PURIFICATION AND CHARACTERIZATION OF GLUCOAMYLASE FROM *Aspergillus niger* ATCC 10864.

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ABSTRACT

The *Aspergillus niger* ATCC 10864 produced substantial amount of glucoamylase activity when grown on rice bran as carbon source. The glucoamylase was purified using a procedure that included 80 % saturated ammonium sulfate precipitation, dialysis, chromatography on Sephacryl S-100 and DEAE-Sepharose. Two fractions of glucoamylase from DEAE-Sepharose (F1a and F2a) were obtained. The purification fold were 26.24 and 19.57 times with 28 and 14.56 percent yields, the specific activities of 842.20 and 628.37 U/mg, and the molecular weight with estimated molecular mass of 80 kDa and 73 kDa, respectively. The optimal pH and temperature of F1a and F2a were 4.0, 60°C and 5.5, 60°C, respectively. F1 was stable at a pH range of 4.0 to 5.5 and at a temperature range of 20 to 50°C, while F2a was stable at a pH range of 4 to 6 and a temperature range of 20 to 40°C. In the presence of rice starch in fresh culture medium, the purified fractions of glucoamylase, F1a and F2a demonstrated apparent K_m and V_{max} values of 10 mg/ml, 0.02 umol/ml/min and 5.6 mg/mL, 0.032 umol/ml/min, respectively, while in the presence of soluble starch, the values of apparent K_m and V_{max} of F1 and F2 were 6.7 mg/ml, 0.02 umol/ml/min and 5.0 mg/ml 0.023 umol/ml/min, respectively.

KEYWORDS: glucoamylase, purification, characterization, Km, Vmax

1. INTRODUCTION

Glucoamylase [1, 4 - α - D – glucan glucohydrolase (E. C. 3.2.1.3)] is an exo-hydrolase that catalyzes and releases β-D-glucose from the non-reducing ends of starch or related oligosaccharides and polysaccharides. Glucoamylase is an important enzyme in the starch processing and ethanol fermentation industries. Most of the glucoamylase are produced by *A. niger* [1]. Isolation and purification of fungal glucoamylase is relatively easy because the enzyme is extracellular and quite stable [2]. In early experiments the glucoamylase of *A. niger* and *Rhizpous delemar* were purified by ammonium sulfate precipitation [2]. Recently, combinations of different procedures such as ammonium sulfate fractionation, gel filtration and ion-exchange chromatography have been successfully applied to the purification of glucoamylase from *A. niger* [3]. We examined purification and characterization of starch degrading glucoamylase from *A. niger* ATCC 10864 in this study in order to study its characterization and application in the following.

2. MATERIALS AND METHODS

2.1 Microorganism and Cultivation

The organism used was a strain of *A. niger* ATCC 10866. For the production of the enzyme, a loopful of actively growing fungal mycelium was transferred from a potato dextrose agar (Sigma) slant to a 250 ml flask containing 70 ml of growth medium. The medium consisted of the following per liter : 20 g rice starch, 10 g peptone, 1.5 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$, 1.0 g NaCl, 0.1 g $CaCl_2.2H_2O$, 7.2 mg $ZnSO_4.7H_2O$, 1.3 mg $CuSO_4.5H_2O$, 0.3 mg $NiCl_2.6H_2O$, 3.5 mg $MnCl_2.4H_2O$ and 6.9 mg $FeSO_4.7H_2O$ (pH 7.0) [4]. The incubation was carried out at 30°C for 4 days at 200 rpm in a rotary shaker. The culture fluid was filtered and centrifuged at 5,000 rpm for 15 min and the supernatant was used as a crude enzyme extract preparation.

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2.2 Purification of glucoamylase.

All steps were carried out at 4°C.

Step 1 : Precipitation with ammonium sulfate

Crude enzyme (100 ml) was treated with solid ammonium sulfate, with continuous stirring and separating into the following saturation ranges : 0-30%, 30-50%, 50-70% and 70-90% [5]. Precipitates were collected after 60 min by centrifugation at 5,000 rpm for 30 min and dissolved in a minimum of 0.05 M sodium acetate buffer pH 4.0. The dialyzed enzyme solution was centrifuged at 5,000 rpm for 30 min. thirty ml of supernatant were then concentrated using ultrafiltration (Amicon) and fitted with cellulose membrane (molecular weight cut-off 10000 dalton) to a volume of 20 ml. The concentrated crude enzyme was filtrated through a 0.45 μm membrane filter.

