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Purification and Characterization of Tannin Acyl Hydrolase from *Aspergillus niger* ATCC 16620

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Summary

Tannin acyl hydrolase produced extracellularly by the fungal strain *Aspergillus niger* ATTC 16620 in solid state fermentation was purified from the cell free culture broth by ammonium sulphate fractionation followed by DEAE–Sephadex A-50 chromatography. SDS-PAGE analysis indicated that the enzyme protein molecular mass was 168 kDa. Enzyme activity was stable up to the temperature of 40 °C and the enzyme activity was optimal at pH=6. Tannase activity was maximal at 0.01 M concentration of the substrate. The addition of metal ions like Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, $Mg^{2+}$and Fe^{2+} inhibited the enzyme activity. Only K^+ ions enhanced tannase activity, and an activity of 4.31 U/mL was reported here. Enzyme activity was maximal after 15–20 min of incubation time, with an activity of 3.9 U/mL. K_m was found to be 1.03 mM and V_{max}=4.25 μmol/min. Since the enzyme is active over a wide range of pH and temperature it could find potential use in the food-processing industry.

Key words: tannase, fermentation, gel filtration, purification, characterization, electrophoresis

Introduction

The enzyme tannase (E.C. 3.1.1.20), also known as tannin acyl hydrolase (TAH), is a hydrolytic enzyme that acts on tannins. Tannase catalyses the hydrolysis of bonds present in the molecules of hydrolysable tannins and gallic acid esters (*1*). Tannins are a group of water-soluble phenolic compounds with different molecular mass, which form hydrogen bonds in solutions (*2*) that result in the formation of tannin-protein complexes.

Tannase is extensively used in the food, feed, beverage, brewing, pharmaceutical and chemical industries. The major commercial applications of tannase are in the manufacture of instant tea and the production of gallic acid (*3*). Gallic acid is a key intermediate required for the synthesis of the antibacterial drug trimethoprim, used in the pharmaceutical industry. Tannase is used as a clarifying agent in wine, fruit juices and coffee flavoured soft drinks (*4,5*). Bacteria (*6,7*), yeast (*8*) and filamentous fungi (*5,9–11*) are known tannase producers. Also, a recombinant *Aspergillus oryzae* tannase was expressed in *Pichia pastoris* and the enzyme was purified and characterized (*12*).

Fungal TAH is a glycoprotein (*8,13*) formed from a mixture of an esterase and depsidase (*14,15*) with a pH stability around 3.5–8.0, pH optimum of 5.5–6.0, temperature stability between 30 and 60 °C, temperature optimum around 30–40 °C, an isoelectric point of 4.0–4.5, and molecular mass between 186 and 300 kDa. TAH is inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Mg^{2+}, and is inactivated by *o*-phenantroline, EDTA, 2-mercaptoethanol, sodium thioglycolate, magnesium sulphate, calcium chloride and magnesium chloride (*8,13,16–18*).

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Since 1970 there have been some reports on TAH purification from plant and microbial sources (*8*). Beverini and Metche (*15*) purified TAH from a commercial product of *A. oryzae.* Rajakumar and Nandy (*13*) purified the enzyme by ammonium sulphate precipitation followed by DEAE-cellulose column chromatography and gel filtration on a Sephadex G-200 column. Barthomeuf *et al.* (*18*) reported that a pure enzyme sample (1.980 nkat/mg) was obtained by physical disruption of the mycelium, ultrafiltration and high-pressure exclusion chromatography.

Yamada *et al.* (*19*) purified a tannase produced by *A. flavus* strain and examined its properties. The optimum temperature for the production of tannase by the *A. flavus* strain was 70 °C, pH optimum was 6 and the molecular mass of tannase was 80–85 kDa. The K_m for tannases from *A. flavus*, *Selenomonas ruminantium* and *Cryphonectria parasitica* using methylgallate as substrate have been found to be 0.86 mM (*19*), 1.6 mM (*20*) and 7.49 mM (*21*). In the present report results obtained from the studies on purification and characterization of tannin acyl hydrolase from *A. niger* ATCC 16620 are described.

