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PURIFICATION AND CHARACTERIZATION OF α-GLUCOSIDASE FROM *MUCOR ROUXII*

A. FLORES-CARREÓN AND J. RUÍZ-HERRERA

Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México 17, D.F. (México)

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SUMMARY

1. α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) from *Mucor rouxii* was purified by treatment which included heat shock, precipitation with protamine sulfate, $(NH_4)_2SO_4$ and ethanol, and chromatography on DEAE-Sephadex and Sephadex G-200.

2. Purity of the enzyme was assessed by gel chromatography and polyacrylamide gel electrophoresis. Only one band of protein was observed.

3. The enzyme appeared to be a glycoprotein containing 40% carbohydrate. Glucose and, to a lesser extent, galactose and fucose were identified in acid hydrolysates of the enzyme. The enzyme behaved as a cation at acid pH but not as an anion at alkaline pH. It is suggested that possibly carboxylic groups from aspartate and/or glutamate are blocked.

4. The optimum pH with phosphate buffer was found to be 3.5 and the optimum temperature 50–60°. The enzyme was highly specific. It failed to hydrolyse several disaccharides and synthetic α-glucosides. It hydrolysed maltose and starch only. The sole product formed from maltose was glucose. Glucose inhibited enzymatic activity, whereas glucosamine had no effect.

INTRODUCTION

Only a few reports have appeared on the purification of α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) from filamentous fungi[1,2]. Investigations of maltose breakdown by bacteria and yeasts are more numerous. Maltose utilization in *Escherichia coli* involves the function of an operon which is apparently under positive control[3] and gives information for the synthesis of a transglycosidase, a phosphorylase and a permease. Transglycosidase has been purified and characterized by WIESMEYER AND COHN[4,5]. α-Glucosidase from *Clostridium acetobutylicum* and *Klebsiella aerogenes* have also been studied[6,7]. In *Candida stellatoidea* maltose is hydrolysed by an inducible α-glucosidase[8]. The enzyme from *Saccharomyces cerevisiae* appears to be constitutive[9], whereas in *S. carlsbergensis* the enzyme is induced by maltose[10,11].

We have previously reported that *Mucor rouxii* possesses an inducible α-glucosidase[12]. The basal and inducible enzymes were partially purified and on comparison their characteristics were found to be essentially the same. Most of the enzyme is cell wall-bound and its kinetic behavior is similar to that of the soluble enzyme[13]. In this paper we describe the characterization of a thoroughly purified preparation of the enzyme.

METHODS

The strain of *M. rouxii* and complex media (YPG and YPM) have been previously described[12]. α-Glucosidase was measured by a modification of the method also previously described[12]. Incubation mixtures of 1 ml containing 800 μg of maltose, 50 μmoles of potassium phosphate, pH 4.5, and enzyme were incubated at 50° for variable periods of time, normally for 30 min. At the end of the incubation period, 0.5 ml of special glucostat (Worthington) were added. This reagent increased the pH to about 7.0. Incubation was continued for another 5 min at 20°. Under the conditions used the increase in pH and the decrease in temperature slowed down α-glucosidase activity to negligible values. A drop of 4 M HCl was added. Absorbance was measured at 400 nm in a Maroc V (Jobin et Ivon) spectrophotometer with the aid of micro cells. Glucose was calculated from the values obtained using simultaneously run controls. A control containing maltose but no enzyme was also simultaneously incubated. Color did not usually develop in this control. Activity measured by this technique was strictly dependent on the amount of enzyme tested and was linear with time for about 30 min. Activity is expressed in units, one unit being the amount of enzyme which hydrolysed 1 μM of maltose per min.

The total amounts of sugars were measured with anthrone[14]. Identification of sugars was carried out by paper chromatography using a solvent mixture of butanol–pyridine–water (6:4:3, by vol.). Spots were developed with aniline phtalate or detected by their radioactivity. When necessary, samples were hydrolysed with 1 M H_2SO_4 for 10 h at 100° and deionized using a mixed-bed Dowex resin.

