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ISOLATION AND PURIFICATION OF GLUCOAMYLASES FROM *ARACHNIOTUS CITRINUS* UNDER SOLID PHASE GROWTH CONDITIONS

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ABSTRACT

Arachniotus citrinus, a fungal strain, was grown for 30 days at 30°C, pH 6 under solid-state growth conditions on wheat bran as carbon source for the production of glucoamylases (glucano glucohydrolases). Specific activity of crude enzyme was 13.26 units mg^{-1}. The enzyme was purified using Fast Protein Liquid Chromatography (FPLC) unit and four-step purification procedure, comprised of ammonium sulfate precipitation, Hiload anion-exchange, hydrophobic interaction and Mono-Q anion-exchange chromatography was used. The onset of glucoamylases precipitation occurred at 60% and completed at 75% saturation of ammonium sulfate at 0°C. The purification after ammonium sulfate precipitation was 2.48-fold and the recovery was 67%. While, recovery after Hiload chromatography was 50% and their purity reached to about 20-fold. During hydrophobic interaction chromatography (HIC) glucoamylases were eluted at 429 mM ammonium sulfate and were 52-fold purified with respect to crude. The recovery of dialyzed glucoamylases after HIC was 33%. Glucoamylases after HIC were further purified to 63-fold on MonoQ column with a recovery of about 33% and their specific activity was 839.1 U mg^{-1} protein. The glucoamylase was monomeric in nature because its native molecular mass (87 kDa) determined on Gel filtration, and the subunit mass (88 kDa) determined on 10% SDS-PAGE were the same.

Key Words: *Arachniotus citrinus*, wheat bran, glucoamylases, purification, molecular weight.

INTRODUCTION

Glucoamylases [(Exo-1,4 alpha-D-glucan glucanohydrolase) (E.C.3.2.1.3)] are classified in the family 15 of hydrolases. Involvement of glucoamylases during industrial bioprocesses is advantageous as they use α-inverting reaction mechanism. In this way, glucoamylases are inverting exo-acting starch hydrolases releasing glucose from non-reducing ends of starch and related substrates, until all the high molecular weight carbohydrate is degraded to glucose (Sauer, *et al.*, 2000).

Starch, a polymer of α-D glucose is the commonest storage carbohydrate in plants. The microbes and higher organisms also use it, consequently, there is a great diversity of enzymes able to catalyze its hydrolysis. For human consumption, it is splitted into its component units. For various industrial processing, acid hydrolysis of starch has been the conventional method. Enzymic processes now largely replace this.

Starch from almost all natural sources is a mixture of amyloses and amylopectins. Of the two components of starch, amylopectin presents the great challenge to hydrolytic enzyme systems. This is due to the residues involved in α-1, 6-glycosidic branch points, which constitute about 4-6% of the glucose present. Most hydrolytic enzymes are specific for α-1, 4-glucosidic links yet the α-1, 6-glucosidic links must also be cleaved for complete hydrolysis of amylopectin to glucose.

The diversity in occurrence and characteristics make the search of a novel glucoamylase an imperative for its industrial application. All the glucoamylases used in food and beverage industries of Pakistan are being imported, which is a source of additional burden on already lean economy. Furthermore, non-traditional use of glucoamylases in detergent industry has a great scope of expansion the world over in the coming years.

Glucoamylases play a tremendous role in the improvement of starch based food products. There is hardly any report on glucoamylases from the genus *Arachniotus*. Therefore, the present study was planed to isolate and purify the glucoamylases from a novel fungal strain *A. citrinus* for their possible industrial application.

MATERIALS AND METHODS

The culture of *A. citrinus* was maintained on malt extract peptone agar slants, which were prepared as described by DSM-catalogue of strains 1989 (method 90).

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Inoculum Preparation

Inoculum was prepared as described by Iqbal *et al*., (1991).

Growth of *Arachniotus citrinus*

Arachniotus citrinus was grown under solid-state growth conditions. Fine particle wheat bran (80 mesh size) was obtained from flour Mill. About 60 conical flasks of 250 ml capacity containing 15 grams of wheat bran as carbon source were soaked with 30 ml of distilled water. The flasks were plugged with cotton, covered with aluminium foil and autoclaved at 121°C (1.1 Kg cm^{-2}) for 20 minutes. 4 ml of inoculum was sprinkled aseptically on the carbon source containing flasks and incubated at 30°C for one month to produce glucoamylases. Thick fungal mat covered the whole carbon source on fourth day.

