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The Effect of Wheat Flour in Enhancing and Stabilizing Intracellular and Extracellular *Bacillus licheniformis* 5A5 β-amylase Activity

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ABSTRACT

The effect of wheat flour on the enhancement of β-amylase activity was studied. Crude intracellular and extracellular *Bacillus licheniformis* 5A5 β-amylases were treated with different wheat flour concentrations (5-20%) for 1-5 days at 4°C. Maximum specific activities were obtained after 4 days at 20% wheat flour addition (183 and 135 U mg^{-1}, respectively). This addition protected the enzymes completely during lyophilization and was considered as a partial purification step. The results were confirmed using a Rapid Visco Analyzer, where 1000 U ml^{-1} of partially purified enzyme led to near complete degradation of raw maize starch (4 g/40 mL) after 10 min. Noticeable splitting of some maize and wheat starch granules, also complete lysis of others was observed by scanning electron microscopy during the incubation of wheat starch granules with the enzyme for only 30 min. SDS gel electrophoresis showed that the wheat flour had removed and added new protein bands to the lyophilized enzyme molecule and the disappearance of bands through the purification steps led to a big loss in enzyme activities. Thermal stability of the pure β-amylases was studied in the presence and absence of 0.01% (w/v) wheat flour filtrate.

Keywords: β-amylase, enzyme stability, starch
Abbreviations: RVA, Rapid Visco Analyzer

INTRODUCTION

Commercially usable β-amylase occurs in higher plants such as barley, sweet potato, soybean, and wheat, and also in certain mesophilic bacteria. It is now well established that microorganisms, especially from the genus *Bacillus*, produce β-amylase very similar to that found in plants such as barley and sweet potatoes. The food and beverage industries use β-amylase [EC. 3.2.1.2] to convert starch to maltose (Fogarty and Kelly 1990; Gupta *et al.* 2003; Hara *et al*. 2009).

Enhancement of enzymatic activity is industrially important, and may be achieved by chemical modifications, immobilization and protein engineering as reported by many authors (e.g. Norin *et al*. 1989; Kise *et al*. 1990; Aldercreutz 1999; Bryjak 2003; Pandya *et al.* 2005; Habibi *et al.* 2006; Gangadharan *et al.* 2009; Montealvo *et al.* 2011). Many authors reported in enzyme purification and partial purification with different methods. Partial purification of *Bacillus polymyxa* No. 26-1 β-amylase which was easily adsorbed onto raw maize starch was reported (Cheon Bae *et al*. 1996; Pessela *et al*. 2003). It was reported in one-step purification using a thermophilic poly-His-tagged β-galactosidase from *Thermus* sp. Histidine-tagged Class II fructose-1,6-bisphosphate aldolase easily purified using immobilized metal affinity chromatography (Rukseree *et al*. 2008). Vigants *et al*. (2001) reported, in a new and efficient method, the purification of levansucrase from cell-free extracts of a flocculant mutant of *Zymomonas mobilis* ATCC 10988, showing that the levansucrase activity was almost completely recovered, and purification by a factor of 15 was achieved, after precipitation with 0.1 m M $MnCl_2$ as a first capturing step.

Although plant β-amylases are widely used in industrial applications, bacterial amylases have yet to be used. Despite the greater activities of bacterial β-amylases (Hirata *et al*. 2004), their thermo-stabilities are less than those of plants (Totsuka and Fukazawa 1993) and their thermo-stabilities should be improved before it could be used in industrial processes.

The present article focuses on the potential role of raw wheat flour in the enhancement of the intracellular and extracellular β-amylase activities from *Bacillus licheniformis* 5A5.

MATERIALS AND METHODS

Experiments to evaluate the enzyme production, activity and stability

1. Microorganism and growth conditions

Bacillus licheniformis 5A5 was obtained from the Department of Biochemistry, Ohio State University, Columbus, OH. Nutrient agar medium (in g l^{-1}: peptone, 5.0; meat extract, 15.0; NaCl, 5.0; agar, 15; the pH was adjusted to 7.0) was used for culture maintenance and stock cultures. Unless otherwise specified, the following optimized culture medium was used for β-amylase production (in g l^{-1}: wheat flour, 20.0; wheat bran, 5.0; casein, 5.0; $(NH4)_2HPO_4$, 5.0; $CaCl_2$, 0.16; Na citrate, 2.0; and $MgSO_4 \cdot 7H_2O$ 5.0).