Protein was measured as described by LOWRY *et al.*[15]. Spectra of the enzyme were measured with the Maroc V spectrophotometer.

Analytical acrylamide gel electrophoresis was carried out using the following systems: Tris–glycine as described by DAVIS[16]; ethanolamine–triethanolamine as described by BRUENING *et al.*[17]; the acetic acid–glycine system of NAGAI *et al.*[18]; and a slight modification of the latter system, adjusting the pH of the reservoir buffer to 4.25, the pH of the spacer gel to 5.0, and the pH of the running gel to 5.5. All electrophoretic experiments were carried out at 5°. Protein bands were stained with amido black, carbohydrate with Schiff's reagent (GOMORI[19]), and α-glucosidase as follows: The whole gel was immersed in a solution containing 0.8 mg phenazine methosulfate, 2 μmoles of *p*-nitro-blue-tetrazolium, 3 μmoles of maltose, 132 units of glucose oxidase and 0.05 M potassium phosphate, pH 4.5, in a final volume of 10 ml. Nitrogen was flushed through the system, the tubes were stoppered and incubated at 37° until a violet precipitate was noticed on the gel.

The radioactivity in paper strips was measured using a Phillips PW 4035 Geiger counter whose absolute efficiency for ^{14}C is 2.5%. No correction for self-absorption was made.

Preparation of crude cell-free extracts from M. rouxii

Mycelia were grown for 15 h at 28° in 30 l of YPM medium contained in New Brunswick Scientific Co. fermentors. The mycelium was recovered by filtration, freeze dried with a Usifroid lyophilizer and ground to a fine powder in a Wiley mill. Aliquots of dried mycelium were resuspended in 0.05 M phosphate buffer, pH 7.2, and disrupted 3–4 times for 10-min periods with a Mixo-labo (Jouan) homogenizer. Refrigeration at this step was achieved by immersing the suspension in an ice salt bath. Suspensions were centrifuged at 10 000 × *g* for 15 min and the supernatant was recovered. The residue was resuspended in more buffer and the process repeated once more. The supernatants were mixed and used as the starting material for the purification of the enzyme.

RESULTS

Purification of the enzyme

Crude cell-free extracts obtained as described above were heated at 50° for 10 min with continuous shaking. The precipitate material was removed by centrifugation. To the supernatant, solid protamine sulfate at a ratio of 380 μg per mg of protein was added. The mixture was shaken in the cold for 60 min and the residue eliminated by centrifugation. 20% acetic acid was added dropwise to the supernatant to lower the pH to a value of 4.5 and the precipitate was removed by centrifugation. The supernatant was dialysed against 30 vol. of 0.05 M phosphate, pH 7.2, for 18 h changing the buffer every 6 h. The material which precipitated at this step was removed by centrifugation. The supernatant was concentrated by pressure dialysis and solid $(NH_4)_2SO_4$ was added to a value of 0.7 saturation. The mixture was shaken in the cold room for 60 min and the insoluble material removed by centrifugation. The supernatant was dialysed as mentioned above and ethanol maintained at −20° was added dropwise at a ratio of 2 vol. of ethanol per volume of enzymatic material. After standing in the cold for one hour the precipitate was centrifuged, resuspended in a small amount of buffer and dialysed against 0.05 M phosphate buffer, pH 8.0.

TABLE I

PURIFICATION OF α-GLUCOSIDASE FROM *M. rouxii*

10 g of dry mycelium powder were treated as described in METHODS. Specific activity is expressed as units per mg of protein. Total activity is expressed as total units in the fraction.

Step	*Volume (ml)*	*Total protein (mg)*	*Specific activity*	*Total activity*	*Yield*
Crude extract	575	3053	0.014	43.05	100
Heated extract	543	1232	0.032	46.70	100
Protamine sulfate	549	1328	0.036	47.90	100
Acidified extract	550	326	0.166	54.20	100
Ethanol	3	6.8	1.043	7.15	16.6
DEAE-Sephadex	41.4	3.18	1.32	4.2	9.8

The results of an experiment carried out using 10 g of dry mycelial powder as the starting material are shown in Table I.

