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MICROSOMAL GLUCOSIDASES ACTING ON THE SACCHARIDE MOIETY OF THE GLUCOSE-CONTAINING DOLICHYL DIPHOSPHATE OLIGOSACCHARIDE

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SUMMARY. The microsomal glucosidases which act on the oligosaccharide that contains 2 N-acetylglucosamine, 9 mannose and 1-3 glucose residues have been studied. Two fractions were separated by differential solubility in detergent and phosphate solutions or by gel filtration. One of the fractions removed glucose from the oligosaccharide containing three glucose residues, and another fraction acted on the compounds containing one and two glucose residues. Both fractions were free of mannosidase. Some properties of the enzymes are described.

A dolichyl diphosphate oligosaccharide containing glucose is known to be an intermediate in the glycosylation of proteins (1, 2). According to present ideas the oligosaccharide, (G-oligosaccharide), which contains 9 mannoses, 2 acetylglucosamines and 1-3 glucoses is transferred to a polypeptide and is then processed by removal of glucose followed by several other changes which lead to the formation of the various asparagine-linked oligosaccharides.

Previous work (3) showed that glucosidases found in the microsomal fraction of liver extracts act on the G-oligosaccharide free, combined to dolichyl diphosphate or as the glycopeptide.

Studies on the glucosidases have now been continued, facilitated by the fact that three oligosaccharides differing in glucose content can be separated

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The glucose containing oligosaccharides with three, two and one glucose residues are referred to as G_3-, G_2- and G_1-oligosaccharide.

Glucosidase 3 refers to the preparation acting on G_3 and glucosidase 2 to that active on G_1 and G_2.

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by paper chromatography. Two different enzymes have been detected up to now, one acting on G_3 oligosaccharide and another which acts on the G_2 and G_1 compounds.

MATERIALS AND METHODS

Rat liver microsomes, dolichyl diphosphate oligosaccharides and free oligosaccharides were prepared as described (4). Separation of the oligosaccharides was effected by paper chromatography with 1-propanol:nitromethane:water (5:2:4) for 7 days. Dolichyl diphosphate G-oligosaccharide labelled in mannose and glucose was obtained from oviduct slices incubated with [^{14}C] mannose (5, 6).

Estimation of the glucosidases. The assay was carried out as described previously (3) with some changes. The standard reaction mixture contained: G-oligosaccharide (1000 cpm), 40 mM phosphate buffer (pH 7), 0.5% Nonidet P-40 and enzyme (50-400 µg protein). Total volume: 50 µl. Plastic tubes of 0.5 ml (Beckman microfuge B) were used. After 10 min at 37ºC, 50 µl of methanol was added and the tubes were centrifuged for 5 min at 11,000 rpm. The supernatant fluid was spotted on 2.5 x 14 cm papers and chromatographed for 3 h with 2-butanol: acetic acid:water (29:4:9). The radioactive zones were located with a scanner and the corresponding pieces of paper were counted with a toluene-based scintillation fluid, in a scintillation counter.

One unit of glucosidase 2 was defined as the amount that liberates 10% of the glucose per min from G_2-oligosaccharide. Since glucosidase 3 liberates only one of the three glucoses of G_3-oligosaccharide, one unit was taken as 3.3% of the glucose set free.

RESULTS

Extraction of the enzymes. Liver microsomes were suspended in 80 mM trimethylamine-acetate buffer of pH 7 (final concentration 30 mg protein per ml) in a Dounce A homogenizer (Kontes Glass Co.). After 20 min at 0ºC the mixture was centrifuged at 100,000 x g for 90 min. The supernatant fluid was removed and an equal volume of fresh buffer containing 2% Nonidet P-40 was added to the pellet. After 20 min the mixture was centrifuged as above. The procedure was repeated four times more. After the fifth centrifugation the pellet was suspended in 160 mM phosphate buffer containing 2% Nonidet P-40. After 20 min at 0ºC the mixture was centrifuged as before. The extraction was repeated once more and the pellet was suspended in a volume of the same buffer equal to the original supernatant fluid. The results of estimating glucosidase activity on the G_3 and