Step 2 : Gel filtration on Sephacryl S-100 column.

Twenty ml of crude enzyme from step 1 was chromotographed on Sephacryl S-100 column (1.6 x 100 cm) from Amersham Biosciences. The fractions containing glucoamylase activity were pooled and dialyzed with 0.05 M sodium acetate buffer pH 4.0 previously equilibrated with the same buffer solution. The column was eluted by using the same buffer with 0.15 M NaCl. Elution was performed at a flow rate of 0.5 ml/min and fractions of 5 ml were colleced.

Step 3 : Chromatography on DEAE – Sepharose.

The dialyzed enzyme solution was applied to the anion – exchange column (0.7 x 2.5 cm) from Amersham Biosciences which was equilibrated with 0.05 M sodium acetate buffer pH 4.0. The enzyme was eluted from the column with a stepwise gradient of 0 – 0.5 M NaCl dissolved in 0.05 M sodium acetate buffer pH 4.0, at a flow rate of 1 ml/min and a a fraction of 5 ml was collected.

2.3 Enzyme activity and protein assay

Glucoamylase activity was determined by using soluble starch as substrate [6]. The assay mixture consisted of 0.5 ml 1% soluble starch (Sigma), 0.4 ml 0.02 M sodium acetate (pH 4.0) and 0.1 ml of appropriate diluted enzyme. After incubation for 10 min at 60°C, the reaction was stopped by adding 1.0 ml of 1% DNS reagent containing the followings per litre : 10 g 3, 5 – dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite, 200 g potassium sodium tartrate and 500 ml 2% NaOH. The mixture was heated for 5 min in boiling water. After cooling, the total volume of the assay mixture was made up to 6 ml with distilled water and measured the adsorbance of 540 nm using glucose as standard solution. One unit of glucoamylase activity was defined as the amount of enzyme that formed 1 μmol glucose in 1 min.

Protein was measured by using the method of Lowry [7] using Folin-ciocalteu's phenol reagent from Sigma, with bovine serum albumin as standard.

2.4 Characterization of glucoamylase

2.4.1 Optimum and stability pH

The optimum pH was determined by assaying the enzyme activity in the following buffer systems : 0.05 M sodium acetate buffer, pH 3-5.5 ; 0.05 M potassium phosphate buffer, pH 6 - 7 [8]. Enzyme stability at different pH values (3 - 7) was determined by measuring the remaining activity after incubating the enzyme for 30 min at 40°C at pH 3-7. The conditions of the assay were similar to that described above.

2.4.2 Optimum temperature and heat stability

The optimum temperature of enzyme activity was determined by assaying the enzyme activity at different temperatures (20 - 80°C) [5]. Heat stability was measured by incubating the enzyme at the op timum pH for 30 min at different temperatures (20 - 80°C). After the treatment, the enzyme solution w as cooled and the remaining activity was determined under standard assay conditions.

2.4.3 Purity and molecular weight determination

Purity of the isolated enzyme and its molecular weight were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by minigel gel electrophoresis (Amersham Biosciences) and standard proteins with a molecular weight ranging from 14,400– 97,000 daltons (Low Molecular Weight Calibration kit, Amersham Biosciences) were used for calibration and Coomassie Brillant Blue R-250 staining method was used.

2.4.4 Determination of glucoamylase kinetics

The effect of soluble starch (Sigma) and rice starch (Tantawan, Thailand) (1, 2.5, 5 and 10 mg/ml) on the reaction rate were determined at the optimum pH and optimum temperature. The samples were withdrawn at different time intervals (5, 10, 15, 15, 20, 25 and 30 min) for the reducing sugar assay.

The values of the Michaelis constant (Km) and the maximum velocity (Vmax) were determined from Lineweaver-Burk plots.

