

Research Article

Biochemical and Structural Characterization of Amy1: An Alpha-Amylase from *Cryptococcus flavus* Expressed in *Saccharomyces cerevisiae*

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An extracellular alpha-amylase (Amy1) whose gene from *Cryptococcus flavus* was previously expressed in *Saccharomyces cerevisiae* was purified to homogeneity (67 kDa) by ion-exchange and molecular exclusion chromatography. The enzyme was activated by NH_4^+ and inhibited by Cu^{+2} and Hg^{+2} . Significant biochemical and structural discrepancies between wild-type and recombinant α -amylase with respect to K_m values, enzyme specificity, and secondary structure content were found. Far-UV CD spectra analysis at pH 7.0 revealed the high thermal stability of both proteins and the difference in folding pattern of Amy1 compared with wild-type amylase from *C. flavus*, which reflected in decrease (10-fold) of enzymatic activity of recombinant protein. Despite the differences, the highest activity of Amy1 towards soluble starch, amylopectin, and amylase, in contrast with the lowest activity of Amy1_w, points to this protein as being of paramount biotechnological importance with many applications ranging from food industry to the production of biofuels.

1. Introduction

Starch is a major storage product of many economically important crops such as wheat, rice, cassava, and potato [1]. A large variety of microorganisms employ extracellular or intracellular enzymes to hydrolyze starch thus enabling its utilization as a source of energy. One of the most important groups of enzymes that process starch is represented by the α -amylase family or family 13 glycosyl hydrolases [2, 3]. Amylases (EC 3.2.1.1, α -1,4-glucan-glucanohydrolase) are enzymes that hydrolyze starch polymers yielding diverse products including dextrans and

smaller polymers of glucose. These enzymes are of great biotechnological interest with many applications ranging from food industry to the production of biofuels. Since each different application requires amylases with unique properties it is often necessary to search the biodiversity for new sources of these enzymes [4]. Several amylases isolated from yeasts such as *Candida antarctica*, *Candida japonica* [4], *Lipomyces kononenkoae*, *Saccharomycopsis fibuligera*, *Schwanniomyces alluvius* [5], *Trichosporon pullulans*, and *Filobasidium capsuligenum* [6] have been described. We have previously reported the characterization of an α -amylase (Amy1) from the basidiomycetous yeast *Cryptococcus flavus*

which exhibited important biochemical properties for its industrial utilization such as high stability at pH 5.5 and optimal temperature at 50°C [7]. The gene encoding this α -amylase (*AMY1*) was cloned and successfully expressed in *Saccharomyces cerevisiae* [8]. In order to assess the use of *S. cerevisiae* as a host for the heterologous production of Amy1 we sought the purification and enzymatic characterization of recombinant enzyme produced in this system.

2. Material and Methods

2.1. Strains. *S. cerevisiae* CENPK2 (MATa/ α *ura3-52/ura3-52 leu 2-3,112 trp1-289/trp1-289 his3-1/his3-1*).

2.2. Enzyme Purification. A colony of *S. cerevisiae* CENPK2 harboring YEpAMY was cultured in SD medium (0.62% YNB, 2% glucose, uracil, tryptophan, and histidine), and 2 mL of this preculture was transferred to 1 L conical flask containing 200 mL of the same medium following incubation at 28°C for 60 h (200 rpm). *S. cerevisiae* cells were harvested by centrifugation (5,000 g/10 min), and the supernatant was dialyzed overnight against water and loaded on a Q-Sepharose fast flow column (0.9 × 18 cm) previously equilibrated in 50 mM sodium acetate (pH 5.5). The column was washed with the same buffer, and proteins were eluted by a linear gradient of 0–0.5 M NaCl. Four fractions of 3 mL were collected and monitored for the presence of proteins and amylase activity. These samples were collected, dialyzed, lyophilized, and resuspended in 1 mL of water and loaded on a Sephacryl S-200 HR (2.5 × 85 cm) column previously equilibrated with 50 mM sodium acetate containing 0.1 M NaCl. The column was washed with the same buffer at a flow rate of 12 mL·h⁻¹. Fractions containing amylase activity were pooled, dialyzed against water, lyophilized, and stored at -20°C. In chromatography experiments, the protein content of each fraction was routinely monitored by measuring the absorbance at 280 nm. Protein concentration was measured using serum albumin as standard [9].