Isolation of Glucoamylases

After one month of growth 15 ml of distilled water was added to each flask, mixed and shaken on an orbital shaker at 100 rpm for 1 h. Soluble contents were extracted by squeezing the mixture through the muslin cloth and centrifuged at 15,600×*g* at 4°C for 30 min. Total proteins and glucoamylases activity in the crude extract were determined.

Crude glucoamylases were again centrifuged at 39,200×*g* at 4°C to increase the clarity and concentrated by freeze-drying. The concentrate was dialyzed to remove the soluble reagents using 15 kDa cut off cellulosic dialysis tubing. The dialyzed enzyme concentrate was further processed for purification.

Protein Estimation

Proteins were estimated by Bradford assay and bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

Glucoamylase Assay

Two ml of 0.5% (w/v) soluble starch in 50 mM MES monohydrate buffer pH 5.5 was taken in each of two test tubes. 100 µl of enzyme extract was added to one of these test tubes. Moreover, the enzyme blank was also made by adding 100 µl of enzyme in 2 ml of buffer. The reaction mixture and the substrate and enzyme blanks were incubated at 40°C for 40 min. with constant shaking at 60 rpm. The reaction was quenched promptly by placing the reaction mixture in boiling water for 5 min. Placing the test tubes in ice-cold water-cooled the quenched mixture.

The released glucose was determined by using glucose-measuring kit (Diasys; Germany) according to the manufacturer's instructions. Briefly, varying amounts of reaction mixture and substrate blank were added separately to 1ml of glucose kit and reaction was allowed to proceed for 10 min. at 37°C in a water bath**.** Finally the absorbance was measured at 500 nm. Values of blanks (substrate and enzyme) were subtracted from the experimental to determine the units of glucoamylases activity.

One unit of glucoamylase activity was the µ□moles of glucose equivalents liberated per min per ml at 40°C pH 5.5.

Ammonium Sulphate Precipitation

Different amounts of solid ammonium sulphate were added separately to one ml of crude dialyzed glucoamylase concentrate in eppendorf tubes to get 10-90% saturation at 0°C and vertimixed. These tubes were left over night at 4°C and centrifuged at 12,000 rpm for 15 min. The supernatant were assayed for remaining glucoamylase activity.

After optimization of ammonium sulphate precipitation, the crude enzyme concentrate was placed in ice and solid ammonium sulphate was dissolved bit by bit to attain initially 60% saturation at 0°C and left at 4°C for overnight. Then it was centrifuged at 18,000 rpm (39,200×*g*) for 30 min. at 4°C. The pellet of precipitated protein was discarded. In supernatant more solid ammonium sulphate was added to attain 75% saturation at 0°C. It was again kept overnight at 4°C and centrifuged as previously. This time pellet was kept and supernatant was discarded. The pellet was dissolved in distilled water and dialyzed against distilled water for 24 h with 4 changes of equal

intervals to remove ammonium sulphate. Total proteins and glucoamylase activity was determined before and after dialysis of precipitated glucoamylases and finally freeze-dried.

Fast Protein Liquid Chromatography (FPLC)

The partially purified and dialyzed glucoamylases after ammonium sulphate precipitation were subjected serially to FPLC for anion-exchange chromatography on Hiload Q-sepharose column, hydrophobic-interaction chromatography on phenyl superose column and anion exchange chromatography on Mono-Q column as described (Deutscher, 1990, Rashid, 1997).

Hiload Anion-exchange Chromatography

The glucoamylases after ammonium sulphate precipitation were loaded on Hiload-Q sepharose column, using superloop of 50 ml with flow rate of 2 ml per minute. The linear gradient of NaCl (0-1 M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer. 4 ml fractions were collected. The fractions containing glucoamylases were pooled, dialyzed against distilled water and partially lyophilised for reduction of volume. Total enzyme activity and proteins were estimated.

Hydrophobic-interaction Chromatography

Active fractions from Hiload column having 2M-ammonium sulphate were loaded on phenyl superose column at a flow rate of 0.5 ml per minute. The elution was carried out with a linear gradient of ammonium sulphate (2-0M) in 50 mM phosphate buffer pH 7.0. Active fractions of 2.5 ml size were pooled, dialyzed and total glucoamylase activity and proteins were estimated.

Mono-Q Anion-exchange Chromatography

The purified glucoamylases from HIC after dialysis were loaded to Mono-Q column at a flow rate of 1 ml per minute and a linear gradient of NaCl (0-1M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer. 2 ml size fractions were collected. Active fractions were pooled, dialyzed, assayed for total enzyme activity and proteins.

