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Original Article

Effect of amino acids on tannase biosynthesis by *Bacillus licheniformis* KBR6

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Background and purpose: Microbial tannase (tannin acyl hydrolase, EC 3.1.1.20), a hydrolysable tannin-degrading enzyme, has gained importance in various industrial processes, and is used extensively in the manufacture of instant tea, beer, wine, and gallic acid. Tannase is an inducible enzyme, and hydrolysable tannin, especially tannic acid, is the sole inducer. This study is of the effect of various amino acids and their analogues on tannase biosynthesis by *Bacillus licheniformis* KBR6 to ascertain the mode of action of these growth factors on tannase biosynthesis from microbial origin.

Methods: Enzyme production was carried out in enriched tannic acid medium through submerged fermentation for 20 h at 35°C. Different amino acids at a concentration of 0.05 g% (w/v) were added to the culture medium immediately after sterilization. Culture supernatant was used as the source of the enzyme and the quantity of tannase was estimated by the colorimetric assay method. Growth of the organism was estimated according to biomass dry weight.

Results: Maximum tannase (2.87-fold that of the control) was synthesized by *B. licheniformis* KBR6 when alanine was added to the culture medium. Other amino acids, such as DL-serine, L-cystine, glycine, L-ornithine, aspartic acid, L-glutamic acid, DL-valine, L-leucine and L-lysine, also induced tannase synthesis. L-Cysteine monohydrochloride and DL-threonine were the most potent inhibitors.

Conclusions: Regulation of tannase biosynthesis by *B. licheniformis* in the presence of various amino acids is shown. This information will be helpful for formulating an enriched culture medium for industrial-scale tannase production.

Key words: Amino acids; *Bacillus licheniformis*; Tannase

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20) can hydrolyze the ester and depside linkages of hydrolysable tannins, especially gallotannins, to produce glucose and gallic acid. Tannase has wide applications in the food, beverage, brewing, cosmetic, and chemical industries [1]. The enzyme is mainly used for the preparation of gallic acid, instant tea, acorn wine, coffee-flavored soft drinks, high-grade leather tannin, clarification of beer and fruit juice, detannification of food, and to clean-up highly polluting tannin from the effluent of the leather industry [1]. Gallic acid (3,4,5 tri-hydroxy benzoic acid) also has a variety of uses in the preparation of trimethoprim, pyrogallol, propyl gallate, dye, and fur [2,3]. Gallic acid has many industrial applications, for example, it is an important substrate for the synthesis of propyl gallate, a food antioxidant [4], trimethoprim, a broad-spectrum antibacterial agent [2], and pyrogallol, a precursor of dyes for staining leather, fur, and hair. Gallic acid is also used as a photosensitive resin in semiconductor production [2].

Amino acids and their analogues are known to stimulate the production of enzymes such as α-amylase [5] and xylanases [6-8]. Each microorganism can tolerate different intracellular amino acid concentrations and, depending on the intracellular regulation and

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control mechanisms, different inhibitions take place. Consequently, insufficient synthesis of some amino acids creates intracellular reaction-rate limitation in the bioreaction network for tannase production. Thus, supplementation of these controlling amino acids to the fermentation broth and the timing of this action are important for the production of tannase enzyme.

This study investigated the effects of various amino acids and their analogues on tannase production by *Bacillus licheniformis* KBR6 through submerged fermentation.

Methods

Microorganism and culture conditions

A Gram-positive endospore former strain of *B. licheniformis* KBR6 was isolated from a forest soil sample in the Department of Microbiology, Vidyasagar University, Midnapore, India. The medium used for tannase production was composed of tannic acid, 10 g/L; K_2HPO_4, 0.5 g/L; KH_2PO_4, 0.5 g/L; magnesium sulfate, 0.5 g/L; and ammonium chloride, 3 g/L. Since the optimum pH for growth of the organism was determined as 5.0, the pH of the sterilized tannase production media was adjusted to 5.0 using sodium hydroxide 0.5 M. The stock solutions of amino acids and their analogues were filter sterilized and added to a final concentration of 0.05% (w/v) to the sterilized medium, where tannic acid was filter sterilized. Fifty mL of the production in a 250 mL Erlen Mayer flask was inoculated with 2% of 20-h grown seed culture and incubated at 35°C under shaking conditions at 200 rpm of 20 h for tannase production. Thereafter, the cell-free supernatant was obtained by centrifugation at 8000 g for 10 min. The tannase yield and total protein contained were determined in the cell-free supernatant. The dry biomass was estimated by drying the cells of the organism in a hot air oven at 60°C for approximately 72 h until a constant weight was attained. The protein content was determined by the Bradford method using bovine serum albumin (BSA) as standard. All the experiments were performed in triplicate and the reported results are the mean of the 3 experiments. The standard deviation was within 10%.

