ELSEVIER

Process Biochemistry 40 (2005) 3251–3254

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori nakazawa*

Kalyani Mahapatra [a], Ranjan Kumar Nanda [a], Subhendu Sekher Bag [b], Rintu Banerjee [a,*], Ashok Pandey [c], George Szakacs [d]

[a] *Microbial Biotechnology and Downstream Processing Laboratory, Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur 721302, India*

[b] *Department of Chemistry, Indian Institute of Technology, Kharagpur 721302, India*

[c] *Biotechnology Division, Regional Research Laboratory, CSIR, Trivandrum 695 019, India*

[d] *Department of Agricultural Chemical Technology, Technical University of Budapest, Hungary*

Received 6 May 2004; received in revised form 20 January 2005; accepted 7 March 2005

Abstract

Tannase (tannin acyl hydrolase EC 3.1.1.20) produced by *Aspergillus awamori nakazawa* was purified and characterized. Optimal conditions of production were determined using varying substrate combinations and studying fermentation on various media combinations. Fermentation was carried out for 46 h for optimum enzyme production. Enzyme samples were obtained from the broth after fermentation by acetone precipitation of the supernatant followed by gel filtration chromatography. The properties of the enzyme were investigated. The optimum conditions of temperature and pH were investigated and the effects of urea, surfactant and chelator were studied. Tannase from this new isolate exhibited optimum activity at 35 °C and at a pH of 5.0. Urea concentrations higher than 3 M were inhibitory. Increasing concentrations of sodium lauryl sulphate also led to decrease in activity. Two percent SLS was inhibitory. Increasing concentrations of EDTA had an inhibitory effect on tannase. Tannase was found to be a glycoprotein. Circular dichroism analysis of purified fractions of tannase indicates that the β-sheet structure in tannase is predominant indicating its globular nature.

Keywords: Fungal tannase; Tannase substrate; Tannase assay; Glycoprotein; GFC; HPLC; CD

1. Introduction

Tannins are phenolic compounds, which can be grouped as hydrolysable and non-hydrolysable tannins. Tannin acyl hydrolase (E.C.3.1.1.20) commonly called tannase, hydrolyses only hydrolysable tannins and catalyses the hydrolysis of ester and depside bonds in tannic acid releasing glucose and gallic acid [1,2].

Production of tannase can be done by various methods like liquid surface, submerged, modified solid-state fermentation and solid-state fermentation [1]. The use of submerged fermentation is advantageous because of ease of sterilization, and process control is easier to engineer in these systems. Purification and characterization of tannase has been attempted earlier owing to its wide applications in various food, feed, leather and pharmaceutical industries. Various media preparations can be used with tannic acid as the sole carbon source for production of microbial tannase but biotransformation of tannin rich agro residue is cost-effective.

The catalytic activity of enzyme is linked with their protein conformations. Proteins are made up of L-amino acids that are optically active. The optical activity of proteins is a resultant of three factors, L-amino acids, arrangement of these amino acids in ordered fashion and asymmetric distribution of charges or dipoles about a chromophore. Circular dichroism (CD) spectroscopy is widely used for secondary structure determination of proteins and to monitor conformational changes in them

* Corresponding author. Tel.: +91 3222 283104; fax: +91 3222 55303.
E-mail address: rb@agfe.iitkgp.ernet.in (R. Banerjee).

1359-5113/$ – see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.procbio.2005.03.034

in the presence of additives. The circular dichroism spectrum may be thought of as the difference between two absorption spectra; one obtained using the left while the other using the right circularly polarized component. Application of CD for conformational studies in peptides can be largely grouped into estimation of secondary structural content and monitoring conformational changes in them.

2. Materials and methods

2.1. Screening and culture conditions

A tannase producing fungus was isolated from the soil of I.I.T. campus and identified as *Aspergillus awamori nakazawa*. A pure culture was maintained in 1% tannic acid agar slants at 4 °C and sub-cultured after every 3 weeks. The new isolate exhibited white cottony mycelia. On microscopic examination, the mycelium was found to be septate, having spores, which were visible only at higher magnifications (400×). The induced inoculum was prepared for tannase production [3].

2.2. Fermentation process

Submerged fermentation in flasks without shaking was carried out using natural tannin rich substrates, which consisted of myrobalan (*Terminalia chebula*) fruit and gallo seed cover (*Caesalpinia digyna*) in a fixed ratio of 1.5:0.25 (gallo seed:myrobalan) in Czapek Dox medium.