Native Molecular Weight

The glucoamylases and different protein standard markers were loaded on Superose Gel filtration column to determine native molecular weight as described by Rashid (1997). The 200 µl/run of protein samples was loaded and 100 mM Tris/HCl, pH 7 having 0.15 M NaCl was used as elution buffer. The flow rate was 0.5 ml/minute. 1 ml size fractions were collected**.**

Subunit Molecular Weight

Sub-unit molecular weight of glucoamylase was determined by using 10% Sodium Dodecyl Sulphate Denaturing Renaturing Polyacrylamide Gel Electrophoresis (SDS-DR-PAGE) and the PAGE was performed as described by Laemmli (1970). The glucoamylases (5 mg ml^{-1}) were mixed in SDS sample buffer containing β-mercaptoethanol and heated at 40°C for 60 min for appropriate binding of SDS to enzyme. The 10-kDa-protein markers ladder (Gibco BRL) was used as standard for SDS-PAGE. The ladder consisted of 12 bands ranging from 10-120 kDa with an increment of 10 kDa plus a 200-kDa band. Moreover, for easy orientation within the ladder, 50-kDa-band was made more intense. The protein markers were applied directly to SDS-polyacrylamide gel after slight warming. The SDS gel was prepared using Hoefer's Mighty Small SE 245 Dual Gel Caster. The PAGE was carried out at a constant voltage of 100 volts and stopped when tracking dye approached to bottom.

The part of gel containing the glucoamylases were separated from the protein standard lane and a cut was applied on the lower side of gel to illustrate the direction of electrophoresis. The gels were treated with 20% (v/v) isopropyl alcohol in 50 mM sodium acetate buffer, pH 5.0 to remove SDS and three washes of 15 minutes each were given. Then the gels were immersed in 50 mM sodium acetate buffer, pH 5 to remove isopropyl alcohol. Three changes of 30 min. each were given.

Staining of Proteins

The polyacrylamide gel containing protein standards was stained with Coomassie blue G-250 fast stain (Merril, 1990) and the gel was soaked in stain and protein bands were observed after ten minutes. The stained gel was rinsed in water to improve the intensity of bands. Gels can be left in the stain for hours with no increase in the background (Coughlan, 1988). Plotting R_f values of marker protein bands Vs their log molecular weights for more precise subunit molecular weight evaluation drew standard curve.

Glucoamylase Activity Staining

Agarose overlay gel (0.8% w/v) was prepared by dissolving 0.16 g agarose in 20 ml 50mM sodium acetate buffer pH 5.0, with constant shaking and warming. Then 0.1 g soluble starch (0.5% w/v) was added to the warmed gel solution, which was further heated to get a clear solution. Finally the pH was adjusted to 5.0 and gel solution was poured into the mould.

The part of polyacrylamide gel containing glucoamylases after removing SDS was overlaid on soluble starch containing agarose gel and incubated at 45°C for appropriate time. The agarose overlay gel was treated with iodine solution for activity band visualization. Colourless band of activity was found in dark blue background. The R_f value for glucoamylase band was calculated and sub unit molecular weight was determined from the standard curve.

RESULTS AND DISCUSSION

Production of Glucoamylases

Solid state fermentation systems offer several economical and practical advantages such as: higher product concentration, improved product recovery, very simple cultivation equipment, reduced waste water output, lower capital investment and lower plant operation costs (Muniswaran *et al*., 1994; Shankarnand *et al*., 1992; Pandey, 1995; Becerra *et al*., 1996). Therefore, *A. citrinus* a mesophillic fungal strain was grown under solid-state fermentation conditions at 30°C. The specific activity of glucoamylases after 30 days was 13.26 units mg^{-1} protein (Table 1). The crude enzyme was dialyzed against distilled water prior to purification. The *Sclerotium rolfsii* yielded 8.33 U mg^{-1} of glucoamylase (Kelkar and Deshpande, 1993). There is no report regarding the production, purification and characterization of glucoamylases from *A. citrinus*.

Purification of Glucoamylases

Crude enzyme was purified to homogeneity after subjecting it to ammonium sulphate precipitation, hiload anion exchange, hydrophobic interaction and mono-Q anion exchange chromatography on Pharmacia's FPLC unit. The four-step purification procedure for glucoamylase resulted into 63.28-fold purification (Table 1). The recovery of purified glucoamylases was 32.6%. The glucoamylases from *Monascus purpureus* have been purified up to 25.3 fold using five-step purification procedure (El-Sayed *et al*., 2000).

