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Comparative studies on glucoamylases from three fungal sources

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Abstract. Five commercial preparations of glucoamylases (three from *Aspergillus niger,* one each from *Aspergillus foetidus* and *Aspergillus candidus*) were purified by ultrafiltration, Sepharose-gel filtration and DEAE-sephadex chromatography. Two forms of the enzyme, namely glucoamylase I and glucoamylase II were obtained from the fungi except from one strain of *A. niger.* All the enzymes appeared homogeneous by electrophoresis and ultracentrifugation. The specific activities varied between 85 and 142 units. The pH and temperature optima were between 4 and 5, and 60° C respectively. The molecular weight as determined by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis ranged from 75,000 to 79,000 for glucoamylase I and 60,000 to 72,000 for glucoamylase II. Only *A. niger* glucoamylases contained phenylalanine at the N-terminal end. The amino acid composition of the enzymes was generally similar. However, *A. niger* and *A. foetidus* glucoamylases, in contrast to *A. candidus* enzymes, contained greater percentage of acidic than of basic amino acids. The enzymes contained 15 to 30% carbohydrate and 49 to 57 residues of monosaccharides per mol. *A. niger* enzymes contained mannose, glucose, galactose, xylose and glucosamine but the *A. candidus* enzyme lacked xylose and glucose and only xylose was absent in *A, foetidus* enzymes.. Majority of the carbohydrate moieties were O-glycosidically linked through mannose to the hydroxyl groups of seline and threonine of the polypeptide chain.

Keywords. Fungal glucoamylases; amyloglucosidases; comparative study.

Introduction

Several authors have described the purification and properties of glucoamylase (α-1,4-glucan glucohydrolase, EC. 3.2.1.3), a glycoprotein from fungal sources. In many instances, multiple forms of the enzyme have been reported. Lineback *et al.* (1969, 1972), Pazur and Ando (1959) and Pazur *et al.* (1971) have thoroughly studied the properties of the multiple forms of glucoamylases from *Aspergillus niger.* However, a comparative study of the purification, properties, immunological behaviour, role of the carbohydrate moiety, the glycoprotein chemistry of glucoamylases from different species of *Aspergillus* has not so far been attempted. In the present communication, a comparative study of a few of these aspects of five preparations of glucoamylase (three from *A. niger* strains, one from *A. foetidus* and another from *A. candidus*) is reported.

Materials and methods

Materials

The following chemicals were purchased from various suppliers: DEAE-Sephadex A-50 (50-120 mesh), 1-fluoro-2,4-dinitrobenzene, bovine serum albumin, ovalbumin, glucose oxidase, peroxidase, cytochromec, and amylose from the Sigma Chemical Company, St. Louis, MO, USA; Sepharose-6B from Pharmacia Fine Chemicals, Uppsala, Sweden; N, N, N′, N′-tetramethylethylenediamine from Eastman Kodak Ltd., Rochester, NY., USA; acrylamide, N-N′-methylene bis-acrylamide, Amido-Black 10B and *p*-dimethylaminobenzaldehyde from E. Merck AG., Darmstadt, Germany; *o*-dianisidine and DNP-amino acids from the Biochemicals Unit of V.P. Chest Institute, Delhi, glucose, maltose, galactose, xylose, soluble starch, phenol (distilled over zinc turnings) and benzidine from the British Drug House, Bombay, India; and sodium dodecyl sulphate from the Pierce Chemical Company, Rockford, Illinois, USA.

Enzyme preparations (tan brown powder/liquid) of glucoamylase were obtained from the following: glucoamylases from *A. niger* 1 of Anil Starch Products, Ahmedabad; *A. niger*-2 of Sarabhai Chemicals, Baroda; *A. niger*-3 of Maize Products, Ahmedabad; Diazyme from *A. foetidus* of Miles Laboratory, Elkhart, Indiana, USA; and the preparation from *A. candidus* of Hindustan Antibiotics Ltd., Pimpri.

Methods

Glucoamylase activity : Glucoamylase activity was assayed using starch as substrate. A fresh solution of starch was made daily. A slurry of soluble starch (2·0 g) was poured into boiling water (60-70 ml), cooled, 10 ml 0·5 M sodium acetate buffer (pH 4·8) added and the volume made up to 100 ml. For the assay, 10-50 μl (5 to 20 μg) of enzyme solution was mixed with 2 ml of the starch solution previously equilibrated to 60° C, and incubated at 60° for 15 min. The reaction was stopped by adding 0·5 ml of 0·1 N NaOH. A 10 μl aliquot of the alkaline mixture was made upto 0·5 ml with water to which was added 3·0 ml tris-glucose oxidase reagent (Dahlquist, 1961, 1964), followed by incubation at 37° C for 30 min. The absorbance of the resulting orange-coloured solution was measured at 420 am.

One unit of enzyme activity releases one μmol of glucose/min under the above conditions. Specific activity is expressed as units/mg protein.

Carbohydrate analysis : Total carbohydrate content of the glucoamylases was determined by phenol-sulphuric acid method as outlined by McKelvy and Lee (1969).

