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Note

# Purification and characterization of a thiol amylase over produced by a non-cereal non-leguminous plant, *Tinospora cordifolia*

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ABSTRACT

A 43 kDa α-amylase was purified from *Tinospora cordifolia* by glycogen precipitation, ammonium sulfate precipitation, gel filtration chromatography, and HPGPLC. The enzyme was optimally active in pH 6.0 at 60 °C and had specific activity of 546.2 U/mg of protein. Activity was stable in the pH range of 4–7 and at temperatures up to 60 °C. PCMB, iodoacetic acid, iodoacetamide, DTNB, and heavy metal ions $Hg^{2+} > Ag^{+} > Cd^{2+}$ inhibited enzyme activity while $Ca^{2+}$ improved both activity and thermostability. The enzyme was a thiol amylase (3 SH group/mole) and DTNB inhibition of activity was released by cysteine. N-terminal sequence of the enzyme had poor similarity (12–24%) with those of plant and microbial amylases. The enzyme was equally active on soluble starch and amylopectin and released maltose as the major end product.

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Large scale industries like textile, food, and pharmaceuticals have huge demand for amylases, next to that of alkaline protease in detergents.[1] Bacteria and fungi, belonging to *Bacillus* and *Aspergillus* species are the common commercial producers, which over produce the enzyme extracellularly during growth.[2] Amylases present in plants are classified as family I (extracellular), family II (cytosolic), and family III (plastidal).[3] α-Amylases (*endo*-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) present in various cereals, leguminous, and other plants have been purified and characterized,[4–6] but none of these enzymes are over produced during plant growth except those produced during germination of cereals. Malted barley is the only commercial plant amylase used in brewing. Plant α-amylases are generally non-thiol enzymes except that obtained from poplar leaves.[7] *Tinospora cordifolia* miers is a medicinal herb, known for a long time in Indian sub-continent. It grows wild on unconditioned soil throughout the year and rapidly produces huge biomass. We reported earlier in a patent[8] that the plant contained large amounts of α-amylase, extractable from the stem. The present study describes purification and characterization of the amylase present in *T. cordifolia*.

The plant contained about 450 ± 50 U of amylase/g of fresh stem (Table 1). Plant stem, dried by lyophilization, retained full activity at least for 18 months (data not shown). Amylase activities reported[9] for other plants (U/g of fresh weight) were: malted barley; 953, soybean cotyledons; 431, sweet potato root; 211, bush bean stem; 37.47, bush bean leaf; 15.65, sweet corn stem; 2.19, alfalfa tap root; 1490, pea cotyledons; 24.1. However, none of the sources except malt are better than *T. cordifolia* amylase in terms of availability and enzyme content. *T. cordifolia* amylase, similar to plant proteases[10] like papain (papaya latex), bromelain (pineapple), and ficin (fig.) required reducing environment for stability as in buffer (pH 5.0) containing 10 mM cysteine, 10 mM EDTA, and 10 mM $CaCl_2$. EDTA was added in the buffer as a heavy metal chelator. The amylase purified subsequently was found to be inhibited by heavy metal ions. Glycogen precipitation (Table 1) recovered more than 56% of enzyme activity with threefold purification from buffer extract of blended plant stem. Subsequent ammonium sulfate precipitation, Bio-gel P-60 chromatography purified the enzyme to HPGPLC homogenous stage. The purified enzyme was homogenous in native-PAGE and gave a single band in SDS–PAGE (figs. not shown). The molecular weight was approximated to be 43 kDa, in both HPGPLC and SDS–PAGE. Amylase was affinity purified from human saliva with 91.1% recovery and 3.5-fold purification, on starch coated super paramagnetic iron oxide modified by epichlorohydrin.[11] The N-terminal sequence of *T. cordifolia* amylase (resolved upto 17 amino acid) had 18% homology with *Aspergillus* amylases, 12–24% with bacterial amylases, and only 12% with other plant amylases (Table 2). The enzyme was active in the pH range of 4–7, with optimum activity at pH 6.0. The enzyme lost full activity at pH ⩽ 2.0 and pH ⩾ 10.0 but retained about 25% and 30% of activity at pH 3 and 8, respectively (fig. not shown). Activity was stable in pH range of 4–7 but not in alkaline pH range ⩾8 (fig. not