Ammonium Sulphate Precipitation

The onset of glucoamylase precipitation occurred at 60%, while complete precipitation was at 75% saturation of ammonium sulphate at 0°C (Fig. 1). The glucoamylases from *Monascus purpureus* has been found to be precipitated completely at 50% ammonium sulphate saturation at 0°C (El-Sayed *et al*., 2000). The ammonium sulphate inhibited glucoamylases activity because after the removal of salt by dialysis, its purification factor and % recovery were increased to 2.48 and 67, respectively (Table 1)

Hiload Anion Exchange Chromatography

Partially purified glucoamylases after ammonium sulphate precipitation were further purified by subjecting them to FPLC Hiload anion exchange chromatography. They elute at about 700 mM NaCl, which indicated that glucoamylases were highly acidic in nature (Fig. 2). While, purification was about 20-fold and their recovery was 50% after Hiload anion exchange chromatography. Glucoamylases, like other glucanases were not inhibited by sodium chloride (Rashid, 1997; Siddiqui *et al.*, 1997) because after dialyzing against water, total activity remained almost the same (Table 1).

Hydrophobic Interaction Chromatography

Purified glucoamylases, after Hiload column, were further purified by applying them on phenyl superose. The elution of glucoamylases was maximum at about 429 mM ammonium sulphate (Fig. 3). The glucoamylases were 52- and 2.52-fold purified with respect to crude and Hiload column, respectively (Table 1).

Mono-Q Anion Exchange Chromatography

The purified glucoamylases from HIC were further subjected to FPLC Mono-Q anion exchange column to get purity to homogeneity level. The glucoamylases showed a 63.28 fold increase in specific activity with respect to crude enzyme and elute just before the onset of sodium chloride gradient (Fig. 4). The recovery of glucoamylases after Mono-Q was 32.6% (Table 1).

Table 1. Purification of glucoamylases from *Arachniotus citrinus*.

Treatment	Total units	Total protein (mg)	Specific activity (units/mg)	Purification factor	% Recovery
Crude	5200*	392*	13.26*	1.00*	100*
$(NH_4)_2SO_4$ precipitation.	2550	102	25.00	1.09	49
	3488*	106	32.90*	2.48*	67*
Q-sepharose Hiload anion	2582	10.0	252.20	19	50
exchange chromatography	2595*	9.8*	264.79*	20*	50*
Phenyl superose:	1188	2.04	582.51	44	29
hydrophobic interaction chromatography	1710*	2.52*	687.57*	52*	33*
Mono-Q: anion exchange	1681	1.99	844.7	63.70	20
chromatography	1695*	2.02*	839.1*	63.28*	33*

Denotes values after dialysis against distilled water.

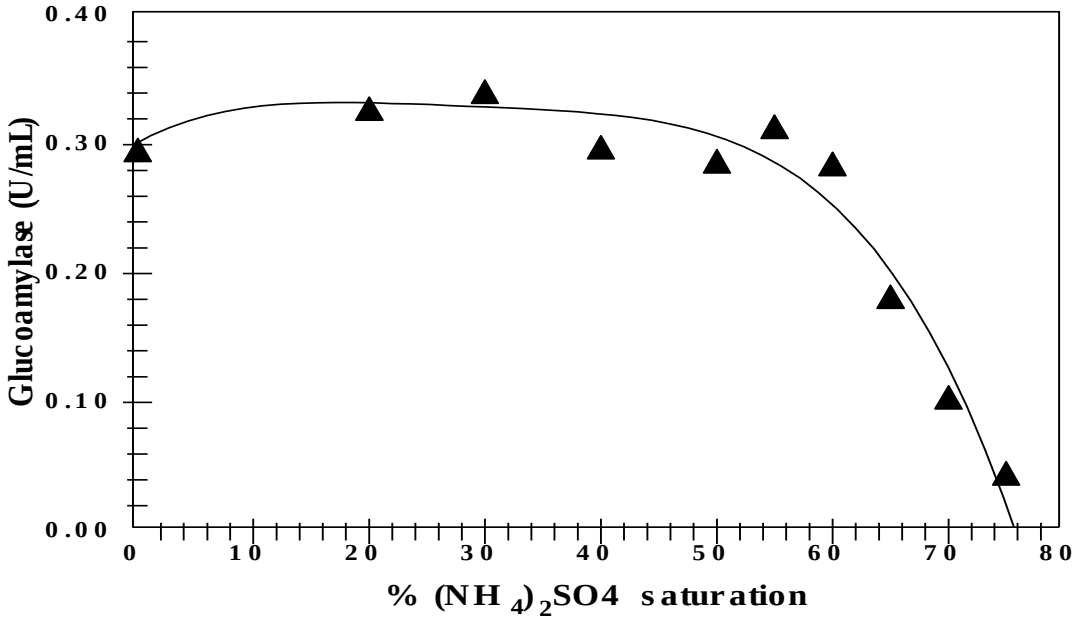

Fig.1. Ammonium sulphate precipitation of glucoamylases.