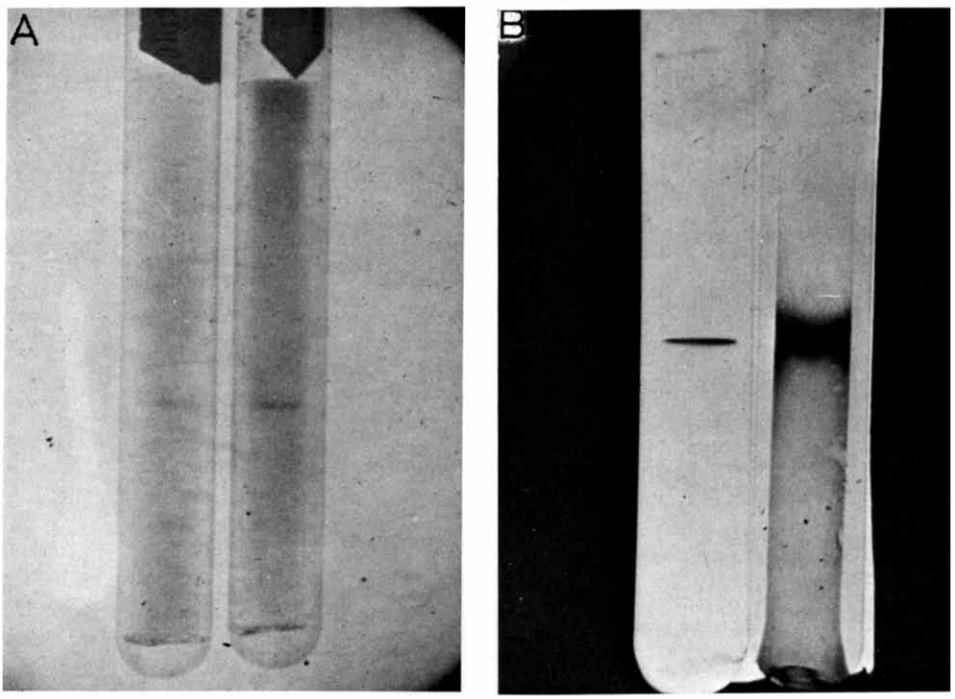

Fig. 1. Polyacrylamide gel electrophoresis of purified α-glucosidase from *M. rouxii*. Gel electrophoresis was performed at acid pH as described in METHODS. A, photograph of gel stained at the left for protein, at the right for carbohydrate. B, Photograph of gels stained at the left for protein, at the right for activity. Cathode at the bottom.

The purity of the enzyme was assessed by analytical electrophoresis on polyacrylamide gels. When the Tris–glycine and ethanolamine–triethanolamine systems were used, no protein bands were detected when the most purified fraction was analyzed. On the other hand, using the acetic acid–glycine system a single band was observed. However, at the low pH used, the enzyme was inactivated. Using a slightly higher pH, activity was preserved. The single protein band gave a positive reaction for carbohydrate. These results are shown in Fig. 1. One protein band which coincided with activity was observed when purified samples were subjected to chromatography in columns of Sephadex G-200.

Characteristics of α-glucosidase

The most purified fraction contained a great proportion of carbohydrate: 40% (measured with anthrone and calculated as glucose). This value was highly reproducible from experiment to experiment. Carbohydrate was eluted in two peaks, one of which coincided with the enzyme when this was subjected to chromatography on Sephadex G-200 and CM-Sephadex at pH 5.0 (Fig. 2). Samples of the enzyme were hydrolyzed with 1 M H_2SO_4 and sugars identified by paper chromatography. The most abundant sugar was glucose. Smaller amounts of galactose and fucose were also detected.