3. RESULTS AND DISCUSSION

3.1 Purification of glucoamylase

Glucoamylase from the culture filtrate of *A. niger* ATCC 10864 was precipitated by 80% ammonium sulfate precipitation. The Sephacryl S-100 gel filtration column separated the enzyme into two distinct peaks (Figure 1). The major portion of enzyme activity was eluted as a sharp peak at fractions 11 – 22, while the minor portion was eluted at fractions 28 - 33. The presence of two enzyme peaks have also been reported by Fogarty and Benson [5] for thermostable glucoamylase from *A. niger*. Fractions 11 – 22 (F1) and 28 -33 (F2) containing glucoamylase activity were pooled, concentrated by ultratiltration and applied to DEAE Sepharose ion-exchange column which equilibrated with 0.05 M sodium acetate buffer pH 4. The column was developed with a stepwise gradient of 0-0.5 M NaCl in the above buffer solution. The proteins from F1 were resolved into two peaks of which one showed glucoamylase activity. The active fraction eluted at 0.2 M NaCl (F1a and Fig 2a) and from F2 gave one peak of protein and enzyme activity which eluted at 0.2 M NaCl (F2a and Fig 2b). The purification of glucoamylase is summarized in Table 1. Two glucoamylase (F1a and F2a) were purified 26.24 and 19.57 times with a yield of 28 and 14.56%, respectively.

3.2 Characterization of glucoamylase

3.2.1 Optimum pH, temperature and pH, temperature stability

Characteristics of the two purified fractions of glucoamylase, F1a and F2a were as follows : F1a exhibited maximum activity at pH 4 (Figure 3a). At below pH 4 and above pH 4.5 the activity decreased rapidly. The enzyme was stable in a pH range of 4.0 to 5.5 for 30 min incubation at 40°C (Figure 4a). Similar finding have been reported by Lineback and Baumann [9] for glucoamylase from *A. phoenicis*. As shown in Figure 5a, the purified enzyme has optimum temperature of 60 °C and is stable at the temperature range of 20 to 50°C for 30 min (Figure 6a). Forgarty and Benxon [5] reported that the glucoamylase from *A. niger* was thermostable between 20 to 50°C for 30 min, for F2a, the optimum pH and temperature values were 5.5 (figure 3b) and 60°C (Figure 5b). The enzyme showed stability at the pH range of 4.0 to 6.0 after 30 min at 40°C (Fig. 4b). The thermal stability was observed at the range of 20 to 40°C for 30 min (Fig. 6b). Similar findings have been reported by Sadhukhan *et al.* [10].

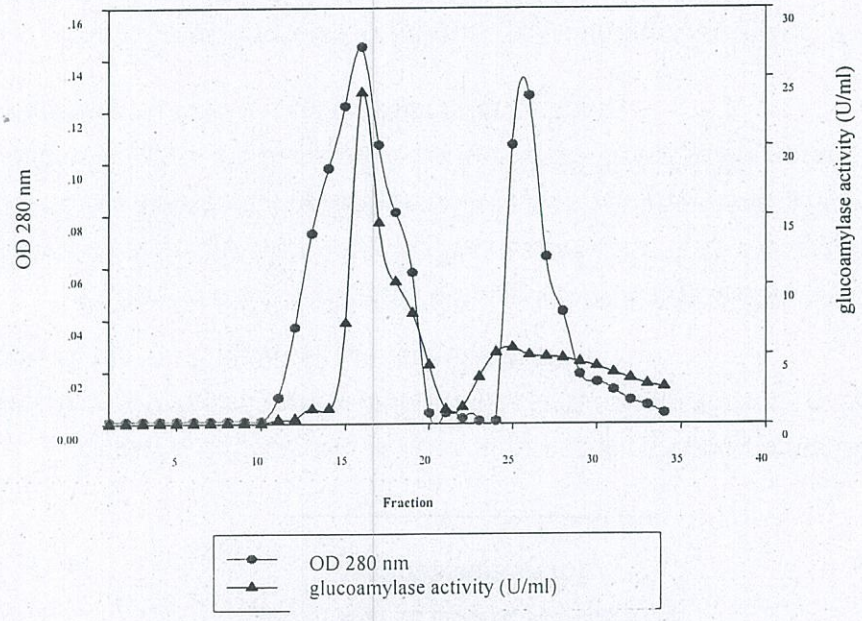

Figure 1 Gel filtration of glucoamylase from *Aspergillus niger* ATCC 10864 on Sephacryl S-100 column (1.6 x 100 cm) equilibrated with 0.05 M sodium acetate buffer pH 4.0. (Fractions of 5 ml were collected at a rate of 0.5 ml/min)