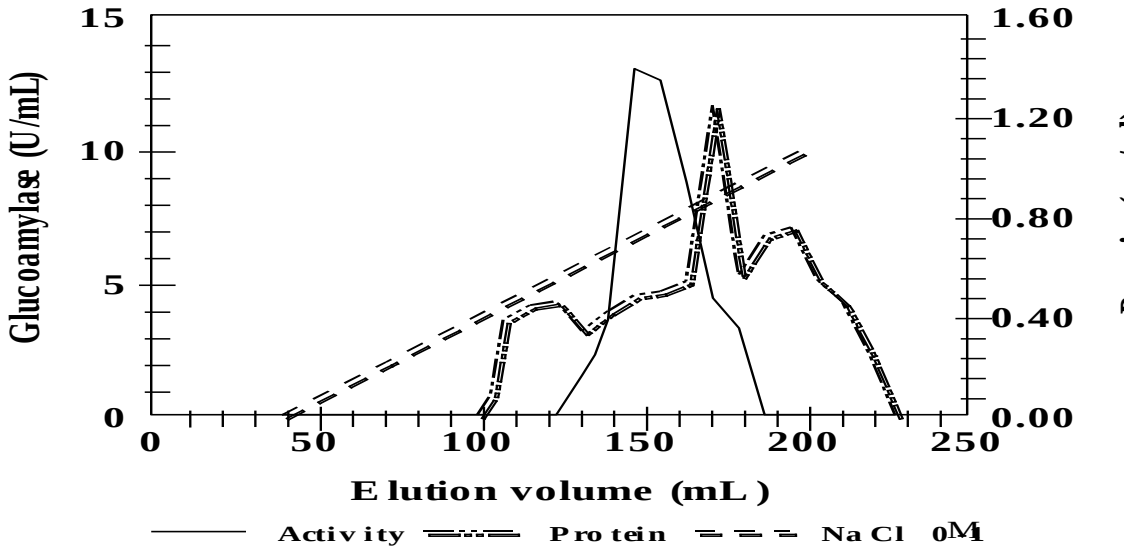

Fig.2. Hiload Anion Exchange Chromatography of glucoamylases on FPLC.

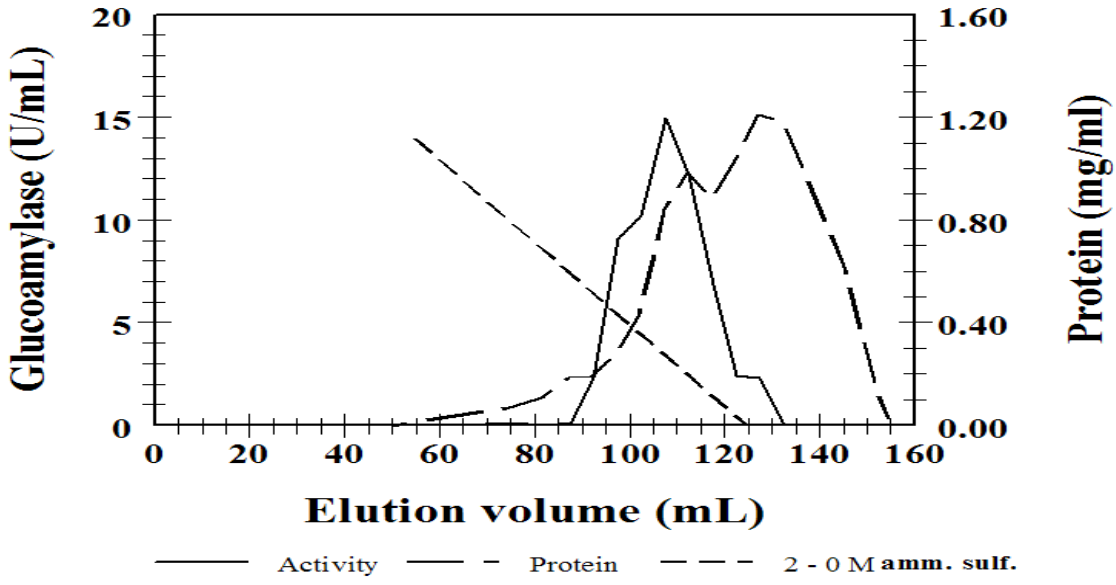

Fig.3. Hydrophobic Interaction Chromatography of glucoamylases on FPLC.

