

## Evidence of Tryptophan at or near Active Site of Glucoamylase I of *Arthrobotrys amerospora*

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### ABSTRACT

*Arthrobotrys amerospora* (ATCC 34468) produced glucoamylase in a semi-synthetic medium containing starch as a sole carbon source. Polyacrylamide gel electrophoresis of crude glucoamylase showed three isoenzymes. They were designated as glu I, glu II and glu III according to their electrophoretic mobility. These iso-glucoamylases were purified by column chromatography using DEAE-Sephadex A-50. The major fraction, namely glu I, was subjected to various group specific reagents like NEM, idoacetamide, PALP, DEP, Rose Bengal, NBS and acarbose. *N*-bromosuccinimide and acarbose totally inhibited glu I.  $Hg^{2+}$  ion did not inhibit glu I activity at 25 m mol concentration. Glu I also showed raw starch activity. *Iran. Biomed. J.* 3 (3 & 4): 103-107, 1999

**Keywords:** Glucoamylase I, Tryptophan, *Arthrobotrys amerospora*

### INTRODUCTION

*Arthrobotrys amerospora* is a nematode trapping or a predacious fungus. This organism belongs to class of Deutromycetes, (family of Molinialacea, genus of *Arthrobotrys*). Generally, nematode trapping fungi plays a major role in recycling the carbon, nitrogen and other important elements from the biomass and nematodes [1]. Schenck *et al.* and Rosenweig and Pramer [2, 3] employed this organism to produce collagenase in a medium devoid of collagen. In the previous study, [4] *Arthrobotrys amerospora* was used to produce glucoamylase in the medium of Schenck *et al.* [2] containing starch as carbon source [3]. Glucoamylase (1,4, -D-glucanglucohydrolase EC 3.2.1.3) is an exo-acting enzyme capable of hydrolysing -1,4 linked glucose residue consecutively from the non-reducing ends of amylose, amylopectin and glycogen producing  $\alpha$ -glucose. Thus, glucoamylase is industrially an important biocatalyst and is mainly produced by fungi of *Aspergilli species* [5, 6]. Therefore there are no other reports indicating that *Arthrobotrys amerospora* produces glucoamylase. In the present work, we have attempted to show the role of tryptophan at or near the active site of purified glu I by chemical modification which may be responsible

for raw starch activity of glu I produced by *Arthrobotrys amerospora*.

### MATERIALS AND METHODS

**Chemicals.** Diethyl pyrocarbonate, histidine, *N*-ethylmaleimide, dithiothreitol, iodoacetamide, *N*-bromosuccinimide, pyridoxal phosphate, tryptophan, 3,5-dinitrosalicylic acid and starch were obtained from Sigma Chemical Company USA. Corn Meal Agar was purchased from Difco. All other reagents were purchased from Merck, Germany. Acarbose was a gift by Bayer Company, Germany.

**Organism.** *Arthrobotrys amerospora* ATCC 34468 was obtained from American Type Culture Collection, Maryland, USA. It was sub-cultured every 4-6 weeks on Corn Meal Agar.

**Enzyme production.** The spore suspension ( $10^7$  spores /ml) was inoculated into a medium containing 0.5 g  $MgSO_4 \cdot 7H_2O$ , 10 mg  $FeSO_4 \cdot 7H_2O$ , 0.5 g  $(NH_4)_2SO_4$ , 10 g peptone, 1.0 g Yeast extract and 10 g starch per liter in 0.05 M phosphate buffer pH 6.5. The inoculated flask was kept on rotary shaker (200 rpm) at 25°C for 6 days.

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**Assay of glucoamylase activity.** The broth of 6 days growth was centrifuged at 10,000 rpm, 4°C for 15 min and used as a source of glucoamylase. The enzyme solution (1 ml) was added to 1 ml 0.05 % (w/v) starch solution prepared in acetate buffer pH 5.6 and incubated at 55°C for 30 min. The amount of reducing sugar was determined by addition of 2 ml 3,5-dinitrosalicylic acid [7]. The tube was kept in boiling water bath for 5 min (to develop the color), cooled and the absorbance of the solution was read at 540 nm against blank that contained thermally inactivated glucoamylase. Glucoamylase was inactivated by placing the solution containing enzyme in boiling water-bath for 15 min. One unit of enzyme was defined as the amount of enzyme that liberates one m mole of glucose from the substrate under the defined assay conditions.

