

# Purification and characterization of thermostable $\alpha$ -amylase from germinating Sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds

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**Abstract** The thermostable  $\alpha$ -amylase from germinating sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds (CgAmy) was successfully purified by a combination of ammonium sulphate fractionation and Epoxy-activated Sepharose 6B affinity chromatography. The purified  $\alpha$ -amylase showed 507.8 fold with a specific activity of 750.0 U/mg. SDS-PAGE of the purified enzyme revealed a single protein band of 50.0 kDa. Purified enzyme was confirmed as  $\alpha$ -amylase type by LC-MS/MS analysis and activity on specific substrate of ethylidene-pNP-G7. The CgAmy revealed extreme activity at a high temperature of 50.0–70.0°C with optimum activity at 70.0°C. The optimal pH of enzyme activity was observed at 6.0. The CgAmy exhibited stability in pH range of 5.0–8.0 and highly thermostable with a temperature of 40.0–60.0°C. The kinetic parameters  $K_m$  for hydrolysis of starch were found to be 3.12 mg/ml. The  $\alpha$ -amylase activity was enhanced in the presence of  $\text{Co}^{2+}$  and  $\beta$ -mercaptoethanol. While,  $\text{Na}^{2+}$ ,  $\text{K}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Cd}^{2+}$  slightly inhibited  $\alpha$ -amylase activity. Interestingly, the CgAmy displayed stability towards some organic solvents and detergents. Stability at high temperature and some metal ions, organic solvents and detergents indicated that this enzyme has potential for various applications.

**Key words:**  $\alpha$ -amylase, characterization, enzymes, purification, Sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds.

## Introduction

$\alpha$ -Amylase (1,4- $\alpha$ -D-glucan-4-glucanohydrolase, EC 3.2.1.1) belongs to glycosyl hydrolase (GH-13 family) class which catalyses random the hydrolysis of internal  $\alpha$ -1,4 glycosidic bonds in polysaccharides resulting in an  $\alpha$ -anomeric configuration of oligosaccharides (van der Maarel et al. 2002). The source of this enzyme is microorganisms, animals, plants, especially in germinating seeds (Muralikrishna and Nirmala 2005; Singh et al. 2017; Sundarram and Murthy 2014). In plants,  $\alpha$ -amylase plays a very important role in starch degradation in stored starch during seeds germination releasing sugars for the proper growth of plants (Xie et al. 2007). Enzymatic processes have been used in various industries due to several advantages such as fast in action, specific of the reaction and save energy, raw materials, chemical and provide more environment and consumer safe solution compared to conventional processes (Jegannathan and Nielsen 2013). Cereal  $\alpha$ -amylases are a good choice for food and biotechnological application (Muralikrishna and Nirmala 2005). The  $\alpha$ -amylases is one of the most critical industrial enzyme which holds the maximum

about 25% of the industrial enzyme market with various applications such as food, detergents, textiles, biorefinery, brewing, fermentation, pharmaceuticals, and paper industries etc. (Gupta et al. 2003; Sindhu et al. 2017). The demand for  $\alpha$ -amylase has increased in various industries but the production and properties of this enzyme have been limited in terms of specificity, stability and catalytic efficiency (Choi et al. 2015; Sivaramakrishnan et al. 2006). Therefore, there is a need to find novel sources that produce  $\alpha$ -amylases with desired characteristics for specific applications. Thermostable enzymes are a desirable characteristic of extreme temperature in various industry processes (Sindhu et al. 2017). In the past, very few researches of  $\alpha$ -amylases from plant sources have been reported. However, thermostable  $\alpha$ -amylases have been reported from *Vicia faba* (65°C), red pitaya (*Hylocereus polyrhizus*) peel (70°C), wheat (*Triticum aestivum*) seeds (68°C), soybean (*Glycine max*) seeds (70°C), and mung bean (*Vigna radiata*) seeds (65°C). Sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds which widely cultivated in the local areas are interesting for  $\alpha$ -amylases source and increasing commercial value. The seed-pods are approximately 20–40 cm long and 3.5–5.0 cm broad including on average 8 to 16 of very large

and high stored starch seeds (Vadivel and Janardhanan 2004). The seed germination process is predicted from  $\alpha$ -amylases activity.

In this research, we report the purification, biochemical characterization, and discovery of thermostable, surfactant and organic solvent stable  $\alpha$ -amylase from newly source germinating sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds.