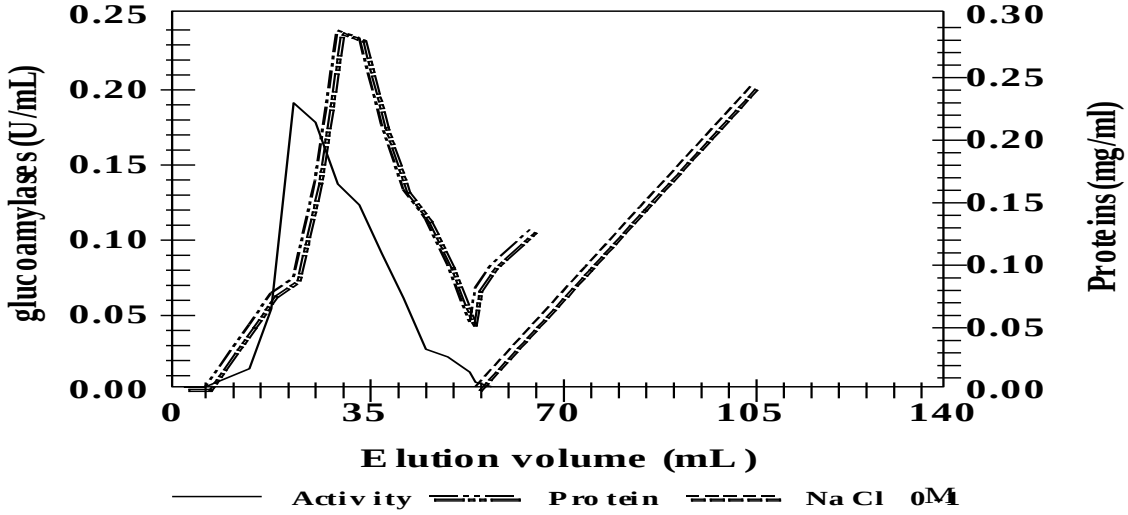

Fig.4. Mono-Q Anion Exchange Chromatography of glucoamylases on FPLC.

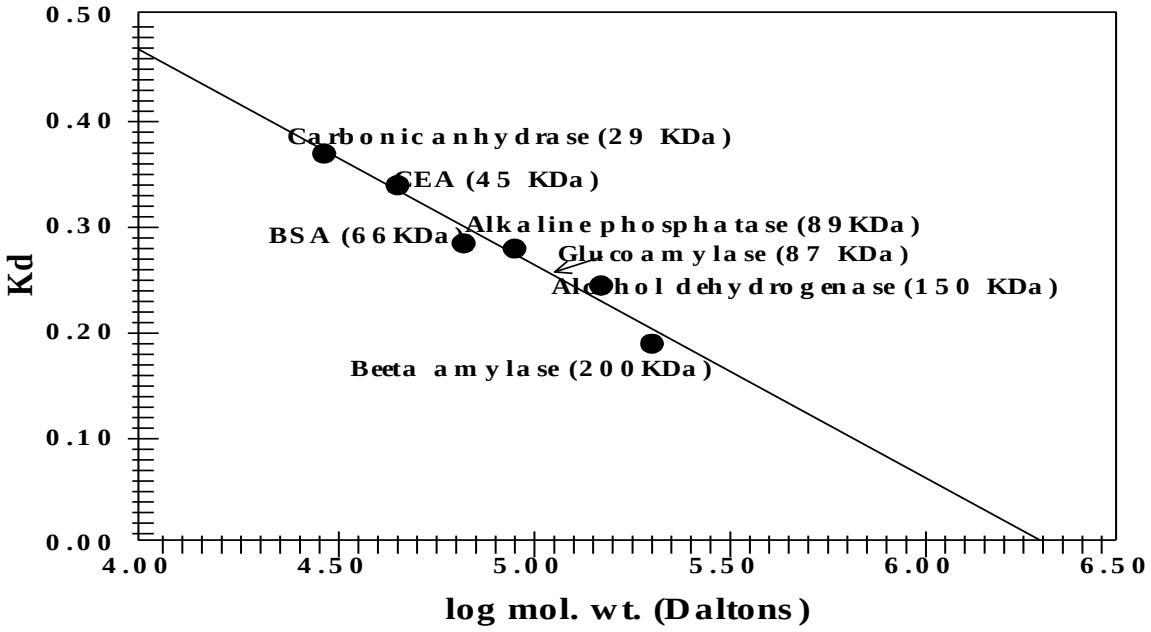

Fig.5. Standard curve for native molecular weight determination of glucoamylases on FPLC Gelfiltration Chromatography.