**Enzyme purification.** Fermentation of glucoamylase was carried out for six days. The broth containing glucoamylase was separated from the fungal mycelia by centrifugation at 10,000 rpm at 4°C for 15 min. The broth then was precipitated by 75% saturated ammonium sulfate solution and desalted on column of Sephadex G-25 (2.5 × 10 cm). Two ml fractions at the flow rate of 2 ml/min were collected. Each fraction was assayed for glucoamylase activity and the protein content was estimated by Lowry's method [8] using bovine serum albumin as standard. The active fractions were pooled together and concentrated by PEG 20,000 and loaded on column of DEAE-Sephadex A-50 (2.5 × 16 cm). This was eluted with linear gradient of NaCl (0 @ 0.5M) at a flow rate of 0.5 ml/min. The size of each fraction was 5 ml. The activity and protein content of each fraction was determined. Active fractions of each peak were pooled separately and concentrated against PEG 20,000 and applied on column of Sephadex G-100 (1.1 × 55 cm, flow rate 0.2 ml/min and fraction size of 2 ml). The fractions containing glucoamylase I activity were pooled, concentrated and used for electrophoresis as well as chemical modification.

**Electrophoresis.** Non-denaturing, discontinuous electrophoresis was performed by method of Davis [9] in 3.75% stacking and 7.5% separating gel at pH 8.3 (Tris-glycine buffer). The electrophoregrams were stained by Coomassie brilliant blue R-250 for protein and Zaccarius method for glycoprotein [10]. The activity of glucoamylase on electrophoregram was shown by incubating the gel in 0.1% starch solution. Gel in starch solution was incubated

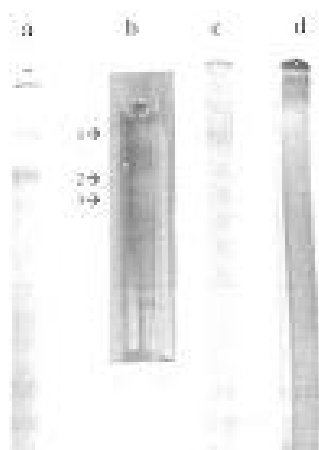
at 37° C overnight. Then, gel was washed with distilled water and stained with 0.01 N iodine solution in order to show the zone of hydrolysis.

**Raw starch activity.** Agar solution 2% in acetate buffer was prepared (helped dissolution of agar in buffer) and to this starch was added to give final concentration of 0.5%. The above solution was poured into Petri dish and allowed to solidify. Plates were punched and to each well 0.1 ml of concentrated glucoamylase solution were added. The plates were kept at 37°C overnight and then the zone of hydrolysis was visualized by 0.01 N iodine solution.

**Effect of group specific reagents on glu I.** The stock solutions of NEM, iodoacetamide, PALP and NBS, at 10 mM concentration were prepared, DEP at 150 mM, HgCl<sub>2</sub> at 50 mM and acarbose at 100 mM concentrations were made. The reaction mixtures were incubated at room temperature for 10 min. The residual activity of glucoamylase was measured using starch as substrate as described in the materials and methods.