## Materials and methods

### Materials

Seeds of sword bean were collected from Nakhon Ratchasima province, Thailand. They were surface sterilized with 1% (v/v) sodium hypochlorite solution for 10 min followed by thoroughly washing with distilled water. Seeds were imbibed 12 h in distilled water and then placed to germinate in dark over moist filter paper on a moist sand bed for 48 h (Singh et al. 2017).

### Enzyme extraction and purification

All purification steps were carried out at 4°C. Germinated seeds (400 g) were homogenized in 1,200 ml chilled extraction buffer (50 mM sodium acetate buffer, pH 5.5) (Singh et al. 2017) using a laboratory blender. The extract was filtered through two layers of pre-washed cheesecloth. The supernatant was obtained by centrifugation at 21,000 × g for 25 min.

### Ammonium sulphate fractionation

The crude extract was precipitated in range 35–65% ammonium sulphate saturation (Kumari et al. 2010) and then stirred for 2.5 h at 4°C. The protein precipitate was collected by centrifugation at 21,000 × g for 25 min. The pellet was dissolved in minimum volume of extraction buffer (50 mM sodium acetate buffer, pH 5.5) and dialyzed with the same buffer for four buffer changes to remove ammonium sulphate.

### Epoxy-activated Sepharose 6B affinity chromatography

The dialyzed supernatant (40 mg protein) were then loaded onto pre-equilibrated epoxy-activated sepharose 6B ligated with  $\beta$ -cyclodextrin columns with 50 mM acetate buffer (pH 6.0) containing 5 mM CaCl<sub>2</sub> (Singh and Kayastha 2014; Vretblad 1974) at flow rate of 0.5 ml/min. The column was washed unbound protein with washing buffer (20 mM acetate buffer (pH 6.0) containing 25 mM CaCl<sub>2</sub> and 200 mM NaCl). Elution was carried out with a  $\beta$ -cyclodextrin (10 mg/ml) in the washing buffer. The active fractions (2 ml each) were pooled and then dialysed with 50 mM acetate buffer (pH 6.0) containing 5 mM CaCl<sub>2</sub>.

### Amylase activity assay

The amylase activity was assayed based on the standard method (Miller 1959) with some modification. The reaction mixture contained 0.5 ml of the enzyme and 0.5 ml of 1% w/v of soluble

starch in 100 mM sodium acetate buffer pH 5.5. The reaction mixture was incubated for 10 min at 37°C. The reaction was stopped by the addition of 1 ml of DNS reagent and then boiled for 5 min. The released reducing sugar was measured using a spectrophotometer (BioMate<sup>TM-3</sup>, Thermo Scientific) at 520 nm by using glucose as a standard reducing sugar. One unit of  $\alpha$ -amylase activity was defined as the amount of enzyme that produced 1  $\mu$ mol of glucose per minute under the assay condition.

### Determination of protein concentration

Protein concentration was determined according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as protein standard. For purification, the protein was determined by measuring the absorbance at 280 nm.

### Determination of purity and molecular weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the previous method (Laemmli 1970). The gel was stained with Coomassie Brilliant Blue R-250. The molecular weight of the enzyme was estimated using a protein marker with sizes ranging from 17 to 250 kDa (ENZMART Biotech).

### Zymographic method

The samples were prepared under non-reducing conditions and separated on 13% polyacrylamide gels containing 0.1% w/v soluble starch (Posoongnoen et al. 2015). The gel was washed with 1% v/v triton X-100 to remove SDS and then incubated with 0.2% w/v starch in 50 mM sodium acetate buffer (pH 5.5) for 6 h at 37°C. The amylase activity band was detected by staining with activity staining solution (1% acetic acid, 10 mM I<sub>2</sub> and 14 mM KI).

### Optimum pH and pH stability

The optimum pH was determined using amylase activity assay at different pH values using various buffers: 100 mM sodium acetate buffer (pH range 3.7–5.6), 100 mM citrate phosphate buffer (pH range 5.0–6.5), 100 mM potassium phosphate buffer (pH range 6.0–7.6), 100 mM Tris-HCl buffer (pH range 7.0–9.0) at 37°C. The pH stability was evaluated by pre-incubating enzyme with 100 mM buffer at different pH values for 1 h at 4°C. The residual amylase activity was determined by amylase activity assay as mentioned above. The activity of the enzyme before incubation was regarded as 100% relative activity.