The enzyme was excluded from columns of Sephadex G-100 but it was imbibed

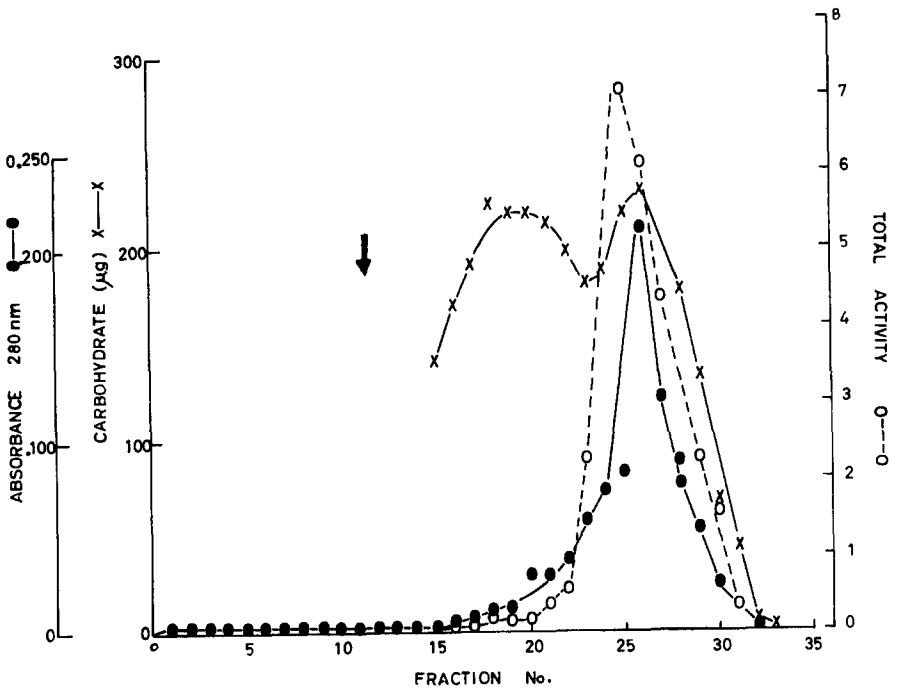

Fig. 2. Chromatography of purified glucosidase using Sephadex G-200. 4 ml of α-glucosidase eluted from DEAE-Sephadex and containing 1.824 mg of protein were subjected to chromatography using a Sephadex G-200 column (26.5 cm × 2.5 cm)). Aliquots of 3 ml were recovered. Absorbance at 280 nm, total carbohydrate and activity was measured as usual. The arrow indicates the void volume of the column.

by columns of Sephadex G-200. Apparently it contains only a small amount of aromatic amino acids since it has a low absorbance at 280 nm (Fig. 3).

Kinetic properties of α-glucosidase

The optimum temperature was found to be 50–60° (Fig. 4). Activity was dependent not only on pH but also on the buffer used. At pH 3.5 there was no activity in acetate buffer, but activity in phosphate was highest at this pH; maximum activity in acetate buffer was obtained at pH 6.0. The K_m value was $3.71 \cdot 10^{-4}$ M. These results, except with respect to optimum pH, are comparable to those previously reported for the partially purified preparations[12,13]. The enzyme was highly specific, it acted on maltose and in lesser amounts on starch. There was no detectable hydrolysis when the following disaccharides were tested as substrates under the normal conditions previously stated: melezitose, cellobiose, trehalose and sucrose. The synthetic α-glucosides, *p*-nitrophenyl-α-D-glucoside and 6-bromo-naphtyl-α-D-glucoside were not hydrolyzed by the enzyme either.