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**Table 1**
Steps of purification of amylase from *T. cordifolia*

| Sample | Total enzyme activity (U) | Total protein (mg) | Specific activity (U/mg) | Fold purification | % Recovery |
|---|---|---|---|---|---|
| Crude extract (100 mL) [obtained from 30 g fresh stem] | 14,250 | 155.6 | 91.58 | 1.00 | 100 |
| Concentrated (ultra filtration and lyophilization) extract (20 mL) | 13,600 | 143.2 | 94.97 | 1.01 | 95.4 |
| Glycogen precipitate (10 mL) | 8250 | 32.5 | 253.89 | 2.87 | 57.8 |
| Ammonium sulfate precipitate (2 mL) | 6245 | 14.3 | 436.71 | 4.76 | 43.8 |
| Bio-Gel P-60 (1 mL) | 5368 | 10.3 | 521.1 | 5.69 | 37.6 |
| HPGPLC | 4710 | 8.8 | 546.20 | 5.84 | 33.0 |

**Table 2**
Comparison of N-terminal sequence of *T. cordifolia* amylase with bacterial, fungal and other plant amylases

| Organism/Acc ID | Sequence | | | | | | | | | | | | | | | | | Identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Tinospora cordifolia* | D | T | P | G | G | N | V | A | D | D | I | Q | K | F | M | D | G | |
| *Aspergillus shirousami* [P 30292] | A | **T** | **P** | A | D | W | R | S | Q | S | **I** | Y | F | L | L | T | D | 18% identity in 17 amino acid overlap |
| *Aspergillus awamori* [Q 02906] | A | **T** | **P** | A | D | W | R | S | Q | S | **I** | Y | F | L | L | T | D | 18% identity in 17 amino acid overlap |
| *Aspergillus oryzae* [POC1B4] | A | **T** | **P** | A | D | W | R | S | Q | S | **I** | Y | F | L | L | T | D | 18% identity in 17 amino acid overlap |
| *Aspergillus niger* [A2QL05] | A | **T** | **P** | A | D | W | R | S | Q | S | **I** | Y | F | L | L | T | D | 18% identity in 17 amino acid overlap |
| $G_4$amylase, *Pseudomonas mendocina* [A4XX23] | **D** | A | **P** | **G** | K | T | A | S | G | V | R | Y | H | G | G | **D** | E | 24% identity in 17 amino acid overlap |
| *Streptomyces lividans* [Q05884] | **D** | **T** | **P** | P | A | P | P | S | **D** | A | K | L | A | K | T | A | A | 24% identity in 17 amino acid overlap |
| G4 amylase *Pseudomonas saccharrophilia* [P22963] | **D** | Q | A | **G** | K | S | P | **A** | G | V | R | Y | H | G | G | **D** | E | 24% identity in 17 amino acid overlap |
| G4 amylase, *Pseudomonas stutzeri* [P13507] | **D** | Q | A | **G** | K | S | P | N | A | V | R | Y | H | G | G | **D** | E | 18% identity in 17 amino acid overlap |
| *Streptomyces limosus* (P09794) | A | P | **P** | **G** | A | K | D | V | T | A | V | L | F | E | W | K | F | 12% identity in 17 amino acid overlap |
| *Bacillus amyloliquefaciens* (P00692) | V | N | G | T | L | M | Q | Y | F | E | W | Y | T | P | N | **D** | **G** | 12% identity in 17 amino acid overlap |
| *Hordeum vulgare* (P04063) [Barley] | Q | V | L | F | Q | G | F | N | W | E | S | W | **K** | H | N | G | **G** | 12% identity in 17 amino acid overlap |
| *Oryza sativa* (A2YGY2) [Rice] | **D** | K | I | L | F | Q | G | F | N | W | E | S | W | R | Q | S | **G** | 12% identity in 17 amino acid overlap |
| *Elusine coracana* (Q7Y1C3) [Finger millet] | Q | I | L | F | Q | G | F | N | W | E | S | W | **K** | Q | N | G | **G** | 12% identity in 17 amino acid overlap |
| *Avena fatua* (CAA09324) [Oat] | Q | V | L | F | Q | G | F | N | W | E | S | W | **K** | Q | N | G | **G** | 12% identity in 17 amino acid overlap |
| Maize (NP-0011055391) | Q | V | L | F | Q | G | F | N | W | E | S | W | **K** | K | Q | G | **G** | 12% identity in 17 amino acid overlap |
| *Cuscuta reflexa* (Q42678) [Giant dodder] | S | **T** | V | L | F | Q | G | F | N | W | E | S | N | K | Q | Q | **G** | 12% identity in 17 amino acid overlap |

