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Full Length Research Paper

Purification profile of β-amylase from *Bacillus species*

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The purified β-amylase had more enzymatic activity than crude samples from *Bacillus species* as shown in this study whereby the activity of crude enzyme from *Bacillus subtilis* (WBS) and *Bacillus licheniformis* (WBL) were 6.24 and 4.2 unit/ml while the purified enzymes had an improved activity of 18 and 18.60 unit/ml, respectively. The protein concentration of the enzyme samples ranged from 264.639 µg in *Bacillus macerans* (MBM) to 627.627 µg in *B. subtilis* (WBS) enzyme filtrates and relatively lower values of 6.418 µg in *B. licheniformis* (WBL) to 77.702 µg in *B.coagulans* (MBC) was observed for the purified enzymes samples. Similarly, the purification process improves the specific activity of the enzyme samples during the study. The crude enzyme samples precipitated by salting with known quantity of Ammonium sulphate [$(NH_4)_2SO_4$] at the range of 50 and 80% fraction improved the activity of the enzyme samples whereby the strains of *B. subtilis* (WBS) and *B. licheniformis* (WBL) had their specific activity improved from the original value of 0.596 and 0.488 U/mg to 2.012 and 2.062 U/mg, respectively. It was also observed that the protein concentration of the enzyme samples decreases gradually with the increase in the specific activity. 570.945 mg of protein was detected in the crude enzyme of *B. coagulans* (MBC) and this value decreases to 92.216 on precipitation with 80% Ammonium sulphate. The use of Sephadex Gel Chromatography further enhances the purification of the enzyme.

Key words: Activity, amylase, *Bacillus*, concentration, purification, starch, substrate.

INTRODUCTION

Amylases degrade starch and related polymers to yield products characteristics of individual amylolytic enzymes (Rose, 1980). There are two main types, that is α- and β-amylase based on their pattern of activity (Street, 1958). The α-amylase is an endo-enzyme while β-amylase is an exo-enzyme and was found to be produced by microorganisms like *Bacillus* species.

The substrate for amylases production is starch (Prescott et al., 2002). According to Rose (1980), starch occurs in form of water insoluble granules as the major reserve carbohydrate in all higher plants. Amylolytic microorganisms are usually cultured in a suitable chemically defined medium for production of amylase en-zyme. The culture filtrate or supernatant obtained at this stage is the crude enzyme. The crude enzymes could be made wholesome and effective for various researches. For example β-amylases produced by *Bacillus cereus* BQ10 – 51 sp11 was purified by salting out with ammonium sulphate and column chromatography on sephadex G-100 or C-50. The purified enzyme was made homogeneous by disc electrophoresis and ultracentrifugation (Nomori et al., 1983). Similar purification method was adopted by Takasaki 1976b in which sixty gram of corn-starch added to 200 ml crude enzyme and was stirred overnight in refrigerator. The starch was collected by filtration and washed with distilled water. The adsorbed β-amylase on starch was extracted with 10% maltose solution (Takasaki, 1976b). The study of Ginsburg and Nuefield (1966) and Takasaki (1976a and b) shows that purification of enzyme improves their activity and preserves their potency for a relatively long period of time. Hence the purification of amylases used for this study is aimed at enhancing their specific activity for desired purposes.

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MATERIALS AND METHODS

Ten isolates of *Bacillus* species from soil, waste-water and some milk were cultured on a special medium constituting 2.0% peptone, 0.5% starch, 0.1% K_2HPO_4 and 0.3% $MgSO_4.7H_2O$ at 30°C on a

rotatory shaker at 150 rpm for 15 min. This enhances amylase production. The samples were centrifuged at 4000 rpm for 15 min and the resultant supernatant was used as the crude enzyme source.

Crude enzyme filtrate

60 g of cornstarch was added to 200 ml of the *Bacillus* strains crude enzyme and stirred overnight in an ice pack container placed on a rotatory shaker. Alternatively the samples were stirred overnight in a cold room. This condition provides cool temperature and thorough mixture. The starch was filtered and washed with distilled water (Takasaki, 1976b).

Ammonium sulphate fractionation

Ammonium sulphate was added to the enzyme solution at a fraction precipitating at 50 and 80% w/v. This mixture was cooled and the temperature maintained at 4°C in the refrigerator, the precipitate allowed to settle several hours, then centrifuged using MSE cold centrifuge and dissolved in 20 ml acetate buffer pH 6.6 (Takasaki, 1976b) containing 5 mM calcium chloride according to the study of Ginsburg and Nuefeld (1966). The solution was again immersed into an ethanol- ice bath and the temperature allowed to drop to 2°C. Two (2) volumes of acetone that was frozen to about -10°C was added drop-wise with stirring, and the temperature was maintained between 2 and 5°C. The process of salting the crude enzyme with ammonium sulfate and centrifugation using appropriate buffers with cold storage temperature helps to remove impurities from the crude enzyme and improves its potency (Ginsburg and Nuefeld, 1966). The solution obtained at this stage was finally centrifuged at 4000 rpm for 15 min, and the precipitate was dissolved in 2 ml of buffer pH 7. The purified enzyme was made homogeneous by disc electrophoresis and ultracentrifugation (Nomori et al, 1983).

