December 2008 *Volume 2 Issue (2-3)*

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 2(2-3), 2008 [91-98]

Purification and characterization of α-amylase from germinating chickpea (*Cicer arietinum* L.) seed

Niranjan Kumar Sana*, Md.Shanawazur Rahman, Md.Mehedi Hasan, Bidhan Chandra Sarkar, Md.Entazul Huque[1], Ranajit Kumar Shaha

Department of Biochemistry, Molecular Biology, University of Rajshahi, Rajshahi-6205, (BANGLADESH)

E-mail : nksana2003@yahoo.com

Received: 20th November, 2008 ; Accepted: 25th November, 2008

ABSTRACT

In germinating chickpea seeds after 72 hours, the abundant amylolytic activity was found to be due to α-amylase. The enzyme was purified from germinating chickpea (*Cicer arietinum* L.) seed by successive 30-50% ammonium sulphate fractionation followed by DEAE-cellulose and Sephadex G-75 gel filtration chromatography to the homogenous state as confirmed by slab gel electrophoresis. The enzyme was purified 68.3-fold with a yield of 72.4% of the total activity. The purified enzyme was found to be a glycoprotein with an apparent molecular mass of 42 and 45 kDa as estimated by sodiumdodecylsulfate-polyacrylamide gel electrophoresis and gel permeation chromatography, respectively. The purified amylase from germinating chickpea seems to be α-type as confirmed by EDTA and glycoprotein in nature. The glycoprotein was found to contain 2.7% sugar. Amylolytic activity of this enzyme was 96% and 63% for amylose and amylopectin, respectively. The enzyme has no effect on maltose and maltotetraose. The optimum pH and temperature of the purified α-amylase were 7.0 and 37^{0}C respectively. Metallic ions like Ca^{2+}, Fe^{3+}, Mn^{2+} and Na^{+} increased amylase activity while Cu^{2+}, Fe^{2+} and Zn^{2+} strongly; Hg^{+}, Ag^{+}, Mg^{2+} and K^{+} moderately and Li^{+}, Cd^{+} slightly inhibited the enzymatic activity. With increasing concentration of EDTA and urea, the activity of the purified enzyme decreased sharply. The K_m value of this enzyme was found to be 0.28% for starch as substrate.

KEYWORDS

Chickpea
(*Cicer arietinum* L.) seed;
α-Amylase;
Starch;
Purification;
Characterization.

INTRODUCTION

Amylase is a digestive enzyme classified as a saccharidase. It is mainly a constituent of pancreatic juice and saliva, needed for the breakdown of long-chain carbohydrates into smaller units. Alpha-amylases hydrolyze alpha-1, 4-glycosidic linkages, randomly yielding dextrins, oligosaccharides and monosaccharides. Alpha-amylases are endo-amylases. It is also a major digestive enzyme[1]. α-Amylase is derived from plant, animal and fungal sources. Plant seeds, tubers and vegetative organs contain amylolytic enzymes. It is synthesized in the fruit of plants during ripening causing them sweeter. α-Amylase digests carbohydrates (polysaccharides) into smaller disaccharide units, eventually converting them into monosaccharides such as

Regular Paper

glucose so that they can be used by the body. α-Amylase, considered as one of the enzymes, which degrade starch, may play a role in the mobilization of starch during germination or sprouting of tubers[2,3]. α-Amylase plays a vital role in human physiology, pathology and in different industries. Salivary α-amylase has been used as a biomarker for stress that does not require a blood draw[4]. Some disease condition may be determined by the increased or decreased level of α-amylase[5]. An inhibitor of α-amylase called phaseolamin has been tested as a potential diet aid[6]. The industrial importance of α-amylase makes it a popular subject for study. It is used in the textile industry for designing textiles, in the laundry industry 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 the removal of starch in jelly production. Responses of sugar metabolism during germination of rice (*O.sativa*), wheat (*T.aestivum*) and rape (*B.chinensis var. oleifera*) seeds to stimulate acid rain were investigated[7]. Prematurity α-amylase is associated with temperature shocks during wheat grain filling was reported[8]. α-Amylase inhibitor from amaranth seeds has been studied and assumed that it may be a better candidate to make genetically modified potatoes resistant to Tecia solanivora larvae than inhibitors from common bean seeds[9]. Extensive studies have been performed on the purification and characterization of α-amylase from different origins such as from honey[10]; ripening bananas[11]; cassava (*Manihot esculenta*)[12]; mung (*Vigna radiata*) beans[13]; *Bacillus sp. PN5*[14]; *Thermobifida fusca*[15]; *Nocardiopsis* sp. 7326[16]; Bacillus subtilis[17]; potato tubers[18]; Eisenia foetida[19] and Geobacillus thermoleovorans[20]. In contrast, relatively little information is available on the purification and characterization of α-amylase from germinating seeds. The amylolytic activities in chickpea seeds have not been studied. This work is an attempt to fill the gap.

