitor constants were 150 mM (maltose) and 250 mM (starch).

The inhibition by glycerol was dependent on the nature of the substrate. Although glycerol is a competitive inhibitor of hydrolysis of starch, it inhibits the hydrolysis of maltose non-competitively. The inhibition constants were 150 mM (maltose) and 174 mM (starch).

Inhibition by sucrose. As mentioned above, the enzyme is devoid of sucrase activity, but sucrose competitively inhibits the hydrolysis of starch and maltose. The inhibitor constants were 50 mM (maltose) and 20 mM (starch).

Inhibition by trehalose. Trehalose competes with starch hydrolysis, with an inhibition constant of 17 mM. It does not inhibit the hydrolysis of maltose.

Among other sugars tested, Schardinger dextrins, raffinose, melibiose and lactose did not inhibit either maltase or glucoamylase activity; cellobiose and melizitose, however, did inhibit both activities to a small extent, but the kinetics were not further investigated.

Inhibition by monosaccharides. Galactose, mannose, fructose and xylose inhibit both maltase and glucoamylase activities (Table 3). Arabinose inhibits only glucoamylase activity. The kinetics of inhibition by mannose and xylose were investigated; both the monosaccharides are uncompetitive inhibitors of maltase and glucoamylase activities.

The inhibition patterns were analysed by replotting the slopes and intercepts of the reciprocal plots against inhibitor concentration, as suggested by

Table 2. *Mixed-substrate incubation studies*

The range of concentration (in mM) of the inhibitors used is given in parentheses. The inhibitor concentrations were chosen so as to obtain 40–60% inhibition.

Substrate	Inhibitor substrate	K_i (mM)
Maltose	Starch (1–2)	3.0
	Glycogen	—
	Palatinose (20–40)	40.0
	Turanose (20–40)	8.0
	Isomaltose	—
Starch	Glycogen (5–10)	9.2
	Palatinose (4–10)	4.0
	Isomaltose (5–10)	2.5
	Turanose (10–20)	5.0

Table 3. *Inhibition by monosaccharides*

The extent of inhibition is expressed as percentage of enzyme activity in control tubes containing no inhibitor. For further details, see the text.

Monosaccharide inhibitor	[Inhibitor] (mM)	Substrate ...	Inhibition (%) Maltose	Starch
Galactose	20		18	26
	40		65	62
Mannose	20		32	22
	40		68	50
Xylose	20		50	74
	40		59	83
Fructose	20		8	5
	40		12	10
Arabinose	20		0	21
	40		0	26

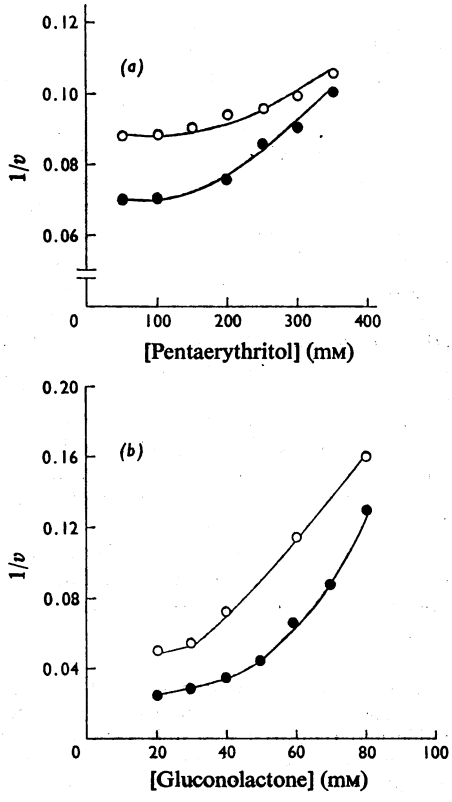

Fig. 5. *Dixon plots for maltose* (●) *and starch* (○), *with pentaerythritol* (*a*) *and glucono-δ-lactone* (*b*) *as inhibitors*

Reaction velocities are expressed as units of enzyme activity/mg of protein. For further details, see the text.

Cleland (1970). With pentaerythritol, which is a non-competitive inhibitor, when the values of intercept are plotted against inhibitor concentration, a parabolic curve is obtained with both substrates. The parabolae thus obtained have been confirmed by Dixon plots of the reciprocal of velocity directly against inhibitor concentration (Cleland, 1970) (Fig. 5*a*). The parabolic pattern, according to Cleland (1970), is caused by the combination of at least two molecules of inhibitor with the enzyme. Glucono-δ-lactone, which is a competitive inhibitor of both substrates, also gives a parabola in Dixon plots (Fig. 5*b*). But trehalose and Tris give straight lines in the plots of $1/v$ versus inhibitor concentration with starch as substrate. Therefore the mutual inhibition between the two competitive inhibitors trehalose and Tris was checked by Dixon plots in the presence of both the inhibitors. The rate equations for mutual interaction between fully competitive and fully non-competitive inhibitors have been given by

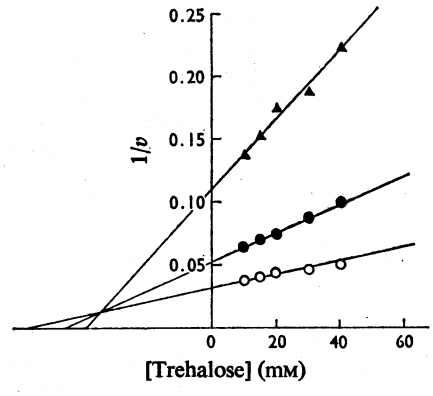

Fig. 6. *Dixon plots showing lack of mutual competition between two competitive inhibitors, Tris and trehalose*

○, No Tris; ●, 25mM-Tris; ▲, 75mM-Tris. Standard assay conditions were used with starch as substrate. Reaction velocities are expressed as units of enzyme activity/mg of protein. For further details, see the text.