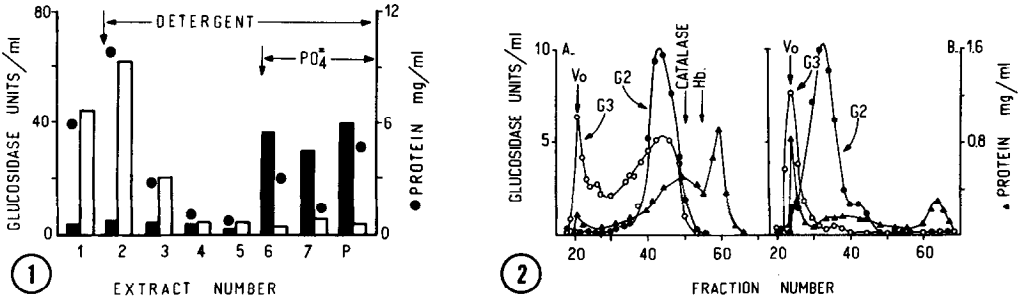


Fig. 1. Extraction of the enzymes. The glucosidases activity of the extracts obtained as indicated in text were estimated on G_3 (full bars) and G_2 (empty bars) oligosaccharides as described in Materials and Methods.

Fig. 2. A. Gel filtration of the glucosidases. Extract Nº 2 (0.5 ml) and Nº 6 (0.5 ml) prepared as indicated in Fig. 1 were mixed and poured into a column of Bio-Gel A 15 m (1.5 x 45 cm). Elution was carried out with 5 mM phosphate of pH 7, 5 mM EDTA, 5 mM 2-mercaptoethanol and 0.1% Nonidet P-40. Fractions of 1 ml were collected. Glucosidases were determined and expressed in units as described in Materials and Methods. B. Gel filtration of the glucosidases with Bio-Gel A 1.5 m. Procedure as in A but with half the amount of extract and 0.5 ml fractions. (O) Glucosidase activity on G_3-oligosaccharide; (●) glucosidase activity on G_2-oligosaccharide; (▲) protein.

G_2 oligosaccharides are shown in Fig. 1. It may be observed that the first solvent extracts mainly the enzyme acting on the G_2 - oligosaccharide (glucosidase 2) while the phosphate containing solvent leads to the extraction of a glucosidase acting on the G_3-oligosaccharide (glucosidase 3). The ratio of activities G_3/G_2 varies from 0.08 in extract 2 to 15.3 in extract 6.

Preparation of the glucosidases. Separation of the enzymes could also be obtained by gel filtration. Use was made of the fact that glucosidase 3 aggregates in low phosphate solution. Under these conditions it can be separated from glucosidase 2 whereas in high phosphate where glucosidase 3 is completely soluble no separation is obtained.

The microsomal enzyme extracted with phosphate and detergent was passed through an agarose gel filtration column having a fractionation range of 40,000 to 15 million daltons. The results are shown in Fig. 2A. Glucosidase 3 was found in the void volume and continued to come out in many fractions including those

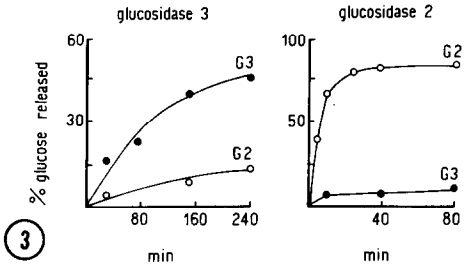
containing glucosidase 2. It seems that at that salt concentration the enzyme had many different states of aggregation. However the first fraction was nearly free of glucosidase 2. A pool of fractions 21-22 was used as purified glucosidase 3.