MATERIALS AND METHODS

Materials

Chickpea (*Cicer arietinum* L.) seeds were collected from Bangladesh Agriculture research Institute (BARI), Ishwardi, Pabna, Bangladesh. The seeds were soaked in distilled water for 6 hours, germinated in a lighted room at 25^0C for 24, 36, 48, 72, 96, 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 (-10^0C) 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.

Enzyme extraction

100 grams of germinated seeds (germinated at 72 h) were taken in a pre-cooled mortar and pasted with a pestle and homogenized with cold de-ionized distilled water in a ratio of 3:5 (weight of dry seeds/water volume) and then centrifuged at 6×10^3rpm (at 2^0C) for 10 minutes by a refrigerated centrifuge machine. After centrifugation, the clear supernatant was taken as a crude extract of the enzyme.

Purification of amylase

Ammonium sulphate fractionation

The crude extract was saturated to 30-50% by the addition of solid ammonium sulphate under constant and gentle stirring at 4^0C. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of pre-cooled distilled water and dialyzed against distilled water for 24 hours at 4^0C. The dialyzed solution was then centrifuged in a refrigerated centrifuge machine at 10000 rpm for 15 minutes to remove insoluble materials. The clear supernatant thus obtained was designated as "crude enzyme solution".

DEAE-cellulose chromatography

The concentrated crude enzyme solution after dialysis in 50 mM phosphate buffer, pH 7.4, 4^0C was loaded onto a DEAE-cellulose column (32×1.0 cm, flow rate 30 ml h^{-1}), which was equilibrated with the same buffer. The column was eluted with 50 mM phosphate buffer, pH 7.4, containing a NaCl linear gradient (100-500 mM). Absorbance of each fraction at 280 nm, amylolytic activities and protein concentration were measured and the active fractions were collected.

Gel filtration chromatography

The enzymatically active protein fractions after DEAE-cellulose column were collected and dialyzed against 50 mM phosphate buffer, pH 7.0 for overnight and then concentrated to its 1/4th volume by freeze dryer and finally applied to a Sephadex G-75 column (2.5×120 cm) previously equilibrated with 50 mM phosphate buffer, pH 7.0 and eluted with the same buffer. Absorbance at 280 nm, protein concentration and amylolytic activities were determined and the active fractions were collected.

Measurement of amylase activity

Amylase activity was assayed following the method as described by Jayaraman[21]. 1% of starch solution was used as substrate (1 gm in 100 ml of 0.1 M phosphate buffer, pH 6.7). The amylase activity was measured by estimating the amount of maltose released by it. 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 1μg of maltose from starch per minute at 37^{0}C.

Molecular weight of α-amylase

(a) The molecular weight of the purified α-amylase was determined by gel filtration on Sephadex G-75 column (150X3.0 cm) equilibrated with 50 mM phosphate buffer, pH 6.8, following the established procedure[22]. Trypsin inhibitor (12 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), phosphorylase-b (97 kDa) and β-galactosidase (116 kDa) were used as marker proteins[23].

(b) Electrophoresis: SDS-PAGE was performed according to the method of Laemmli[24] on a Bio-rad mini electrophoresis system. The standard proteins used were β-lactoglobulin (18.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa) and phosphorylase-b (97.4 kDa). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

Test for glycoprotein and estimation of sugar

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois et al.[25]. The method was also employed for detecting the presence of sugar in protein.