When the enzyme was incubated with maltose, the reaction rate was linear for about 30 min until about 30% of the theoretical amount of glucose was formed (Fig. 5). This behavior was not due to inactivation of the enzyme by the high incubation temperature (50°) since fresh enzyme added after the plateau had been reached, showed only slight activity. Addition of more maltose also did not lead to the release

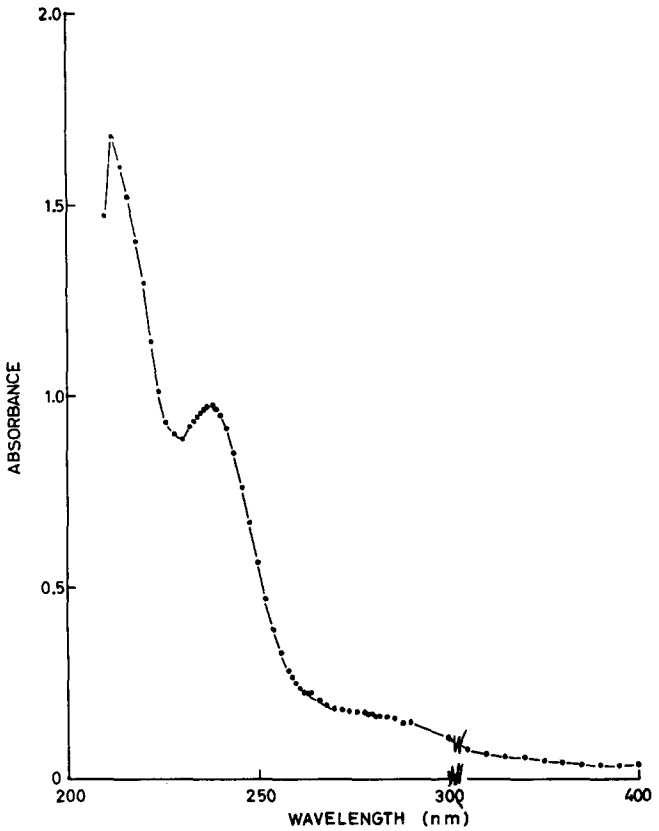

Fig. 3. Spectrum of α-glucosidase from *M. rouxii*. α-glucosidase was dissolved in 0.05 M phosphate buffer, pH 7.2. Its spectrum was recorded using a Maroc V spectrophotometer.

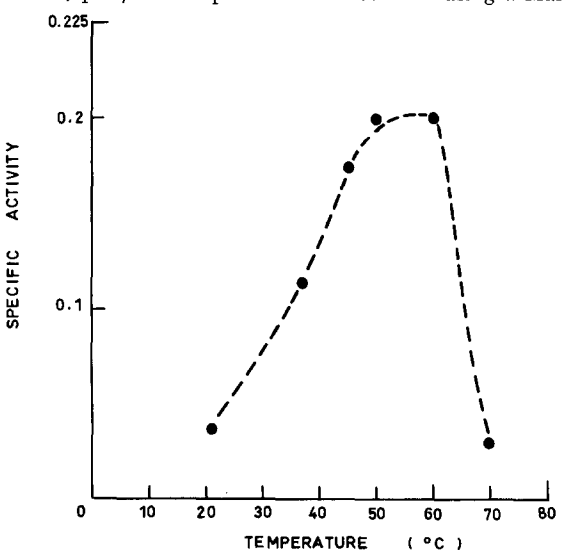

Fig. 4. Effect of incubation temperature on α-glucosidase. Aliquots of 26 μg of α-glucosidase were incubated under the standard assay conditions at different temperatures for 20 min. The amount of glucose released was measured with glucostat reagent.