Materials and Methods

Microorganism and maintenance of culture

A strain of *Aspergillus niger* ATCC 16620, given by the Technical University of Budapest, was used for the study. Potato dextrose agar slants were used for the maintenance of *A. niger*. Fully sporulated slants were stored at 4 °C in a refrigerator and subcultured once every three weeks.

Preparation of spore inoculum

Fungal spore inoculum was prepared by adding 2.5 mL of sterile distilled water containing 0.1 % Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and the number of viable spores in the suspension was determined using the plate count method. The volume of 1 mL of the prepared spore suspension was used as the inoculum, with concentration of $44 \cdot 10^9$ spores.

Production of tannase under SSF

A mass of 5 g of wheat bran was taken in 250-mL Erlenmeyer flasks and moistened with 5 mL of salt solution. The composition of the salt solution was NH_4NO_3 0.5 %, NaCl 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 % and gallic acid 4 % at pH=5.5. The contents were sterilized by autoclaving at 103.4 kPa for 20 min. After cooling the sterilized solid substrate was inoculated with 1 mL of the spore inoculum. The contents were mixed properly and incubated at 30 °C for 96 h.

Extraction and analysis of crude enzyme

Tannase was extracted from the fermented substrate by adding 50 mL of distilled water containing 0.01 % Tween 80. Contents were mixed well for 10 min using a magnetic stirrer. Crude enzyme was separated from the fermented matter by centrifugation at 8000 rpm at 4 °C for 20 min in a refrigerated centrifuge. The filtrate was collected in bottles and preserved for further studies.

Tannase assay was performed by the method of Sharma *et al.* (*22*). Enzyme activity was expressed in international units (IU). One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions. Total soluble protein was determined by the method of Lowry *et al.* (*23*) and was expressed in mg/mL.

Purification and characterization

Ammonium sulphate fractionation and dialysis

A volume of 100 mL of crude tannase was taken initially and then the required quantity of ammonium sulphate was added slowly to obtain various saturation levels (0–40, 40–60 and 60–80 %). The addition of ammonium sulphate was done under constant stirring at 4 °C for 30 min and then stirring was continued for another 30 min. After this the mixture was kept to settle for 3 h at 4 °C. Proteins were precipitated and then separated by centrifugation at 8000 rpm at 4 °C for 20 min. The separated proteins were then dissolved in minimum amount of 0.05 M citrate buffer (pH=5) and refrigerated for further analysis.

Precipitated proteins were transferred into a dialysis tube (Sigma) using a micropipette and dialyzed against citrate buffer (0.05 M, pH=5) at 4 °C. The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was conducted over night and the buffer was changed several times to increase the efficiency of the dialysis.

DEAE–Sephadex A-50 chromatography

A chromatographic column (Pharmacia) was packed with DEAE–Sephadex A-50 to a bed size of 2.5 x 10 cm and was equilibrated with 0.05 M citrate buffer (pH=5). The dialyzed enzyme was applied to the column and the elution was done with citrate buffer (pH=5). Twenty fractions were collected and absorbance was measured at 520 nm. Fractions which showed absorbance greater than 0.2 were pooled and used for further investigations.

Enzyme characterization

The effect of different temperatures, pH, substrate concentration, metal ions and incubation time on the enzyme fractions obtained after DEAE-Sephadex A–50 chromatography was studied. The K_m and V_{max} of the purified enzyme were also determined. All the experiments were done in triplicate and the mean values with standard errors are reported.

Effect of temperature

To determine the effect of temperature on tannase activity the enzymatic reaction was carried out at different temperatures ranging from 25 to 85 °C. Purified enzyme and substrate were preincubated at various reaction temperatures before starting the experiment and the enzyme assay was performed as described earlier to determine the optimal incubation temperature.

Effect of pH

Effect of pH on tannase activity was studied by conducting the enzymatic reaction at various pH ranging from 3 to 9. The pH of the reaction mixture was varied using different buffers (0.05 M citrate buffer for pH=3–6, 0.05 M phosphate buffer for pH=7–8 and 0.05 M Tris HCl for pH=9). The substrate solution (0.01 M methyl gallate) was also prepared in buffers with different pH values (3–9). Tannase assay was performed as described earlier to determine the pH optimum.