Neutral sugars were released from samples of glycoproteins (10 mg) by hydrolysis in two ml of 2N H_2SO_4 in evacuated sealed ampoules in boiling water for 6-7 h (Boas, 1953) and the hexosamines were determined by the Elson-Morgan reaction as described by Davidson (1966). Amino sugars were qualitatively identified by paper chromatography (Fischer and Nebel, 1955) and the ninhydrin oxidation (Stoffyn and Jeanloz, 1954) techniques.

Descending paper chromatography of neutral sugars was performed on Whatman No. 1 paper in the following systems: (*a*) 1-butanol-pyridine-water, 6 : 4 : 3 (v/v); (b) ethylacetate-pyridine-water, 8 : 1 : 2 (v/v); (c) 1-butanol-ethanol-water, 10 :1 :2

(v/v). Carbohydrates were detected on paper by the modified silver nitrate reagent (Mayer and Larner, 1959) or by the periodate-benzidine reagent (Gordon *et al.,* 1956).

Amino sugars were chromatographed on Whatman No. 1 paper using the Fischer and Nebel solvent systems (Fischer and Nebel, 1955), and detected by the alkaline silver nitrate (Mayer and Larner, 1959) or by Morgan-Elson reagent (Partridge, 1948).

Thin layer chromatography of sugars was conducted on microcrystalline cellulose (Avicel) previously reduced with $NaBH_4$ (Wolfrom *et al.,* 1966) using ethyl acetate-pyridine-water, 10 : 3 :3 (v/v) as the solvent system. Carbohydrates were detected by spraying with aniline-phthalate reagent (Partridge, 1949).

Gas-liquid chromatography of alditol acetates of sugars was carried out as described by Kannan *et al.* (1974) using Varian Aerograph Model 1,400 gas chromatograph equipped with a flame ionisation detector. A stainless steel column (240 cm × 2 mm) packed with 1% OV-225 on Gas Chrome *Q* was used.

Analysis of amino acids and protein : Proteins were hydrolysed for 24 h at 110° C with 5·8 N HCl in evacuated tubes. Amino acids were analysed in a Beckman Model 120B amino acid analyser using M-72 type resins (Assay No. B 30. 20408) and lithium buffer system (Kedenburg, 1971).

N-terminal amino acid was determined by the Sanger method as outlined by Narita (1970). Protein concentrations were determined according to Lowry *et al.,* (1951) using crystalline bovine serum albumin as the standard.

Alkaline borohydride treatment : The glycoprotein (1 mg) was treated with alkaline borohydride (1ml) according to Carlson (1966) at 37° C for 50 h. Borate was removed as the volatile methyl borate complex by repeated solubilisation in methanol and evaporation. The residue obtained was dried over P_2O_5 and prepared for amino acid and sugar analysis as described previously.

Polyacrylamide gel electrophoresis : Polyacrylamide gel electrophoresis was carried out on 7% acrylamide gel at pH 8·6 with tris-glycine buffer (Davis, 1964) or at pH 4·3 in ß-alanine-acetic acid buffer (Reisfeld *et al.,* 1962). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on 12% polyacrylamide gels (using 0 · 1% sodium dodecyl sulphate as outlined by Segrest and Jackson (1972)).

Enzyme activity was made visible on the gels by using polyacrylamide gels containing 0·1% soluble starch. After electrophoresis, the gels were immersed in 50 mM acetate buffer pH 4·8 at 50° C for 1 h, and then in 1% iodine solution. The gels became blue with clear colourless zones where the enzyme was localised.

Analytical ultracentrifugation : All ultracentrifugal studies were performed using a Spinco Model E Analytical Ultracentrifuge. Sedimentation velocity experiments were conducted on solutions of purified glucoamylases equilibrated by dialysis with 0 · 1 M NaCl at concentrations of 1%(w/v) protein. A standard 12 mm duraluminium cell centre piece was used at room temperature (25° C) at a speed of 59,780 rpm. Sedimentation coefficient values were calculated by the standard procedure (Schachman, 1959).

Purification

Purification of A. niger and A. foetidus glucoamylases

Step 1—*Extraction and concentration* : Ten g of the commercial enzyme preparations were stirred with 50 ml of 0·05 M potassium phosphate buffer, pH 7·0 at 4° for 30 min. The resulting suspension was centrifuged at 10,000 g for 15 min. The sediment was stirred with 25 ml of buffer and centrifuged. This process was repeated once more and the supernatant fractions were pooled and concentrated to about 5 ml using an Amicon ultrafiltration cell (Amicon Ltd., High Wycombe, Bucks, UK) equipped with PM-10 membrane. This extraction procedure eliminated most of the inactive chromogenic compounds while ensuring 97% recovery of activity. *A. foetidus* glucoamylase (Diazyme) supplied as a liquid concentrate was diluted 20 times with 50 mM phosphate buffer (pH 7·0) and ultrafiltered.

Step 2—*Gel filtration on Sepharose-6B column:* About 400 mg of the enzyme from step 1 was chromatographed on a Sepharose-6B column (2 × 120 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7·0. The fractions containing glucoamylase activity were pooled, dialysed against distilled water and lyophilised.

