April 2010 ISSN : 0974 - 7427 Volume 4 Issue 1

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 4(1), 2010 [30-37]

Purification and characterization of β-amylase from germinating wheat *(Triticum aestivum L.)* seed

Niranjan Kumar Sana[1], Bidhan Chandra Sarkar*[1], Goutam Kumar Sarker[1], Md.Entazul Huque[2], Shaha Ranajit Kumar[1]

[1]Department of Biochemistry & Molecular Biology, The University of Rajshahi, Rajshahi - 6205, (BANGLADESH)

[2]Department of Chemistry, The University of Rajshahi, Rajshahi - 6205, (BANGLADESH)

E-mail: bidhan_sar@yahoo.com

Received: 26th December, 2009 ; Accepted: 5th January, 2010

ABSTRACT

In germinating wheat (*Triticum aestivum* L) seeds at 42 hours the abundant amylolytic activity was found to be due to -amylase (α-1-4-glucan maltohydrolase). The enzyme was purified to homogeneity by ammonium sulphate precipitation followed by gel filtration on Sephadex G-75, and DEAE-cellulose chromatography. The enzyme was found to be more active against starch (pea) and amylopectin than soluble starch used as substrate. The β-amylase showed maximum activity at pH 6.0 and at 45°C. The enzyme was stable at a pH range of 4.0-8.0 and at 30-60°C for 15 min. The molecular weight of the enzyme was estimated to be 88kDa by Sephadex G-75 column chromatography and 89kDa by sodiumdodecylsulfate gel electrophoresis (SDS-PAGE). The K_m value for β-amylase with soluble starch as substrate was found to be 1.47mg/ml. The enzyme was completely inactivated by Cu^{2+}, Hg^{2+}, Pb^{2+}, Urea and Ag^{+} at 0.5mM concentration and its activity was increased by the addition of Fe^{3+}, Mn^{2+} and EDTA. The study indicates the importance of β- amylase as a starch-degrading enzyme.

KEYWORDS

Wheat seeds (*Triticum aestivum* L);
β-amylase;
Starch;
Purification;
Characterization.

INTRODUCTION

Amylolytic enzymes are widely distributed in plant tissues e.g. in storage tissues such as seeds and tubers, and in vegetative organs such as leaves[1]. There exits two types of amylases in some species of plants, -(α-1-4-glucan glucohydrolase) and β-(α-1-4-glucan maltohydrolase) amylases[2,3]. It has long been known that the mature ungerminated kernels of cereals (barley and rye) contain activities of β-amylase. When the seed germinate, this activity increases considerably[4,5]. β-Amylase, considered as one of the enzymes, which degrade starch, may play a role in the mobilization of starch during germination or the sprouting of tubers[6,7]. The primary function of the enzyme amylase is to break down starches in food so that the body can use them. It is involved in anti-inflammatory reactions such as those caused by the release of histamine and similar substances.

They are extensively used in bread making to break down complex sugars such as starch (found in flour) into simple sugars. It is used in detergents to dissolve starches from fabrics. It is also used in the textile industry for designing textiles, in the laundry industry in a mixture with protease and lipase to launder clothes, in the paper industry for sizing and in the food industry for preparation of sweet syrups, to increase diastase content of flour, for modification of food for infants and for the removal of starch in jelly production. The saccharifying activity of cereal seed β-amylases is also exploited in bread making and in the use of malt as an additive in other foodstuffs and even as a 'digestive'[8]. Cereal β-amylases also find applications in the production of maltose and maltose-rich syrups, sweeteners and vaccines[9]. Several researchers have been studied on the isolation of β-amylase enzyme from seeds[10,11], tubers[12,13], leaves[14,1], soybean[15,16] and microorganisms such as bacillus[17,18] and fungal strains[19]. There are few overviews dealing extensively with cereal β-amylases[20,21] and a few research works is concerned exclusively with these enzymes[22]. Studies on the purification of β-amylases from cereals are relatively few. Barley and millet β-amylases have been isolated and characterized by several workers[23,24]. The literature on wheat *beta*-amylase is particularly scanty. The accumulation of *beta*-amylase in the seeds of cereals and the activity increases during germination is interesting from both physiological and biotechnological standpoint. We present here the characterization of β-amylase from germinating wheat seeds.