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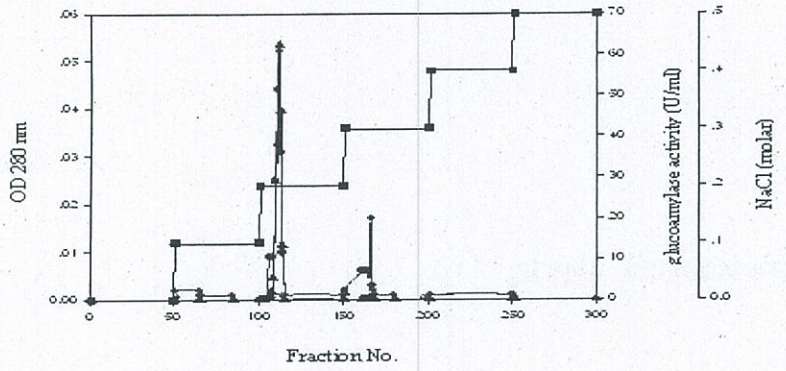

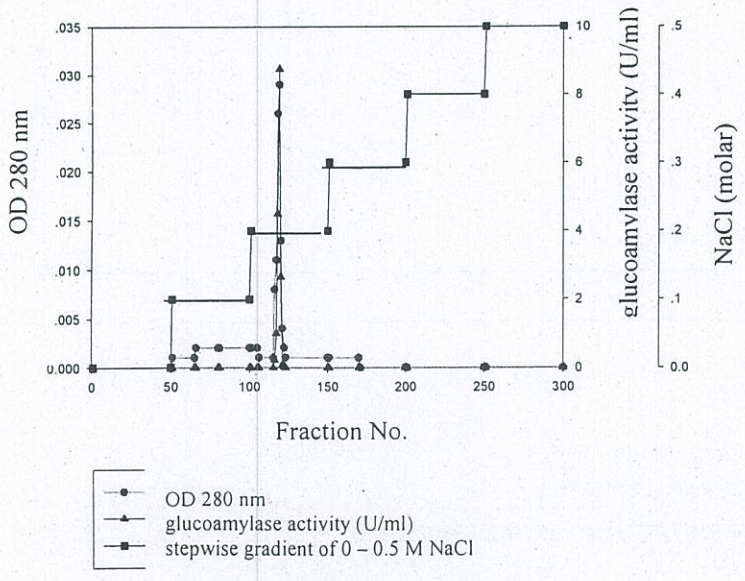

Figure 2 a, b. DEAE Sepharose chromatography of glucoamylase (F1a and F2a) from *Aspergillus niger* ATCC 10864. A DEAE Sepharose column (0.7 x 2.54 cm) was equilibrated with 0.05 M sodium acetate buffer pH 4.0. The enzyme was eluted from the column with a stepwise gradient of 0 – 0.5 M NaCl dissolved in 0.05 M sodium acetate buffer pH 4.0, at flow rate of 1 ml/min and a fraction of 5 ml were collected (a, F1a and b, F2a).

3.2.2 Purity and molecular weight determination

Showing simple protein species on SDS-PAGE, both purified fraction were purified to apparent homogeneity. From these electrophoresis results the molecular weight was estimated from the relative mobility of standard proteins on SDS-PAGE to be 80000 and 73000 daltons (Figure 7).