Effect of substrate concentration

Methyl gallate solution of different concentrations (0.005 to 0.75 M) was prepared in 0.05 M citrate buffer (pH=5) and the effect of substrate concentration on tannase activity was determined.

Effect of metal ions

Different metal ions like Mg^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Ca^{2+}, Zn^{2+} and Mn^{2+} were dissolved in 0.05 M citrate buffer (pH=5) at a concentration of 0.1 M. Tannase assay was performed using this buffer and the effect of metal ions on tannase activity was studied.

Effect of incubation time

Tannase assay was performed using the purified sample for various incubation times (5 to 30 min) and the effect of incubation time on tannase activity was determined. Enzyme assay was done as described earlier.

Determination of K_m and V_{max}

K_m and V_{max} were determined by plotting velocity against substrate concentration [S]. K_m is equal to [S] when initial velocity (v) is equal to ½ V_{max}. K_m is a property of ES complex; it does not depend on the concentration of enzyme or substrate.

Molecular mass determination by SDS–PAGE

SDS–PAGE was conducted with the purified tannase for elucidation of the molecular mass of enzyme protein. Electrophoresis was done on 12 % gel according to Laemmli (*24*) and the separated protein band was detected by Coomassie blue staining. The separating gel consisted of 12 % polyacrylamide and the stacking gel consisted of 5 % polyacrylamide. Sample of 12 μL and 8 μL of sample buffer with 50 mM Tris HCl (pH=6.8), 10 % SDS, 0.1 % bromophenol blue, β-mercaptoethanol and glycerol were loaded into the well. Molecular mass markers were purchased from Bangalore Genei (India) and were run parallel to the samples. Electric current of 50 V was supplied using a standard power pack. The gel was stained using Coomassie brilliant blue and then destained using a mixture of methanol, glacial acetic acid and distilled water.

Results and Discussion

Tannase was produced extracellularly by *A. niger* ATCC 16620 in solid state fermentation on wheat bran. Crude tannase was concentrated by ammonium sulphate fractionation. At 40–60 % of ammonium sulphate most of the tannase protein was precipitated. After dialysis of the 40–60 % fraction, a specific activity of 0.916 U/mg protein was obtained, which was a four-fold enhancement in activity when compared to the crude enzyme. The 40–60 % fraction was further purified through DEAE-Sephadex A–50 chromatography and the eluted fractions, which showed absorbance of more than 0.2 at 520 nm, was pooled and used for further analysis (Table 1).

Table 1. Tannase activity after different purification steps

Purification step	Tannase activity/(U/mg)
Crude enzyme	0.27
40–60 % fraction	0.916
DEAE–Sephadex A–50 chromatography	1.6

Tannase assay was performed at various incubation temperatures ranging from 25 to 85 °C (Fig. 1a). The optimum temperature for the enzyme activity was found to be 30–40 °C, at which the enzyme activity was the highest (2.2 U/mL). Similar observations were reported for tannase from *A. oryzae*, *Aspergillus* sp. and *Penicillium chrysogenum* (*1,13,25*). With further increase in temperature tannase activity was found to decrease. Increase in temperature increases the rate of denaturation of the enzyme, with the loss of secondary and tertiary structure. There was considerably good activity even at 65 °C (1.4 U/mL) and this was considered as an additional advantage, since most of the processes assisted by tannase are performed at increased temperatures.

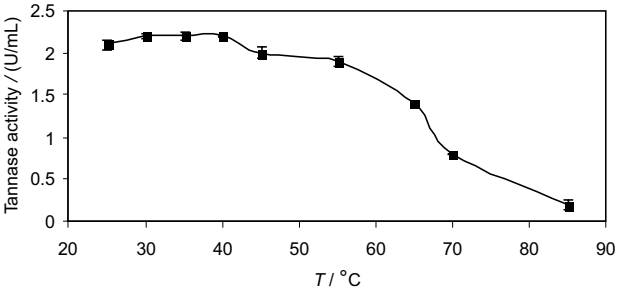

Fig. 1a. Effect of temperature on tannase activity

Enzyme assay was performed at different pH values (3–9) and the results obtained were described in Fig. 1b. In the present study, tannase activity was found to increase with the increase in pH and the optimum activity was at pH=6 (3.2 U/mL). The enzyme was active over a wide range of pH (pH=4–8) and this is a positive aspect regarding its utilization in the brewing industry. Similar results were reported for tannase from *A. niger* LCF8 (*14,18,21,26*). At pH values below 3.5 the enzyme was unstable. The effect of pH on the enzyme activity is determined by the nature of the aminoacids at the active site, which undergoes protonation and deprotonation, and by the conformational changes induced by the ionization of other amino acids. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum.