MATERIALS AND METHODS

Materials

Three varieties of wheat seeds (Akbar, Agrani and Kanchan) were collected from Bangladesh Agricultural Research Institute (BARI), Irshardi, Pabna, Bangladesh. The seeds were soaked in distilled water for 6 hours, germinated in a lighted room at 25°C for 24, 36, 48, 72, 96 and 120 hours including soaking time. The germinated seeds at different hours were separated from seedling, rinsed with distilled water and stored separately in a deep freeze (-10°C) for further analysis. Sephadex G-75, BSA, and reagents for SDS-PAGE were purchased from Sigma Chemicals Ltd., USA. Standard proteins, DEAE-cellulose were purchased from Pharmacia Fine Chemicals Ltd., Sweden. All other chemicals used were of analytical grade.

Preparation of crude enzyme extract

About 30 grams of germinated wheat seeds were taken in a small pot of homogenizer and homogenized well with cold 0.1M phosphate buffer, pH 7. The extracts were filtered by few layer of cheesecloth and further clarified by centrifugation at 6000 rpm, for 15 minutes at 4°C. The supernatants was collected and precipitated by $(NH_4)_2SO_4$ at different concentration and finally collected the precipitate at 100% with high amylase activity as previously described[25]. The precipitate was redissolved in small amount of pre-cooled distilled water and dialyzed (first in distilled H_2O for 24 hours and then in 50mM phosphate buffer, for overnight, pH 7.8, 4°C). After centrifugation, the clear supernatant obtained was used as crude enzyme extract.

Gel filtration column chromatography

The crude extract after dialysis with 50mM phosphate buffer, pH 7.8, 4°C was loaded onto a Sephadex G-75 gel column (2.5 × 120cm), which was equilibrated with the same buffer. The column was eluted with 50 mM phosphate buffer, pH 7.8, at a flow rate of 1.0ml min^{-1}. Absorbance of each fraction at 280nm, amylase activities and protein concentration were measured. The active fractions were collected.

DEAE-cellulose column chromatography

The enzymatically active protein fractions after gel filtration were collected and dialyzed against 50mM phosphate buffer, pH 7.8 for overnight and then concentrated to its $1/4^{th}$ volume by freeze dryer and applied to a DEAE-cellulose column (32 × 1.0cm, flow rate 25ml h^{-1}) previously equilibrated with 50mM phosphate buffer, pH 7.8 and eluted with the same buffer containing a linear gradient of NaCl (100-500mM). Absorbance at 280nm, protein concentration and amylase activities were determined. The active fractions were collected.

Measurement of amylase activity

Amylase activity was assayed following the method described by Jayaraman[26]. One percent of starch so-

Regular Paper

lution (soluble) was used as substrate (1gm in 100ml of 0.1M phosphate buffer, pH 6.7). The amylase activity was measured by estimating the release of maltose. The amount of maltose released was calculated from the standard curve prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1mg of maltose in 15 minutes at 37°C.

Molecular weight determination of β-amylase

The molecular weight of the purified β-amylase was determined by gel filtration on Sephadex G-75 column (150 × 3.0cm) equilibrated with 50mM phosphate buffer, pH 7.8, following the established procedure[27]. Trypsin inhibitor (12kDa), carbonic anhydrase (29kDa), ovalbumin (43kDa), bovine serum albumin (67kDa), phosphorylase-b (97.4kDa) and β-galactosidase (116kDa) were used as marker proteins[28].

Electrophoresis

SDS-PAGE was performed according to the method of Laemmili[29] on a Bio-rad mini electrophoresis system. The standard proteins used were β-lactoglobulin (18.4kDa), carbonic anhydrase (29.0kDa), ovalbumin (43.0kDa), bovine serum albumin (67.0kDa) and phosphorylase-b (97.4kDa). PAGE was performed with 7% gels and the electrophoresis was run at 2000V and 50 A.

Optimum pH of the enzyme

To study the effect of pH on enzyme activity, the enzyme solutions (0.6%) were dialyzed against 50mM buffer of different pH (pH 2.0-3.0, CH_3COONa-HCl; pH 4.0-5.0, CH_3COONa-CH_3COOH; pH 5.5 - 8.0, NaH_2PO_4-Na_2HPO_4; pH 8.5 - 9.0, $Na_2B_4O_7$ - HCl; pH 9.5, $Na_2B_4O_7$ - Na_2CO_3) for 24 hours with frequent change of buffers. After necessary adjustment of pH by adding 0.1N HCl or 0.1N NaOH, the enzyme activities were assayed using starch as substrate.