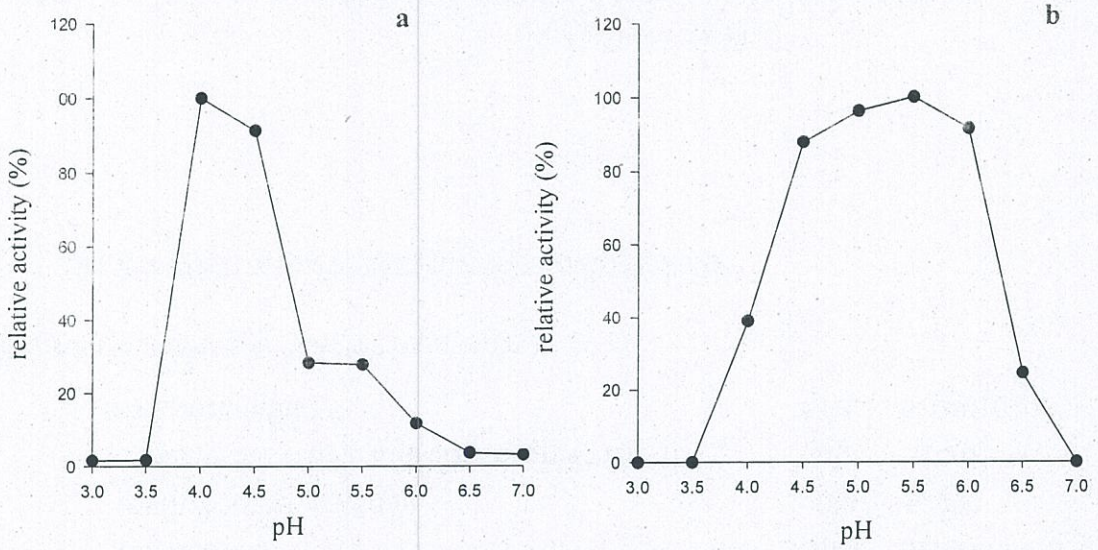

Figure 3 a, b The optimum pH of purified glucoamylase from *Aspergillus niger* ATCC 10864 at 40 ° C at difference pH (3.0-7.0) (a, F1a and b, F2a)

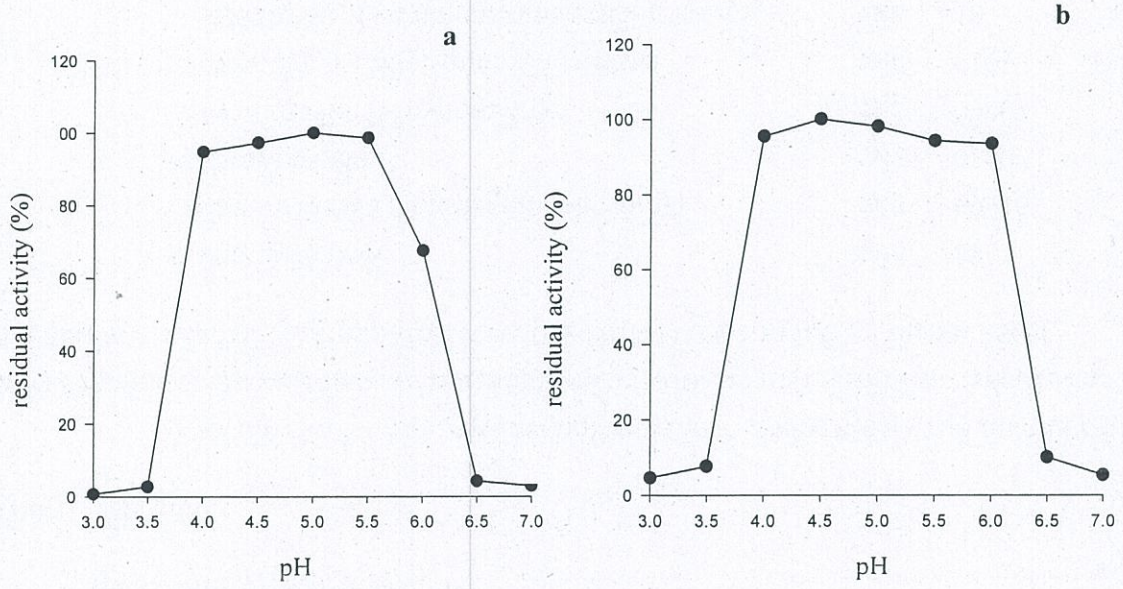

Figure 4 a, b. The pH stability of purified glucoamylase from *Aspergillus niger* ATCC 10864 at difference temperatures (20-80 °C) for 30 min (a, F1a and b, F2a).