For the preparation of G_2-glucosidase the procedure was as described before but with a column of agarose of fractionation range of 10,000 to 1.5 million daltons. As shown in Fig. 2B the two enzymes appeared as fairly sharp peaks. Some of the fractions were rich in G_2 and had hardly any G_3 activity. These fractions (32-34) were pooled and used as purified glucosidase 2.

Some properties of enzymes. Both glucosidases were found to be fairly stable. No loss of activity occurred on incubation with Nonidet P-40 (0.5%) for 4 h at 37ºC. With deoxycholate (0.25%) the activity decreased to 50% in 15 min. Frozen samples were active for weeks.

The pH optimum for both glucosidases was found to be 6.5 - 7. Tris (hydroxymethyl)-aminomethane inhibited completely both glucosidases at 0.1 M concentration. Total inhibition was produced by 1 mM p-chloromercuriphenylsulfonate.

The action of the glucosidases on the G_3 and G_2 oligosaccharides. In order to confirm the specificity of the enzymes separated by gel permeation they were allowed to act for different lengths of time on the G_3 and G_2 oligosaccharides. As shown in Fig. 3A the action of glucosidase 3 on the G_3 oligosaccharide leads to a rapid liberation of glucose which reaches about 50% of the total. This is more than the theoretical maximum since if all three glucoses are equally labelled the maximum would be 33%. It seems that in some G_3-oligosaccharide samples the external glucose is more highly labelled than the internal. The action on G_2 was much slower. When glucosidase 2 was incubated with G_2-oligosaccharide glucose was rapidly liberated (Fig. 3B) but there was little release when glucosidase 2 was incubated with G_3-oligosaccharide. Similar results were obtained with glucosidase 3 and 2 acting on a G_3 and G_2-oligosaccharides



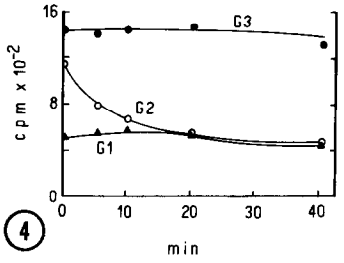


Fig. 3. The action of glucosidases 3 and 2 on G-oligosaccharides 3 and 2. The procedure was as described in Materials and Methods for the estimation of glucosidases. A. Action of glucosidase 3 (fractions 21-22, Fig. 2A). B. Action of glucosidase 2 (fractions 32-34, Fig. 2B). (●) G_3-oligosaccharide; (○) G_2-oligosaccharide.

Fig. 4. Action of glucosidase 2 on the G-oligosaccharides. Samples containing the three saccharides were incubated with glucosidase 2 (fractions 32-34, Fig. 2B) for different lengths of time at 37ºC. The reaction mixture and separation of the products were as described in text for glucosidase estimation. The G-oligosaccharides were eluted from the paper and chromatographed with 1-propanol-nitromethane-water (5:2:4) for 7 days. The radioactivity in each oligosaccharide was counted by scintillation. (●) G_3-oligosaccharide; (○) G_2-oligosaccharide; (▲) G_1-oligosaccharide.

respectively, labelled in both glucose and mannose. The hexose liberated was checked to be glucose by paper chromatography in butanol:pyridine:water (6:4:3). No mannose was detectable showing that glucosidase preparation 3 and 2 were free from mannosidase.

The action of glucosidase 2 on mixed G-oligosaccharides. The action of glucosidase 2 on the oligosaccharides was studied in an experiment similar to that shown in Fig. 3B but instead of using pure G_2-oligosaccharide a mixture of glucose labelled G_1, G_2 and G_3 was used. When a relatively high concentration of glucosidase 2 (30 μg protein) was used the G_3 oligosaccharide remained unaffected but G_2 and G_1 disappeared completely in 5 min. With a lower amount of enzyme the changes in the oligosaccharides were those shown in Fig. 4: G_3 did not change, there was a rapid decrease of G_2 oligosaccharide while G_1 remained constant. This is understandable if G_1 is formed from G_2 at the same rate as G_1 disappears. Under these conditions, since the glucosidase 2 was very diluted,