Step 3—*Chromatography on DEAE-Sephadex* : About 150 mg protein from step 2 was applied to a DEAE-Sephadex column (2·2 × 28 cm) equilibrated with 0·01 M potassium phosphate buffer, pH 7·0 (buffer B). The column was developed with a linear gradient of 0 to 0·7 M NaCl in buffer B. In all other sources except in *A. niger*-3 (Maize Products) the proteins were resolved into three distinct peaks of which the last two were active (figure 1). The first active fraction was eluted at 0·22–0·32 M NaCl and the second one at 0·37–0·46 M. These active eluates were designated according to the recommendations for the nomenclature of multiple forms of enzymes (Webb, 1964). Thus the second active fraction, which moved faster towards the anode in analytical polyacrylamide gel electrophoresis than the first was designated glucoamylase I and first one glucoamylase II. *A. niger*-3 gave only one active fraction which was eluted at 0·3 M NaCl and therefore corresponds to glucoamylase II from other sources.

Glucoamylases I and II were separately pooled, dialysed against buffer B and rechromatographed on DEAE-Sephadex as before.

Purification of A. Candidus enzyme

This enzyme was purified upto the Sepharose filtration step by the same procedure as that described for *A. niger* and *A. foetidus* enzymes. Subsequently, the following modifications were used. About 150 mg protein from the Sepharose step was applied on to a DEAE-Sephadex column (2·2 × 28 cm) which was previously equilibrated with 10 mM potassium phosphate buffer, pH 7·5. A linear pH gradient of 6·5-7·5 (10 mM potassium phosphate buffer) was used for elution. Two active fractions were eluted from the column, one at pH 7·3 designated glucoamylase II and another at pH 7·0 designated glucoamylase I. The glucoamylases I and II were separately pooled, dialysed against 0·01M potassium phosphate buffer, pH 7·5 and rechromatographed on DEAE-Sephadex as described above.

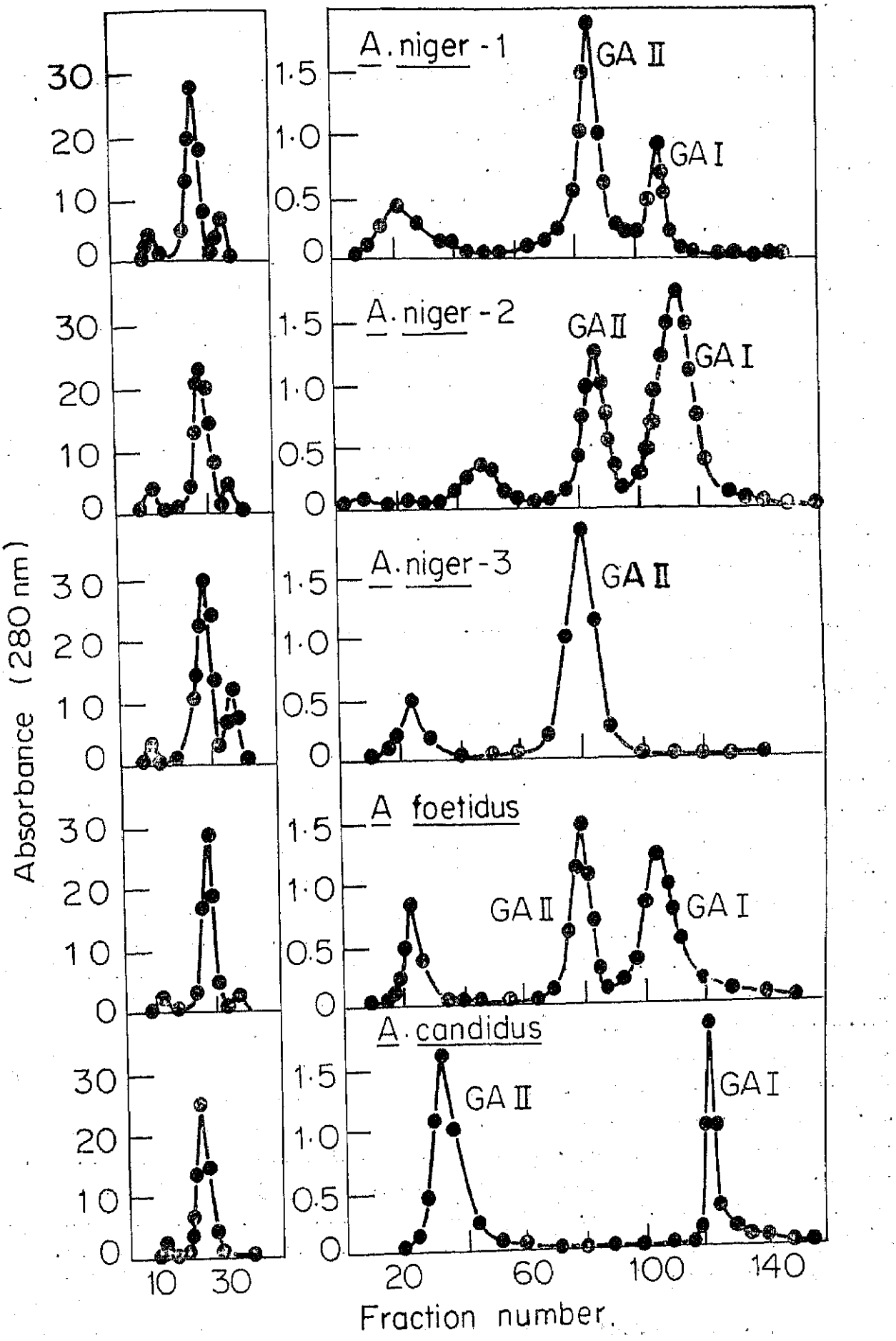

Figure 1. Sepharose and DEAE-Sephadex chromatography patterns. The chromatography was performed as described in the text. The *A. candidus* enzyme was eluted with a linear pH gradient (7·5–6·6, 0·01 M potassium phosphate buffer).