2.3. Electrophoresis and Zymogram Analysis. Protein integrity and the molecular mass calculation were performed by running samples on 12% sodium dodecyl sulfate-polyacrylamide gels [10]. Proteins were silver-stained as described by manufacturer [11] (1987). Molecular mass markers (Fermentas Life Sciences) were as follows: β -galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β -lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa) by manufacturer. Activity gels (zymogram) were performed after running samples on 12% nondenaturing PAGE. Gels were washed with distilled water, incubated with 50 mM sodium acetate (pH 5.5) for 60 min, and then incubated at 4°C for 12 h in a solution containing 0.5% of starch (in 50 mM sodium acetate buffer [pH 5.5]). The gel was then incubated at 37°C for 2 h, and bands with amylase activity were detected after staining with iodine solution (1 mM I₂ in 0.5 M KI).

2.4. Amylase Assay. α -amylase dextrinizing activity was assayed in a reaction system containing 100 μ L 0.5% (w/v) soluble starch, 40 μ L 0.5 M acetate buffer (pH 5.5), enzyme solution (0–60 μ L), and water to a total volume of 200 μ L. After 10 min at 60°C, the reaction was stopped with 200 μ L 1.0 M acetic acid. Iodine reagent was then added to determine dextrinizing activity [12]. Saccharifying activity was determined by measuring the production of reducing sugars from starch by using the dinitrosalicylic acid (DNS) method [13]. One unit of dextrinizing activity was defined as the amount of enzyme necessary to hydrolyse 0.1 mg starch/min. One unit of saccharifying activity was defined as the amount of enzyme necessary to produce 1 mg glucose equivalent/min.

2.5. Enzyme Characterization. The optimum pH of the recombinant amylase was determined by varying the pH of the reaction mixtures using the following buffers (50 mM): glycine-HCl (pH 1.0–3.0), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.0), and Tris-HCl (pH 8.0–10) at 60°C. In order to determine pH stability, the enzyme was preincubated in different buffers for 60 min at 60°C. The residual enzyme activity was assayed in 50 mM sodium acetate buffer (pH 5.5). The optimum estimated temperature of the enzyme was evaluated by measuring amylase activity at different temperatures (30°C to 70°C) in 50 mM sodium acetate (pH 5.5). The effect of temperature on enzyme stability was determined by measuring the residual activity after 15, 30, 45, and 60 min of preincubation in 50 mM sodium acetate (pH 5.5) at 55 and 60°C. In order to determine the effect of metal ions, the assay was performed after preincubation of the enzyme with various metal ions at a final concentration of 4 mM. The effect of 10 mM CaCl₂ and DTT (5–10 mM) was also evaluated. K_m values for the purified enzyme were determined by incubating the enzyme with 0–0.8 mg·mL⁻¹ soluble starch in 50 mM sodium acetate (pH 5.5) at 60°C. The data obtained were fitted to a standard Lineweaver-Burk model using linear least squares regression.

2.6. Thin Layer Chromatography Analysis. Briefly, purified amylase was incubated with 0.5% starch, glycogen, pullulan, amylase, and amylopectin, in 50 mM sodium acetate (pH 5.5) at 40°C. Aliquots (10 μ L) were removed after incubation for 6 and 12 h. The chromatogram was developed with *n*-butanol/methanol/H₂O (4:2:1). Sugars were detected by thin-layer chromatography [14].

2.7. Circular Dichroism Spectroscopy. Circular Dichroism (CD) assays were carried out using Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature cuvette holder. Far-UV spectra were recorded using 0.1 cm path length quartz cuvette. Proteins (0.085 to 0.100 mg/mL) were analyzed in 2 mM glycine pH 3.0 and 4.0, 2 mM sodium acetate pH 5.5, 2 mM Tris-HCl pH 7.0 and 8.0. We used CD data over the wavelength range 260–200 nm to ensure a satisfactory CD signal and to prevent the high signal-noise ratio. Four consecutive measurements were

TABLE 1: Summary of the purification steps of Amy1.