Optimum temperature of the enzyme

In order to determine the optimum temperature, the enzyme solutions (0.5%) in 50mM phosphate buffer, pH 7.0, were incubated at various temperatures ranging from 10C - 90°C for 15 minutes in a temperature controlled water bath and the enzyme activities were assayed.

Substrate specificity

To determine the substrate specificity of the enzyme amylose, amylopectin, maltotetraose and maltose were used as substrate instead of starch during the assay. In the procedure, 2.5 ml of 100mM phosphate buffer pH 6.7, 2.5ml of substrate (1%) of different types and 0.5 ml of enzyme solution were taken in different test tubes and incubated at 37°C for 10 min and the enzyme reaction was stopped by adding 0.5ml of 2N NaOH. Then 0.5ml of dinitrosalicylic acid (DNS) reagent was mixed to each test tube. The tubes were heated in a boiling water bath for 5 minutes. After cooling at room temperature the absorbance was measured at 520nm.

Influence of metal ions and chemical reagents

The effects of metal ions and chemical reagents on the enzyme activity were examined by incubating the enzyme solution at room temperature in the presence of different ion or compound for 5 minutes and aliquots were withdrawn and assayed under standard reaction conditions (pH 7.0, Tem. 37°C).

Measurement of K_m of enzyme

Michaelis constant (K_m) was determined by the assay of enzyme activity for various concentration of the substrate (starch 0.1-2.0mM) at definite interval. Initial velocities of respective substrate concentration were calculated. K_m was calculated from Lineweaver-Burk double reciprocal plot[30].

Protein assay

Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280nm. The amount of protein was estimated by the published method of Lowry et al.[31] using BSA as standard substrate.

RESULTS

The amylolytic activity of β–amylase from the three varieties of germinating wheat seeds (Agrani, Kanchan & Akbar) showed their maximum activity at 42 hours of germination. After that the activity declined rapidly (Figure 1). The activity of Agrani variety of amylase was found higher than that of the other two varieties.

Regular Paper

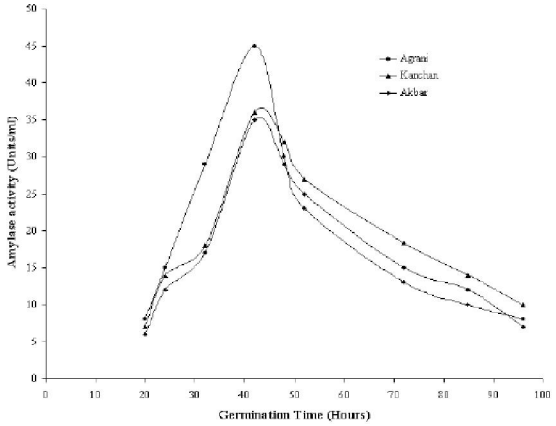

Figure 1 : Time course of the three varieties of wheat seeds amylase activities during germination

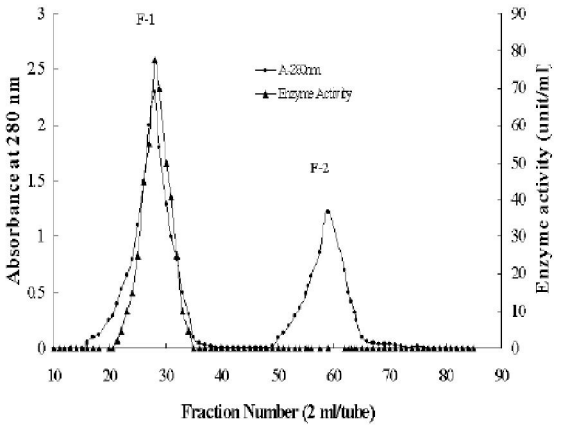

Figure 2 : Gel-filtration pattern of crude extract from germinating wheat seeds on shephadex G-75 column chromatography (2.5 × 120cm). The column was pre-equilibrated with 50mM sodium phosphate buffer, pH 7.8 and the column was eluted with same buffer. The flow rate of the column was 1ml/min. (•) absorbance at 280nm, (▲) enzyme activity.