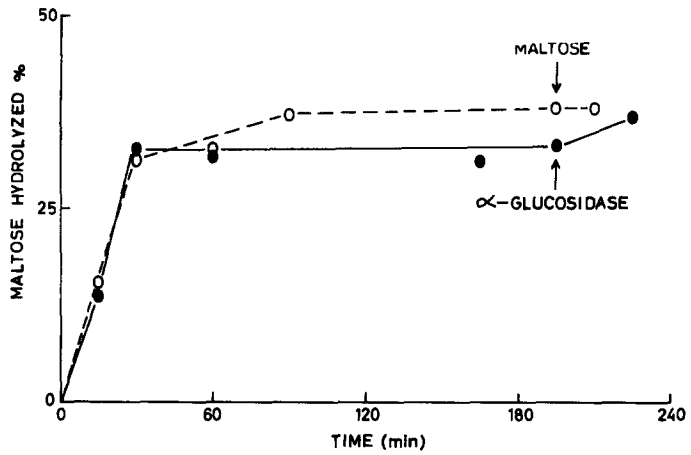

Fig. 5. Kinetics of maltose hydrolysis by α-glucosidase. The enzyme (0.3 ml containing 0.485 mg of protein) was incubated with 4 mg of maltose. The final volume was 1 ml, pH 4.5, and the temperature 50°. At intervals, 0.1-ml aliquots were removed and released glucose was measured. The arrows indicate when either 2 mg of maltose or 0.15 ml of enzyme were added.

of more glucose. It was assumed that maltose was not the only product and that dextrins could be formed by transglycosidase activity. However, when the enzyme was incubated with [1-^{14}C]maltose (Calbiochem), glucose was the only product detected. Controls incubated without enzyme revealed the presence of maltose only (Fig.6).

In a further experiment the enzyme was incubated with [1-^{14}C]maltose in the

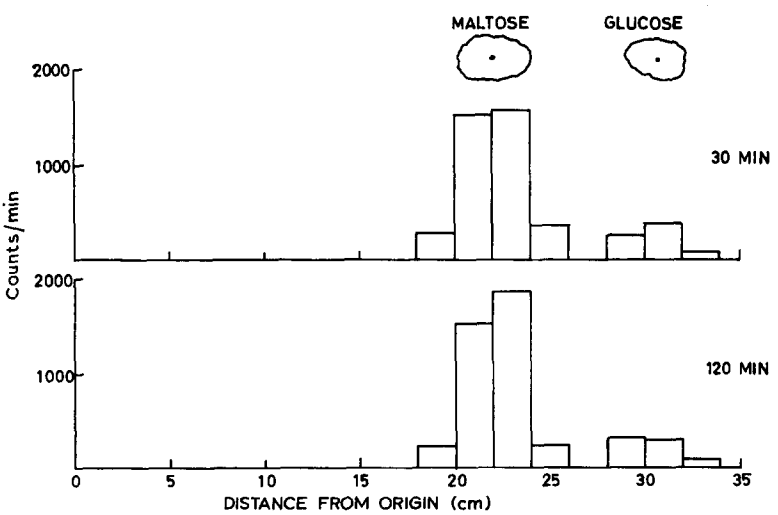

Fig. 6. Identification of the products formed by the action of α-glucosidase on maltose. 0.05 ml of α-glucosidase (64 μg of protein) were incubated with 80 μg [1-C^{14}]maltose (specific activity 0.21 μC/μmole) in a final volume of 0.1 ml for 30 or 120 min at 50°. The sample was spotted onto Whatman paper. Non-radioactive maltose and glucose were spotted on the same paper sheet. The material was subjected to paper chromatography, non-radioactive spots were developed with aniline phtalate and the radioactivity present in 2-cm segments was measured using a Geiger counter.

TABLE II

INHIBITION OF MALTOSE HYDROLYSIS BY GLUCOSE

0.05 ml of α-glucosidase containing 63 μg of protein were incubated with 80 μg of [1-C^{14}]maltose (specific activity 0.21 μC/μmole) and the indicated amounts of glucose, for 30 min at 50°. Total volume was 0.1 ml. The whole sample was applied as a spot to No. 1 Whatman chromatography paper and subjected to chromatography using butanol–pyridine–water (6:4:3, by vol.) as solvent for 36 h. A guide strip containing a mixture of non-radioactive glucose and maltose was cut and developed with aniline phtalate. The radioactivity of the corresponding regions under investigation was measured with a Geiger counter.