Results and discussion

Purification

The elution patterns during Sepharose filtration and during DEAE-Sephadex chromatography show that enzymes from *A. niger*-1, -2 and -3; *A. foetidus* and *A. Candidus* all eluted similarly (figure 1). By DEAE-Sephadex chromatography, enzymes from *A. niger*-1 and -2 and *A. foetidus* were eluted in two active fractions while enzyme from *A. niger*-3 was eluted in a single active peak. The two glucoamylases I and II from *A. candidus* behaved differently during DEAE-Sephadex chromatography. The *A. candidus* enzyme was held on the DEAE-Sephadex column at pH 7·5 but not at pH 7. Obviously, the *A. candidus* enzymes possess electrochemical properties different from the rest. It was also found that application of salt gradients, as in the other cases, to the *A. candidus* enzymes on DEAE-Sephadex did not lead to a clear separation of glucoamylase I from II whereas the pH gradient was very effective.

In all, nine purified glucoamylase preparations were obtained (two each from *A. niger*-1, -2, *A. foetidus* and *A. candidus* and one from *A. niger*-3). Results of the purification experiments are given in table 1. The specific activities of the different homogeneous preparations varied from 85 to 101 for the *A. niger* and *A. foetidus* enzyme, and 112 to 142 for *A. candidus* enzyme when assayed at the optimum temperature (60° C). Lineback *et al.* (1969) reported a specific activity of 12 and 16 for glucoamylases I and II of diazyme respectively at 30°. Under similar assay conditions (*viz.,* at 30° C), specific activities of 12 and 18 were obtained for glucoamylases I and II of *A. niger*-1 and the respective values for two enzyme forms of *A. candidus* were 19 and 32.

Glucoamylases from *A. niger* generally have been resolved into two forms (Lineback *et al.,* 1969; Pazur and Ando, 1959; Venkataramu *et al.,* 1975; Fleming and Stone, 1965; Pazur *et al.,* 1963; Smiley *et al.,* 1971). In the present investigation, two forms of glucoamylase were obtained in all cases except *A. niger*-3 which had only one. It is difficult to explain why only one enzyme form was obtained from *A. niger*-3 in contrast to two forms in all other sources now studied. The enzyme from *A. niger*-3 is a product essentially for the manufacturer's use and it is not clear whether it has been subjected to any specific treatment or obtained under special growth conditions. Other species of *Aspergilli* such as *A. awamori* (Watanabe and Fukimbara, 1965) and *A. phoenicis* (Lineback and Baumann, 1970), *Penicillium oxalicum* (Yamasaki *et al.,* 1977), *Mucor rouxianus* (Tsuboi *et al.,* 1974) and *Rhizopus* species (Ueda and Kano, 1975) have all been reported to synthesise two forms of glucoamylase. In two instances even more forms of this enzyme were reported: three in *A. niger* (Venkataramu *et al.,* 1975; Fleming and Stone, 1965) and four in *A. oryzae* (Morita *et al.,* 1966). Composition of the growth medium (Barton *et al.,* 1969; Kawamura and Sawai, 1968; Hayashida, 1975) and the purification procedure (Fleming and Stone, 1965) are known to influence the number of discrete forms of glucoamylase.

Homogeneity : The purified preparations from all the five sources studied gave a single coincident peak of protein, activity and carbohydrate during rechromato-

Table 1. Purification of glucoamylases.*

Source	Purification steps		Sp. act.[a] (Units/mg protein)	Carbohydrate[b] (%)
A. niger-1	Crude		69	ND
	Extract-concentrate		79	ND
	Sepharose-6B		84	27·0
	DEAE-Sephadex	Glucoamylase I	86	15·2
		Glucoamylase II	90	18·0
	Rechromatography	Glucoamylase I	86	15·2
		Glucoamylase II	91	18·0
A. niger-2	Crude		64	ND
	Extract-concentrate		79	ND
	Sepharose-6B		92	30·0
	DEAE-Sephadex	Glucoamylase I	100	18·0
		Glucoamylase II	95	20·5
	Rechromatography	Glucoamylase I	101	16·0
		Glucoamylase II	95	19·5
A. niger-3	Crude		60	ND
	Extract-concentrate		75	ND
	Sepharose-6B		84	27·0
	DEAE-Sephadex		90	20·0
	Rechromatography		91	20·0
A. foetidus	Crude		67	ND
	Extract-concentrate		75	ND
	Sepharose-6B		82	25·0
	DEAE-Sephadex	Glucoamylase I	92	15·6
		Glucoamylase II	90	19·1
	Rechromatography	Glucoamylase I	92	15·4
		Glucoamylase II	90	19·1
A. candidus	Crude		73	ND
	Extract-concentrate		82	ND
	Sepharose-6B		99	39·0
	DEAE-Sephadex	Glucoamylase I	112	30·7
		Glucoamylase II	141	25·6
	Rechromatography	Glucoamylase I	112	30·1
		Glucoamylase II	142	24·8

* 10 g of the enzyme preparation were used in each case: 10ml in the case of *A. foetidus* enzyme. The protein contents of the initial materials were 1,470; 1,420; 1,680; 1,230 and 1,104 mg respectively. 95-98% of the activity and protein were recovered in steps 1 and 2: the final recovery of activity ranged from 60 to 100%.