2. Extraction of intracellular enzymes (cell free extract)

Weighed sample of a fresh wet cell pellet was suspended in fixed volume of buffer (0.1M, pH 7.0), and ultrasonic extraction was done using a Branson Ultrasonic Sonifier W-250 (Branson Ultrasonics, Danbury, CT) for 18 cycles (3 min $cycle^{-1}$). The homogeneous extract was centrifuged in a cooling centrifuge for 60 min at 10,000 rpm and 4°C.

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3. Assay for β-amylase activity

β-Amylase activity was determined according to Mathewson and Seabourn (1983). One unit of β-amylase activity was defined as the amount of enzyme required in the presence of excess α-glucosidase to release one micromole *p*-nitrophenol from PNPG5 in one minute under the defined conditions.

4. Estimation of protein content

The protein content of enzyme preparation was determined by a Coomassie blue binding assay following Murphey *et al*. (1989).

5. Chromatography

Paper chromatography was performed to investigate the original products of β-amylase formed by fermentation. The descending technique was adopted using Whatman No. 1 mm paper using the solvent mixture *n*-butanol: acetone: water (4: 5: 1, by volume) (Block *et al*. 1955). The chromatograms were sprayed with aniline phthalate.

6. Rapid Visco Analysis (RVA)

For viscosity analysis (Anon 1998) a heating, holding, cooling profile was used to characterize the apparent viscosity of starch or starch with β-amylase and the apparent viscosity vs time curve obtained was used as an index of β-amylase activity. The viscosity changes were continually recorded, using a Rapid Visco Analyzer model 3-D (RVA) (Newport Scientific, Warriewood, Australia). Maize starch (4 g) was added to 25 mL malate buffer (pH 7, 0.01 M) and heated at 95°C until it reached its peak viscosity. The temperature of the RVA was adjusted to 40°C (the optimum temperature for enzyme activity), and then a known amount of β-amylase was added. Viscosity decline after β-amylase addition was recorded.

7. Partial purification

The enzyme was partially purified by the addition of 20 g wheat flour to 100 mL β-amylase culture filtrate or cell free extract (with addition of a drop of 1% Na azide). The mixtures were incubated for 4 days at 4°C and then left for occasional shaking for 30 min at 4°C. After centrifugation at 6,000 rpm, the enzymes were lyophilized.

8. Purification procedure

Further purification was done by gel filtration on Sephadex G-150. The gel was soaked in 20 mM malate buffer, pH 7.0. The column (50 × 2.5 cm) was packed and the bed was stabilized with three column volumes of buffer. One mL partially purified enzyme sample was applied to the column. The buffer flow rate was adjusted to 60 mL/hr and 5 mL fractions were collected. Protein content was estimated. Peak fractions from the column were pooled. Mixed and lyophilized β-amylase activity and protein content were determined. Dialyzed sample was applied to DEAE-Sephadex A-50 column (column × 2 mL) which was pre-equilibrated with malate buffer (pH 7, 0.01 M). Unbound proteins were washed from the column with the same buffer and stepwise elution was done at a flow rate of 60 mL hr^{-1} with linear gradient molarity of the malate buffer (20-100 mM) and 5-mL fractions were collected. The peak fractions were pooled and assayed for protein content and β-amylase activity.

9. Gel electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970).

10. Raw starch granule degradation

Raw starch granule degradation was started by adding 0.5 mL enzyme solution (containing 100 U mL^{-1} to 0.5 mL of 1% (w/v) maize and wheat starch in malate buffer pH 7.0, 100 mM). Enzyme substrate mixtures were incubated for 30 min then centrifuged and all starch granules were washed twice with pure ethanol and *n*-butyl alcohol. They were examined by scanning electron microscopy following routine techniques.

Table 1 Specific activity (U mg^{-1}) of crude extracellular β-amylase in the absence and presence of wheat flour at different concentrations over 5 days.

Plain flour conc.	Activity (U mg^{-1})				
(%)	Day 1	Day 2	Day 3	Day 4	Day 5
0	2.4	2.4	2.4	2.4	2.4
5	24	33	49	77	75
10	39	46	58	85	86
15	50	60	72	99	95
20	58	73	79	135	122
25	66	74	80	124	121
30	66	73	77	120	120

Table 2 Specific activity (U mg^{-1}) of crude intracellular β-amylase in the absence and presence of wheat flour at different concentrations over 5 days.