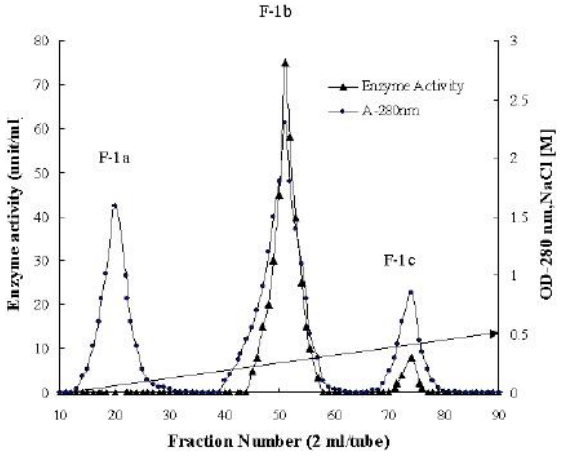

Figure 3 : DEAE-cellulose column chromatography of β-amylase from F-2 fraction obtained from gel filtration. The column (32 × 1.0cm) was pre-equilibrated with 50mM phosphate buffer, pH 7.8 and was eluted with the same buffer. The column was then eluted with a linear gradient of NaCl (0.1 to 0.5M) in the same buffer. The flow rate of the column was 25ml/h. (•) absorbance at 280nm, (▲) enzyme activity and (▶) NaCl gradient

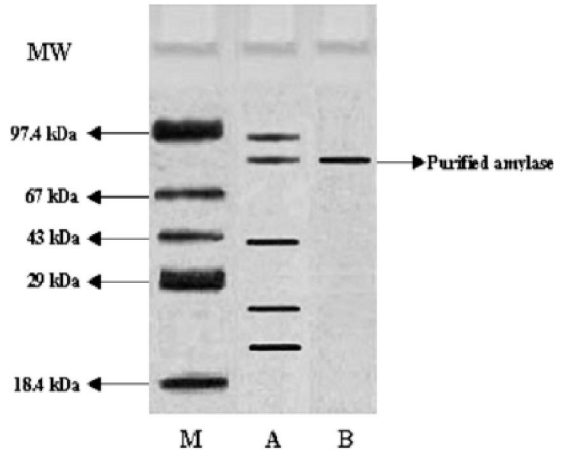

Figure 4 : Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified β-amylase and marker proteins for the determination of purity and molecular weight of the enzyme. Lane-A: After gel filtration column, Lane-B: After DEAE-cellulose column, Lane-M: Marker protein solution containing phosphorylase B (97.4kDa), Bovin serum albumin (67.0kDa), Ovalbumin (43.0kDa), Carbonic anhydrase (29.0kDa), β-lactoglobulin (18.4kDa)

So, in further studies, we used the extracts of Agrani variety of wheat seeds germinated at 42 hours.

The crude enzyme solution from Agrani variety of germinating wheat seeds showed two main peaks F-1 and F-2 (Figure 2) on Sephadex G-75 column. Only the F-1 fraction showed the amylolytic activity while the F-2 fraction showed no activity. The F-1 fraction was pooled, concentrated by freeze dryer and further purified by DEAE-cellulose column chromatography.

The enzyme active fraction F-1 obtained after Gel filtration when applied to a DEAE-cellulose column separated into three peaks: F-1a, F-1b and F-1c (Figure 3). Fraction F-1a eluted with the buffer showed no amylolytic activity. The other two fractions F-1b and F-1c eluted with salt gradient showed amylolytic activity. The minor peak (F-1c) was not collected for further study because of low amylolytic activity. Only major peak (F-1b) having high amylolytic activity was collected separately, dialyzed against same buffer for over night at 4°C and concentrated by freeze dryer and then rechromatographed on DEAE-cellulose under identical conditions. The fraction was eluted only as a sharp single peak (figure not shown) and its purity was judged

Regular Paper

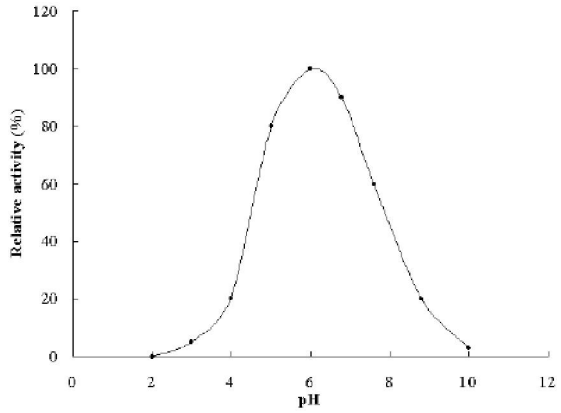

Figure 5 : Effect of pH on the activity of β-amylase from germinating wheat seeds