Total glucose (μg)	*Counts/min in glucose spot*	*% of radioactivity as glucose*	*% of inhibition of maltose hydrolysis*
0	448	11.9	—
50	247	7.2	39.5
100	192	5.6	53.0

presence of variable amounts of non-radioactive glucose. The extent of hydrolysis of the substrate was measured by separation of the radioactive glucose by paper chromatography. The results are shown in Table II. Glucose inhbited the enzyme. When glucosamine was tested as a potential inhibitor, no effect on enzymatic activity was observed.

DISCUSSION

α-Glucosidase from *M. rouxii* appears to be a glycoprotein. The polysaccharide moiety consists mostly of glucose with much lower amounts of galactose and fucose. It is interesting to mention that BARTNICKI-GARCÍA AND NICKERSON[20] did not report the presence of glucose in the cell walls from *M. rouxii*. JONES *et al.*[21] on the other hand described the existence of small amounts of glucose on cell walls from *Mucor ramannianus*. Considering the electrophoretic behavior of the enzyme, it is interesting to point out that in alkaline media it does not behave as an anion. We are tempted to suggest that anionic residues (aspartate and/or glutamate) of the enzyme are responsible for forming the bonds with the carbohydrate moiety. Several microbial enzymes have been described as glycoproteins: PAZUR *et al.*[22] reported that glucose oxidase, glucoamylases A and B and α-amylase from Aspergilli contained between 5 and 18% carbohydrate, D-mannose being the sugar present in higher amounts. According to these authors, α-amylase contained only mannose. On the other hand, ANAI *et al.*[23] reported that α-amylase from *Aspergillus oryzae* (Taka-amylase) contained mannose, galactose, xylose and *N*-acetylglucosamine in a ratio of 6:0.8:0.5:2%. The sugar moiety was bound *via* *N*-acetylglucosamine to the β-carboxyl group of the polypeptide chain. PAZUR *et al.*[24] in a further report mentioned that glucose oxidase contained 16% of carbohydrate, including mannose, galactose and *N*-acetylglucosamine in a ratio of 13.9:2:0.3%. METZENBERG[25] described that invertase from *Neurospora crassa* contained small amounts of glucosamine. BOER AND STEYN-PARVE[26] found that purified acid phosphatase from yeast contained mannan. Similarly, external invertase from *Saccharomyces cerevisiae* contains about 50% mannose[27]. A further example of glycoprotein enzymes from microorganisms is chloroperoxidase from *Caldariomyces fumago* which contains 25–30% glucosamine and arabinose[28]. It is worthwhile to

recall that all the enzymes referred to above are external enzymes. It is not known what the relationship between the presence of carbohydrate and the secretion of the enzyme is, but it is interesting to mention that EYLAR[29] and SOODAK[30] have emphasized that all external enzymes are glycoproteins. SPIRO[31] has described that during the synthesis of glycoproteins, the polypeptide moiety is synthesized on the polysomes by the usual mechanism, and as the protein moves along the channels of the endoplasmic reticulum, it is glycosylated.

Characteristics of purified α-glucosidase from *M. rouxii* are similar to those obtained with more crude preparations[12,13]. α-Glucosidase from *M. rouxii* is highly specific and does not hydrolyse synthetic α-glucosides. In this respect it differs from yeast α-glucosidase which hydrolyses *p*-nitrophenyl-α-D-glucoside[32]. The enzyme from *M. rouxii* is similar to amyloglucosidase from *Aspergillus niger*[1] in its general properties. Amyloglucosidase from crude preparations of taka-diastase has transglycosidase action on maltose whereas the purified enzyme produces only glucose from maltose[1], as does α-glucosidase from *M. rouxii*.

With regard to the inhibitory effect of glucose on the enzymatic activity we have not tested whether the inhibition brought about by glucose is competitive or not, but it may constitute a mechanism by which the fungus could control maltose hydrolysis so as not to exceed its capacity of glucose uptake.

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