Optimum pH of the enzyme

To study the effect of pH on enzyme activity, the enzyme solutions (0.6%) were dialyzed against 50 mM 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_2CO3.) for 24 hours with frequent change of buffers. After necessary adjustment of pH by adding 0.1 N HCl or 0.1 N 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 50 mM phosphate buffer, pH 7.0, were incubated at various temperatures ranging from 10^{0}C - 90^{0}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 100 mM phosphate buffer pH 6.7, 2.5 ml of substrate (1%) of different types and 0.5 ml of enzyme solution were taken in different test tubes and incubated at 37^{0}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^{0}C).

Measurement of K_m of α-amylase

Michaelis constant (K_m) was determined by the

Regular Paper

assay of enzyme activity for various concentration of the substrate (starch 0.1 - 2.0 mM) at definite interval. Initial velocities of respective substrate concentration were calculated[26]. Km was calculated from Lineweaver-Burk double reciprocal plot.

Protein assay

Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280 nm. The amount of protein was estimated by the published method of Lowry et al.[27], using BSA as standard substrate.

RESULTS AND DISCUSSION

The amylolytic activity of α-amylase from the germinating chickpea seeds showed their maximum activity at 72 hours of germination. After that the activity declined rapidly. So, in further studies, we used the extracts of chickpea seeds germinated at 72 hours.

Purification of amylase

In DEAE-cellulose column, the proteins of crude enzyme extract from germinating chickpea were eluted as one major peak (F-1) and three minor peaks (F-0, F-2, F-3) (**figure** 1). The major fraction F-1 contained the amylase activity while the other three minor fractions had no amylase activity. The active fraction F-1 was pooled separately and the purity was checked by the slab gel electrophoresis. As shown in the figure, F-1 fraction gave more than one band indicating that F-1 fraction contained more than one protein.

The active fraction F-1 from DEAE-cellulose column chromatography was dialyzed against 50 mM phosphate buffer, pH 7.0 for overnight and charged onto gel-filtration column previously equilibrated with the same buffer at 4^0C. The contents of F-1 fraction were eluted with 50 mM phosphate buffer; pH 7.0.The F-1 fraction separated into three protein peaks F-1a, F-1b and F-1c (figure 2). Of these, only F-1b contained the amylase activity. The active fraction (F-1b) obtained from gel-filtration chromatography was homogenous on slab gel electrophoresis and showed single protein band (Figure 3) indicating that the enzyme was in pure form.

The data on purification of germinating chickpea

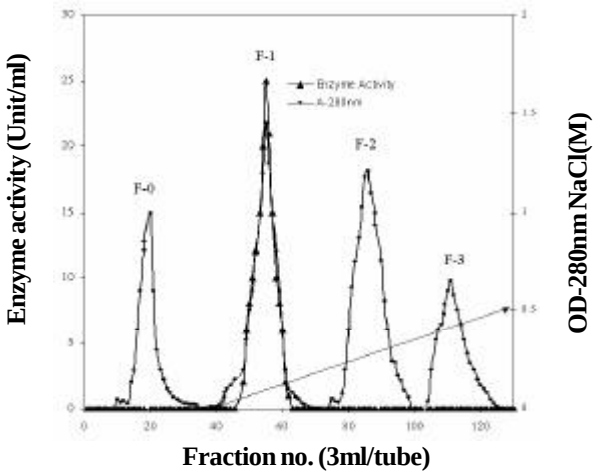

Figure 1: Elution profile of crude enzyme from DEAE-cellulose column. The column (32×1.0 cm) was pre-equilibrated with 50 mM phosphate buffer, pH 7.4 and was eluted with the same buffer. The column was then eluted with a linear gradient of NaCl (0.1 to 0.5 M) in the same buffer. The flow rate of the column was 30 ml/h. Symbols: (•) absorbance at 280 nm, (▲) enzyme activity and (→) NaCl gradient