According to Ginsburg and Nuefeld, 1966, if the enzyme is to be kept for long periods, it is precipitated with 50% ammonium sulfate and the precipitate is stored at 4°C as an ammonium sulfate suspension. The enzyme has maintained its activity, stored in this way, for over two years.

Column gel filterate

The enzyme dialysate was applied on sephadex G100 column equilibrated with acetate buffer (pH 6.6). Each of the purified enzyme samples from *Bacillus* species at this stage was assayed for the enzymatic activity. Few weeks later 50% ammonium sulphate fraction was added to some samples to preserve them for long period of up to two years according to the method of Ginsburg and Nuefeld (1966).

Enzyme assay

Each of the enzyme samples was assayed by adding 0.1 ml of the enzyme solution to 1 ml of the phosphate buffer (pH 7) into which 1% soluble starch substrate was added. This solution was incubated at 37°C for 10 min and the reaction mixture was stopped by adding 10 ml dinitro-salicyclic acid (DNSA) reagent (Murao et al., 1979). It was heated at 100°C for 10 min and cooled. 17 ml of water was added and allowed to cool down to room temperature for 15 min before the optical density (O.D) was determined using a spectrophotometer at wavelength 530 nm. The enzyme activity was therefore the amount of amylase that will release 1 µMole of maltose per sec during the assay (Murao et al., 1979; Bailey, 1988).

Protein concentration determination

The amount of protein in the enzyme samples for the *Bacillus species* was estimated by the method of Lowry et al (1951). A reagent for the detection of phenolic group known as Folin and Ciocalteu reagent was used in the quantitation of proteins in this study.

The assay was performed by adding 2.1 ml of the alkaline copper reagent which was freshly prepared by mixing 2% Na_2CO_3 in 0.1 M NaOH / 1% $CuSO_4$ $5H_2O$ / 1% Na, K tartrate.$4H_2O$ (98:1:1, by volume) to 0.2 ml of the test sample in a test tube. The mixture was vortexed and allowed to stand for 10 min after which 0.2 ml of diluted Folin-Ciocalteu colour reagent was added. The resulting reaction mixture was vortexed and allowed to stand at room temperature in a dark locker for about an hour after which the absorbance of the mixture was read at 660 nm against a reagent blank. The blank was made up of 0.2 ml of distilled water and appropriate volume of the diluent and colour reagent. When the reagents were separately prepared, 0.3 ml of the enzyme was pipetted into test tube. 3 ml of reagent C {Reagent A: 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) preparation: 0.4 g of NaOH pellets in 100 ml of dil. H2O + 2 g of Na_2CO_3. Reagent B: 0.5% Copper sulphate in 1% sodium potassium tartarate. Preparation: 1.0 g of Sodium potassium tartarate in 100 ml of dil. H_2O + 0.5g $CuSO_4$. Reagent C: 1 ml of Reagent B + 50 ml of reagent A}, was added to it, shaken thoroughly and allowed to stand for about 10 min. Then 0.3 ml of reagent D was added to it and mixed. This was allowed to stand for 30 min and the optical density at 660 nm was read. Blank was prepared with 0.3 ml dilution H_2O as the samples.

The protein concentrations of the test samples were estimated from a standard curve obtained using bovine serum albumin (BSA). To 0.2 ml of five different concentrations (40 – 200 µg/ml) of BSA were added appropriate volumes of diluent and colour reagent as given above. The values obtained were then used to plot a curve of absorbance against BSA concentration using linear regression.

RESULT AND DISCUSSION

The purification of the enzyme samples improves their amylolytic and protects their potency for a relatively long period of time compared with the crude enzyme with lower enzymatic values. For example, the amylolytic activity of the purified enzyme of *B. subtilis* (WBS) was 18.72 unit/ml, which is an improvement of the original value of crude enzyme at 6.24 unit/ml (Table 1).