[a] Enzymatic activity according to standard assay.

[b] By the phenol-H_2SO_4 reaction using glucose as standard; percentage based on protein, ND, not determined.

graphy on a DEAE-Sephadex column. Polyacrylamide gel electrophoresis at pH 8·6 and pH 4·3 of each of the glucoamylases revealed only one band after staining with Amido Black or with the Schiff's reagent which detects glycoprotein. These bands had glucoamylase activity as evident by incubating the 0·1% starch containing gels at pH 4·8 at 50° C for 1 h and then staining with 1% iodine solution. Figure 2 shows the pattern obtained during polyacrylamide gel electrophoresis at pH 8·6. Glucoamylases from *A. Candidus* had lower mobilities compared to glucoamylase from other sources.

Examination of the purified enzymes from all the sources in an analytical ultracentrifuge showed a single component which sedimented as a symmetrical peak in each instance. The values for sedimentation coefficient varied between 4·3 and 4·5 S for glucoamylase II and 4·4 and 4·6 S for glucoamylase I.

Fungal glucoamylases often occur in association with α-amylases (Marshall and Whelan, 1970, 1971). Such contaminants can be tested by the very sensitive test of Marshall and Whelan (1971). All enzyme preparations in the present investigation failed to release glucose (as observed by chromatography and by specific enzyme test) from the enzyme-modified oxidised-amylose, thereby indicating that they are free from *a*-amylase contamination.

pH and temperature optima

Glucoamylases have pH activity optima from pH 4·5 to 5 (Lineback *et al.,* 1969; Pazur and Ando, 1959; Pazur *et al.,* 1971; Venkataramu *et al.,* 1975). The present finding also conforms to this pattern. All the enzyme preparations including *A. Candidus* had maximal activity between pH 4 and 5. The enzymes from *A. niger* were quite stable during the assay even at pH 2 retaining more than 60% activity, whereas the enzymes from *A. candidus* lost 90% of its activity at pH 2 (and 75% at pH 3) and *A. foetidus* lost more than 50% activity below pH 3. These results on *A. candidus* and *A. foetidus* are thus similar to the observations of Pazur *et al.* (1959) who reported more than 50% inactivation of diazyme glucoamylase below pH 3.

Maximal activities were observed at 60° for these enzyme forms. Glucoamylase of *A. niger*-1 was stable at 60° C whereas the same enzyme from *A. candidus* lost 50% of its activity during 20 min exposure. The glucoamylase II from the two sources are less stable than their counterparts (*i.e.,* the enzyme from *A. niger*-1 and *A. candidus*). In this respect also, they resemble other glucoamylases produced by *Aspergillus* species reported so far (Lineback *et al.,* 1969; Pazur and Ando, 1959; Venkataramu *et al.,* 1975). Glucoamylase from a thermophilic fungus *Humicola lanuginosa* has a higher pH optimum of 6·6 and shows a remarkable heat stability in the presence of starch: only 30% of the activity was lost on boiling the enzyme in 0·5% starch for 10 min (Taylor *et al.,* 1978).

Molecular weight and subunit structure

Pazur *et al.* (1962) reported that glucoamylase I of *A. niger* had a M_r of 97,000 by ultracentrifugal analysis; according to a later report, it was 110,000 (Pazur *et al.,* 1970). Lineback *et al.* (1972) obtained M_r values of 74,900 (± 1,500) for glucoamylase I and 54,300 (± 6,690) for glucoamylase II by ultracentrifugal analysis. Previous investigations from this laboratory showed (sodium dodecyl sulphate-