Step	Total activity (U)	Total protein (mg $\times 10^{-3}$)	Specific activity (U/mg $\times 10^{-3}$)	Purification (fold)	Yield (%)
Supernatant	1922.2	1.750	1098.5	1.00	100.0
Q-Sepharose	490.8	0.120	4090.0	3.72	25.4
Sephacryl S-200HR	199.9	0.048	4164.5	3.79	10.3

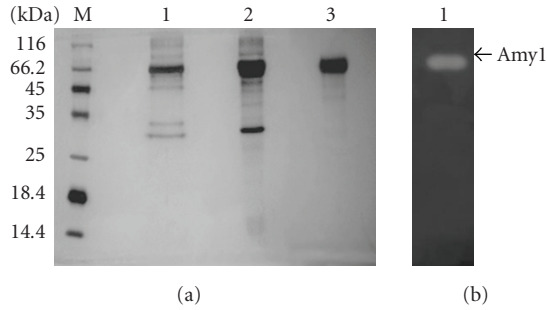


FIGURE 1: (a) Analysis of purified recombinant Amy1 on 12% SDS-PAGE; M, standard marker proteins; lane 1, lane 2, protein profile after ion exchange on Q-Sepharose column; lane 3, purified recombinant amylase after gel filtration on Sephacryl S-200HR column. (b) Detection of amylase activity on non-denaturing PAGE.

accumulated, and the mean spectra were corrected for the baseline contribution of the buffer. Thermal denaturation assays were performed raising the temperature from 25 to 95°C. The observed ellipticities were converted into molar ellipticity $[\theta]$ based on molecular mass per residue of 115 Da. Protein structure and thermal unfolding curves were tracked by changes in $[\theta]$ at 222 or 206 nm.

The temperature dependence of the secondary structure was estimated from the Far-UV CD curves adjustments using the CDNN deconvolution software (Version 2.1, Bioinformatik.biochemtech.uni-halle.de/cdnn) [15].

3. Results and Discussion

3.1. Purification of Amy1. Amy1 was purified from the supernatant of *S. cerevisiae* cultures in a two-step chromatographic procedure. Elution profiles of both Q-Sepharose and Sephacryl-S200HR chromatography showed one peak with amylase activity (data not shown). This fraction was collected, dialyzed, and concentrated by lyophilization. The enzyme was purified to homogeneity with 3.79-fold increase in specific activity with a yield of ~10.3% as compared to the supernatant (Table 1). Comparing with the recombinant enzyme, wild-type Amy1 was purified from *C. fluvus* cultures in only a single purification step [7]. SDS-PAGE analysis of the purified recombinant amylase showed a single protein band corresponding to ~67 kDa (Figure 1(a)) which showed α -amylase activity after zymogram analyses (Figure 1(b)). Among the amylase-producing yeasts and filamentous fungi, the α -amylases from *Cryptococcus* sp S-2 [16],

TABLE 2: Effect of ions on purified Amy1.

Compound (4 mM)	Relative activity (%)
Control	100
MgSO ₄	88.6
MnSO ₄	77.9
CaCl ₂	71.6
(NH ₄) ₂ SO ₄	111
ZnCl ₂	20.1
CuSO ₄	0
HgSO ₄	0

Aspergillus fumigatus [17], *Lipomyces starkeyi* [18], and *Lipomyces kononenkoae* [19] showed similar molecular masses as Amy1 produced in *S. cerevisiae*.

3.2. Enzyme Characterization. The optimum pH for the recombinant enzyme was determined as pH 5.5 (Figure 2(a)). Amy1 was tested for pH stability by preincubating the purified enzyme in appropriate buffers for 1 hour. The data suggest that Amy1 is tolerant to wide pH range (Figure 2(b)). This result was similar to that found for α -amylases from a variety of yeast strains [20, 21]. The optimum pH for yeast α -amylase activity is usually in the range of 4.0 and 6.0 [5, 7, 16, 21].

Amylase activity measured as a function of temperature from 30 to 70°C shows that the activity was the highest at 60°C (Figure 2(c)). This optimum temperature is in agreement with the α -amylases from *Cryptococcus* sp S-2 treated with 1 mM CaCl₂ [16] and *Aureobasidium pullulans* N13d [20]. Thermostability is considered an important and useful criterion for industrial application of α -amylase from microorganisms. As shown in Figure 2(d), the residual amylase activity is practically constant at 55°C after 1 h of incubation. However, at 60°C 50% of the residual activity was lost. Iefuji et al. [16] proposed that the C-terminal raw starch binding motifs present in *Cryptococcus* sp S-2 amylase (AMY-CS2) might be related to the thermostability presented by this amylase. Because Amy1 has 97% sequence identity with AMY-CS2 [8] it is possible that the same explanation applies to Amy1.