### Optimum temperature and Heat stability

The optimum temperature was analysed by assaying amylase activity in 100 mM sodium acetate buffer pH 5.5 at temperatures ranging from 30 to 90°C. The heat stability was determined by pre-incubating enzyme at different temperature range in 30 to 90°C in 100 mM sodium acetate buffer pH 5.5 for 1 h. Then, the residual amylase activity was determined by the amylase activity assay. The activity of the enzyme before incubation was regarded as 100% relative activity.

### Kinetic studies

The enzyme was determined activity in the presence of varying concentration of starch in a range of 3.0 to 9.0 mg/ml soluble starch in 100 mM potassium phosphate buffer pH 6.0 at 70°C using amylase activity assay. The data was determined of Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) using Lineweaver-Burk plot.

### Effect of metal ions and chemical reagents on the $\alpha$ -amylase activity

The influence of various metal ions (5 mM  $CaCl_2$ ,  $MgCl_2$ ,  $MnCl_2$ ,  $BaCl_2$ ,  $CoCl_2$ ,  $CuCl_2$ ,  $ZnCl_2$ , NaCl and KCl) on enzyme activity was analysed. The metal ions were prepared by dissolving the metal ions in deionised water. The reaction mixture of 0.5 ml of purified enzyme with 0.5 ml of metal ion solution was pre-incubated at 4°C and pH 5.5 for 1 h. Then, the residual enzyme activity was determined using amylase activity assay.

For chemical reagents, the enzyme was pre-incubated with chemical reagents of 10 mM  $\beta$ -mercaptoethanol, 10 mM ethylene-diaminetetraacetic acid (EDTA), 5% v/v Triton X-100, 5% v/v Tween 20, 5% w/v SDS and 10% v/v of organic solvent (methanol, ethanol, acetone and hexane) at 4°C and pH 5.5 for 1 h. The residual enzyme activity was measured by the amylase activity assay. The activity of amylase in the absence of any additives was taken as 100% relative activity.

### Substrate specificity

The activity of the enzyme against different substrates was determined using amylase activity assay. The enzyme was incubated with 0.1% w/v of substrates (starch, amylopectin, glycogen,  $\beta$ -cyclodextrin, and dextran) in 100 mM sodium acetate buffer (pH 5.5) for 10 min at 37°C. The released reducing sugar was analysed using the DNS method. Soluble starch was used as a reference substrate for enzyme activity comparisons.

### Liquid chromatography electrospray ionization tandem mass spectroscopy (LC-ESI-MS/MS) and database searching

The single gel band of purified  $\alpha$ -amylase was tryptic digested and analysed with a nano-liquid chromatography system (EASY-nLC II, Bruker) coupled to an ion trap mass spectrometer (Amazon Speed ETD, Bruker) equipped with an ESI nano-sprayer. Tryptic digest of bovine serum albumin (BSA) was used as a standard control of the experiment. Services were provided by Research Instrument Center, Khon Kaen University, Thailand. Proteins identification was performed by searching against the protein database from SwissProt using MASCOT MS/MS Ion Search program ([www.matrixscience.com](http://www.matrixscience.com)) with the initial searching parameters; Enzyme: Trypsin; carbamidomethylation (C) as fixed modification, and oxidation (HW) and oxidation (M) as variable modification; peptide mass tolerance of 0.5 Da and fragment mass tolerance of 0.5 Da; a peptide charge state of +1,

+2, +3; instrument type: ESI-TRAP; and report top: Auto.

### Statistical analysis

All samples were analysed three replicates. The results were reported as mean  $\pm$  standard error (SE). Variance was statistical determined by one-way ANOVA. The significant differences between means were defined at  $p \leq 0.05$ .

## Results

### Purification of $\alpha$ -amylase from sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds

$\alpha$ -Amylase from germinated sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds (CgAmy) was purified using ammonium sulphate fractionation and affinity chromatography. Ammonium sulphate fractionation revealed a purification fold of 2.92. Then, CgAmy was purified using Epoxy-activated Sepharose 6B affinity chromatography which was eluted as a single peak by  $\beta$ -cyclodextrin (Figure 1(A)). Finally,  $\alpha$ -amylase was purified up to 507.78 folds with a specific activity of 750.00 U/mg and a yield of 14.05% (Table 1). The CgAmy was also confirmed  $\alpha$ -amylase activity using specific substrate (ethylidene-pNP-G7) which is specifically cleaved by  $\alpha$ -amylase. The result revealed that the purified enzyme catalyzed 736.57 nmol/min/ml of ethylidene-pNP-G7 indicating amylase from sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds is  $\alpha$ -type of amylase.