shown). The optimal activity (60 °C) and stability (up to 55 °C) of the enzyme was also similar to those of *Aspergillus oryzae* amylase. *T. cordifolia* amylase retained 30% activity in 8 M urea and 4 M-guanidine hydrochloride but lost 100% activity in 0.4% (w/v) SDS. $Zn^{2+}$, $Fe^{3+}$, $Mg^{2+}$, $Mn^{2+}$, and $Cl^-$ at 5 mM concentration did not affect enzyme activity. $Hg^{2+}$ (0.1 mM) inhibited 90% while $Ag^+$ (1 mM) and $Cd^{2+}$ (1 mM) inhibited about 75% of enzyme activity similar to those reported for tulip amylase.[12]

The activity of purified *T. cordifolia* amylase was unaffected in the presence of $Ca^{2+}$ (upto 20 mM). However, $Ca^{2+}$ (10 mM) increased the activity of pre-dialyzed enzyme solution (dialyzed against 1 mM EDTA for 48 h) by about 35% (fig. not shown). At higher concentration (>20 mM), $Ca^{2+}$ inhibited about 10–12% of activity of the pre-dialyzed enzyme. Thermostability of the enzyme at 70 °C and 80 °C was, respectively, 35% and 20% higher in the presence of 10 mM $Ca^{2+}$ (fig. not shown). It has been reported that removal of $Ca^{2+}$ from fungal amylase (*A. oryzae*) by EDTA caused reversible inactivation, recoverable by addition of the metal ion.[13,14] *T. cordifolia* enzyme appeared to have tightly bound $Ca^{2+}$ like hog pancreatic amylase.[15] Trypsin, chymotrypsin, pepsin, and proteinase K could not inactivate *T. cordifolia* amylase as reported for hog pancreatic, *Bacillus subtilis*, and *A. oryzae* amylases.[16] Unlike cereal α-amylases,[17] *T. cordifolia* amylase was sensitive to SH reacting reagents (DTNB > PCMB > *N*-ethylmalemide > iodoacetic acid > iodoacetamide) which strongly inhibited enzyme activity (Fig. 1). However, loss of about 90% of activity after 10 min of incubation of the enzyme with 5 mM DTNB was regained within 20 min by the addition of 5 mM cysteine. SH group present in enzyme protein (43 kDa) was estimated (by Ellman's reagent) to be 3 groups/mole. *T. cordifolia* amylase was suggested to be a thiol enzyme whose thiol groups are possibly involved in the exhibition of catalytic activity. Water-soluble carbodiimide (20 mM) had no significant effect on the activity of the enzyme (data not shown). It has been reported that poplar leaf amylase was reversibly inactivated by oxidation in the absence of reducing agents.[7] α-Amylases of germinated common millet (*Panicum milaceu*) was inhibited by iodoacetic acid and PCMB[18] while that of *B. subtilis* KCC103 was inhibited by $Hg^{2+}$, $Ag^+$, and PCMB.[19] Unlike fungal amylases, *T. cordifolia* enzyme was equally active on soluble starch and amylopectin and reasonably active on glycogen, showing specific activities as 545,

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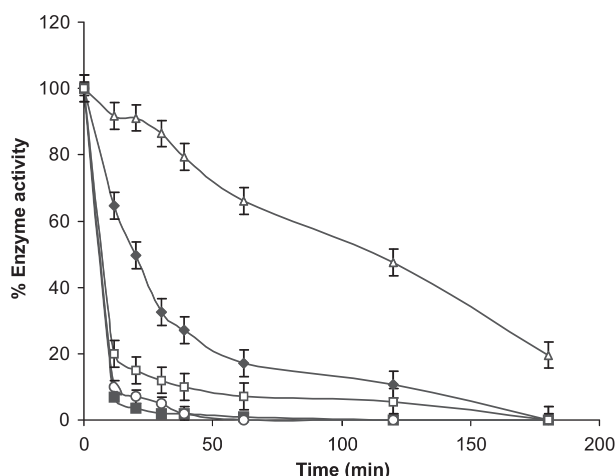


**Figure 1.** Effect of different thiol group inhibitors on purified *T. cordifolia* amylase. Experimental details have been mentioned in the text. Data expressed are the average of best triplicate values. 50 mM Iodoacetic acid at pH 8.8 (—△—), 50 mM iodoacetamide at pH 8.8 (—◆—), 50 mM NEM at pH 7.0 (—□—), 5 mM DTNB at pH 8.0 (—○—), and 5 mM PCMB at pH 6.0 (—■—).