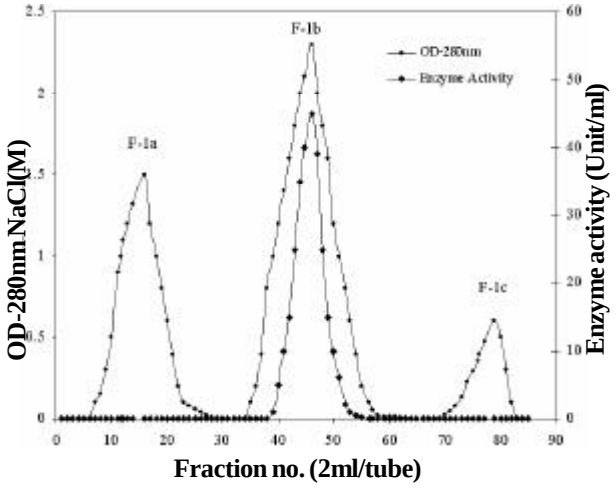

Figure 2: Elution Profile of F-1 fraction from DEAE-cellulose column. Fraction F-1 was applied to a Gel-filtration column (2.5×12 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0 and the column was eluted with same buffer. The flow rate of the column was 20 ml/h. Symbols: (•) absorbance at 280 nm, (♦) enzyme activity

amylase are presented in TABLE 1. The purity of the enzyme from chickpea seeds increased 68.3 fold with an overall yield of 72% with specific activity of 125.4 unit/mg. The decrease in yield may be due to denaturation of the enzyme during purification or to some other reasons. The specific activity of the purified chickpea α-amylase compares well with other highly purified

Regular Paper

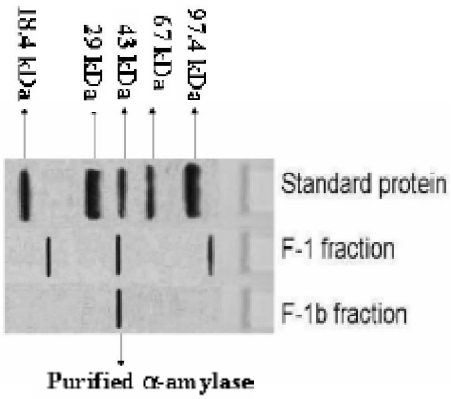

Figure 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified α-amylase and marker proteins for the determination of purity and molecular weight of the enzyme. Standard = Phosphorylase B (MW 97.4 kDa), Bovin Serum Albumin (MW 67.0 kDa), Ovalbumin (MW 43.0 kDa), Carbonic anhydrase (MW 29.0 kDa), β-lactoglobulin (18.4 kDa). F-1 fraction = After DEAE-cellulose column chromatography. F-1b fraction = After gel filtration column chromatography

TABLE 1: Summary of purification of α-amylase from germinating chickpea seeds

Steps	Total protein (mg)	Total activity (Unit/ mg)	Specific activity (Unit/ mg)	Yield (%)	Purification fold
Crude extract	965	1775.6	1.84	100	1
Salting out and dialysis	380	1592.2	4.19	89.67	2.28
DEAE-cellulose	25.32	1433.36	56.61	80.72	30.77
Gel-filtration chromatography	10.23	1286.42	125.73	72.45	68.33

endoamylases[28,29].

Characterization of amylase

Determination of the type of purified amylase

The purified amylase gave 100% hydrolytic activity when incubated with the substrate in the absence of EDTA, but no hydrolytic activity was found when the enzyme was pre-incubated with 30 mM EDTA, an inhibitor of α-amylase[30]. Further the hydrolytic activity of the purified enzyme was found to remain unchanged in the presence or absence of 10 mM cysteine and 20 mM $HgCl_2$ (SH-dependent, β-amylase inhibitor). This finding clearly revealed that the purified chickpea amylase was of α-type.

Determination of molecular weight

The molecular weight of purified α-amylase (F-1b) as determined by gel filtration on Sephadex G-75 to be 45 kDa. The molecular weight of the chickpea seeds α-amylase obtained in this investigation is in reasonable agreement with the molecular weight estimated for α-amylase from other sources. Chang et al.[31] purified α-amylase from Aspergillus oryzae having a molecular mass of 52 kDa by gel filtration. The molecular weight of the purified α-amylase was also determined by SDS-polyacrylamide slab gel electrophoresis and was found to be 42 kDa (figure 3). The molecular weight of chickpea α-amylase is in the same range (38-45 kDa) of other previously reported amylases from plant sources[32,33]. The molecular weight of the enzyme was found to be unchanged in the presence or absence of β-mercaptoethanol indicating that the α-amylase contained no subunit.