### Electrophoresis and LC-ESI tandem mass spectroscopy

The purified enzyme revealed a single protein band on SDS-PAGE with a mobility equivalent to a molecular mass of 50.0 kDa (Figure 1(B)) indicating its homogeneity. The zymographic method exhibited a single clear band of amylase activity associated with the single protein band on SDS-PAGE (Figure 2 lane B) which confirmed the starch hydrolysis by the  $\alpha$ -amylase. Single purified  $\alpha$ -amylase band with a molecular mass of 50.0 kDa obtained from SDS-PAGE was excised, tryptic digested and submitted for LC-ESI-MS/MS analysis, resulted in high quality peptide mass fingerprint spectrum. The  $m/z$  ratio showed significant matches with mass data of  $\alpha$ -amylase from *Vigna mungo* in the protein database (Figure 1(C)). The results clearly demonstrate that the purified enzyme is  $\alpha$ -amylase.

### Effect of temperature on $\alpha$ -amylase activity

The CgAmy was highly activity in a wide range of temperatures from 50 to 70°C with optimum activity at 70°C (Figure 2(A)). The enzyme has activity more than 35% relative activity in the range of 80–90°C. The CgAmy also exhibited high stability at a wide temperature range of 30–70°C which retained more than 50% of the initial

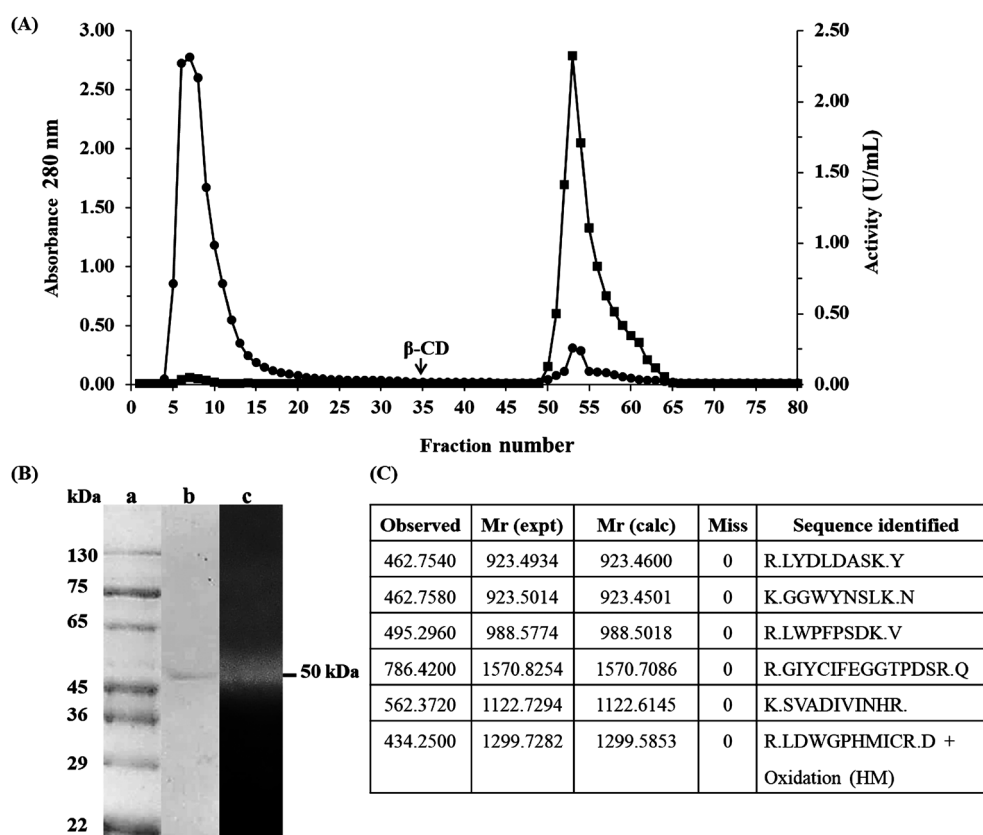


Figure 1. Purification and characterization of  $\alpha$ -amylase: (A) Elution profile of purification using epoxy-activated Sepharose 6B linked with  $\beta$ -cyclodextrin ( $\beta$ -CD) for affinity chromatography. The fractions were analyzed enzyme activity (■) and protein concentration at A280 nm (●). (B) Electrophoresis pattern and amylase activity. Molecular weight markers (lane a) and purified enzyme (lane b) using SDS-PAGE, amylase activity by Zymographic method (lane c). (C) Digested peptides which showed match with  $\alpha$ -amylase sequence from plant  $\alpha$ -amylases.