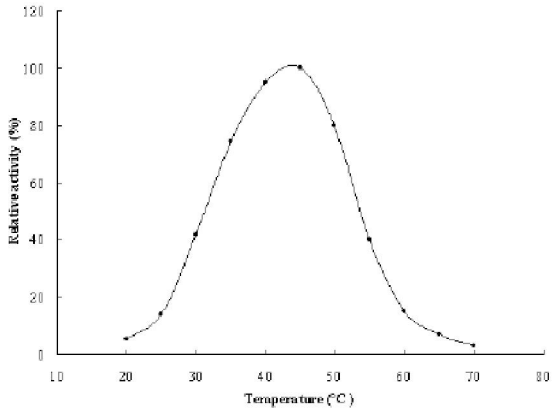

Figure 6 : Effect of temperature on the activity of β-amylase from germinating wheat seeds

TABLE 1 : Purification summary of germinating wheat seeds β–amylase (Agrani variety)

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (folds)
Crude extract	924	1915.00	2.07	100	1.00
Salting out and dialysis	690	1504.20	2.18	78.50	1.05
Gel filtration	24.35	1230.89	50.55	64.27	24.42
DEAE-cellulose	5.23	710.07	135.77	37.07	65.59

TABLE 2 : Substrate specificity of the purified β–amylase from germinating wheat seeds

Substrate	Relative activity (%)
Soluble starch	100
Amylopectin	108
Amylose	60
Maltose	0
Maltotriose	0
Starch (pea)	214
Starch (potato)	56

by polyacrylamide slab gel electrophoresis. This fraction contained pure amylase as it gave single band on polyacrylamide gel electrophoresis (Figure 4).

The purification results of amylase from germinating wheat seeds are summarized in TABLE 1. The specific activities of extracted enzyme increases at each step and the purification fold was achieved from crude extract nearly 65.59. The specific activity of the final preparation was 135.77-unit mg^{-1} with 37.07 % overall yield of enzyme where soluble starch used as a substrate.

The purified amylase enzyme gave 100% hydrolytic activity when treated with the substrate in absence of $HgCl_2$, but no hydrolytic activity was found if the substrate solution was pre-mixed with 2×10^{-3}M $HgCl_2$, an inhibitor of β-amylase. Further the hydrolytic activity of the purified enzyme was found to be unchanged in the presence of 2×10^{-3}M EDTA (α-amylase inhibitor)[32]. These findings clearly indicated that the purified amylase was of β-type.

The activity of β–amylase at various pH values from 3.0 to 10.0 were shown in figure 5. The activity of the enzyme was greatly influenced by pH changes. The pH optimum of the purified enzyme was found in a range of 5.5-6.5, with a maximum at pH 6.0. The enzyme was found to be stable between pH 4.0-8.0 and completely inactive below and above the range.

The activity of the purified β–amylase was measured at various temperatures (25-80°C) at optimum pH 6.0. The activity was found to increase with increasing temperature to 40-50°C, with an optimum temperature of 45°C (Figure 6). The effect of temperature on the stability of the enzyme was studied by keeping the enzyme at various temperatures (25-80°C) for 30 min and the remaining activities were measured. The enzyme was stable below 65ºC and its activity significantly decreased at and above 80°C.

The molecular weight of the purified enzyme was determined by comparing their elution volume on shephadex G-75, with those of the marker proteins, Tripsin inhibitor (12.028kDa), Carbonic anhydrase (29kDa), α-amylase (58 kDa), Serum albumin (66kDa) and β-galactosidase (116kDa) under identical experimental conditions. A logarithmic plot of molecular weight against relative elution volume gave a linear relationship, and the molecular weight of the enzyme was found

TABLE 3 : Effects of metal ions and chemical reagents on β–amylase activity from germinating wheat seeds

Metal or chemical reagent	% Relative activity
Control (soluble starch)	100
$CuCl_2$	4.0
$HgCl_2$	2.6
$FeCl_3$	104
NaCl	94
$CaCl_2$	93
MgC_2	91
$ZnCl_2$	80
$MnCl_2$	105
$BaCl_2$	79
$CoCl_2$	71
$NiCl_2$	63
$PbCl_2$	5.0
$SnCl_2$	20
AgCl	7.5
EDTA	110
Urea	2.5

to be 88kDa. In order to elucidate the sub-unit structure of the enzyme, SDS-polyacrylamide gel electrophoresis was performed.