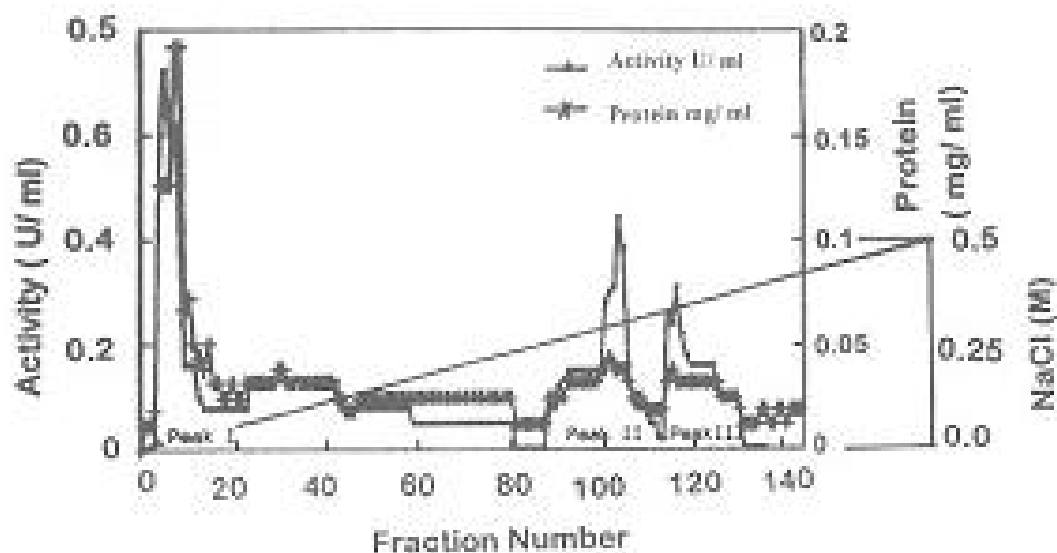
## RESULTS AND DISCUSSION

The fermentation of *Arthrobotrys amerospora* was carried out for 6 days to produce glucoamylase. The crude broth was obtained as described in the materials and methods. Broth containing glucoamylase was subjected to analytical polyacrylamide gel electrophoresis (PAGE) using 7.5 % separating gel, on native PAGE three bands were identified as having enzyme activity. According to their electrophoretic mobility, they were named as glu I, II and III (Fig. 1a and 1b). All the three glucoamylases were found to be glycoprotein. Crude glucoamylase was precipitated by 75% saturated ammonium sulfate solution and then desalted on column of Sephadex G-25. Summary of purification is given in Table 1 and Figure 2. The purity of major glucoamylase *i.e.* glu I was checked by native PAGE (Fig. 1c and 1d). There are a number of reports indicating that glucoamylases from various fungi are in multiple forms [11-14]. The major glucoamylase I was used to study the effect of chemical probes on the enzyme molecule and efforts were made to conclude the



**Fig. 1.** (a) Electrophoregram of crude glucoamylase on native 7.5% PAGE. (b) Activity of crude glucoamylase on electrophoregram, 1- glu I, 2- glu II, 3- glu III. (c) Purified glu I stained by Coomassie blue. (d) Activity of purified glu I, using 0.1% (w/v) starch solution prepared in acetate buffer pH 5.6.

presence of probable amino acid residues at or near the active site of glu I. Group specific reagents were used in order to rule out the presence of amino acid at or near the active site of glu I. [15]. When glu I was reacted with sulphydryl reagents like iodoacetamide, NEM and  $\text{Hg}^{2+}$  at specific pH, no changes in enzyme activity were observed. NEM is highly specific reagents for sulphydryl groups present in proteins at pH 6-7 but at higher pH values the reaction with amino group becomes significant [16]. This clearly rules out the absence of cysteine in the involvement of enzyme-substrate complex formation. Glu I reacted with iodoacetamide at pH 8, at this pH the concentration of thiolate anion becomes large enough and then cysteine is the most reactive amino acid whereas at low pH methionine is the most reactive group.



**Fig. 2.** Fractionation of crude glucoamylase by DEAE-Sephadex A-50.

**Table 1.** Summary of purification of glucoamylase produced by *Arthrobotrys amerospora* ATCC 34468.

Purification steps	Total unit	Total protein (mg)	Specific activity (u/mg)	Fold purification	% Recovery
Culture broth	126	79.50	1.58	1.0	100
75% $(\text{NH}_4)_2\text{SO}_4$	118	21.70	5.50	3.5	94
Sephadex G-25	88	16.48	5.30	3.2	70
DEAE-Sephadex A-50					
Peak I (Glu I)	20	1.22	16.00	10.3	16
Peak II (Glu II)	9	0.50	18.00	11.4	7
Peak III (Glu III)	5.75	0.37	15.30	9.7	4.5
Sephadex G-100					
Peak I (Glu I)	7.50	0.20	37.50	24	6
Peak II (Glu II)	3.40	0.12	28.30	18	2.6
Peak III (Glu III)	4.06	0.14	29.00	18	3.2

**Table 2.** Effect of group specific reagents on glucoamylase I activity: The experiments were run in duplicate.

Group or amino acid	Reagent used	Final conc. mM	pH*	% Residual activity
Sulphydryl/Cysteine	NEM	0.400	6.0	100
	Iodoacetamide	0.125	8.0	100
Lysine	PALP	0.100	8.0	100
Histidine	DEP	30.00	7.0	100
	Rose Bengal	10m g/ml	7.0	100
Tryptophan	NBS	0.350	8.0	0.0

\*pH at which the reagent is specific

Table 2 shows the specificity of each group reagent at particular environmental condition. By proving the absence of cystein residue in the involvement of enzyme-substrate complex formation, *N*-bromo-succinimide at various concentrations was used, because in the absence of sulphydryl groups, NBS is relatively specific for tryptophan. NBS at 0.35 mM concentration totally inhibited the enzyme activity (Table 3).

**Table 3.** Effect of NBS at various concentrations on purified glucoamylase I activity. A= control, given figures are final concentration of NBS in assay mixture.

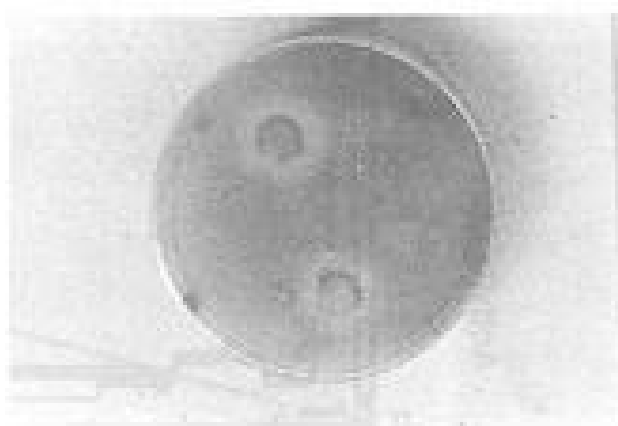
Tube no.	Conc. of NBS (mM)	% Residual activity
A	0.00	100
B	0.17	45
C	0.25	18
D	0.3	10
E	0.35	0.0