Table 1. Purification of  $\alpha$ -amylase from germinated sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	6,279.00	4,251.10	1.48	1	100
35–65% Ammonium sulphate fractionation	1,853.18	429.14	4.32	2.92	29.51
Epoxy activated sepharose 6B Affinity chromatography	882.00	1.18	750.00	507.78	14.05

activity (Figure 2(B)). Furthermore, the residual activity of  $\alpha$ -amylase at a high temperature of 70°C and 80°C was 54.58% and 20.91% of residual activity, respectively. The result demonstrated that the CgAmy is a thermostable amylase.

#### Effect of pH on $\alpha$ -amylase activity

According to the optimum pH profile (Figure 2(C)), the CgAmy revealed appropriate activity in a broad range of pH 5.0–7.0 with an optimum activity at pH 6.0. This enzyme has more than 50% activity at pH 4.0–8.0. The CgAmy displayed highly stable activity in the pH range of 5.0–8.0 retaining over 50% residual activity (Figure 2(D)). The enzyme retained about 80.31% of its maximal activity at pH 6.0.

#### Enzyme kinetic constants

Kinetic studies were carried out under standard conditions using soluble starch as substrate.  $K_m$  and  $V_{max}$  of the CgAmy which was determined for starch as the substrate from Lineweaver-Burk plots were 3.70  $\mu$ moles/min/ml and 3.12 mg/ml, respectively (Figure 2(E)). The lower  $K_m$  value is related to the higher affinity to the substrate.

#### Substrate specificity

The substrate specificity of the  $\alpha$ -amylase using different polysaccharides as substrates revealed that the CgAmy has the highest activity with starch as a substrate. The enzyme also was active on amylopectin and glycogen at 96.59% and 59.58% activity, respectively (Table 2). The enzyme showed no hydrolysis activity of dextran and  $\beta$ -cyclodextrin. Substrates containing the  $\alpha$ -1,4-linkage