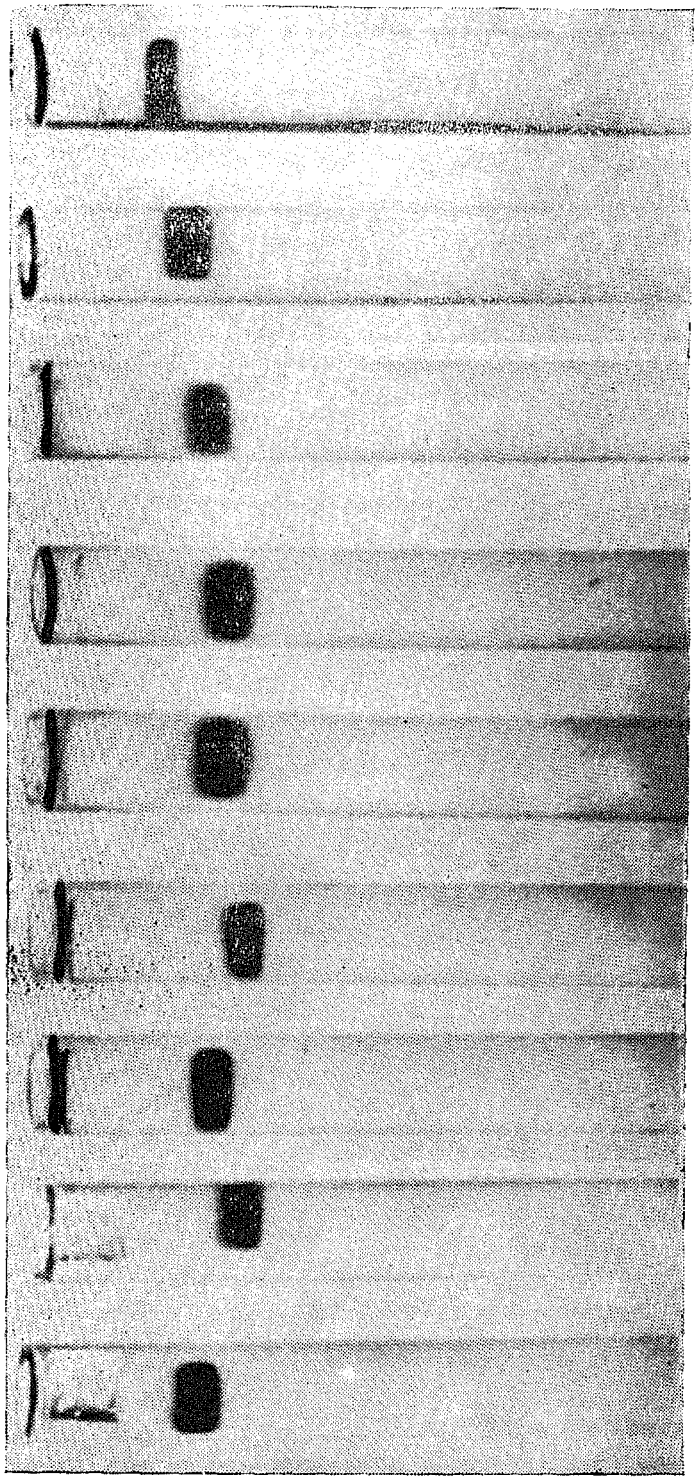

Figure 2. Polyacrylamide gel electrophoresis of the purified glucoamylases: 150 μg protein at pH 8·6.
The sources of the pure enzymes are: (from left to right) *A. niger*-1, forms I and II; *A. niger*-2, forms I and II; *A. foetidus*, forms I and II; *A. niger*-3, form II; *A. candidus*, forms I and II.

polyacrylamide gel electrophoresis) that the three glucoamylases from *A. niger* NRRL 330 had M_r of 90,000, 71,000 and 70,000 (Venkataramu *et al.*, 1975).

The M_r of the purified enzymes in the present investigation was determined by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis method (Segrest and Jackson, 1972) using cytochrome *c* (13,000), myoglobin (17,000), *α*-chymotrypsin (23,000), ovalbumin (45,000) and bovine serum albumin (66,000 and 132,000) as standards. The values extrapolated from the graph are given in table 2. Glucoamylase I from all the sources studied was found to have a M_r between 75,000 and 79,000. On the other hand, glucoamylase II from all but *A. niger*-1 had a M_r of 60,000 (± 3%) and that from *A. niger*-1 72,000. Thus the M_r values reported here are very close to those reported by Lineback *et al.* (1972).

M_r of other glucoamylases range from 57,000 to 110,000. Glucoamylase II of *A. phoenicis* has a M_r of about 63,000 (Lineback and Bauman, 1970), one of the four glucoamylases of *A. oryzae* 69,000 (Morita *et al.*, 1966), and two of the enzyme forms from *A. niger* NRRL 3112 have 71,000 and 57,000 (Smiley *et al.*, 1971). Glucoamylase I of *A. niger* was reported by Pazur and Kleppe (1962) to have a M_r of 97,000 to 110,000 (Pazur *et al.*, 1970).

In all instances reported here except one (*A. niger*-3), the two forms of glucoamylase possess a single polypeptide chain since only one band was observed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and since only phenylalanine was the N-terminal amino acid in most cases (see next section). Only in one case (*A. niger*-3) such electrophoresis gave two bands: the major one corresponding to M_r of 60,000 and a minor one with $M_r \simeq 30,000$. This enzyme from *A. niger*-3 may therefore consist of two chains which are presumably identical. The detection of only one N-terminal amino acid (phenylalanine) (see next section) indicates but does not prove, this tentative conclusion. Freedberg *et al.* (1975) also reported a "subunit"/multichain structure for the enzyme from *A. foetidus* after reduction and carboxymethylation. Strictly speaking such treatment and also sodium dodecyl-sulphate-polyacrylamide gel electrophoresis yield results indicative of a multichain nature and not a subunit structure, since a subunit structure by definition does not involve covalent bonds.

Table 2. M_r of glucoamylases.

Source	Glucoamylase I	Glucoamylase II
A. niger-1	79,000	72,000
A. niger-2	79,000	60,000
A. niger-3	..	60,000
A. foetidus	78,000	60,000
A. candidus	75,000	60,000

The molecular weights were determined by the sodium dodecyl sulphate–polyacrylamide gel electrophoresis procedure: the details are given in the text.