540, and 282 U/mg protein, respectively. TLC analysis showed accumulation of maltose as the major end product from both starch and amylopectin during 1–3 h of digestion of 2% (w/v) substrates (fig. not given). The enzyme could not hydrolyze methyl and *p*-nitrophenyl-α-D-glucose, maltose, isomaltose, and sucrose but was active on panose (specific activity 55.5 U/mg protein). $K_m$ values (mg/mL) determined for soluble starch, amylopectin, and glycogen using Lineweaver–Burk plot were 4.1, 4.3, and 10.0, respectively. The $V_{max}$. value (μmol of maltose equiv/min) obtained was 0.13 (soluble starch), 0.137 (amylopectin), and 0.25 (glycogen). Dextrinizing activity, determined iodometrically, showed that purified *T. cordifolia* amylase (10 U/mL) caused a decrease of 81–84%, 77–80%, and 72–75% of iodine color of (1%w/v) soluble starch, amylopectin, and gelatinized corn flour, respectively, in 30 min at 50 °C, compared to the decrease of 52–55%, 35–38%, and 27–30% of iodine color by fungal amylase under similar conditions (fig. not shown). *T. cordifolia* amylase hydrolyzed gelatinized cereal flours (corn, wheat, gram, rice), amylopectin, and glycogen much more efficiently than fungal enzyme (Figs. 2 and 3). Malted barley amylase hydrolyzed amylopectin at half the rate than that for soluble starch and was inactive on glycogen.[20] *T. cordifolia* amylase appears to be a better saccharifying amylase compared to fungal or barley amylase.