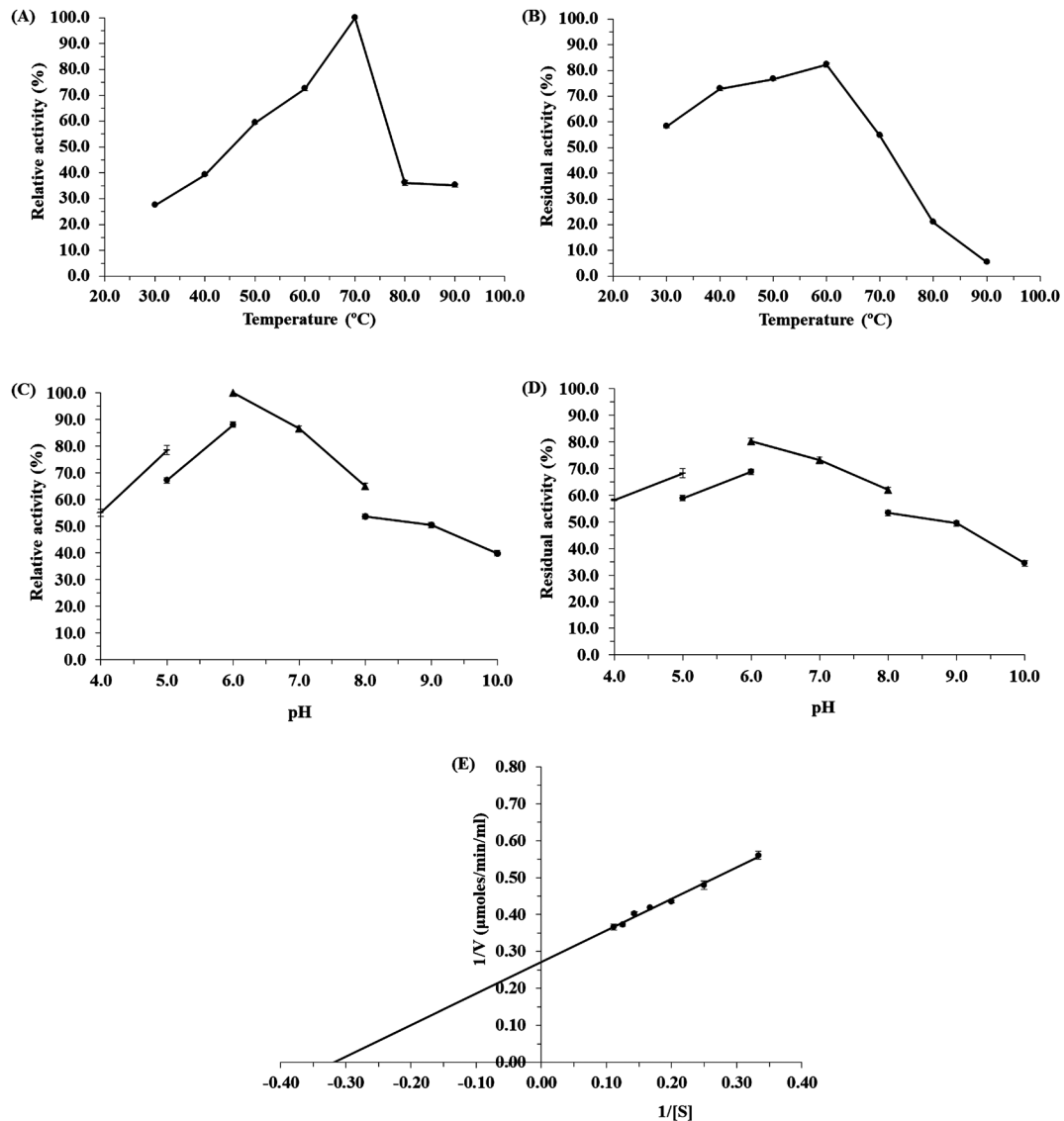


Figure 2. Biochemical properties of  $\alpha$ -amylase: (A) The effect of temperature on amylase activity at different temperature ranging from 30.0 to 90.0°C. (B) The effect of temperature on amylase stability. (C) The effect of pH on amylase activity, the pH conditions were adjusted using the following 100 mM sodium acetate buffer pH range 4.0–5.0, 100 mM citrate phosphate buffer pH 5.0–6.0, 100 mM potassium phosphate buffer pH range 6.0–7.0, 100 mM Tris-HCl buffer pH range 7.0–8.0. (D) The effect of pH on amylase stability. (E) Lineweaver-Burk plots in the presence of different concentration of soluble starch. The data represent the mean  $\pm$  standard deviation from three replicates.

Table 2. Substrate specificity of  $\alpha$ -amylase.

Substrates	Activity (%)
Starch	100.00 $\pm$ 0.01 <sup>e</sup>
Amylopectin	96.59 $\pm$ 0.13 <sup>d</sup>
Glycogen	59.58 $\pm$ 0.20 <sup>c</sup>
$\beta$ -cyclodextrin	0.00 $\pm$ 0.00 <sup>a</sup>
Dextran	0.51 $\pm$ 0.10 <sup>b</sup>

Mean value followed by different letters differs significantly ( $p \leq 0.05$ ).

were better substrates for  $\alpha$ -amylase.

#### Effect of metal ions and chemical reagents on $\alpha$ -amylase activity

The metal ions and chemical reagents had different effects on the enzyme activity. The activity of the

CgAmy was slightly activated in the presence of  $\text{Co}^{2+}$  ions (107.84%) (Table 3). The metal ions of 5 mM  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Cd}^{2+}$  were slightly affected to  $\alpha$ -amylase activity retaining over 70% residual activity. However, the CgAmy activity was inhibited in the presence of 5 mM  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  with 35.77% and 53.27% of its activity, respectively.  $\beta$ -Mercaptoethanol had no significant inhibit on the enzyme activity. However, the chelating agent EDTA displays significant ( $p \leq 0.05$ ) inhibited the enzyme. The stability of the CgAmy was also investigated in the presence of surfactant (SDS, Triton X-100 and Tween-20). The enzyme was highly tolerance in the presence of the non-ionic surfactants of Triton X-100 (95.17%) and Tween-20 (114.10%) but in the presence of SDS, the enzyme was

inactivated. Furthermore, the CgAmy was highly stable in the presence of methanol, ethanol, acetone, and hexane, retaining 84.06%, 82.87%, 117.64% and 92.80% of initial activity, respectively.