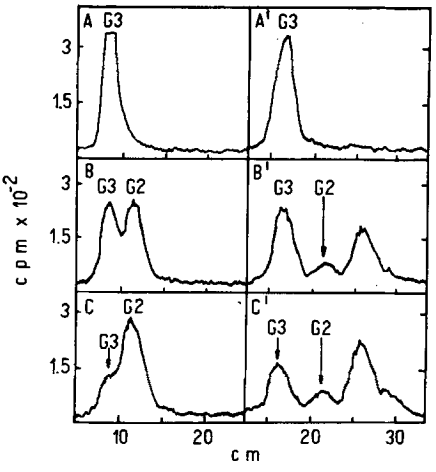


Fig. 5. Action of glucosidases 3 and 2 on the G-oligosaccharide labelled in glucose and mannose. A, B and C: G_3-oligosaccharide treated for 0, 2 h and 4 h, respectively, with glucosidase 3 (fractions 21-22, Fig. 2A) (as in Fig. 4). The radioactive substances were eluted from the paper treated with glucosidase 2 (fractions 32-34, Fig. 2B) (as in Fig. 4) for 60 min and rechromatographed. Results in A', B' and C'. In order to obtain a better separation the second chromatographic run was longer.

the G_2 and G_1-oligosaccharides were not completely degraded, likely due to partial inactivation of the enzyme. In other experiments it was checked that G_1 disappears when it is the only substrate.

The action of the glucosidases on G-oligosaccharides labelled in glucose and mannose. Experiments were carried out with a G-oligosaccharide labelled on glucose and mannose in order to be able to detect any liberation of mannose and determine the mobility of the glucose-free compound. An experiment of this type is shown in Fig. 5. The result of incubating G_3-oligosaccharide (glucose and mannose labelled) with glucosidase 3 is shown in Fig. 5 A, B, C. A new peak corresponding to G_2-oligosaccharide is formed progressively while the peak of G_3- decreases. The results confirm the fact that glucosidase 3 preparation was nearly inactive on the G_2-oligosaccharide. Paper chromatography of the free sugar liberated showed that it was only glucose.

The oligosaccharides of Fig. 5 A, B and C were eluted from the paper and treated with glucosidase 2. The results are shown in Fig. 5 A', B', C'. No ac-

tion was detectable on the G_3-oligosaccharide (A and A') whereas the peak of G_2-oligosaccharide disappeared and was transformed into a compound with a faster mobility than the G_2-oligosaccharide. This compound was presumably free glucose since we know glucosidase 2 removes the two inner glucoses. Therefore the fastest peak would have the composition of $Man_9\text{-}GlcNAc_2$.

DISCUSSION

The transfer of G-oligosaccharide to protein is followed by a rapid removal of the glucose residues (7, 8). The glucosidases involved in this process should be specific for protein linked G-oligosaccharide but since this is not an available substrate studies have been carried out with free G-oligosaccharide. This substrate can be easily obtained labelled and can be separated in the 1, 2 and 3 glucose-containing forms. The lipid-bound form and the glycopeptide have been found to be acted upon by microsomal glucosidases but are not so convenient as substrates.

Using the free oligosaccharide as substrate it has been possible to prove that at least two enzymes are involved in the removal of glucose, both seem to be membrane-bound and require a detergent for solubilization. It remains to be established whether the removal of the two internal glucoses is brought about by one or two enzymes. Another point which requires further work consists in assigning unequivocally the anomeric configuration of the glucosyl residues, these have been considered to be α by some workers (3, 9) and β by others (10). The product which remains after removing all the glucoses has a chromatographic mobility similar to the largest oligosaccharide which becomes labelled by incubation of GDP-[^{14}C]Man with liver microsomes. This shows that the addition of mannoses ceases when the oligosaccharide reaches the exact size (with 9 mannoses) and therefore that the mannosyl transferases are very specific.

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