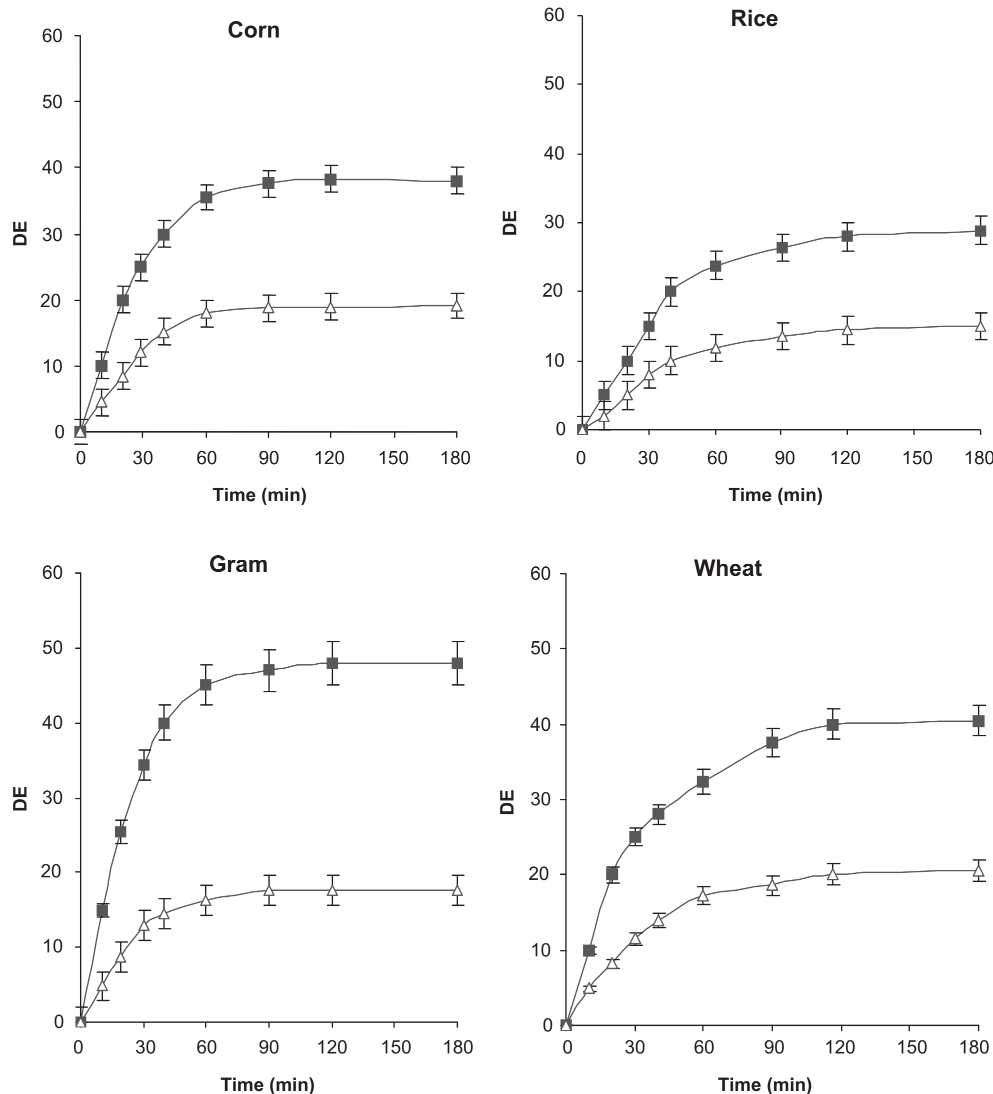


**Figure 2.** Hydrolysis of various crude gelatinized cereal flours by purified plant and fungal (*A. oryzae*) amylase. Experimental details have been mentioned in the text. —■— *T. cordifolia* amylase, —△— Fungal (*Aspergillus oryzae*) amylase. Data expressed are the average of the best triplicate values.

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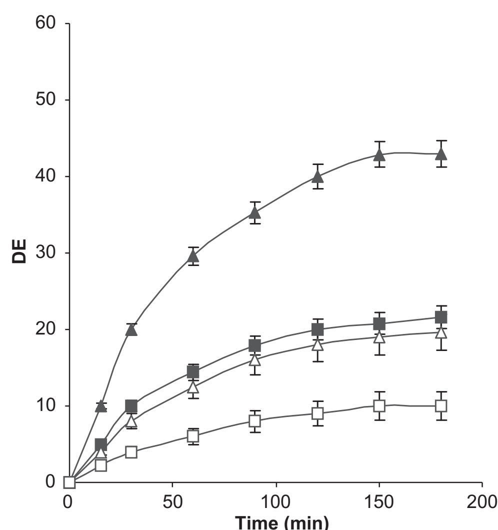


**Figure 3.** Amylopectin and glycogen hydrolysis by purified plant and fungal amylase. Experimental details have been mentioned in the text. Data expressed are the average of the best triplicate values. ▲ Amylopectin hydrolysis by plant enzyme, ■ glycogen hydrolysis by plant enzyme, △ amylopectin hydrolysis by fungal amylase, □ glycogen hydrolysis by fungal amylase.

## 1. Experimental

### 1.1. Extraction of enzyme

Cleaned and washed *T. cordifolia* stems were chopped into 3–4 cm long pieces (30 g). The pieces were immediately dropped in 90 mL of buffer A (0.1 M-acetate buffer, pH 5.0 containing 10 mM $CaCl_2$, 10 mM cysteine and 10 mM EDTA) kept at 45–50 °C. The mixture was blended for 5 min in a waring blender and the mass was squeezed through a nylon cloth to collect the extract. The residue was further mixed with 50 mL of buffer A and processed similarly. The total extract was centrifuged at 10,000 rpm at 4 °C and the clear supernatant (100 mL) was used as the source of enzyme.