Plain flour conc.	Activity (U mg^{-1})				
(%)	Day 1	Day 2	Day 3	Day 4	Day 5
0	5.7	5.7	5.7	5.7	5.7
5	34	40	65	146	133
10	47	56	87	164	159
15	50	61	112	175	170
20	55	77	154	183	183
25	54	76	126	177	169
30	54	75	126	176	167

11. Thermal stability

The pure enzymes with and without 0.01% wheat flour filtrate were incubated with malate buffer (100 mM, pH 7.0) at the designated temperature [50-70°C] for 15 to 60 min and the residual activities were assayed under the standard conditions.

RESULTS AND DISCUSSION

In this study, *Bacillus licheniformis* 5A5 produced both extracellular and intracellular β-amylases, which were characterised as β-amylases (1,4-α-D-glucan maltohydrolase) based on demonstration of exocleavage activity and production of maltose from starch which was detected by paper chromatography and confirmed with control spot. The effect of wheat flour on the enhancement of β-amylase activity was studied. The addition of 5-20% (w/v) wheat flour to the crude extracellular or intracellular β-amylase for 1-5 days at 4°C led to a significant increase in β-amylase specific activities (**Tables 1, 2**). The maximum activities were obtained after 4 days using 20% (w/v) wheat flour concentration (137 and 183 U mg^{-1} for extracellular and intracellular enzyme, respectively). On the other hand, the β-amylase activity test, for wheat flour alone, was negative. Physical immobilization of contaminated proteins on a micro space leads to micro channel reaction system took place between enzyme and substrate provide large surface and interface areas which differs from macro-scale systems (free enzyme) with regards to the strict control of reaction time. This rapid mass transfer is the key advantage of micro reaction technology (Zhang *et al.* 2004; Miyazaki and Maeda 2006). The addition of wheat flour protected the enzymes from denaturation during the lyophilization process. The efficiency of *Bacillus licheniformis* 5A5 β-amylase was confirmed by viscoamylography using an RVA in which the addition of 1000 U ml^{-1} could degrade 4 g gelatinized wheat starch in 25 mL solution over 10 min (**Fig. 1**). The comparison between the efficiency of 100 U ml^{-1} partially purified β-amylase from Sigma showed that our β-amylase (100 U ml^{-1}) had more degradation ability on raw maize starch granules (**Fig. 2**). The examination of maize and wheat starch granules by scanning electron microscopy after incu-

Table 3 Purification of extracellular (Ex) and interacellular (In) *Bacillus licheniformis* 5A5 β-amylase.

Purification steps	Activity (U)		Protein (mg)		Sp.act (U mg^{-1})		Purification (fold)	
	Ex	In	Ex	In	Ex	In	Ex	In
Crude enzyme+20% plain flour	541,689	795,800	3,957	8,410	137	94.6	1	1
Sephadex G-150	38,218	254,232	128	982	300	259	2.18	2.74
DEAE Sephadex A-50	5,579	24,511	13.2	75.6	423	324	3.08	3.40