As shown in figure 4, the enzyme gave a single band of the same mobility with or without SDS and β-mercaptoethanol treatment. The result indicated that the enzyme has no subunit structure. A logarithmic plot of the molecular weight against relative mobility indicated that the molecular weight of the enzyme was 88-89kDa.

The affinity of the enzyme for substrate was investigated. Soluble starch was used as the substrate. The K_m value of β–amylase was estimated from Lineweaver-Burk plots using various concentrations of starch. The K_m value for the hydrolysis of starch was found to be 1.45mg/ml.

Ultra-violet absorbance of the pure enzyme in aqueous solution was maximum at 262-266nm and minimum at 240-244nm. No significant absorbance was detected above 320nm, indicating the absence of chromophore[33].

As given in TABLE 2, the β–amylase gave about 100% activity when soluble starch and amylopectin were used but gave more than 200% activity when starch grains from pea were used. It was also shown that when amylose and starch grains from potato were used, low activity was found. On the other hand, no hydrolytic activity was observed when the enzyme was incubated with maltose and maltotriose.

The effects of various metallic salts and chemical reagents on the activity of β–amylase were examined. Enzyme was added to the substrate solution pre-incubated with a reagent at 45°C for 5 min, and residual activity (%) was determined. As shown in the TABLE 3, the presence of Fe^{3+}, Mn^{2+}, Mg^{2+} and EDTA increased the activity of the enzyme while the presence of Cu^{+2}, Hg^{+2}, Pb^{+2} and Urea reduced the activity of the enzyme remarkably.

DISCUSSION

The enzyme amylase has high amylolytic activity. It rapidly hydrolyzes poly- and oligoglucans from the nonreducing ends of the chains releasing successive maltose units. Although the enzyme was purified with an increase of purification fold of about 65 but the yield was found to be about 37% only. This decrease in yield may be due to denaturation of enzyme during the lengthy purification procedures or for some other unknown reasons.

The molecular weight of β–amylase from germinating wheat seeds determined by shephadex G-75 gel filtration column was 88 kDa. This is in good agreement with the molecular weight (89 kDa) determined by SDS-PAGE (Figure 4). Hence, wheat seeds β–amylase is a monomer. The molecular weight of wheat seeds β–amylase is similar to that of β–amylases from bulbs of Klattianus[34], Sorghum bicolor moench[35] and Bacillus megaterium B (6)[36]. Larger multimeric β–amylases have been reported from vicia faba leaves (107kDa)[37], leaves of potato (111kDa)[14], C. thermosulphurogenes (210kDa)[38] and β–amylase produced by Xanthophyllomyces dendrorhous (240kDa)[39].

β–amylase from germinating wheat seeds has a pH optimum for starch hydrolysis 6.0 (Figure 5) which is higher than that reported by Fan[40], for potato tuber β–amylase (pH 5.1-5.5), Chen-Tien et al.[13], for sweet potatoes β–amylase. However, the optimum pH of amylase activity in extracts of sprouted potato tubers reported by Ross and Davies[41] as 6.0.

Regular Paper

Hydrolysis of soluble starch by β–amylase isolated from pea epicotyl and leaves of Arabidopsis had optima at pH 6.0 and at 6.0-6.5 respectively[42,43], Cereal β–amylases have pH optima at 5.0-6.0 reported by Yamamoto[44], with which our result is in good agreement.

The optimum temperature for β–amylase activity from germinating wheat seeds was 45°C (Figure 6). Shen et al.[38] reported that the temperature optimum was as 75°C for Clostridium thermosulphurogenes β–amylase, Dicko et al.[34] reported as 55°C for bulbs of G. Klattianus β–amylase and Rashap et al.[45] reported as 50°C for Bacillus polymyxa N3 β–amylase, which are higher than our result but our result is very close to those reported by Lizotte et al.[43]; Serafimova et al.[46] as 42°C, and Rashad et al.[12] as 45°C.

The K_m value of β–amylase using starch as substrate was calculated from Lineweaver-Burk plot and found to be 1.45mg/ml. This result is close to those reported by Diaz et al.[39]; Pauline et al.[47]; Shen et al.[38] and is lower than that reported by Kotha and Rameswar[48], Katsuhiro et al.[49], Rashad et al.[12].