Molecular Weight of Glucoamylases

Native molecular weight of gluoamylases from *A. citrinus* determined on gel filtration column of Pharmacia's FPLC unit was 87 kDa (Fig. 5). The glucoamylase was monomeric in nature because subunit molecular weight (88 kDa), which was determined on 10% SDS-DR-PAGE, using standard curve was also equal to native molecular weight (Fig. 6 A, B).

The molecular weights of glucoamylases have been evaluated by number of workers from variety of microbes. Kelkar and Deshpande (1993) found that glucoamylases from *S. rolfsii* were monomeric in nature. Its SDS molecular weight was 64 kDa, while native weight on gel filtration was 66 kDa. On the other hand, Marlida *et al*. (2000) reported glucamylase from *Acremonium* sp. comprised of two subunits (22 and 39kDa). Glucoamylases produced by *Scytalidium thermophilum* showed molecular weights 75 kDa on SDS-PAGE and 60 kDa on gel filtration (Acquino *et al.*, 2001).

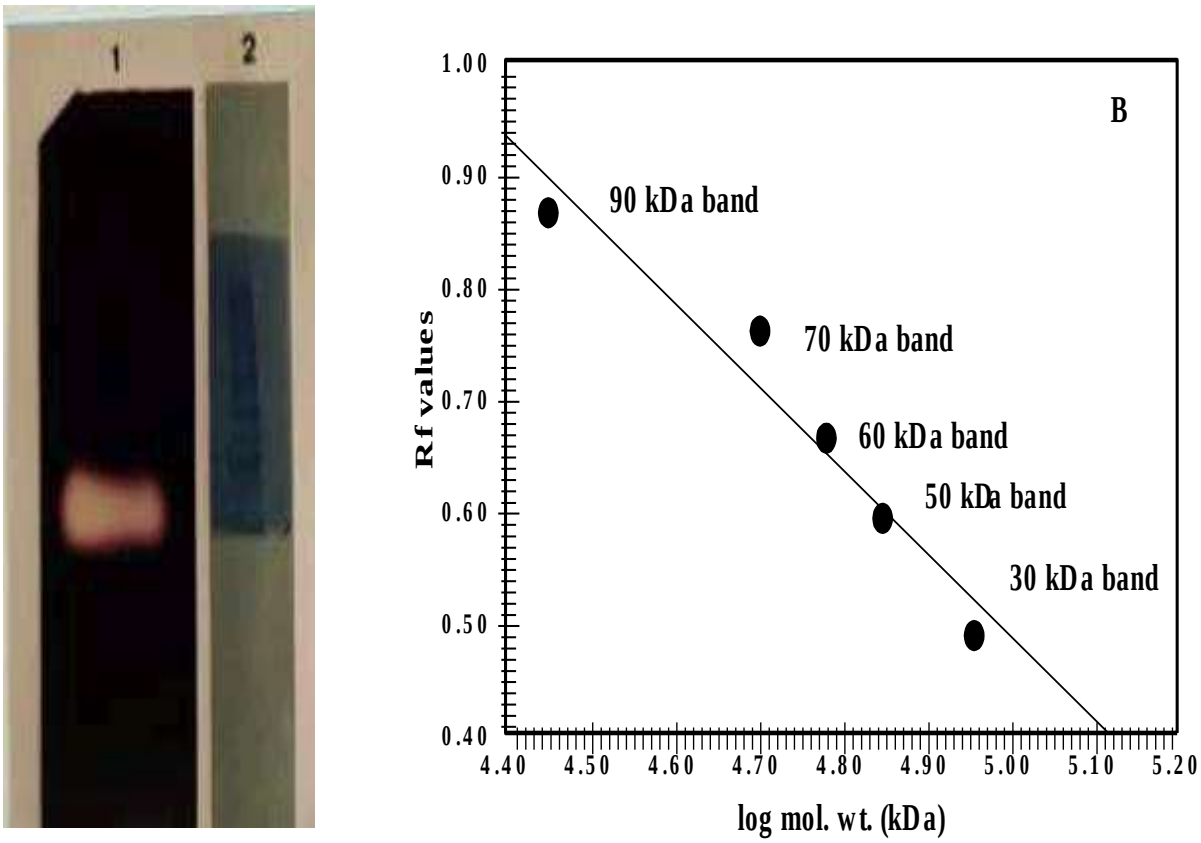

Fig-6. (A) 10% SDS-DR-PAGE for subunit molecular weight determination: lane-1, glucoamylase activity staining, lane-2, Gibco BRL protein markers (B) Standard curve for Subunit molecular weight determination.

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REFERENCES

Acquino, A.C.M.M., J.A. Jorge, H.F. Terenzi and M.L.T.M. Poliziv (2001). Thermostable glucose-tolerant glucoamylase produced by the thermophilic fungus *Scytalidium thermophilum*. *Folia Microbiologica*, 46: 11-16.

Becerra, M. and M.I.G., Siso (1996). Yeast ß-galactosidase in solid-state fermentation. *Enzyme Microb. Technol*., 19**:**39-44.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal. Biochem*., 72**:** 248-254.

Coughlan, M.P. (1988). Staining techniques for the detection of the individual components of cellulolytic enzyme system. *Methods in Enzymology*, 160: 135-144.

Deutscher, M.P. (1990). *Guide to protein purification. Methods in Enzymology*, Academic Press San Deigo. 182

DSM-Deushe Sammlung Von Mikroorganism und Zullkurturen GmbH (German Collection of microorganisms and cell cultures.) 1989. *Catalogue of strains. 4th Edition*. D-3300 Braunschweig, Federal Republic of Germany. p 286.