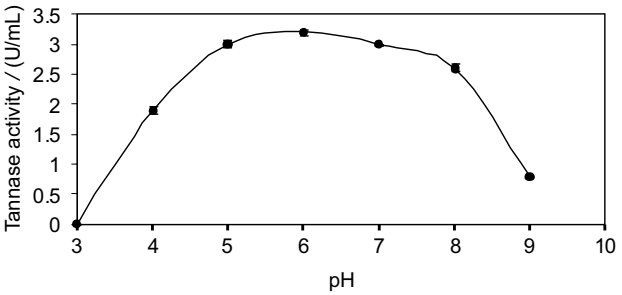

Fig.1b. Effect of pH on the activity of purified tannase

To see the effect of substrate concentration on tannase activity, assay was performed at various concentrations of methyl gallate. Enzyme activity was maximal at 0.01 M methyl gallate, when the activity was 3.8 U/mL. Further increase in substrate concentration was found to reduce enzyme activity. A lower substrate level for the maximal activity of the enzyme was considered as a very positive factor for industrial applications. Results obtained in the experiment to optimize the incubation time are presented in Fig. 1c, which shows that there was an increase in enzyme activity along with the increase in incubation time and the maximum activity (3.9 U/mL) was obtained at 15 and 20 min of incubation. Tannase activity was found to decrease with further increase in incubation time. Tannase from *A. niger* ATCC 16620 showed a K_m value of 1.03 mM and V_{max} of 4.25 μmol/min.

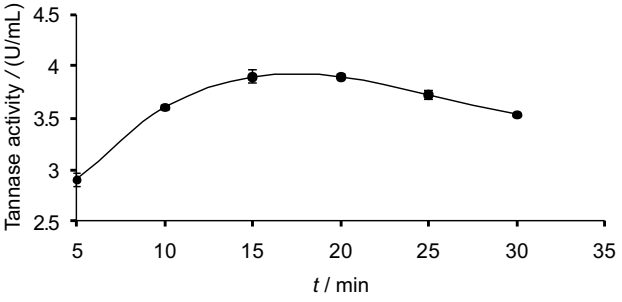

Fig. 1c. Effect of incubation time on tannase activity

Metal ions like Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+} inhibit the activity of tannase. Tannase was found to be inactivated by metal ions like Mg^{2+} and Ca^{2+}, but the enzyme was activated by K^+ ions (Fig. 1d). Similar results were reported previously (*8*,*13*,*17*,*18*,*27*,*28*). The activity was found to increase with the addition of potassium.

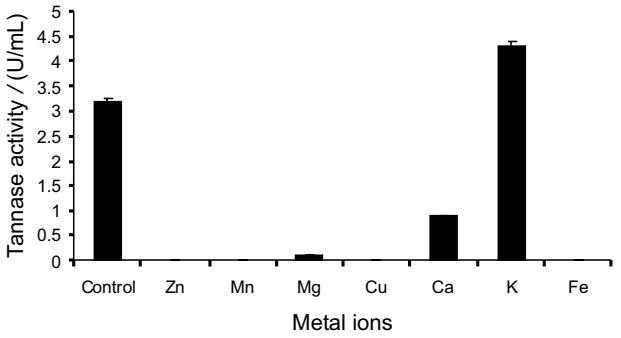

Fig. 1d. Effect of metal ions on tannase activity

After conducting SDS-PAGE a single band of 168--kDa protein was separated (Fig. 2). Reports reveal that all tannases purified so far are multimeric with the molecular masses ranging from 186–300 kDa (*29*,*30*). Table 2 gives a comparative description of characteristics of tannase produced from different sources with that of tannase from the present study.