2.3. Isolation of tannase

The extracellular tannase was isolated and collected after 46 h of fermentation. The culture was filtered through cheesecloth followed by centrifugation at 10,000 rpm for 10 min to remove debris. This supernatant was subjected to acetone precipitation using 1:2 volumes of acetone, which was kept at 4 °C for complete precipitation (3 h).

2.4. Enzyme assay

Tannase assay was determined spectrophotometrically by the method of Ibuchi et al. [4]. One unit of enyme activity is defined as the amount of enzyme required to hydrolyse 1 μmol of the ester in 1 min under the reaction conditions. Enzyme activity is expressed as U/ml.

2.5. Enzyme purification

The acetone-precipitated fraction was further purified using a GF 250 column (4.5 mm × 250 mm, 4 μm pore size). The flow rate was fixed at 1 ml/min. The solvent system used to elute the protein based on molecular size was 0.2 M acetate buffer (pH 5).

2.6. Effect of surfactant, denaturants, and chelator on tannase activity

The effect of the anionic surfactant sodium lauryl sulphate (0.1–5%, w/v) and the effect of the protein denaturant urea (1.0–8.0 M) on the activity of tannase obtained from the isolate were studied. The influence of the chelator, ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt, 1–10 mM) concentration was also studied.

3. Results and discussion

3.1. Purification of tannase

Acetone precipitation was reported as the first step for tannase purification by Beverini and Metche [5]. The partially purified (acetone precipitated fraction) was further purified by GFC (using G-100 Sephadex) column. DEAE-Sephadex has been used for purification of tannase obtained from *Aspergillus flavus*, and *Penicillium chrysogenum* [6,7]. HPLC (using GF-250 column) of the partially purified (acetone precipitated) tannase from the new isolate showed single major peak and the elution time being 6.8 min. The purified fraction was further characterized.

3.2. Effect of pH on purified tannase activity

To determine the effect of pH on purified tannase, the pH of acetate buffer was varied from 3.5 to 6.0. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range. The optimum activity was recorded at pH 5.0 (Fig. 1). Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability. It could be concluded from the results that tannase from the new isolate needed an acidic environment to be active. Fungal tannase is an acidic protein in general. There are reports describing the optimum pH as 5.5 in case of tannase obtained from *A. flavus*, *Aspergillus oryzae*, [6,8,9] and 6.0 in case of tannase obtained from *P. chrysogenum* and *Aspergillus niger* [7,10].

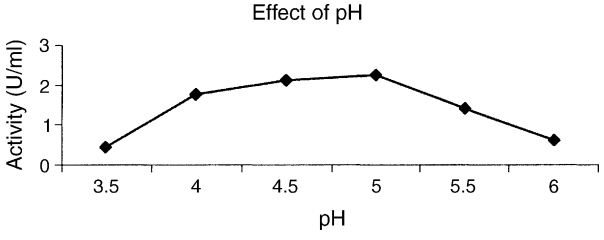

Fig. 1. Effect of pH on purified tannase.

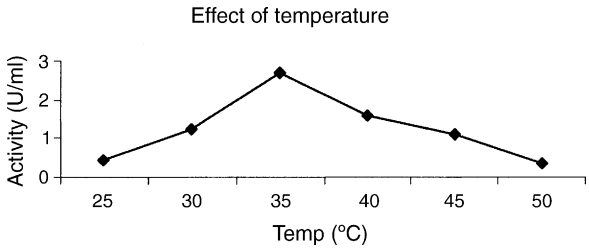

Fig. 2. Effect of temperature on purified tannase.

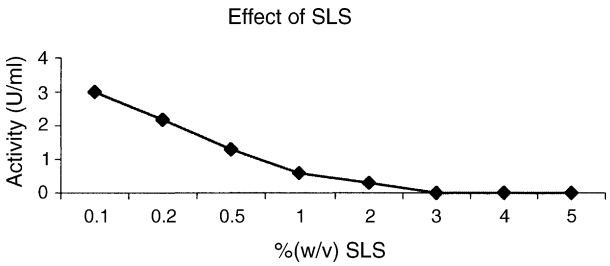

Fig. 4. Effect of sodium lauryl sulphate on purified tannase.