Test for glycoprotein and estimation of percentage of sugar

The purified enzyme gave yellow-orange color in the presence of phenol sulfuric acid indicating that the enzyme contained sugar and hence a glycoprotein. The percentage of sugar in α-amylase was calculated from the standard graph of glucose to be 2.7%. Kuzovlev et al.[34], reported that the α-amylase in germinating maize seeds was a glycoprotein. Beaupoil-abadie et al.[35], also observed the presence of carbohydrate in porcine pancreatic amylase.

Effect of pH on the enzyme activity

The optimum pH of α-amylase was 7.0 (figure 4). Very similar pattern of pH profile have been reported for the α- amylases from pear fruits[36] and crude enzymes from banana pulp[37]. The activity was found to decrease gradually in the acidic pH, but in the alkaline pH it decreased rapidly. Very negligible enzyme activity was observed below pH 3.0 and above pH 10. Berbezy et al.[38] observed the optimum pH 7 for α-amylase from vine shoot inter-nodes, which is nearly similar to the present finding. Beers and Duke[39] reported the optimum pH 5.5-6.5 for α-amylase from shoots and cotyledons of pea (Pisum sativam L.) seedling, which is lower than the present finding.

Effect of temperature on the amylase activity

The effect of temperature on the activity of α-amylase of germinating chickpea was examined in the range

Regular Paper

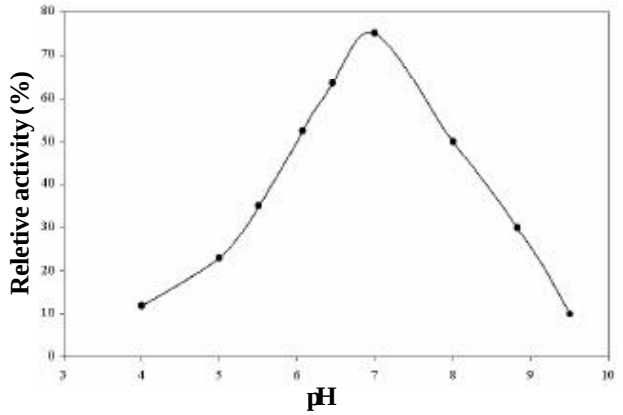

Figure 4: Effect of pH on the activity of chickpea α-amylase

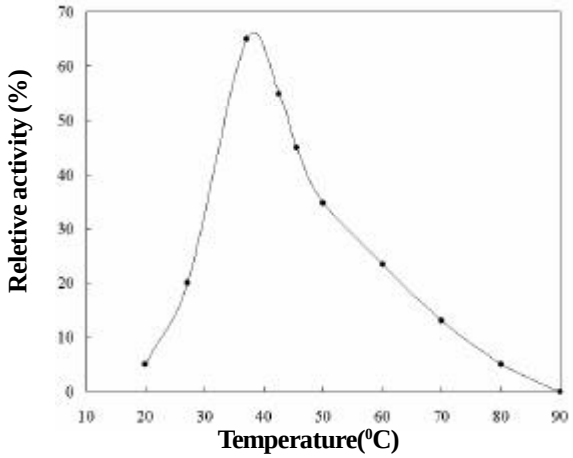

Figure 5: Effect of temperature on the activity of chickpea α-amylase

of 10-90^{0}C. As shown in figure 5, the optimum activity of amylase was observed at 37^{0}C. There was a sharp increase in activity with gradual increase in temperature up to 37^{0}C while the activity gradually decreased with further rise in temperature indicating the loss in active conformation of the enzyme. The enzyme was only about 20% active at temperature 70^{0}C. These results are in accordance with those previously published data for α-amylase from Moringa oleifera seeds[40] and α-amylase from Allernaria alternata[41]. Berbezy et al.[38] observed that the optimum temperature of 57^{0}C for α-amylase from vine shoot internodes which is higher than our result.