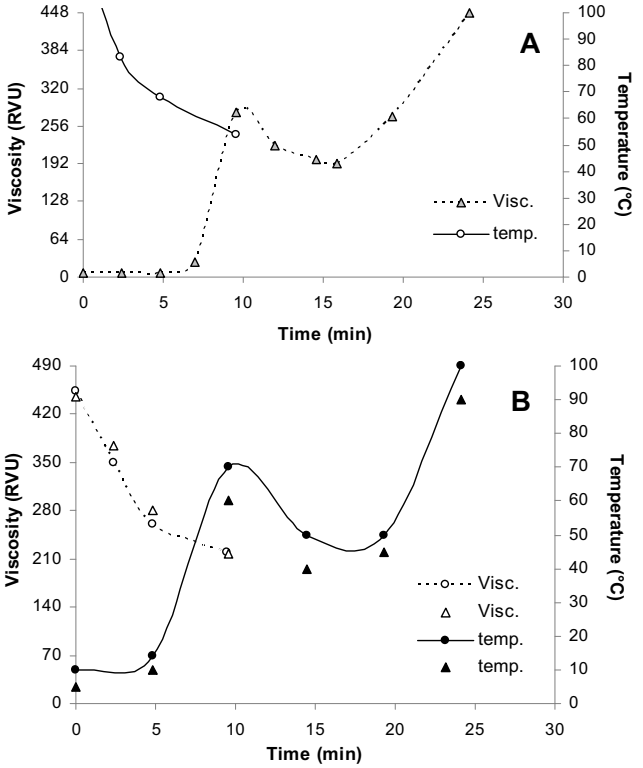

Fig. 1 (**A**) Rapid Visco Analyzer profile showing that partially purified *Bacillus licheniformis* 5A5 (1000 U ml^{-1}) could degrade the starch granules through 10 min. *Bacillus licheniformis* 5A5 β-amylase with maize starch (●), maize starch only (►). (**B**) Rapid Visco Analyzer profile showing that 100 U ml^{-1} partially purified *Bacillus licheniformis* 5A5 β-amylase (●) had more degradation ability on raw maize starch compared to Sigma partially purified β-amylase (▲).

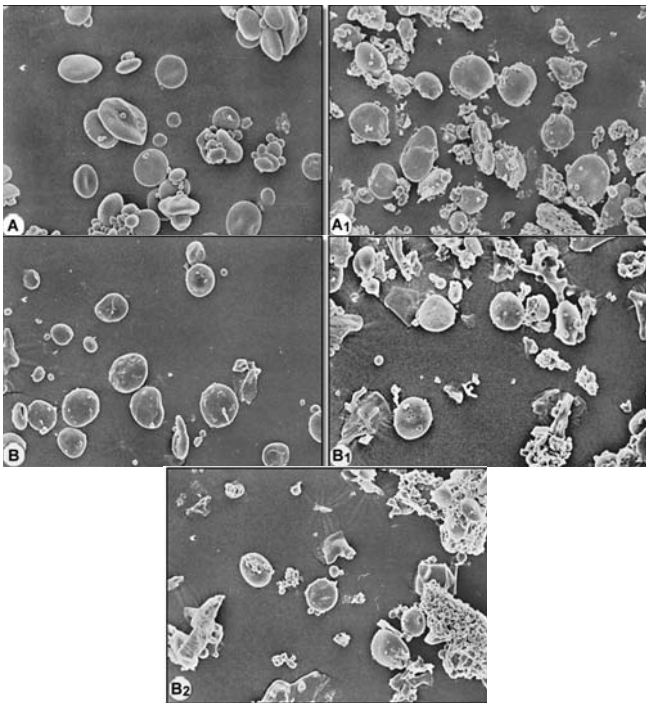

Fig. 2 Native maize and wheat starch granules (untreated) (**A, B**). Maize starch (**A1**) and wheat starch (**B_1, B_2**) granules after incubation for 30 min with partially purified β-amylase. All images under scanning elec-tron microscopy.

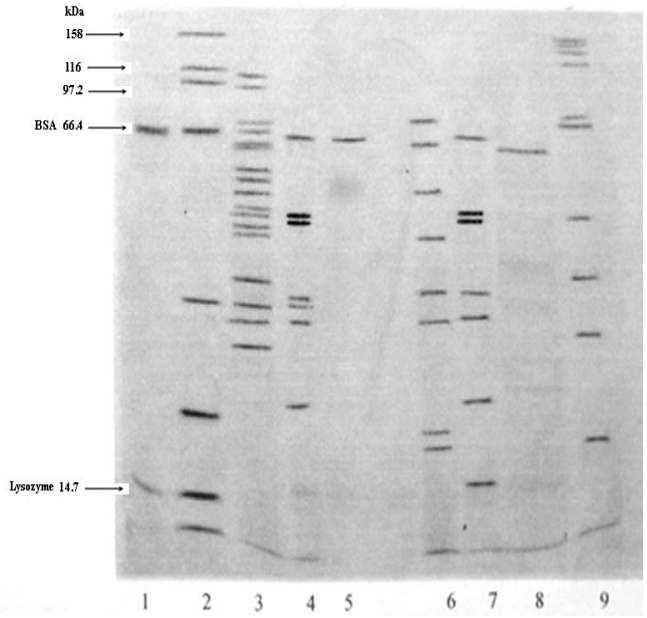

Fig. 3 Gel electrophoresis. Lane 1, BSA (bovine serum albumin) + lysozyme; lane 2, molecular weight marker; lane 3, crude intracellular β-amylase; lane 4, partially purified intracellular β-amylase; Lane 5 pure β-amylase, lane 6 crude extracellular β-amylase; lane 7, partially purified extracellular β-amylase; lane 8, extracellular pure β-amylase; lane 9, prestain molecular weight marker.

bation with the intracellular β-amylase for 30 min showed that most of the granules were completely lysed. It was also observed that clear splitting appeared in the remaining wheat starch granules.