### 1.2. Purification of the enzyme

The plant extract (100 mL) was concentrated to 20 mL by ultra filtration using PM-10 membrane. Amylase was precipitated from the extract by glycogen according to the method of Loyter and Schramm,[21] later modified by Machaiah and Vakil.[22] Ethanol (95%, w/v) was added to the concentrated enzyme solution to reach 40% (v/v) concentration. 2% (w/v) glycogen solution was added at the rate of 0.2 mL/$10^3$ U of enzyme with thorough mixing and the mixture was kept at −10 °C for 15 min. The precipitate formed was centrifuged down, washed twice with 40% ethanol and suspended in 10 mL of Buffer A at 45 °C. The mixture was centrifuged after 1 h and the clear supernatant was dialyzed against buffer A. The enzyme was precipitated from the dialysate at 80% ammonium sulfate saturation, dissolved in buffer A (2 mL), and finally dialyzed. Enzyme solution (1 mL) containing 2 mg of protein was charged each time on a Bio-Gel P-60 column (3.0 cm × 100 cm), pre-equilibrated with 0.05 mM acetate buffer, pH 5.0. The column was eluted with the same buffer and eluted fractions (1.5 mL) were assayed for amylase activity. Active fractions were pooled, concentrated by lyophilization, and subjected to high performance gel permeation chromatography (HPGPLC) using protein Pak TSK2000 SW (7.5 × 600 mm) column, pre-equilibrated with 0.05 M acetate buffer pH 5.0. A sample of 100 µl (0.05 mg protein) was applied to the column and the column was eluted with same buffer at a flow rate of 0.5 mL/min. Eluted fractions (0.5 mL) were assayed for amylase activity.

### 1.3. Polyacrylamide gel electrophoresis

Purified protein sample (10 µg) from HPGPLC column was subjected to native gel electrophoresis according to the method of Gabriell.[23] Denaturing SDS–PAGE of the HPGPLC purified protein sample was carried out at pH 8.3 according to the method of Laemmli[24] using 12.5% gel and 1% (w/v) SDS in the LKB Midget unit (10 cm × 10 cm × 1.5 mm slab).

### 1.4. Molecular weight determination

The native molecular weight of the purified protein was determined by gel filtration on protein Pak TSK 2000 SW (7.5 × 600 mm) HPLC column, pre-calibrated with pure marker proteins (Pharmacia). Protein molecular weight markers (Sigma Chemicals, US) also were run parallel in SDS–PAGE.

### 1.5. N-Terminal sequence

Enzyme obtained from HPGPLC column was subjected to SDS PAGE analysis as mentioned before. The protein resolved in the gel was then transferred to a PVDF membrane in the presence of 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer, pH 11.0 with constant current of 400 mA for 5–6 h at 8–10 °C in Mighty Small Transphore unit (Hoefer, USA) as per the manufacturer's instructions. The membrane was removed from the transblotting sandwich and transferred protein bands on PVDF were stained and identified with Ponceau S solution. The identified protein band was excised for N-terminal sequencing studies by N-terminal sequencing model 491 Procise protein/peptide sequencer from Applied Biosystems, USA. Fifty picomoles of the sample were charged. The sampling rate was 4.0 Hz. The N-terminal sequence of the purified *T. cordifolia* amylase was compared with those of other amylases using BLAST search on universal protein database servers (NCBI, UniProt)

### 1.6. Enzyme assay

α-Amylase activity was determined in 0.1 M phosphate buffer, pH 6.0 (Buffer B) at 50 °C using soluble starch (1% w/v) as substrate. Reducing sugar formed was estimated by dinitro salicylic acid reagent (DNSA) according to Sengupta et al.[25] Enzyme activity (U) was expressed in terms of µmol of maltose equivalent liberated per min under the assay conditions. Pullulanse, sucrase, and 1-0-methyl-α-D-glucosidase activities were determined in the same way using 1% (w/v) each of pullulan (from *Aureobasidium pullulans*), sucrose, and 1-0-methyl-α-D-glucoside, respectively, as substrates. Maltase and isomaltase activities were determined at pH 5.0 at 50 °C using 1% (w/v) each of maltose and isomaltose, respectively, as substrates and glucose formed was estimated by glucose oxidase–peroxidase (GOD-POD) reagent as described by Bergmeyer and Bent.[26] One unit of enzyme activity (U) was expressed as the amount of enzyme that could liberate 1 µmol of glucose per minute under the assay conditions. Isoamylase activity was determined at pH 5.0 at 50 °C using glycogen (from oyster; type II) as substrate according to the method of Martins.[27] *p*-nitrophenyl α-D-glucopyranosidase activity was measured[28] at pH 5.0. Dextrinization kinetics of the enzyme at pH 5.0 was followed iodometrically[29] using 2% (w/v) gelatinized soluble starch, amylopectin, and corn flour incubated for 120 min at 50 °C.