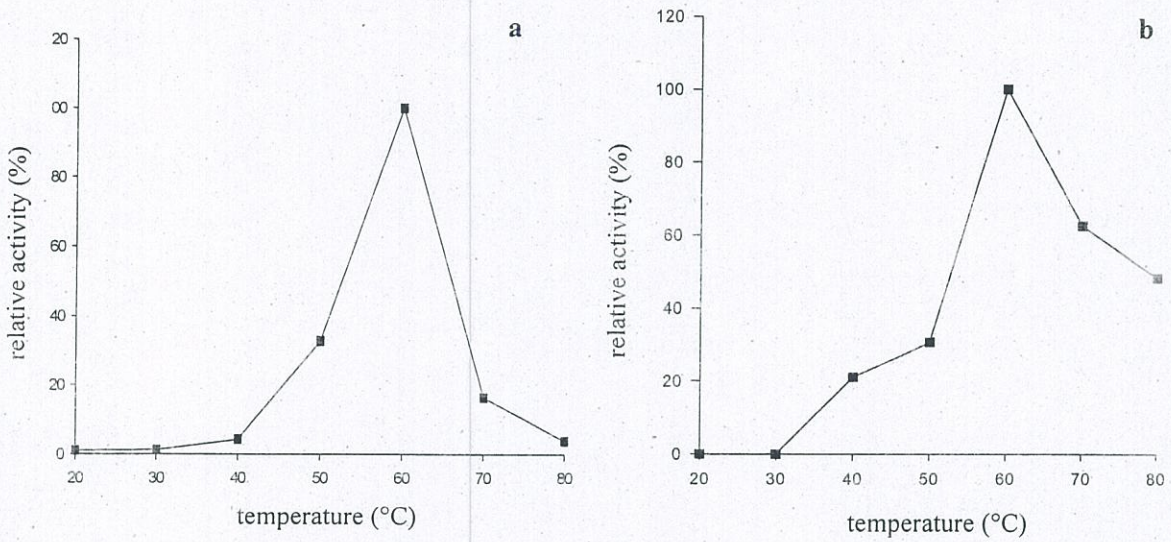

Figure 5 a,b. The optimum temperature of purified glucoamylase from *Aspergillus niger* ATCC 10864 at 40 °C for 30 min. (a, F1a and b, F2a)

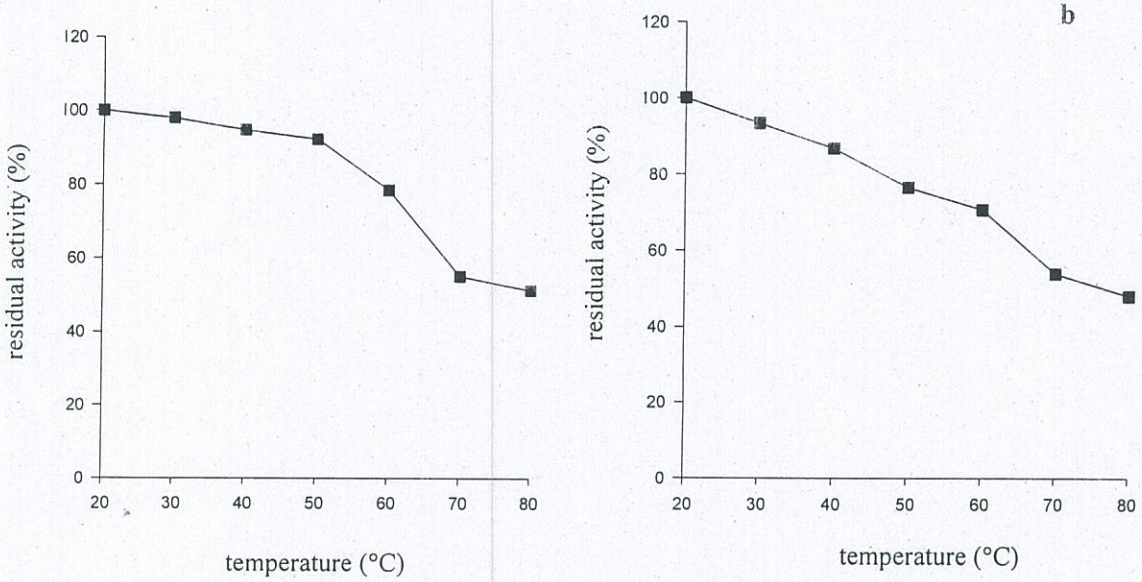

Figure 6. Temperature stability of purified glucoamylase from *Aspergillus niger* ATCC 10864 at different temperatures for 30 min (a, F1a and b, F2a).