N-terminal amino acid

All the enzyme forms from *A. niger* contained phenylalanine at the N-terminal except in the enzymes of Diazyme (*A. foetidus)* and of *A. Candidus.* Apparently the N-terminal is blocked in the latter two cases and no attempt was made to identify the blocking group. The present results on diazyme enzyme are in conformity with those of Freedberg *et al.* (1975), who also failed to detect any free N-terminal end in Diazyme glucoamylase. However, these results are at variance with the report of Lineback *et al.* (1969) that alanine was at the N-terminal end in both the forms of glucoamylases from Diazyme. Previously it was reported from this laboratory that the three glucoamylases of *A. niger* NRRL 330 contained phenylalanine at the N-terminal end (Venkataramu *et al.,* 1975), whereas Pazur *et al.* (1971) found alanine as the N-terminal amino acid in *A. niger* glucoamylases. Thus the N-terminal amino acid of the glucoamylases of *A. niger* appears to vary. Whether this is due to proteolysis or to genetic changes in different strains is a problem requiring further investigation. Glucoamylases from other species differ from *A. niger* enzymes in this respect. Valine is reported to be the N-terminal amino acid in the enzyme from *A. awamori* (Durmishidze and Kresitadze, 1974) and according to another report (Hayashida and Yoshino, 1978) it has alanine at the N-terminal end.

Amino acid composition

There are minor differences in the amino acid composition (table 3) amongthe various glucoamylases tested. All of them contained high proportions of the hydroxy amino acids (serine and threonine), aspartic acid, glutamic acid, alanine and glycine. Sulphur containing amino acids were low. Aromatic amino acids content was comparable but branched-chain amino acids were higher in *A. niger* and *A. foetidus* than for *A. candidus* enzymes. *A. candidus* enzymes had more basic than acidic amino acids. On the other hand, *A. niger* and *A. foetidus* enzymes had more of acidic than basic amino acids. This probably accounts for the observed slower electrophoretic migration and weaker adsorption on DEAE-Sephadex of *A. candidus* enzyme than of *A. niger or A. foetidus* enzymes.

Pazur *et al.* (1971) found that the two forms of *A. niger* glucoamylases had approximately the same amino acid composition. In contrast, Lineback *et al.* (1972) reported a distinctly different amino acid content for the two forms from Diazyme glucoamylases. The present results for amino acid composition of the enzyme pairs from the same source seem to be intermediate between these results. Whether these pronounced or slight differences are genetic or arise by proteolysis at different sites (Hayashida, 1975) depending upon growth condition is not clear.

Carbohydrate analysis

All the enzyme preparations in the present investigation were identified as glycoproteins. The carbohydrate content ranged from 15 to 30% (table 4). *A. candidus* enzymes contained a significantly higher content, about 25 to 30%. Lineback *et al.* (1972) reported that inexplicable variations in carbohydrate content occurred without dissolution of enzyme in 1 M acetic acid. No such variations were observed

Table 3. Amino acid composition of glucoamylases[a].

Amino acid[b]	*A. niger* 1		*A. niger*-2		*A. niger*-3	*A. foetidus*		*A. candidus*	
	I	II	I	II		I	II	I	II
Lys	12	8	11	12	13	12	9	19	17
His	4	4	3	4	4	3	3	5	4
Ammonia	58	59	65	59	60	56	52	55	60
Arg	16	16	17	17	16	16	17	19	19
Asp	62	57	67	58	62	61	53	49	51
Thr	70	62	63	57	60	70	58	66	64
Ser	76	66	79	68	69	76	65	84	71
Glu	42	38	41	37	41	39	35	42	39
Pro	18	17	12	15	13	17	23	28	27
Gly	44	36	50	40	43	45	37	37	34
Ala	62	54	64	55	51	48	41	48	38
Val	28	24	30	18	22	29	21	30	25
Ile	18	9	11	15	18	17	19	16	16
Leu	45	43	36	40	35	40	38	16	18
Tyr	14	14	22	17	21	11	16	17	18
Phe	20	19	19	19	19	9	15	14	16
Basic amino acids minus acidic amino acids	−14	− 8	−12	− 3	−10	−13	− 7	+ 7	+10

[a] Expressed as mol/mol protein: calculated utilising M_r values given in table 2.
[b] Recovery 83-89%, on the basis of nitrogen. Cys/2, Met and Trp not determined.

in the present investigation, when the carbohydrate was determined either directly (by dissolving in water) or after solution in 1 M acetic acid.

The carbohydrate composition was also determined (table 4). It was found that the *A. niger* enzymes contain xylose, and the enzymes from *A. niger* and *A. foetidus* have glucosamine in addition to mannose, glucose and galactose; these are first reports of the presence of xylose and glucosamine in these enzymes. The *A. Candidus* enzymes did not contain any xylose or glucose but only mannose, galactose and glucosamine: glucosamine was also found in the enzymes of *A. oryzae* (Morita *et al.,* 1966) and *A. awamori* (Pazur and Okada, 1967). The *Rhizopus* enzymes were reported to contain only mannose and glucosamine (Watanabe and Fukimbara, 1973). Thus the enzymes of the black *Aspergilli* and *Rhizopus* species appeared to differ from one another in their monosaccharide content.

Although the composition of the total carbohydrate was significantly different, the two resolved enzyme forms from the same source contained approximately the

Table 4. Carbohydrate content of glucoamylases.