The crude enzyme samples precipitated by salting with known quantity of ammonium sulphate at the range of 50 and 80% fraction improved the activity of the enzyme samples as observed in (Table 2b and 2c) whereby the strains of *B. subtilis* (WBS) and *B. licheniformis* (WBL) had their specific activity improved from the original value of 0.596 and 0.488 U/mg to 2.012 and 2.062 U/mg, respectively. It was also observed that the protein concentration of the enzyme samples decreases gradually with the increase in the specific activity. 570.945 mg of protein was detected in the crude enzyme of *B. coagulans* (MBC) and this value decreases to 92.216 on precipitation with with 80% ammonium sulphate. The protein concentrations of enzyme also decrease with increase in specific activity for all the studied *Bacillus strains* during purification systems through gel filteration (Table 2a-g).

The purified enzymes showed more enzymatic activity

Table 1. Amylolytic activity of crude and purified enzyme samples.

Starch conc. (g/100 ml buffer)	0.1% Starch in phosphate buffer (pH 7)			
	Crude amylase activity		Purified amylase activity	
	O.D at 530 nm	Amylase unit	O.D at 530 nm	Amylase unit
B. macerans (SBM1)	0.13	1.56	1.39	16.68
B. macerans (MBM)	0.25	3.0	1.40	16.84
B. licheniformis (SBL)	0.01	0.12	1.37	16.42
B. licheniformis (WBL)	0.35	4.2	1.55	18.60
B. subtilis (WBS)	0.52	6.24	1.55	18.72
B. coagulans (MBC)	0.07	0.84	1.16	13.92
B. coagulans (WBC)	0.03	0.36	1.13	3.61
B. circulans (SBC)	0.01	0.12	1.40	16.84
B. circulans (WBC1)	0.06	0.72	1.36	16.32
B. polymyxa (WBP)	0.01	0.12	1.30	15.60

Table 2. Purification profile of the enzyme.

(a) B. macerans (SBM)

Enzyme sample purification stages	Protein conc. (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture Filterate	402.024	40.32	0.100	100	1
50%$(NH_4)_2SO_4$Precipitation	200.450	36.48	0.181	90.47	1.81
80%$(NH_4)_2SO_4$Precipitation	155.405	33.51	0.215	83.11	2.15
Sephadex Gel Chromatography	61.819	31.44	4.503	77.97	45.03

(b) *B. subtilis* (WBS)

Enzyme sample purification stages	Protein conc. (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture Filterate	627.627	371.4	0.596	100	1
50%$(NH_4)_2SO_4$ Precipitation	171.171	328.8	1.921	87.83	3.22
80%$(NH_4)_2SO_4$ Precipitation	147.522	296.8	2.012	66.02	3.37
Sephadex Gel Chromatography	6.644	159.6	24.02	42.62	40.30

(c) *B. licheniformis* (WBL).

Enzyme sample purification stages	Protein conc. (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture Filterate	532.657	260.4	0.488	100	1
50%$(NH_4)_2SO_4$ Precipitation	210.585	244.8	1.162	96.54	2.38
80%$(NH_4)_2SO_4$ Precipitation	104.729	261.0	2.062	82.96	4.225
Sephadex Gel Chromatography	56.316	124.8	22.163	47.92	45.41

then the crude samples as previously discussed (Table 1). The gel filtration increases the purification fold for

(d) B. coagulans (MBC)

Enzyme sample purification stages	Protein conc. (mg	Total activity (U)	Specific activity (U/mg	Yield (%)	Purification (fold)
Culture Filterate	570.945	52.08	0.091	100	1
50%$(NH_4)_2SO_4$ Precipitation	208.33	50.28	0.241	96.54	2.64
80%$(NH_4)_2SO_4$ Precipitation	92.216	40.8	0.442	78.34	4.85
Sephadex Gel Chromatography	77.702	33.0	4.247	63.36	46.67

(e) B. macerans (MBM)

Enzyme sample purification stages	Protein conc.(mg)	Total activity (U)	Specific activity (U/mg	Yield (%)	Purification (fold)
Culture Filterate	264.639	37.44	0.110	100	1
50%$(NH_4)_2SO_4$ Precipitation	204.954	36.70	0.179	98.02	0.61
80%$(NH_4)_2SO_4$ Precipitation	168.918	33.12	0.196	88.46	1.78
Sephadex Gel Chromatography	7.882	16.90	2.14	45.13	19.49

(f) B**.** *circulans* (SBC)

Enzyme sample purification stages	Protein conc.(mg)	Total activity (U)	Specific activity (U/mg	Yield (%)	Purification (fold)
Culture Filterate	377.252	40.32	0.106	100	1
50%$(NH_4)_2SO_4$ Precipitation	192.567	39.00	0.202	96.72	1.90
80%$(NH_4)_2SO_4$ Precipitation	188.068	38.97	0.207	96.65	1.95
Sephadex Gel Chromatography	69.816	20.4	2.922	50.59	27.56

(g) B. licheniformis (SBL)