Some disaccharides and polysaccharides were tested as substrate for β–amylase activity shown in TABLE 2. From the results it is clear that the rate of hydrolysis is highest in case of starch (pea) followed by amylopectin and soluble starch (potato). This result is very similar in substrate specificity for pea epicotyl β-amylase as reported earlier[47].

The effects of metal ions and chemical reagents on the wheat seeds β–amylase enzyme activity were studied (TABLE 3). The purified enzyme was inactivated in presence of Cu^{2+}, Hg^{2+}, Pb^{2+}, Ag^{+} and Urea, and was strongly activated in presence of Fe^{3+}, Mn^{2+} and EDTA. These results are in agreement with those reported by Okamato and Akazawa[3], from rice seed β–amylase, Rashad et al.[12] from radish root and Tsao et al.[50] from small abalone.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the research facilities provided by the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh.

REFERENCES

[1] C.F.Daniel, S.Michaela, M.Tabea, K.V.Cara, P.F.Jing Li, H.R.Manuel Gil, E.Simona, l.M.Gae, D.Gary, H.Karen, M.S.Alison, M.S.Steven, C.Z.Samuel; The Plant Cell, **20**, 1040–1058 (**2008**).
[2] C.T.Green Wood, E.A.Milne; Adv.Carbohydr. Chem., **23**, 281 (**1968**).
[3] K.Okamato, T.Akazawa; Agric.Biol.Chem., **42**, 1379 (**1978**).
[4] D.E.Evans, W.Wallace, R.C.M.Lance, L.C.MacLeod; Journal of Cereal Science, **26**, 241-250 (**1997**).
[5] T.Sopanen, C.Lauriere; Plant Physiology, **134**, 678-684 (**1989**).
[6] D.J.Manners, P.M.Dey, R.A.Dixon; 'Starch: In Biochemistry of Storage Carbohydrates in Green Plants', Ed., Academic Press, New York, 149 (**1985**).
[7] E.Beck, P.Ziegler; Ann.Rev.Plant Physiol., **40**, 95 (**1989**).
[8] Amylase Research Society of Japan (Ed.); 'Handbook of Amylases and Related enzymes. Their Sourches, Isolation Methods, Properties and Application', Pergamon Press, Oxford, (**1988**).
[9] P.K.Nehere, N.K.Shah, V.Ramamurthy, R.M.Kothari; World Journal of Microbiology and Biotechnology, **8**, 446-450 (**1992**).
[10] B.Mikami, S.Aibara, Y.Morita; Agric.Biol.Chem., **46**, 943 (**1982**).
[11] K.Ubbaramaiah, R.Sharma; Phytochem., **29**, 1417 (**1990**).
[12] M.M.Rashad, E.W.Jwanny, S.T.El-Sayed, A.E.Mahmoud; Bioresource Technology, **51**, 183-186 (**1995**).
[13] C.Chen-Tien, L.Huey-Yu, T.Huey-Ling, S.Hsien-Yi; Biotechnol.Appl.Biochem., **24**, 13-18 (**1996**).
[14] A.Vikso-Nielsen, M.I.E.Tove, M.B.Christensen, M.Jan; Physiologia Plantarum., **99**, 190-196 (**1997**).
[15] A.Hirata, M.Adachi, A.Sekine, Y.N.Kang, S.Utsumi, B.Mikami; J.Biol.Chem., **279**, 7287-7295 (**2004**).
[16] Y.N.Kang, A.Tanabe, M.Adachi, S.Utsumi, B.Mikami; Biochemistry, **44(13)**, 5106-16 (**2005**).
[17] Z.Ye, H.Miyake, M.Tatsumi, S.Nishimura, Y.Nitta; J.Biochem., **135(3)**, 355-63 (**2004**).
[18] A.Hirata, M.Adachi, S.Utsumi, B.Mikami; Biochemistry, **43(39),** 12523-31 (**2004**).
[19] R.R.Ray; Acta Microbiol Immunol Hung., **51(1-2),** 85-95 (**2004**).
[20] J.A.Thoma, J.E.Spradlin, S.Dygert; 'Plant and Animal Amylases. In The Enzymes', (3rd edition), Academic Press, New York, **5**, 115-189 (**1971**).