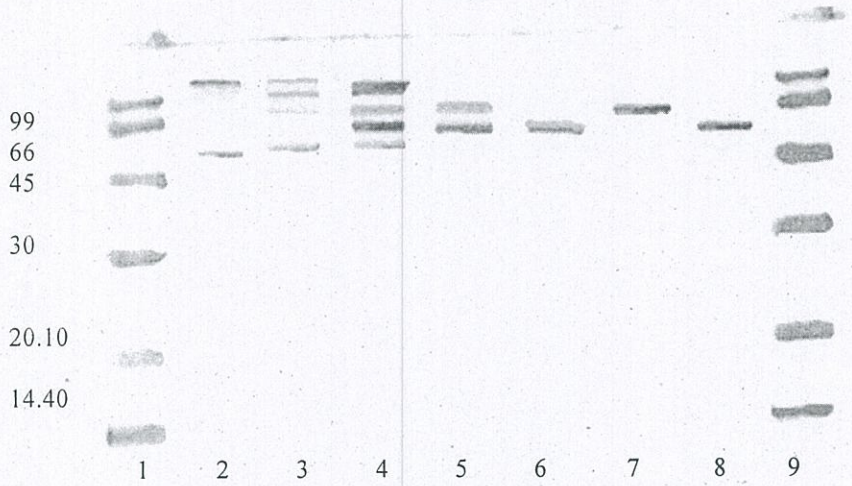

Figure 7. SDS-PAGE of purified glucoamylase from *Aspergillus niger* ATCC 10864. Lane 1 (66000), ovalbumin (45000), carbonic anhydrase (30000), trypsin inhibitor (211000) and α-lactalbumin (14400)). Lane 2, crude enzyme. Lane 3, $(NH_4)_2SO_4$ precipitation. Lane 4, dialysis. Lane 5 and 6, Sephacryl S-100 chromatography (F1 and F2). Lane 7 and 8, DEAE-Sepharose chromatography (F1a and F2a)

Table 1. Summary of the purification of glucoamylase from *Aspergillus niger* ATCC 10864.

Purification step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	% Yield	Purification (fold)
Cude enzyme	100	6,087	289.55	32.11	100	1
80% sat. $(NH_4)_2SO_4$	100	5,410.2	190.91	38.97	88.88	1.64
Dialysis	30	3,766.5	44.32	84.98	61.88	3.58
Ultrafiltration	20	2,036	10	203.60	33.45	6.34
Sephacryl S-100						
F1	25	2,003	6.82	293.70	33.00	9.15
F2	35	1,614.55	7.32	220.57	26.52	6.34
Ultrafiltration						
F1	10	2,000	5.21	384	32.86	12.00
F2	10	1,597	5.95	268.4	26.24	8.40
DEAE Sepharose						
F1a	20	1,800	2.40	750	29.57	23.36
F2a	10	1,000	1.80	555.56	16.43	17.30
Ultrafiltration						
F1a	10	1.685	2.00	842.50	28.00	26.24
F2a	10	886	1.41	628.37	14.56	19.57

2.3 Determination of glucoamylase kinetics

Kinetic of two purified fractions of glucoamylase, F1a and F2a were determined from Lineweaver-Burk plots. The Km and Vmax for F1a using rice starch and soluble starch were 10 mg/ml, 0.02 μmol/ml/min and 6.7 mg/ml and 0.02 μmol/ml/min, respectively. For F2a, the K_m and V_{max} values using rice starch and soluble starch were 5.6 mg/ml, 0.032 and 5 mg/ml, 0.023 μmol/ml/min, respectively (Figure 8 and 9).

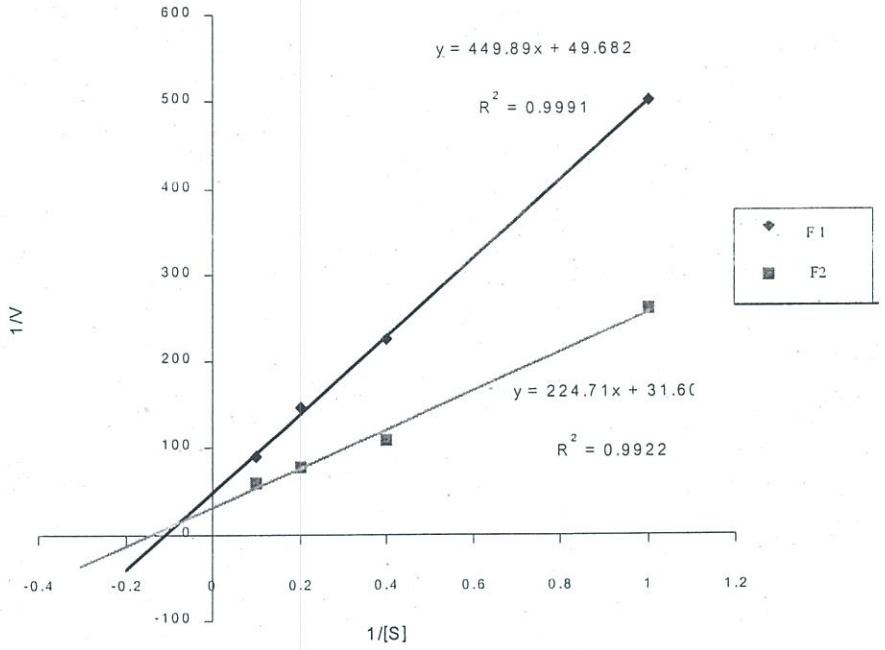

Figure 8. Lineweaver-Burk plots of two fractions purified glucoamylase using rice starch as substrate