**Table 4.** Substrate (starch) protection of glucoamylase I against NBS action.

Tube	Conc. of NBS (mM)	%Residual activity
A	Enzyme + substrate + NBS (0.35 mM)	100
B	Enzyme + substrate	100
C	Enzyme + NBS	0.00

Protection by substrates or related compounds has been used to selectively prevent the modification of amino acid residues at active center of many enzymes. Glu I was reacted with starch at 0°C for 10 minutes then NBS was added in a way to give the final concentration of 0.35 mM in assay mixture. If enzymatic activity is retained following modification in the presence of substrate but is lost in its absence, it is usually said that group in the active site has

been protected by substrate (Table 4). In this way, NBS has modified the indol ring of tryptophanyl residue present in or near the active site of glu I, to oxindol derivatives. Finally, acarbose at 5 mM concentration fully inhibited glu I activity. Acarbose is a substrate analogue and is a pseudo-tetrasaccharide. Svenson *et al.* [17] showed acarbose strongly and reversibly bound to glu I & II of *Aspergillus niger*. They also showed that glu I and II of *Aspergillus niger* contained two tryptophanyl residues which were responsible for raw starch activity. Figure 3 shows raw starch activity of glu I produced by *Arthrobotrys amerospora*.

**Fig. 3.** Raw starch activity of purified glu I on starch agar plate.

Our findings are on the similar line of Svenson *et al.* Therefore, tryptophanyl residue can be present at or near the active site of glu I produced by a nematophagus fungus *Arthrobotrys amerospora*. Therefore this organism may further be used to produce glucose from complex polysaccharide like starch.

## REFERENCES

1. Mankau, R. (1980) Biocontrol: Fungi as nematode control agents. *J. Nematology* 12 (4): 244-252.
2. Schenck, S., Chase, T.J.R., Rosenweig, W.D. and Pramer, D. (1980) Collagenase production by nematod trapping fungi. *J. Appl. Environ. Microbiol.* 40 (3): 567-570.
3. Rosenweig, W.D. and Pramer, D. (1980) Influence of cadmium, zinc & lead on growth, trap formation and collagenase activity of nematode trapping fungi. *J. Appl. Environ. Microbiol.* 40 (4): 694-696.
4. Norouzian, D. and Jaffar, M.B. (1993) Immobilization of glucoamylase of *Arthrobotrys amerospora*. *Indian. J. Exp. Biol.* 31: 680-681.
5. Bhumibhamon, O. (1986) Glucoamylase of fungus isolated from a rotting cassava tuber. *MIRCEN J.* 4: 473 - 482.
6. Yushiki, T., Vichupora, V. and Jaroon, K. (1986) Raw cassava starch digestion by glucoamylase of *Aspergillus* species N<sub>2</sub> isolated from cassava chips. *J. Ferment. Technol.* 64 (5): 405-410.
7. Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for detection of reducing sugar. *Anal. Chem.* 31: 427-431.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.I. (1951) Protein measurment with the Folin-Phenol reagent. *J. Biochem.* 193: 263-275.
9. Davis, B.J. (1964) Disc electrophoresis II, method and application to human serum protein. *Annal. NY. Acad. sci.* 121: 404-427.
10. Zaccharius, R.M. (1969) Glycoprotein staining by Schiff's reagent. *Anal. Biochem.* 1: 409-425.
11. Manjunath, P. and Rao, M.R.R. (1979) Comparative studies on glucoamylase from three fungal sources. *J. Bioscience* 1:409-425.
12. Manjunath, P. and Raghavandra, M.R.R. (1983) Fangal glucoamylase. *J. Appl. Biochem.* 5: 235-260.
13. Venkataraman, K., Manjunath, P. and Raghavandra, M.R.R. (1975) Glucoamylases of *Aspergillus niger* NRRL 330. *Ind. J. Biochem. Biophys.* 12:107-114.
14. Ueda, S. (1981) Fungal glucoamylases and raw starch digestion. *T.I.B.S. (March)* 89-90.
15. Zaime Eyzaguire (1987) *Chemical modification of enzyme: Active site studies* .John Wiley & Sons, NewYork. pp. 5-32.
16. Kyte, J. (1995) *Structure protein chemistry: Chemical probes*. Garland publishing Inc. New York and London.
17. Svenson, B., Kjeld, Larsen., and Alp., Gunnarsson. (1986) Characterization of glucoamylase GII from *Aspergillus niger*. *Eur. J. Biochem.* 154: 497-502.