## Discussion

The CgAmy was successfully purified using two steps of ammonium sulphate fractionation and affinity chromatography.  $\alpha$ -Amylase inhibitor in crude extract was possibly removed by ammonium sulphate fractionation step (Wisessing et al. 2010). The less number of enzyme purification steps showed high fold and yield of enzyme purification. The affinity chromatography used in this study has also been

reported successfully for other plant amylases (Kumari et al. 2010; Singh et al. 2017; Tripathi et al. 2007). The purified  $\alpha$ -amylase from *Vigna radiata* was purified 599 fold to a final specific activity of 437 U/mg and an overall recovery of 9% (Tripathi et al. 2007). Kumari et al. (2010) purified  $\alpha$ -amylase from *glycine max* having specific activity of 384.61 Units/mg, 6.6% yield and purification of 400.63 fold. The purification procedure in this report is a simple and effective method for  $\alpha$ -amylase purification. Recently, Singh et al. (2017) reported  $\alpha$ -amylase from *Vicia faba* with a specific activity of 369 U/mg, purification fold of 2050 and 24% yield. The purified enzyme revealed a molecular mass of 50.0 kDa which is very close to  $\alpha$ -amylase from *Vigna radiata* (46 kDa) (Tripathi et al. 2007). In plants (Table 4),  $\alpha$ -amylases have been reported molecular mass range from 32 kDa to 84 kDa. The CgAmy was in accordance with this range.

The optimum temperature of the CgAmy was at a high temperature of 70°C that is consistent with purified  $\alpha$ -amylase from *Glycine max* (70°C) and is higher compared to *Vigna radiata* (65°C) and safflower (55°C) (Table 4). The CgAmy also revealed high stability at high temperature range of 30–70°C. The results demonstrated that the  $\alpha$ -amylase is a thermostable amylase which can be used to degrade gelatinized starch at high temperature of the starch industry. After pre-incubation at higher temperatures of 90°C, the CgAmy activity dropped rapidly which was probably due to denaturation of the enzyme. The optimum pH (pH 6.0) of the CgAmy is in accordance with the data reported for other plant  $\alpha$ -amylases (Table 4) and values reported for  $\alpha$ -amylase from *Glycine max* (pH 5.5), *Vigna radiata* (pH 5.6) and *Vicia faba* (pH 6.0) (Table 4). In the starch hydrolysis industry, most of the  $\alpha$ -amylase is inactive at acidic pH of 3.2–6.0 of native starch slurry for starch processing (Sivaramakrishnan et al. 2006). Amylase with low pH value can be used as a substitute for currently liquefied amylase (optimum pH of 6.8) and reduced expenses and time of pH adjusting from its native pH to match with the optimum pH of the enzyme (Wu et al. 2018). The  $K_m$  value of  $\alpha$ -amylase from other plants has been

Table 3. Effect of metal ions and chemical reagents on the  $\alpha$ -amylase activity.

Different additives	Concentration	Relative activity (%)
Control (Non-component)	—	100±0.02 <sup>i</sup>
Metal ions		
NaCl	5 mM	95.40±1.00 <sup>gh</sup>
KCl	5 mM	90.71±0.81 <sup>f</sup>
CaCl <sub>2</sub>	5 mM	89.47±0.97 <sup>j</sup>
MgCl <sub>2</sub>	5 mM	82.04±1.21 <sup>e</sup>
ZnCl <sub>2</sub>	5 mM	81.57±0.64 <sup>e</sup>
BaCl <sub>2</sub>	5 mM	90.48±0.33 <sup>f</sup>
FeCl <sub>2</sub>	5 mM	78.88±0.48 <sup>e</sup>
CdCl <sub>2</sub>	5 mM	74.56±1.74 <sup>d</sup>
CoCl <sub>2</sub>	5 mM	107.84±1.14 <sup>m</sup>
CuCl <sub>2</sub>	5 mM	35.77±0.36 <sup>b</sup>
NiCl <sub>2</sub>	5 mM	53.27±0.23 <sup>c</sup>
Chemical reagents		
EDTA	10 mM	0.00±0.00 <sup>a</sup>
$\beta$ -mercaptoethanol	10 mM	117.65±2.58 <sup>l</sup>
SDS	5%	0.00±0.00 <sup>a</sup>
Triton X-100	5%	95.17±0.54 <sup>gh</sup>
Tween-20	5%	114.10±1.04 <sup>k</sup>
Methanol	10%	84.06±1.56 <sup>fg</sup>
Ethanol	10%	82.87±0.69 <sup>h</sup>
Acetone	10%	117.64±2.36 <sup>l</sup>
Hexane	10%	92.80±0.35 <sup>fg</sup>

Mean value followed by different letters differs significantly ( $p \leq 0.05$ ).

Table 4. Biochemical properties of some plant  $\alpha$ -amylases.