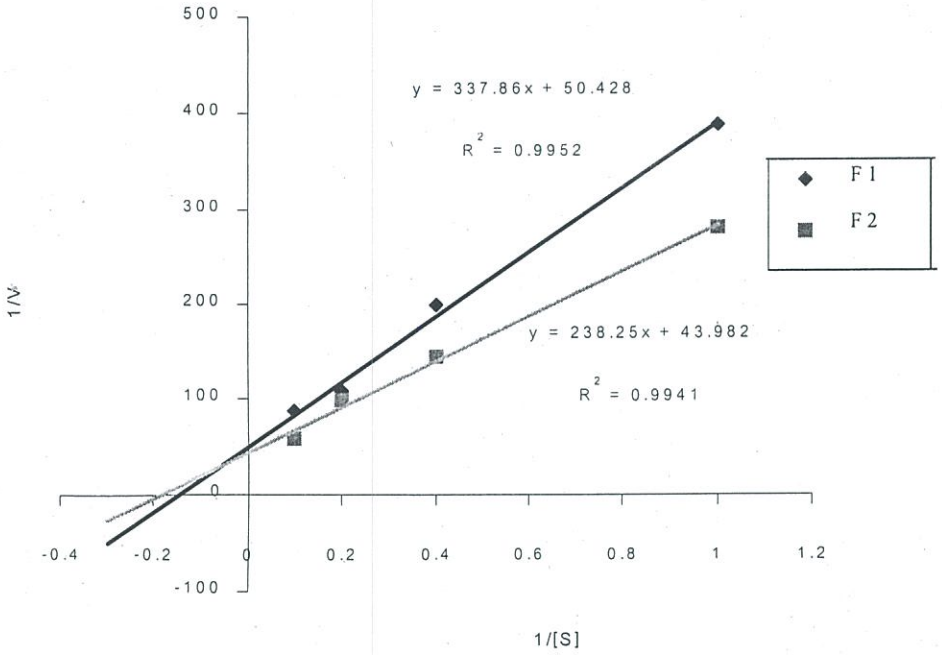

Figure 9. Lineweaver-Burk plots of two fractions purified glucoamylase using soluble starch as substrate

4. CONCLUSIONS

1. Glucoamylase from the culture filtrate of *A. niger* ATCC 10864 was precipitated by 80% ammonium sulfate precipitation. The Sephacryl S-100 gel filtration column and DEAE Sepharose ion-exchange column were resolved into two peaks of glucoamylase activities. Two glucoamylase (F1a and F2a) were purified 26.24 and 19.57 times with a yield of 28 and 14.56%, respectively.
2. Characteristics of the two purified fraction of glucoamylase, F1a and F2a were as follows : F1a exhibited maximum activity at pH 4.0. The enzyme was stable in a pH range of 4.0 to 5.5 for 30 min incubation at 40°C. The purified enzyme has optimum temperature of 60 °C and was stable at the temp erature range of 20 to 50 °C for 30 min, F2a, the optimum pH and temperature values were 5.5 and 60 °C. The enzyme showed stability at a pH range of 4.0 to 6.0 after 30 min at 40°C. The thermal stabilit y was observed at the range of 20 to 40°C for 30 min.
3. The molecular weights of the two purified fractions of glucoamylase, estimated from the relavtive mobility of standard proteins on SDS-PAGE were 80000 and 73000 daltons respectively. Kinetic values of the two purified fractions of glucoamylase, F1a and F2a were as follows : for F1a, the K_m and V_{max} values determined from Lineweave-Burk plots were 10 mg/ml and 0.02 µmol/ml/min for rice starch and 6.7 mg/ml and 0.02 µmol/ml/min for soluble starch, respectively. For F2a, the K 1983_m and V_{max} values were 5.6 mg/ml and 0.032 µmol/ml/min for rice starch and 5 mg/ml and 0.023 µmol/ml/min for soluble starch, respectively

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