Substrate specificities

The substrate specificity of the α-amylase was studied using some disaccharides and polysaccharides as substrate and the results are summarized in TABLE 2. The enzyme was able to hydrolyze a wide range of carbohydrate containing α-1,4-glycosidic bond. In general, high-molecular-mass substrates containing α-1,4-linkage were better substrate for the enzyme. The relative rate of hydrolysis of the polymeric substrate decreased with decreasing percentage of α-1, 4-linkages and increasing percentage of α-1,6-linkages in the substrate, suggesting that the enzyme prefers high-molecular-mass, amylose type material as the substrate. α-Amylase hydrolyzed amylose at rates similar to those obtained with soluble starch, but it was considerably less active with amylopectin as substrate. This enzyme has no effect on maltose and Maltotetraose. Beers and Duke[39] reported the similar substrate specificity of pea α-amylase. Morgan and Priest[42] and Nakakuki et al.[43], reported that *B. licheniformis* α-amylase were capable of polymerization of 6 (maltohexose). Saito[44], on the other hand, reported that *B.licheniformis* α-amylase was capable of cleaving oligosaccharides with

TABLE 2: Substrate specificity of germinating chickpeas α-amylase

Substrate	Glycosidic bond	Relative activity (%)
Soluble starch	Glu α-1,4 Glu-Glu 1,6 Glu.	100
Amylose	Glu α -1,4 Glu	96
Aylopectin	Glu α -1,4 Glu- Glu 1,6 Glu	63
Maltotetraose	Glu α -1,4 Glu	0
Maltose	Glu α -1,4 Glu	0

The activity for soluble starch was taken as 100%

TABLE 3: Effect of various metallic salts and chemicals on the activity of α–amylase purified from chickpea seed

Reagent	Relative activity (%)		
	1mM	3mM	5mM
None	100	100	100
$FeCl_2$	90	74	35
$MgCl_2$	93	85	78
AgCl	87	81	75
LiCl	85	78	55
$MnCl_2$	125	135	145
NaCl	101	103	105
KCl	89	82	76
$CuCl_2$	73	65	43
$ZnCl_2$	65	61	51
$HgCl_2$	96	86	80
$CdCl_2$	84	79	62
$FeCl_3$	109	118	127
$CaCl_2$	148	170	180
EDTA	9.0	3.0	1.5
Urea	55	22	5.0

a minimum degree of polymerization of 4 (maltotetrose).

Effect of metal ions and organic compounds

The effect of calcium as metal salt on the activity of purified amylase from germinating chickpea is presented in TABLE 3. The activity of the enzyme gradually increased with the increase in concentration of calcium. In the presence of 0.1 M Ca^{2+}, the activity of amylase was almost double. From the result it may be suggested that calcium is needed for maintaining the enzyme molecule in the optimum configuration for maximum activity and stability. A variety of α-amylase, mostly from microbial, mammalian and cereal sources are well characterized. These enzymes belong to a large family of Ca^{2+}-proteins, which share several structural features[45]. Calcium has been shown to be essential for the activity of a number of amylases obtained from mesophilic sources[46]. Buisson et al.[47] reported the role of calcium in structure and activity of porcine pancreatic α-amylase.

The effects of various metal salts on the activity of α-amylase purified from germinating chckpea were studied (TABLE 3). From the table, it was evident that the presence of Li^+, K^+, Ag^+, Hg^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Mg^{2+} and Zn^{2+} reduced the enzyme activity while the presence of Na^+ Mn^{2+}, Fe^{3+} increase the activity of the enzyme. These results are in good agreement with those reported by Shaw et al.[48] for α-amylase from *Thermus sp.* and Takeuchi et al.[49], from the traditional starter "murcha" in Nepal.

The effects of urea and EDTA on the activity of α-amylase purified from germinating chickpea were also demonstrated in the TABLE 3. The activities of the enzyme were found to be decreased significantly in the presence of urea and EDTA. The decreased activities in the presence of urea and EDTA might be due to the denaturation of the enzyme or changes in the conformation of the active sites. Landerman et al.[50] reported at a urea concentration of 2M there was a significant decrease in activity of thermostable α-amylase from the hyperthermophilic archaeobacterium, *Pyrococcus furiosus*. Toralballa and Etington[51] also reported the inhibitory effect of urea and other amide reagents on the activity of crystalline porcine pancreatic amylase. Berbezy et al.[38] also reported that amylase was completely inhibited by EDTA.

K_m value of the amylase enzyme

The K_m value of the purified enzyme was determined by Lineweaver-Burk double reciprocal plot and was found to be 0.28% for starch as substrate which is similar to that reported by Baker[52] for two allozymes (was 0.25%) from *S.oryzae*. K_m value for the amylase preparation from *R.donfinicia was* 0.13%, which is lower than the present finding[53]. K_m values of purified amylases from other granivorous coleopterans for soluble starch ranged from 0.18% for *T.molitor*[54] to 0.23% for *C.chinensis*[55].