3.3. Effect of temperature on tannase activity

To evaluate the effect of different temperatures on activity of purified tannase, the temperature range was varied from 25 to 50 °C. With a rise in temperature, the tannase activity increased and optimum activity was recorded at 35 °C (Fig. 2). With a further increase in temperature, there was a decrease in activity. The optimum temperature for tannase activity was 35 °C. An optimum temperature around 30 °C has been reported for tannase activity in case of *A. oryzae* [8,9] and *P. chrysogenum* [7], around 35 °C in case of *A. niger* [10], and 50 °C in case of *Candida* sp. [3].

3.4. Effect of denaturant on tannase activity

Urea acts as denaturing agent at about 6–8 M concentrations, by breaking all hydrogen bonds present in the protein structure. Enzyme activity in the presence of 1–8 M urea was studied and gradually decreased with increasing concentration of urea (Fig. 3). At higher concentrations, urea denatures the enzyme by causing a conformational change in the tertiary structure of the enzyme, which it was unable to bring about at a low concentration [11].

3.5. Effect of surfactant on tannase activity

Enzyme activity was studied by varying the concentration of SLS (0.1–2.0%, w/v) and gradually decreased with increasing percentage of SLS. Two percent SLS was inhibitory to enzyme activity (Fig. 4). This inhibition may be the result of reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interactions with the protein molecule [12].

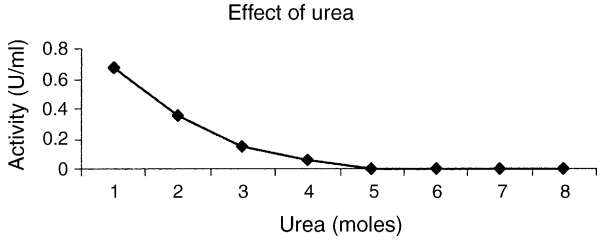

Fig. 3. Effect of urea on purified tannase.

3.6. Effect of chelator on tannase activity

The effect of EDTA on tannase activity was studied from 1 to 10 mM concentration and a concentration of 5 mM was found to be completely inhibitory for tannase activity (Fig. 5). Tannase from *A. niger* [10] was inactivated by both the chelators and *A. oryzae* [8,9] was also inactivated by EDTA.

3.7. Test for glycoprotein

Tannase from various fungal sources has been reported to be glycoprotein in nature where the percentage of carbohydrate varied from species to species [1,16]. Using phenol sulphuric acid method, it was found that tannase from *A. awamori nakazawa* is a glycoprotein containing l4.2% carbohydrate.

3.8. Studies on protein structure using circular dichroism

Tannase from *A. awamori nakazawa* was extracted from the media having madhuca:galloseed:myrobalan in the ratio 2:1.5:1.75 and fermentation was carried out for 46 h (optimized for maximum enzyme production). The extracted tannase was further concentrated using acetone precipitation (1:2). CD analysis was carried out using fractions of GFC having tannase activity as shown in Table 1.

The two fractions obtained after GFC had different structural properties. Fraction 1 is rich in turns, whereas fraction 2 is rich in β-sheets. The protein sample was kept under ice-cold conditions to prevent denaturation and loss of activity. As a result, turns may be predominant in fraction 1. As per cited literature, the helices and turns involve

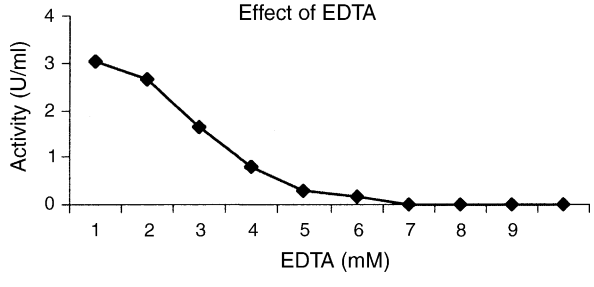

Fig. 5. Effect of EDTA on tannase activity.

Table 1
Secondary structure of tannase as predicted from CD analysis

Enzyme	% α	% β	% Turn	% Random	Activity (U/ml)
Fraction 1	–	–	100	–	0.948
Fraction 2	–	77.1	–	22.9	3.09
Fraction 1 + 2		82.4		17.6	0.894
Acetone ppt	15.6	13.5	24.9	45.9	3.23

Table 2
Effect of urea on the secondary structure of tannase

Enzyme	Urea (M)	% α	% β	% Turn	% Random	Activity (U/ml)
Fraction 1	1	–	–	100	–	0.107
	6	87.8	–	12.2	–	–
Fraction 2	1	–	95.5	–	4.5	0.178
	6	6.7	65.8	5.4	22.2	–

favourable interactions, which are predominantly local and are thus stabilized at low temperature. It has been suggested that turn regions act as nucleation centres during protein folding [13,14].