The results presented on Table 2 indicate that recombinant Amy1 is poorly affected by most of the 4 mM ions tested (Mg²⁺, Mn²⁺, and Ca²⁺) since the relative activity was higher than 70%. In addition, it was also found that in the presence of 10 mM CaCl₂, Amy1 still maintained its original activity (data not shown) which shows that

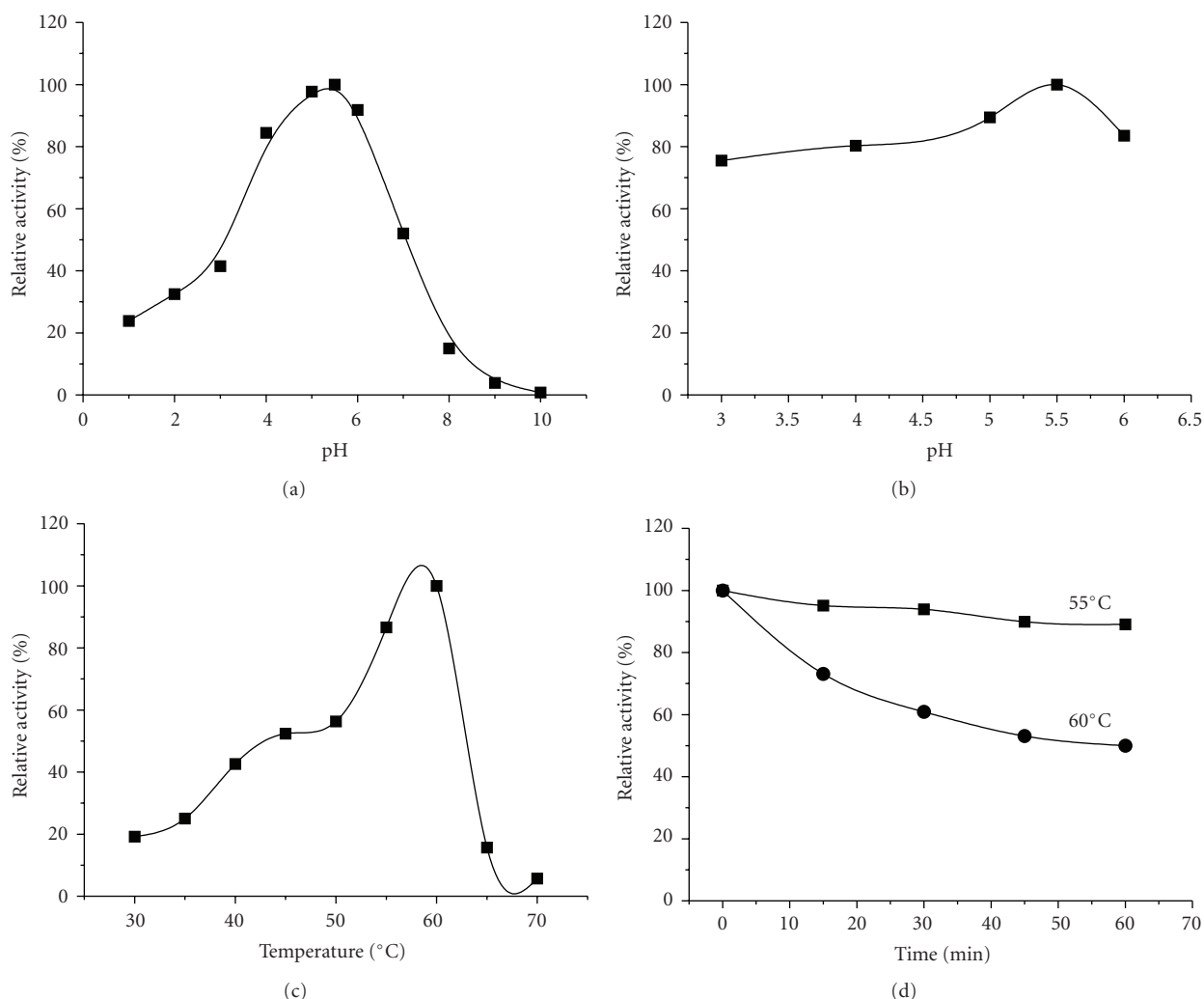


FIGURE 2: Effect of pH (a-b) and temperature (c-d) on Amy1 activity and stability, respectively. The pH optimum and pH stability were determined by varying the pH of the reaction mixtures and preincubating the enzyme in different buffers for 60 min at 55°C (square) and 60°C (circle). The temperature optimum was evaluated by measuring amylase activity at different temperatures, and the effect of temperature on enzyme stability was determined by measuring the residual activity after 15, 30, 45, and 60 min of preincubation in 50 mM sodium acetate (pH 5.5) at 55 and 60°C.