El-Sayed, S. M., S. A. El-Aassar and D. I. Adel Maguid (2000). Leaching, purification and some properties of a glucoamylase from solid state cultures of *Monascus purpureus* ATCC 16437. *African J. Myco. and Biotech*., 8: 1-18.

Iqbal, J.J., M.I. Rajoka and K.A. Malik (1991). Production of a thermostable β-glucosidase by a mesophilic fungus *Aspergillus niger* NIAB-280, In: *Biotechnology for Energy.* K.A. Malik, S.H.M. Naqvi and M.I.H. Aleem, (eds). pp.157-165, NIAB/NIBGE, Faisalabad, Pakistan.

Kelkar, H. S. and M. V. Deshpande (1993). Purification and characterization of a pullulan hydrolyzing glucoamylase from *Sclerotium rolfsii. Starch Strake*, 45: 361-368.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage. *Nature*, 227: 680-685.

Marlida, Y., N. Saari, Z. Hussain, S. Radu and J. Baker (2000). Purification and characterization of sago-dagrading glucoamylase from *Acremonium* sp. Indophylic fungus. *Food Chemistry*, 71: 221-227.

Merril, C.R. (1990). Gel staining techniques. In*: Methods in Enzymology*. 182: 477-488, Academic Press, San Diego.

Muniswarran, P.K.A., P. Selvakumar and N.C.L.N. Charyulu (1994). Production of glucoamylases from coconut coir pith in solid-state fermentation. *Chem. Technol. Bioechnol*., 60: 147-15.

Pandey A. (1995). Glucoamylase research: An overview. *Starch Starke*, 47 : 439-445.

Rashid, M.H. (1997). *Purification and characterization of native, proteolytically nicked and chemically modified ß-glucosidase from Aspergillus niger*. Ph.D thesis, University of the Punjab, Lahore, Pakistan.

Sauer J., B.W. Sigurskjold, U. Christensen, T.P. Frandsen, E. Mirgorodskaya, M. Harrison, R. Roepstorff and B. Svensson (2000). Glucoamylase: Structure/function relationship and protein engineering. *Biochimica et Biophysica Acta –Protein Structure and Enzymology*. 1543: 275-293.

Shankaranand, V.S., M.V. Ramesh and B.K Lonsane (1992). Idiosyncrasies of solid state fermentation system in the biosynthesis of metabolites by some bacterial and fungal cultures. *Process Biochem*., 27**:** 33-36.

Siddiqui, K.S., M.J Azhar, M.H. Rashid and M.I. Rajoka (1997). Stability and identification of Active site residues of carboxymethyl glucoamylase from *Aspergillus niger* and *Cellulomonas biazotea. Folia Microbiol*., 42: 312-318.

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