Webb (1963), Yonetani & Theorell (1964) and Semenza & von Balthazar (1974). In our experiments, we found that there was no mutual competition between the two competitive inhibitors Tris and trehalose, since the two straight lines cross at a point to the left of the ordinate (Fig. 6). This leads us to envisage a situation where the starch site is big enough to accommodate both Tris and trehalose independently and simultaneously. Probably the starch-binding site is further differentiated into subsites where smaller molecules such as trehalose and Tris bind.

Discussion

The pH/activity profile shows that two states of ionization of the enzyme, namely those at pH6.2 and 7.0, hydrolyse maltose efficiently. However, pH7.0 is the optimum for maltose, the activity at that pH being highest. The hydrolysis of starch is also maximum at pH7.0, though the rate of hydrolysis is very similar in the pH range 6.2–6.8.

The variation of K_m and $V_{max.}$ with pH shows that a group with a pK of about 5.7 is involved in the binding of maltose, and in the hydrolysis of both maltose and starch. But an acidic group with a pK of about 4.3 is responsible for the binding of starch. The difference in the mode of binding may be due to the large differences in size and structure between maltose and starch.

The hydrolysis of the maltosaccharides seems to be different from the other substrates in two respects. First, there is no distinct optimum pH for hydrolysis, and, secondly, the presence of substrate inhibition, which has not been observed with maltose and starch.

The results of mixed-substrate incubation studies indicate that there is one site at which polysaccharides such as starch and glycogen and their inhibitory substrates are hydrolysed. In addition there is probably a second site, structurally related and in close proximity to the first and which is responsible for the hydrolysis of maltose. This would explain the lack of inhibition with maltose/glycogen, and maltose/isomaltose. The competition between maltose and starch can then be related to the close proximity and consequent interaction between the sites. Alternatively, owing to the structural differences between starch and glycogen, the mechanism of hydrolysis of the two polysaccharides by the enzyme may be different. By assuming that the hydrolysis of starch takes place in two consecutive and very fast steps involving both the sites, competition between maltose and starch could result by an intermediate oligosaccharide arising from the interaction of starch with one site and competing with maltose at the second site. With the available data we are unable to distinguish between these two possibilities.

The results of the inhibition studies using non-substrate inhibitors are rather more clear-cut. In particular, it was found that trehalose competes with starch hydrolysis but has no effect on maltose hydrolysis. The lack of mutual inhibition between Tris and trehalose shows that the catalytic site for the hydrolysis of starch is probably large enough to have more than one centre for binding. Within the catalytic site maltose can be expected to bind at one centre or subsite and compounds such as trehalose, isomaltose and arabinose, which do not inhibit maltase activity, bind at another centre. The binding and hydrolysis of starch probably involves the entire catalytic site, resulting in a competition between maltose and starch. This situation is similar to that visualized from the results of the mixed-substrate incubation studies, because the two substrate-binding centres could be either separate sites or subsites within a larger catalytic site. This may be relevant to the observation that a number of compounds inhibit starch hydrolysis but do not inhibit maltose hydrolysis. The reverse situation has not been observed with any of the inhibitors.

The parabolic Dixon plot for a non-competitive inhibitor such as pentaerythritol indicates that, apart from the catalytic site, there is more than one inhibitor-binding site. Taken together the data indicate the presence of more than one substrate- and inhibitor-binding site.

It is noteworthy that the glucoamylase of the rabbit small intestine differs from human intestinal enzyme (Kelly & Alpers, 1973). There is probably only one catalytic site in the human enzyme, and it is also most active on oligosaccharides containing five to nine glucose residues. An increase in the number of glucose residues above nine, or a decrease below five, increases the K_m value. The rabbit intestinal enzyme, on the contrary, is most active with starch and maltose, and oligosaccharides with three or five glucose residues have a significantly higher K_m when compared with maltose or starch. But the $V_{max.}$ values do not alter very much with the size of the polymer with the rabbit enzyme. The human intestinal glucoamylase, however, showed a steady decrease in $V_{max.}$ with increase in polymer length.

The intestinal glucoamylase is associated with maltase activity in all the animal species studied, namely the rat (Schlegel-Haueter *et al.*, 1972), rabbit (Sivakami & Radhakrishnan, 1973, 1975), monkey (Seetharam *et al.*, 1970) and the human (Kelly & Alpers, 1973). The maltase and glucoamylase partial activities show identical behaviour on heat inactivation and during ion-exchange column chromatography, and have not been separated so far in any of the animal species studied. However, the maltase and glucoamylase activities of the acid α-glucosidase of rabbit liver have been separated by electrophoresis in the presence of urea (Belenki & Rosenfeld, 1972). Probably a separation of the subunits of these complexes would reveal more information about the catalytic and binding sites.

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