this enzyme is not affected by Ca^{+2} . Similar behavior was found for wild-type Amy1. However, Takeuchi et al. [22] related that the *Pichia burtonii* α -amylase was activated by Ca^{+2} (135%). These authors proposed that the activation of enzyme by calcium ions probably happened during the purification procedure when the enzyme lost activity. The *Bacillus halodurans* 38C-21 α -amylase also had its activity increased in presence of Ca^{+2} [23]. The presence of NH_4^+ ions had the most prominent activating effect (111%) on recombinant Amy1. However, Zn^{+2} , Cu^{+2} , and Hg^{+2} acted as inhibitors of amylase activity, with Cu^{+2} and Hg^{+2} showing a complete inhibition (Table 2). The inhibition by Hg^{+2} may indicate the importance of indole group of amino acid residues in enzyme function [4, 24]. The wild-type amylase is inhibited by Hg^{+2} , Ag^+ , Cu^{+2} , and Mg^{+2} [16] while that from *L. kononenkoae* CBS 5608 is inhibited by Ag^+ and Cu^{+2} [21]. Interestingly, the amylase from yeast *Aureobasidium pullulans*

N13d is not inhibited by Cu^{+2} which had an activating effect on the purified enzyme [20]. DTT is frequently used to reduce and prevent intramolecular and intermolecular disulfide bonds. The enzyme's activity was practically constant when DTT (4–25 mM) was used (data not shown). This result was different from that observed with the *Thermotoga maritima* MSB8 amylase expressed in *E. coli* which showed increased activity in presence of DDT ranging from 5 to 10 mM [25].

Wanderley et al. [7] reported that the K_m for wild-type Amy1 was $0.056 \text{ mg} \cdot \text{mL}^{-1}$ which is considerably smaller than the K_m observed for the recombinant amylase ($0.37 \text{ mg} \cdot \text{mL}^{-1}$) which showed Michaelis-Menten type kinetics with soluble starch as substrate. The K_m of the recombinant enzyme is similar to other yeast amylases such as *Schwanniomyces alluvius* [5] and *Thermomonospora curvata* [26].

In order to establish the specificity of recombinant Amy1, the enzyme was incubated with starch, amylopectin, amylose, glycogen, and pullulan. These substrates consist of different glucose polymers containing α -1,4 linkages or a mixture of α -1,4- and α -1,6-glucosidic linkages. Amy1 showed the highest activity towards soluble starch (100%) amylopectin (97.9%), amylose (50.6%) but no activity was observed on pullulan. This result is similar to that observed for the *Bacillus subtilis* truncated α -amylase produced in *E. coli* [27], but different from that observed for wild-type Amy1 which showed the highest activity towards starch (100%), glycogen (18.9%), amylopectin (16.7%), and amylose (7.1%). The recombinant enzyme displayed typical α -amylase properties based on the evidence that oligosaccharides from several sizes were the main products from the digestion of glycogen, amylose, amylopectin, and starch (Figure 3).

3.3. Conformational Stability of Amy. Structural characterization of recombinant (Amy1) and wild-type (Amy_w) amylase from *C. flavus* was carried out by circular dichroism spectroscopy for comparison. Far-UV CD spectra of Amy1 and Amy1_w at pH 7.0 and 25°C are quite different showing typical features of beta/unordered and beta/ α -helix secondary structure pattern, respectively (Figure 4(a)). While the 222 nm dichroic band is predominantly associated with α -helical structure, the dichroic band at 198 and 206 nm may arise from changes in other secondary structure elements in the protein, as unordered or beta structure. The secondary structure content of both proteins slightly differs on helical (Amy1, 5.7% and Amy1_w, 9.5%) and antiparallel β -sheet (Amy1, 42.1% and Amy1_w, 32.4%) and is similar for other secondary structures.