Source	Man[a]	Glc[a]	Gal[a]	Xyl[a]	GlcN[b]	Total CH_2O[c]
	(mol/mol enzyme)					
A. niger-1						
Glucoamylase I	41·3	8·0	3·5	0·8	2·9	56·5
Glucoamylase II	44·8	4·2	3·3	1·7	3·4	57·4
A. niger-2						
Glucoamylase I	43·4	5·0	2·8	0·6	3·3	55·1
Glucoamylase II	34·7	5·0	2·1	3·8	2·7	47·6
A. niger-3						
Glucoamylase II	36·0	7·2	2·6	1·4	3·2	50·4
A. foetidus						
Glucoamylase I	39·1	6·9	2·2	..	4·0	52·2
Glucoamylase II	37·3	4·8	2·7	..	3·7	48·5
A. candidus						
Glucoamylase I	44·0	..	1·8	..	7·1	53·0
Glucoamylase II	43·0	..	3·0	..	6·9	53·0

[a] Man, mannose; GIc, glucose; Gal, galactose: Xyl, xylose: by gas chromatography as alditol acetates.

[b] GlcN, glucosamine: by Elson-Morgan reaction.

[c] CH_2O, carbohydrate: by the phenol-H_2SO_4.
Number of mol of monosaccharide/mol enzyme was calculated utilising molecular weight values from table 2.

same number of monosaccharide residues per mol. For example, glucoamylases I and II of *A. niger* contained 56 to 57 residues and those of *A. candidus* contained 53 residues each. These results are similar to that of Lineback *et al.* (1972) who reported the presence of approximately 60 monosaccharide residues in both enzyme forms of Diazyme. Contrary to this, Pazur *et al.* (1971) reported that multiple forms (glucoamylases I and II) of *A. niger* differ in total monosaccharide residues: glucoamylase II containing 151 residues of monosaccharides had twice as much carbohydrate as glucoamylase I containing 87 residues.

When the present enzymes were treated with alkaline borohydride, there was a decrease in the number of threonine and serine residues, an increase in the content of alanine, and *de novo* appearance of *a*-aminobutyric acid and mannitol (table 5). No sugar alcohol other than mannitol was formed. These results indicated that most of the carbohydrate moieties in the glucoamylase from all the fungi tested here were O-glyscosidically linked through mannose to the hydroxyl groups of

Table 5. Serine, threonine, alanine and α-aminobutyric acid composition[a] and carbohydrate content[b] of glucoamylase before and after treatment with alkaline borohydride.

Source	Initial					After alkaline borohydride treatment					Difference				
	Ser	Thr	Ala	α-ABA	CH_2O	Ser	Thr	Ala	α-ABA	CH_2O	Ser	Thr	Ala	α-ABA	CH_2O
1. *A. niger*-1															
Glucoamylase I	76	70	62	0	15·2	65	55	70	4	3·1	−11	−15	+8	+4	−12·1
Glucoamylase II	66	62	54	0	18·0	56	46	61	3	5·7	−10	−16	+7	+3	−12·3
2. *A. niger*-2															
Glucoamylase I	79	63	64	0	16·0	67	44	72	4	5·6	−12	−19	+8	+8	−10·4
Glucoamylase II	68	57	55	0	19·5	56	47	64	4	6·4	−12	−20	+9	+4	−13·1
3. *A. niger*-3															
Glucoamylase II	69	60	51	0	20·0	55	44	61	2	6·0	−14	−14	+8	+2	−14·0
4. *A. foetidus*															
Glucoamylase I	76	70	48	0	15·4	66	51	56	3	4·8	−10	−19	+8	+3	−10·6
Glucoamylase II	61	58	41	0	19·1	55	43	49	3	5·8	−10	−15	+8	+3	−13·3
5. *A. candidus*															
Glucoamylase I	84	66	48	0	24·8	73	56	56	2	9·9	−11	−10	+8	+2	−14·9
Glucoamylase II	71	64	38	0	30·1	61	54	46	2	11·4	−10	−10	+8	+2	−18·7

[a] expressed as mol/mol protein; calculated utilising M_r values given in table 2.

[b] expressed as g/100 g protein.

− Residues lost or decomposed; + Extra residues formed.

α-ABA—α-aminobutyric acid.

CH_2O—Carbohydrate.

serine and threonine as reported by several investigators for other glycoproteins (Tanaka *et al.,* 1964; Anderson *et al.,* 1963; Anderson *et al.,* 1964; Hashimoto *et al.,* 1963). These results are very similar to those of Lineback *et al.* (1972) and of Pazur *et al.* (1970). However, the present results do not completely eliminate the possibility of other types of linkages. Nearly 20-40% of the carbohydrate was still resistant to ß-elimination, suggesting that carbohydrate-peptide linkages such as N-acetyl-glucosamine-asparagine which are resistant to alkaline treatment might be present.

It is clear that there are differences in the amino acid composition and in their carbohydrate content of glucoamylase from *Aspergilli.* These differences do not seem to affect the catalytic activities and the pH and temperature optima which are similar. It is unlikely therefore that there will be any advantage of using the enzyme from one source over another. Results of studies on their immunological properties and the distribution and sequence of carbohydrate moieties will be reported elsewhere.

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