Plant sources	Molecular weight (kDa)	Optimum pH (% relative activity)	Optimum temperature (% relative activity)	$K_m$	Reference
Safflower ( <i>Carthamus tinctorius</i> L.) seeds	35.0	6.0	55	—	Ben Elarbi et al. 2009
Apple ( <i>Malus pumila</i> )	51.2	6.8	37	$2.0 \times 10^{-3}$ g/ml	Kanwal et al. 2004
<i>Pachyrhizus erosus</i> L. tuber	40.0	7.3	37	0.29%	Noman et al. 2006
Mung bean ( <i>Vigna radiata</i> ) seeds	46.0	5.6	65	1.60 mg/ml	Tripathi et al. 2007
Soybean ( <i>Glycine max</i> ) seeds	84.0	5.5	70	0.71 mg/ml	Kumari et al. 2010
Wheat ( <i>Triticum aestivum</i> ) seeds	32.0	5.0	68	1.56 mg/ml	Singh and Kayastha 2014
Red pitaya ( <i>Hylocereus polyrhizus</i> ) peel	42.1	5.0	70	2.70 mg/ml	Amid and Manap 2014
<i>Vicia faba</i>	45.0	6.0	65	4.60 mg/ml	Singh et al. 2017
Sword bean ( <i>Canavalia gladiata</i> (Jacq.) DC.) seeds	50.0	6.0	70	3.12 mg/ml	This study

reported ranging from 0.71 to 4.6 mg/ml (Table 4). However, the kinetic values of the amylases are difficult to compare with other amylases reports because of variation in substrates and assay conditions. The CgAmy also was active on amylopectin and glycogen. Amid and Manap (2014) reported that  $\alpha$ -amylase from pitaya peel revealed the highest activity toward starch followed by amylopectin (90%) and glycogen (82%). The CgAmy showed no hydrolysis of dextran indicating that the enzyme has the capability of cleaving  $\alpha$ -1,4 bonds but not  $\alpha$ -1,6 bonds. The enzyme also did not hydrolyse  $\beta$ -cyclodextrin with was reported as a competitive inhibitor of  $\alpha$ -amylase (Hamilton et al. 2000). The no hydrolytic activity with  $\beta$ -cyclodextrin and dextran supported that CgAmy prefers  $\alpha$ -1,4 glycosidic linkage for cleaving and is  $\alpha$ -amylase type. The  $\alpha$ -amylase from pitaya peel (Amid and Manap 2014) and mung beans (Tripathi et al. 2007) also reported no significant hydrolysis of  $\beta$ -cyclodextrin.

The activity of CgAmy was slightly activated in the presence of  $\text{Co}^{2+}$  ions. The  $\alpha$ -amylase from *Aspergillus oryzae* IFO-30103 also found  $\text{Co}^{2+}$  as an activator (Bhanja Dey and Banerjee 2015). The inactivation of the enzyme by  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  may be due to their binding to the catalytic residues in the active site of the enzyme. The chelating agent EDTA showed the enzyme inhibition, suggesting that CgAmy is a metalloenzyme. This result is in agreement with  $\alpha$ -amylase from *Pachyrhizus erosus* L. tuber, soybean, mung bean, wheat which was inhibited in the presence of EDTA (Kumari et al. 2010; Noman et al. 2006; Singh and Kayastha 2014; Tripathi et al. 2007). The CgAmy was inactivated in the presence SDS which suggested that SDS disrupts the protein's higher ordered structure resulting in inactivation of the enzyme (Singh and Kayastha 2014). The CgAmy also was highly stable in some organic solvents and detergents. The thermostable enzymes have been reported that is inconsistent with more resistant to organic solvents and detergents (Klibanov 2001). The resistant in the reagents suggested that the enzyme has a well-packed structure and rigid native conformation (Chakraborty et al. 2011). Due to the good enzymatic properties of the enzyme in organic solvents, it may have a wide range of industrial applications such as the treatment of carbohydrate-polluted industrial wastewater contaminated with organic solvent (Wu et al. 2018), alcohol production from starch material, brewing of alcoholic beverages.

In the present investigation, thermostable  $\alpha$ -amylase from germinating sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds (CgAmy) have discovered. Furthermore, it exhibited good stability in the presence of some detergents and organic solvents. Therefore, sword bean (*Canavalia gladiata* (Jacq.) DC.) seeds should be considered as novel sources and an important candidate for producing thermostable  $\alpha$ -amylase that are desirable

characteristics in various industries.

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