Gel electrophoresis (**Fig. 3**) of the crude and lyophilized enzyme preparation showed that the wheat flour removed and added new bands of proteins which could be responsible for the enhancement of β-amylase activity. This, step could be considered as a partial purification process. Many authors reported the improvement of β-amylase through different techniques (Kihara *et al.* 2000; Kim *et al*. 2002; Kumar *et al*. 2006; Yamashiro *et al*. 2010). Dabulls and Klibanov (1993) reported on the dramatic enhancement of enzymatic activity in organic solvents where proteases and lipases were lyophilized from aqueous solution containing a ligand, N-Ac-Phe-NH2, and their catalytic activity in anhydrous solvents was much bigger (one to two orders of magnitude) than that of the enzymes lyophilized without the ligand.

The lyophilized β-amylase was easily purified by gel filtration on Sephadex G-150 followed by ion exchange chromatography on DEAE Sephadex A-50. The pure enzyme was eluted as a single protein component and contributed only 1.03 and 3.08% of extracellular and intracellular β-amylases activities, respectively (**Table 3**). The specific activities of the pure extracellular and intracellular β-amylase were 423 and 324 U mg^{-1}, respectively. Gel electrophoresis (**Fig. 3**) of pure β-amylase showed that the removal of the wheat flour bands in the purification process led to a drastic decrease in enzyme activities, also approxi-

Table 4 Thermal stability of pure β-amylase with (A) and without (B) plain flour. Relative values of β-amylase activity expressed in % and obtained by Berridge's method.

Temp.	Exposure time (min)							
(°C)	15		30		45		60	
	A	B	A	B	A	B	A	B
50	100	81.6	100	46.8	100	35.7	100	35.7
60	100	27.4	100	16	100	13.5	100	4.4
70	100	zero	100	zero	100	zero	100	zero

mating the original level (before wheat flour addition). According to this result, it was deduced that the new bands which appeared in the lyophilized β-amylase (**Fig. 3**) had an important role in activating the enzyme, while the removal of these bands during the purification process led to the marked reduction in activity. It was reported that α-amylase entrapped on alginate beads was recoverable by addition of 0.5 M NaCl containing 0.2 M Ca^{2+}. The enzyme was homogenous (recovery of 76%) with a specific activity of 1764 U mg^{-1} (Kumar *et al*. 2006).

The addition of 0.01% wheat flour filtrate to the pure intracellular and extracellular enzymes maintained their stabilities completely up to 70°C while the native enzyme lost 30% of its stability at 50°C after 60 min (**Table 4**). Many authors have reported on the stabilization of enzymes by immobilization and conjugation. El-Batal *et al.* (2005) reported on the stabilization of α-amylase by using anionic surfactant during the immobilization process. Alpha-amylase immobilized on super porous CELBEADS showed better thermal stability than the free enzyme (Shewale and Pandit 2007). Thermal stabilities of free and immobilized α-, β-, and glucoamylases were tested. The immobilization support was prepared with equal weights of chitosan and activated clay and cross-linked with glutaraldehyde. It was also reported that the relative activities of immobilized enzymes are higher than that of the free enzymes (Yun and Shin 2005). Khajeh *et al*. (2001) reported in the usefulness of combination of medium and chemical modification for protein stabilization and enhancement of catalytic potential. Finally Srivastava (1991) showed that stabilization of amylase could be achieved by conjugation with modified dextran, but higher stability was achieved by conjugating the protein with hydrophilic polysaccharides or acetylated dextran, than by hydrophobic polysaccharide or methylated dextran.

CONCLUSIONS

We report on the role of wheat flour in activation, partial purification and stabilization of β-amylase. This draws attention to the importance of crude biomaterials as safe and cheap adjuvants to enhance the mechanisms of enzyme activity. This has potential direct application in the food industry.

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