Enzyme sample purification stages	Protein conc.(mg)	Total activity (U)	Specific activity (U/mg	Yield (%)	Purification (fold)
Culture Filterate	296.171	41.76	0.183	100	1
50%$(NH_4)_2SO_4$ Precipitation	113.738	28.8	0.253	68.96	1.39
80%$(NH_4)_2SO_4$ Precipitation	106.981	28.25	0.264	67.64	1.45
Sephadex Gel Chromatography	6.418	24.63	3.837	58.97	21.00

many enzyme samples tremendously to the range of 45.41 and 46.67 for the strains of *B. licheniformis* (WBL) and *B. coagulans* (MBC) respectively (Table 2c-d). Generally, it is important to note that some culture conditions including the substrate nutrient sources, pH, temperature and other assay conditions are valuable for this enzyme

production and purification processes.

The purification of amylase using appropriate measures improved the enzymatic activity of crude enzyme (Table 1) for example the strains of *B. subtilis* (WBS) and *B. licheniformis* (WBL) had their activity improved from 6.24 and 4.2 unit/ml to 18.72 and 18.60 unit/ml, respectively. This is consistent with the study of Takasaki (1976b) and Ginsburg and Nuefeld (1966). The attainment of optimum cultural and assay conditions using the appropriate substrate such as starch for amylase enhance good enzymetic activity as intensified in this study. Suckling (1998) and Srivastava and Baruah (1986) also reported this kind of enzymatic attributes.

The protein concentration of the enzyme samples ranged from 264.639 µg in *B. macerans* (MBM) to 627.627 µg in *B. subtilis* (WBS) enzyme filtrates and relatively lower values of 5.631 µg in *B. licheniformis* (WBL) to 9.121 µg in *B. circulans* (SBC) was observed for the purified enzymes samples (Table 2a-g). This is in consistence with the study of Takasaki, (1976b), which Showed that the amount of protein content of β-amylase decrease, during purification process. Similarly, the purifi-cation process improves the specific activity of the enzy-me samples during the study. The Lowry et al (1951), method used had been proved to be more sensitive than others in use like the biuret method and has an analytical range from 10 µg to1.0 mg of protein (Holme and Peck, 1998). Similar biotechnological approach was intensified in some related scientific fields like that of Oigiangbe et al (2006) who demonstrated the activity of some biological active substances using appropriate valuable solvents for extraction and reagents for biocontrol purposes in preference to others. The observations made in this study are valuable for determination of appropriate *Bacillus* strains and cultural conditions that can enhance good purified enzyme source for large scale production of β-amylase.

REFERENCES

Bailey MJ (1988). A note on the use of dinitrosalicylic acid for determining the Products of enzymatic reactions. Appl. Microbiol. Biotechnol. 29:494– 496.

Ginsburg V, Nuefeld EF (1966). Complex Carbohydrate methods in Enzymology VIII.. Academic Press, New York and London.

Holme DJ, Peck H (1998). Analytical Biochemistry. Addison Wesley Longman Limited, U.S.A. p. 392.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein concentration determination. J. Biol. Chem. (193): 265.

Murao S, Ohyama K, Arai M (1979). β-Amylase from *Bacillus polymyxa* no. 72. Agric. Biol. Chem., 43(4):719 - 726.

Nommori TR, Shinke K, Aoki P, Nishira H (1983). Purification and characterization of □-amylase from *Bacillus cereus* BQ10- 51 spc 11. Agric. Biol. Chem. 47:941- 947.

Oigiangbe ON, Jackai LEN, Ewete FK, Lajide L (2006). Bioactive fractions of n-hexane extracts from *Vigna* pods against the legume pod borer, *Maruca vitrata* Fabricius. Afr. J. Biotechnol. 5(19):1846-1850.

Prescott ML, Harley PJ, Klein AD (2005). Microbiology (6th Edition) Wm C. Brown Publishers. pp. 900-910.

Rose AH (1980). Microbial Enzymes and Bioconversions. Academic Press London, England.

Srivastava RAK, Baruah JN (1986). Cultural conditions for production of thermostable amylase by *B.stearothermophilus*.Appl. Env. Microbiol. 52(1): 179-184.

Street HV (1958). Clinica Chim. Acta, 3{501.

Suckling CJ (1990). Enzyme Chemistry (Impact and Application) Second Edition. Chapman and Hall, Publication, London. pp.12-30.

Takasaki Y. (1976a). Production and utilization of β-amylase and Pullunase from *Bacillus cereus* var.*mycoides* Agric. Biol. Chem. 40(8):1515-1522.

Takasaki Y. (1976b). Purification and Enzymatic properties of β-amylase from *B.cereus* var *mycoides* . Agric. Biol. Chem. 40(8):1523-1530.