Thermal stability of the proteins at pH 7.0 was assessed upon raising the temperature from 20 to 95°C (Figures 4(b) and 4(d)). The thermal denaturation curves analyzed from Far-UV CD measurements at 206 and 222 nm at pH 7.0 strongly indicated the Amy1_w and Amy1 as thermally stable proteins since no pattern of protein denaturation could be verified (data not shown). Indeed, although the CD spectra of the Amy1_w in this condition show a gradual increase (downward) of the dichroic bands at 206 nm and slight at 222 nm (Figure 4(d)), no secondary structure changes were detected suggesting only conformational changes of protein or secondary structure rearrangement as a function of temperature. In contrast, CD spectra of the Amy1 show a gradual increase and decrease of the dichroic bands at 222 and 200 nm, respectively, suggesting the alteration of helical and β -sheet structures content (Figure 4(b)). A slight increase was observed in terms of α -helix (from 5.7% to 6.8%) and decrease of β -sheet (from 42.1% to 38.8%) when raising the temperature from 25 to 95°C. Altogether, these results revealed the high thermal stability of both proteins and the difference in folding pattern of Amy1 compared with wild-type amylase from *C. flavus* reflecting in decrease (10-fold) of enzymatic activity (K_m values) of recombinant protein using a soluble starch as substrate, as discussed above.

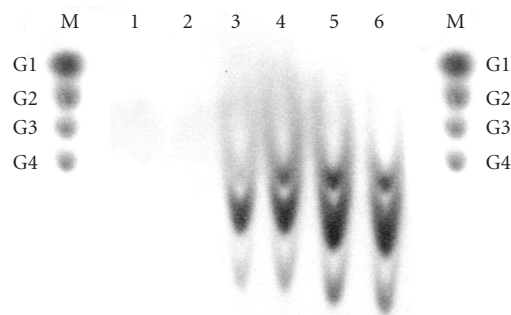


FIGURE 3: Thin-layer chromatogram of products released from starch after treatment with recombinant Amy1. The standards used were glucose (G1), maltose (G2), maltotriose (G3), and maltotetraose (G4). Lane 1, control (starch without enzyme); lane 2, pullulan; lane 3, glycogen; lane 4, amylose; lane 5, amylopectin, and lane 6, soluble starch.

Besides significant distinction in pH region where the maximum enzymatic activity of Amy1 occurred (Figure 2(a)), the protein achieved similar secondary structure content (helical 5.7%–6.5% and β -sheet ~42%) in a broad range of pH (pH 3.0, 5.5, and 7.0), as indicated by the CD spectra (Figure 4(c)) and CDNN estimative. At pH 8.0 as well as pH 4.0, it was verified the mainly increase in the CD signal at 200 nm (Figure 4(c)), compatible with minor β -sheet structures content (~37%) compared with pH 3.0, 5.5, and 7.0 (~42%). Noteworthy, although no secondary structure changes have been observed, analogous or different conformation of Amy1 side chains could occur as a function of those pHs, as judged by the relative activity of the enzyme in these condition (Figures 2(a) and 2(b)). In these cases, some important structural changes related with side chains ionization of aspartate, glutamate, and histidine residues at pH 4.0 and 5.5 could occur and are fundamental to enzyme activity.

In this work we have observed some significant biochemical and slight structural discrepancies between wild-type and recombinant Amy1 especially with respect to K_m values, enzyme specificity, and helical and β -sheet structure content. These could be explained by small folding alterations in the structure of recombinant amylase during its expression. Different patterns of protein glycosylation between *C. flavus* and *S. cerevisiae* could also account for these differences. In fact, three putative *N*-glycosylation sites have been identified in Amy1 [8]. Work is under way to assess the role of glycosylation on the activity of Amy1. Because recombinant Amy1 showed considerable thermostability, its use in biotechnological processes should be considered.

4. Conclusion

In conclusion, the enzymatic and structural features of Amy1, the highest activity towards soluble starch, amylopectin, and amylase, in contrast with the lowest activity of Amy1_w, points out this protein as being of paramount