Fraction 2 exhibits β-sheets, which are very common in globular proteins so the higher percentage of such secondary structure indicate tannase to be a globular protein. Turns are very abundant in globular proteins and generally occur at the surface of the molecule.

Upon mixing fractions 1 and 2 in equal proportions, β-sheets predominated; so, from this it can be further confirmed that tannase is a globular protein. The concentrated protein (after acetone precipitation of the crude broth) exhibits 15.6% α-helix, 13.5% β-sheets, 24.9% turns and 45.9% randomness.

3.8.1. Circular dichroism as a tool to study conformational change in secondary structure in the presence of urea

The phenomenon of circular dichroism is very sensitive to the secondary structure of proteins and responsive to conformational changes in polypeptides. Therefore, this technique is extensively used to study conformational changes in proteins. The two fractions of tannase fraction 1 and 2 when purified and separated showed the denaturation phenomena by increase in the % α-helix as shown in Table 2. It was further observed that the % turn and % randomness decreased and increased, respectively, with the increase in urea concentration. TFE is a denaturing agent like urea and increase in alpha helicity is reported as denaturation proceeds [15].

Acknowledgement

Financial support by the Council of Scientific and Industrial Research (CSIR), New Delhi, India is thankfully acknowledged.

References

[1] Lekha PK, Lonsane BK. Production and application of tannin acyl hydrolase; state of the art. Adv Appl Microbiol 1997;44:215–60.
[2] Mukherjee G, Banerjee R. Production of gallic acid. Biotechnological routes (Part 1). Chim Oggi Chem Today 2003;21(1/2):59–62.
[3] Aoki K, Shinke R, Nishira H. Purification and some properties of yeast tannase. Agric Biol Chem 1976;40(1):79–85.
[4] Ibuchi S, Minoda Y, Yamada K. Studies on tannin acyl hydrolase of the microorganism. III. A new method of determining the enzyme activity using change in the ultraviolet absorption. J Agric Biol Chem 1967; 31:513–8.
[5] Beverini M, Metche M. Identification, purification, physio-chemical properties of tannase from *Aspergillus oryzae*. Sci Aliments 1990; 10:807–16.
[6] Yamada H, Adachi M, Watanabe M, Sato N. Studies on fungal tannase Part I. Formation, purification and catalytic properties of tannase of *Aspergillus flavus*. Agric Biol Chem 1968;32(9):1070–8.
[7] Rajakumar S, Nandy SC. Isolation, purification and some properties of *Penicillium chrysogenum* tannase. Appl Environ Microbiol 1983; 46(2):525–7.
[8] Ibuchi S, Minoda Y, Yamada K. Studies on tannin acyl hydrolase of microorganisms. Part III. Purification of the enzyme and some properties of it. Agric Biol Chem 1968;32(7):803–9.
[9] Ibuchi S, Minoda Y, Yamada K. Hydrolyzing pathway. Substrate specificity and inhibition of tannin acyl hydrolase. Agric Biol Chem 1972;36(9):1553–62.
[10] Barthomeuf C, Regerat F, Pourrat H. Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. J Ferment Bioeng 1994;77(3):320–3.
[11] Laidler KJ, Bunting PS. The steady state in enzyme kinetics. In: The chemical kinetics of enzyme action2nd ed., Oxford: Clarendon Press, 1973. pp. 68–113.
[12] Creighton TE. Protein function: a practical approach. Oxford: IRL Press, 1989.
[13] Trinh XH, Marek C, Banawar JR, Amos M. Prediction of protein secondary structure from conformational biases. Protein Struct Funct Genet 2002;48:558–65.
[14] Renugopalakrishnan V, Balasubrarnaniam A, Dobbs JC, Carreira LA, Rapaka RS. Secondary structure of neuropeptide Y. Indian J Pharmacol 1981;19:174–8.
[15] Pompea DV, Giuseppe G, Vincenzo G, Guido B, Luigi M, Mose R, et al. Effect of trifluroethanol on the conformatioanl stability of a hyperthermophilic esterase: a CD study. Biophys Chem 2003;(104): 407–15.
[16] Hatamoto O, Watarai T, Kikuchi M, Mizusawa K, Sekine H. Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. Gene 1996;(175):215–21.