### 1.7. Determination of physicochemical properties of the enzyme

The effects of pH and temperature on the activity and stability of amylase were determined in the range of 2.0–9.0 and 30–

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90 °C, respectively. The effect of various cations and anions was studied by pre-incubating the reaction mixtures (2 mL) containing 0.5 U of enzyme and various cations (1 mM) and anions (1 mM) in buffer B for 15 min at 37 °C followed by the addition of 1% (w/v) substrate and subsequent determination of residual enzyme activity. Effect of $Ca^{2+}$ on enzyme activity was studied by dialyzing amylase solution (100 U/mL of 0.05 mM acetate buffer, pH 5.0) against 1 mM EDTA for 48 h at 25 °C, pre-incubating the dialyzed enzyme solution with 0–50 mM $CaCl_2$ for 15 min and determining the residual activity. Effect of $Ca^{2+}$ on thermostability of the purified enzyme was studied by pre-incubating 2 U of dialyzed enzyme in buffer B with 0–10 mM $CaCl_2$ for 20 min in the temperature range of 50–80 °C followed by determination of residual enzyme activity. Action of proteolytic enzymes on stability of amylase was determined by the assay of residual enzyme activity of the incubation mixtures (1 mL), containing 20 U of amylase, pre-incubated separately with pepsin (200 U/mL) in 0.1 M glycine–HCl pH 3.0, and trypsin (200 U/mL), chymotrypsin (132 U/mL), and proteinase K (40 U/mL) in 0.1 M phosphate buffer, pH 7 for 180 min at 37 °C. Stability of enzyme in the presence of SDS, urea, and guanidine hydrochloride was determined in an incubation mixture (0.5 mL), containing 10 U of enzyme in buffer B incubated for 60 min at 37 °C.

### 1.8. Effect of different chemical modifiers on amylase activity

In an incubation mixture (1 mL), 20 U of enzyme was pre-incubated at 37 °C with iodoacetic acid (50 mM), iodoacetamide (50 mM), *N*-ethylmaleimide (NEM; 50 mM), *p*-chloromercuri benzoate (PCMB; 5 mM), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; 5 mM), and water soluble carbodiimide (50 mM) at 37 °C in their respective recommended buffers.[30] Inactivation kinetics was monitored till 3 h of incubation. Reactivation kinetics of DTNB inactivated enzyme was studied by addition of equal volume of 5 mM cysteine followed by determination of residual enzyme activity at different time intervals. Free thiol of the purified enzyme was determined using DTNB (Ellman's reagent) according to Ellman[31] using *N*-acetyl cysteine as standard.

### 1.9. Substrate specificity and kinetics of different starch hydrolysis

The activity of the enzyme on various substrates (starch, glycogen, *p*-nitrophenyl-α-D-glucopyranoside, maltose, isomaltose, sucrose, panose, amylopectin, 1-O-methyl-α-D-glucopyranoside) was determined in 0.1 M-acetate buffer, pH 5.0 at 50 °C. The kinetics of soluble starch hydrolysis was studied in terms of liberation of reducing groups and glucose. Reaction mixture (10 mL) containing 2% (w/v) substrate and 100 U of purified amylase in 0.1 M-acetate buffer, pH 5.0 was incubated at 50 °C for 3 h. End products were detected by TLC.[32] Lineweaver–Burk plot for the determination of $K_m$ value was prepared by measuring amylase activity for increasing substrate concentration followed by regression analysis to determine the slope of the best fitting line. Cereal flours (corn, wheat, rice and gram), purchased from local market were well gelatinized with water at 100 °C for 5 min with constant stirring. Reaction mixtures (10 mL) containing 2% (w/v) gelatinized cereal flours in 0.1 M acetate buffer, pH 5.0 were separately incubated with 100 U of both plant and fungal amylase (purified α-amylase from *A. oryzae;* Mol. wt ~51000; Sigma Cat. No. 10065) at 50 °C for 180 min. Reducing sugar formed at different time intervals was estimated and results were expressed in terms of Dextrose Equivalent [DE = (Reducing sugar expressed as glucose/total carbohydrate) × 100].

### 1.10. Analytical methods

Protein was determined using coomassie blue (Bradford) protein assay reagent[33] and total carbohydrate by anthrone reagent.[34]

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