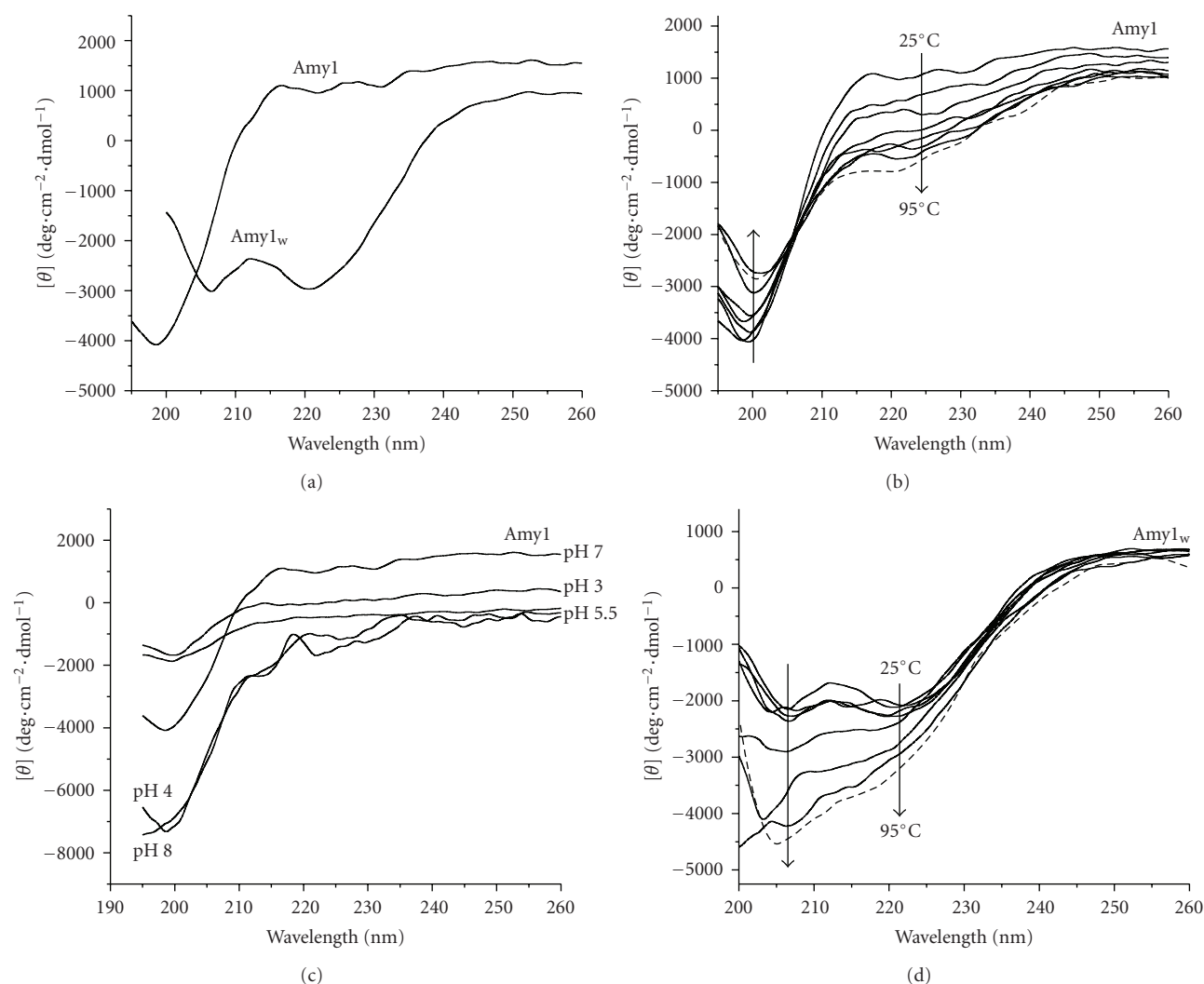


FIGURE 4: Far-UV circular dichroism analyses. Residual molar ellipticity $[\theta]$ was measured from 200 to 260 nm at 25°C or temperature arising from 25 to 95°C. (a) The distinct spectra of wild-type (Amy1_w) and recombinant (Amy1) amylase are shown, indicating that they had different amount of secondary structure (see Section 3). (b) Effect of temperature on secondary structure of Amy1 . Changes in molar ellipticity at 222 and 200 nm are shown as a function of temperature. (c) Effect of pH on secondary structure of Amy1 . Note the slight differences of CD spectra at pH 7.0, 3.0, and 5.5 and similarity of CD spectra at pH 4.0 and 8.0. (d) Effect of temperature on secondary structure of Amy1_w . Changes in molar ellipticity at 222 and 206 nm are shown as a function of temperature.

biotechnological importance with many applications ranging from food industry to the production of biofuels.

Acronyms

SD: Synthetic dextrose minimal media
 YNB: Yeast nitrogen base
 REase: Ribonuclease
 PAGE: Polyacrylamide gel electrophoresis
 DTT: Dithiothreitol
 CDNN: Neural network circular dichroism.

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