[21] J.Daussam, A.Skakoun; Isozymes: Current Topics in Biological and Medical Research, New York, **5**, 175-218 (**1981**).
[22] J.Daussant, J.Sadowski, P.Ziegler; Journal of Plant Physiology, **143**, 585-590 (**1994**).
[23] Y.Yamasaki; Phytochemistry, **64(5)**, 935-939 (**2003**).
[24] V.Heinz, R.Buckow, D.Knorr; Biotechnol Prog, **21(6)**, 1632-8 (**2005**).
[25] S.T.El-Sayed, E.W.Jwanny, M.M.Rashad, A.E.Mahmoud, N.M.Abdullah; Appl.Biochem. Biotechnol., **3(55)**, 219-230 (**1995**).
[26] J.Jayaraman; 'Laboratory Manual in Biochemistry', 1st Ed., Wiley Eastern Ltd., New Delhi, 75 (**1985**).
[27] P.Andrews; Biochem.J., **91**, 222 (**1974**).
[28] K.Weber, M.Osborn; J.Biol.Chem., **244**, 406 (**1966**).
[29] U.K.Laemmli; Nature, **227**, 680-685 (**1970**).
[30] T.F.Robyt, D.J.White; 'Biochemical Techniques: Theory and Practical', Waveland Press, Inc., Illinois, USA, 295-296 and 304-305 (**1990**).
[31] O.H.Lowry, N.J.Rosenbrough, A.L.Farr, R.J. Rendall; Biol.Chem., **183**, 265-275 (**1951**).
[32] E.Garcia, F.M.Lajolo; J.Food Sci., **53(4)**, 1181-1186 (**1988**).
[33] H.Inoue, F.Suzuki, K.A.Fukunishi, K.Abachi, Y.Takeda; J.Biochem., **60**, 543 (**1966**).
[34] M.H.Dicko, M.J.F.Searle-van Leeuwen, R.Hilhorst, A.S.Traore; Bioresource Technology, **73**, 183-185 (**2000**).
[35] U.O.Ekong, O.U.Anthony; J.Agric.Food Chem., **32**, 11-14 (**1984**).
[36] R.R.Ray; Acta Microbiol Immunol Hung., **47(1)**, 29-40 (**2000**).
[37] G.W.Chapman, J.E.Pallas, J.Mendicine; Biochemica et Biophysica Acta, **276**, 491 (**1972**).
[38] G.J.Shen, B.C.Saha, Y.E.Lee, L.Bhatnagar, J.G.Zeikus; Biochem.J., **254**, 835-840 (**1988**).
[39] A.Diaz, C.Sieiro, T.G.Villa; Lett. Appl Microbiol., **36(4)**, 203-7 (**2003**).
[40] M.L.Fan; Taiwania, **20**, 132-138 (**1975**).
[41] H.A.Ross, H.V.Davies; Potato Res., **30**, 675-678 (**1987**).
[42] T.P.Lin, S.R.Spilatro, J.Preiss; Plant Physiol., **86**, 251-259 (**1988**).
[43] P.A.Lizotte, C.A.Henson, S.H.Duke; Plant Physiol., **92**, 615-621 (**1990**).
[44] T.Yamamoto; 'B-amylases (EC 3.2.1.2.a-1,4-glucan maltohydrolase), In Handbook of Amylases and Related Enzymes. Their Sources, Isolation Methods, Properties and Applications' (The Amylase Research Society of Japan, Eds.)', Pergamon Press, Tokyo, 81-104 (**1988**).
[45] R.K.Rashap, Z.A.Iushkaite, A.A.Glemzha; Prikl Biokhim Mikrobiol., **17(2)**, 225-32 (**1981**).
[46] F.Serafimova, A.Franz, E.Werries; Mol.Biochem. Parasitol., **83(2)**, 175-81 (**1996**).
[47] A.L.Pauline, A.H.Cynthia, H.D.Stanley; Plant Physiol, **92**, 615-621 (**1990**).
[48] S.Kotha, S.Rameshwar; Phytochemistry, **29(5)**, 1417-1419 (**1990**).
[49] Y.Katsuhiro, Y.Hiroshi, M.Keiichi, N.Yoshiki, H.Masataka, K.Sumio, Y.Kaisuke, U.Chisae; Chem.Pharm.Bull., **37(4)**, 973-978 (**1989**).
[50] C.Y.Tsao, Y.Z.Pan, S.T.Jiang; J.Agric Food Chem., **51(4)**, 1064-70 (**2003**).