ACKNOWLEDGMENTS

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

REFERENCES

[1] Burtis Carl, R.Ashwood Edward; 'Teitz Textbook of Clinical Chemistry', 3rd Ed., W.B.Saunders Company, Philadelphia, **(1999)**.

[2] T.U.Kim, B.G.Gu, J.Y.Jeong, S.M.Byun, Y.C.Shin; Appl.Env.Microbiol., USA, **61 (8)**, 3105-3112 **(1995)**.

[3] E.Beck, P.Ziegler; Plant Physiol.Plant Mol.Biol., **40**, 95 **(1989)**.

[4] N.Yuka, T.Sato, M.Kudo, K.Kurata, K.Hirota; Anesth.Analg., **101(6)**, 1873-1876 **(2005)**.

[5] K.Jenifer, M.D.Lehrer; Department of Gastroenterology, Frankford-Torresdale Hospital, Jefferson Health System, Philadelphia, PA. Review provided by VeriMed Healthcare Network, ADAM Health illustrated Encyclopedia, **(2007)**.

[6] J.Udani, M.Hardy, D.C.Madsen; Altern Med Rev., **9(1)**, 63-9 **(2004)**.

[7] L.H.Wang, Q.Zhou, Q.L.Zeng; Huan Jing Ke Xue., **29(3)**, 799-803 **(2008)**.

[8] A.D.Farrell, P.S.Kettlewell; Ann.Bot.Lond., **102(2)**, 287-93 **(2008)**.

[9] Valencia-Jimenez, V.J.W.Arboleda, A.Lopez Avila, M.F.Grossi-de-Sa; Bull.Entomol. Res., **1**, 1-5 **(2008)**.

[10] S.Babacan; A.G.J.Food Science, **72(1)**, 50-55 **(2007)**.

[11] A.V.Junior, J.R.Nascimento, F.M.Lajolo; J.Agric. Food.Chem., **54(21)**, 8222-8 **(2006)**.

Regular Paper

[12] S.Tangphatsornruang, M.Naconsie, C.Thammaro ngtham, J.Narangajavana; Plant Physiol Biochem., **43(9)**, 821-7 **(2005)**.

[13] P.Tripathi, L.Lo Leggio, J.Mansfeld, R.Ulbrich-Hofmann, A.M.Kayastha; Photochemistry, **68(12)**, 1623-31 **(2007)**.

[14] R.K.Saxena, K.Dutt, L.Agarwal, P.Nayyar; Bioresour.Technol., **98 (2)**, 260-5 **(2007)**.

[15] C.H.Yang, W.H.Liu; J.Ind.Microbiol.Biotechnol., **34(4)**, 325-30 **(2007)**.

[16] J.W.Zhang, R.Y.Zeng; Mar.Biotechnol., NY., **10(1)**, 75-82 **(2008)**.

[17] M.R.Swain, S.Kar, G.Padmaja, R.C.Pol; J. Microbiol., **55(4)**, 289-96 **(2006)**.

[18] W.Witt, J.J.Sauter; J.Exp.Bot., **47(304)**, 1789-1795 **(1996)**.

[19] M.Ueda, T.Asano, M.Nakazawa, K.Miyatake, K.Inouve; Comp.Biochem.Physiol.B Biochem.Mol. Biol., **150(1)**, 125-30 **(2008)**.

[20] J.L.Uma Maheswar Rao, T.Satyanarayana; Appl Biochem Biotechnol., **142(2)**, 179-93 **(2007)**.

[21] J.Jayaraman; 'Laboratory Manual in Biochemistry', 1st Ed., Wiley Eastern Ltd., New Delhi, **(1985)**.

[22] P.Andrews; Biochem.J., **91**, 222 **(1974)**.

[23] K.Weber, M.Osborn; J.Biol.Chem., **244**, 406 **(1966)**.

[24] U.K.Laemmli; Nature, **227**, 680-685 **(1970)**.