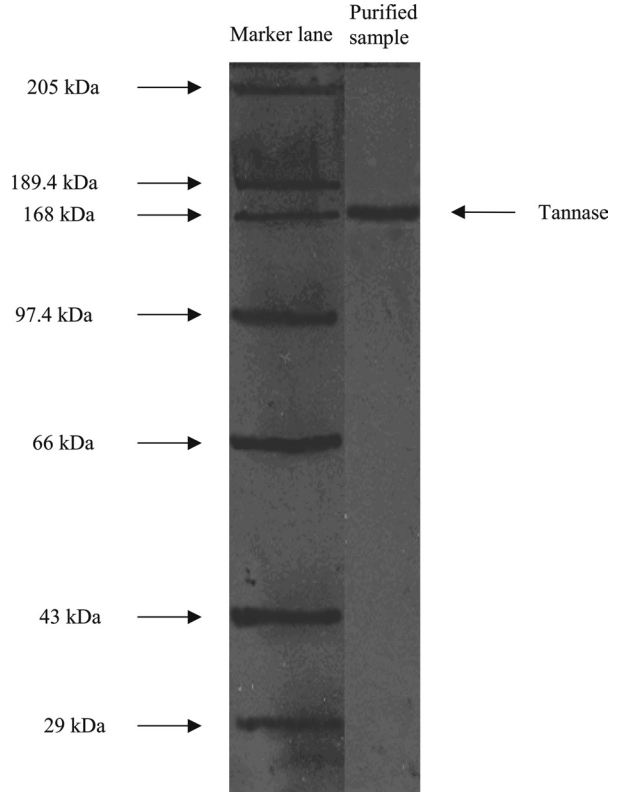

Fig. 2. Tannase protein separated on SDS-PAGE

Table 2. Characteristics of microbial tannase from different sources

Microorganism	Molecular mass/ kDa	Optimum pH	Optimum temperature/ °C	Reference
Aspergillus flavus	194	5.0–5.5	50–60	*28*
Aspergillus flavus	80–85	6	70	*19*
Aspergillus niger	–	5.5	60	5
Aspergillus niger N 888	165	6	70	*26*
Aspergillus niger	90, 180	6	60–70	*11*
Aspergillus niger ATCC 16620	168	6	30–40	*Present report*

Conclusions

Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. As the range of applications of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse fields of applications. The present paper reports the purification of a tannase from *A. niger* ATCC 16620. The purified enzyme is a monomeric protein of 168 kDa and it is found to give its optimum activity at temperature between 30 and 40 °C. Furthermore, it was noted that the enzyme was active with nearly 60 % of its activity even at 65 °C. Similarly, the enzyme was active over a wide range of pH values and inhibited by the increase of substrate concentration. The enzyme was inhibited by Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+}, while the enzyme activity was enhanced by K^+ ions. The purified tannase from *A. niger* is a unique one with low K_m. All these characteristics are considered favourable for industrial processing, especially in the food-processing industry.

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Purifikacija i karakterizacija tanin acilhidrolaze iz *Aspergillus niger* ATTC 16620

Sažetak

Tanin acilhidrolaza (tanaza), dobivena fermentacijom na čvrstoj podlozi iz plijesni *Aspergillus niger* ATTC 16620, pročišćena je iz tekuće podloge frakcioniranjem s amonijevim sulfatom i naknadno kromatografijom na DEAE-Sephadex A-50. SDS-PAGE analizom utvrđena je molekularna masa enzimskog proteina od 168 kDa. Enzim je bio aktivan na temperaturi do 40 °C, a pH=6 optimalan. Tanazna je aktivnost bila najveća pri koncentraciji supstrata od 0,01 M. Dodatak iona metala, kao Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+} i Fe^{2+} inhibira enzimsku aktivnost. Aktivnost tanaze povećavaju samo ioni K^{+}, a dobivena aktivnost iznosila je 4,31 U/mL. Enzimska aktivnost bila je maksimalna nakon 15–20 minuta inkubacije (3,9 U/mL). Vrijednost K_m je 1,03 mM, a V_{max} 4,25 mol/min. Budući da je enzim aktivan u širokom rasponu pH i temperature, mogao bi se koristiti u preradi prehrambenih proizvoda.