[25] M.Dubois, K.A.Gilles, J.K.Hamilton, P.A.Rebers, F.Smith; Anal.Chem., **28**, 350-356 **(1856)**.

[26] T.F.Robyt, D.J.White; 'Biochemical Techniques: Theory and Practical', Waveland press, Inc., Illinois, USA, 295-296/304-305, **(1990)**.

[27] Lowry, N.J.Rosenbrough, A.L.Farr, R.J.Rendall; Biol.Chem., **183**, 265-275 **(1951)**.

[28] P.Ziegler; Plant Physiol., **86**, 659-666 **(1988)**.

[29] W.Witt, J.J.Sauter; Phytochem., **41**, 365-372 **(1996)**.

[30] E.Garcia, F.M.Lajolo; J.Food Sci., **53(4)**, 1181-1186 **(1988)**.

[31] C.T.Chang, M.S.Tang, C.F.Lin; Biochem.Mol.Biol. Int., **36(1)**, 185-93 **(1995)**.

[32] W.Witt, J.J.Sauter; J.Bot., **47**, 1789-1795 **(1996)**.

[33] F.Hirasawa, S.Yamamoto; Planta, **184**, 438-4422 **(1991)**.

[34] V.A.Kuzovlev, O.V.Fursov; J.Biochem., **23**, 92-96 **(1991)**.

[35] Beaupoil-abadie, M.Raffalli, P.Cozzone, G.Marchis-Moureii; Biochem.Biol.Phyc.Acia., **297**, 436-476 **(1973)**.

[36] G.W.F.Maris McArthur-Hespe; Acta Botanica Neeflandica, **5**, 200 **(1956)**.

[37] W.W.Mao, J.E.Kinsella; J.Food.Sci., **46**, 1400-1409 **(1981)**.

[38] P.Berbezy, L.Legendre, A.MaLkjean; Plant Physiol. Biochem., **34(3)**, 353-361 **(1996)**.

[39] E.P.Beers, S.H.Duke; Plant Physiol., **92**, 1154-63 **(1990)**.

[40] M.U.Dahot, A.A.Saboury, S.Ghobadi, A.A.Moosavi -Movahedi; J.Biol.Sci., **1(8)**, 747-749 **(2001)**.

[41] S.J.Chung, B.Hwang; Korn.J.Mycol., **24(1)**, 816-984 **(1996)**.

[42] F.J.Morgan, F.G.Priest; J.Appl.Bacteriol., **50**, 107-114 **(1981)**.

[43] T.Nakakuki, K.Azurna, K.KainLinia; Carbohydr. Res., **128**, 297-310 **(1985)**.

[44] N.Saito; Arch.Biochem.Biophys., **15**5, 290-298 **(1973)**.

[45] MacGregor; Starch, **45**, 232-237 **(1993)**.

[46] B.L.Vallee, E.A.Stein, W.N.Sumerwell, E.H.Fischer; J.Biol.Chem., **234**, 2901-2905 **(1959)**.

[47] G.Buisson, E.Duce, R.Haser, F.Payan; J.Embro., **6**, 3909 **(1987)**.

[48] J.F.Shaw, F.P.Lin, S.C.Chen, H.C.Chen; Bol.Bull. Acad.Sin., **36**, 195-200 **(1995)**.

[49] Takeuchi, A.Shimizu-Ibuka, Y.Nishiyama, K.Mura, S.Okada, A.S.Tokue; Biosci Biotechnol Biochem., **70(12)**, 3019-24 **(2006)**.

[50] K.A.Landerman, B.R.Davis, H.C.Krutzsch, M.S.Lewis, Y.V.Griko, P.L.Privalov, C.B.Anfinsen; The Journal of Biological Chemistry, **266(32)**, 24394-24401 **(1993)**.

[51] G.Toralballa, M.Eitingon; Arch.Biochem.Biophys., **119**, 519 **(1967)**.

[52] J.E.Baker; Insect Biochem., **17**, 37-44 **(1987)**.

[53] J.E.Baker; Insect Biochem., **21**, 303-311 **(1991)**.

[54] V.Buonocore, E.Poerio, V.Silano, M.Thomasi; J.Biochem., **153**, 6221-6225 **(1976a)**.

[55] H.Podoler, S.W.Applebaum; J.Biochem., **121**, 321-325 **(1971)**.