Tannase assay

The activity of extracellular tannase from *B. licheniformis* was determined by the newly developed colorimetric method of Mondal et al [9]. For each assay, 0.1 mL of the sample was mixed with 0.3 mL of the tannic acid substrate solution (1.0% w/v in 0.2 M citrate buffer; pH 5.0), and incubated at 50°C for 30 min. The reaction was terminated by the addition of BSA solution (1 mg/mL), which precipitated residual tannic acid. A control reaction with heat-denatured enzyme was performed concomitantly. The tubes were then centrifuged at 5000 g for 10 min, and the precipitate was dissolved in 2 mL of sodium dodecyl sulfate (SDS)–triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. The absorbance was measured at 530 nm after addition of 1 mL of 0.13 M aqueous solution of ferric chloride. One unit of tannase activity was defined as the amount of enzyme that was able to hydrolyze 1 μmol of ester linkage of tannic acid in 1 min under specific conditions.

Results

There was a significant level of stimulation of tannase production by *B. licheniformis* in the presence of specific amino acids. DL-Serine, L-cystine, glycine, L-ornithine monohydrochloride, DL-aspartic acid, DL-alanine, L-glutamic acid, DL-valine, L-leucine, and L-lysine monohydrochloride stimulated tannase production up to 2.87 fold (Table 1), whereas L-proline and DL-tryptophan showed only a marginal effect on tannase production. DL-Methionine, L-tyrosine, L-isoleucine, DL-phenylalanine, and L-histidine monohydrochloride showed no stimulation or inhibition of tannase production. In the presence of L-arginine monohydrochloride, tannase production was decreased, where the yield index was 0.78 fold.

Discussion

This study ascertained the effect of amino acids and their analogues on an inducible hydrolytic enzyme, tannase, from *B. licheniformis* KBR6. The striking feature of the study was that tannase production was inhibited in the presence of L-cysteine monohydrochloride and DL-threonine. Ikura and Horikoshi reported that 0.5% (w/v) glycine enhanced xylanase production by 1.8-fold in *Bacillus* No. C-125 [6]. Balakrishnan et al reported a 2- to 5-fold enhancement in xylanase production from *Bacillus* spp. (NCL-87-6-10) using DL-norvalin, glycine and casamino acids [7]. Gupta et al observed the stimulation of xylanase production up to 5.5-fold in *Staphylococcus* spp. SG-13 using DL-2-amino-n-butyric acid, DL-alanine, L-lysine monohydrochloride, DL-valine, and L-proline [8].

Table 1. Effect of amino acids on tannase production from *Bacillus licheniformis* KBR6 at pH 5.0 and 35°C under shaking conditions at 200 rpm.

Amino acid (0.05% w/v)	Dry biomass (mg/mL)	Extracellular protein (mg/mL)	Tannase yield (U/mL)	Yield index of tannase (fold)
Control	0.27	0.72	0.372	1.00
L-Proline	0.41	0.76	0.456	1.23
DL-Serine	0.24	0.81	0.621	1.67
L-Cysteine monohydrochloride	0.08	0.35	ND	ND
L-Cystine	0.23	0.77	0.621	1.67
DL-Threonine	0.23	0.32	ND	ND
Glycine	0.28	0.88	0.704	1.70
DL-Alanine	0.33	1.30	1.068	2.87
DL-Valine	0.30	0.85	0.704	1.70
DL-Methionine	0.30	0.74	0.373	1.00
DL-Tryptophan	0.41	0.77	0.456	1.23
L-Tyrosine	0.29	0.73	0.372	1.00
L-Glutamic acid	0.43	0.65	0.538	1.45
L-Isoleucine	0.30	0.76	0.372	1.00
L-Leucine	0.39	0.72	0.538	1.45
DL-Phenylalanine	0.23	0.81	0.372	1.00
DL-Aspartic acid	0.38	0.83	0.703	1.89
L-Arginine monohydrochloride	0.38	0.74	0.289	0.78
L-Lysine monohydrochloride	0.37	0.77	0.538	1.45
L-Histidine monohydrochloride	0.26	0.87	0.372	1.00
L-Ornithine monohydrochloride	0.45	0.77	0.620	1.67

Abbreviation: ND = not detectable.

Some reports have shown that glycine, DL-norvaline and casamino acids cause a significant decrease in extracellular protease yield [6,7]. In this study, enzyme synthesis was greater (2.87-fold) in presence of alanine. This may be due to the fact that the glucose consumption rate is increased with the addition of alanine. Consequently, the glucose is only available in the medium as a core molecule in the tannic acid structure. To utilize the glucose, the organism may synthesize more tannase, by which the ester and depside bonds are hydrolyzed and glucose is available for the organism.

Enzyme production by the organism is related to growth, but slight differences were observed, in that the greatest growth occurred in the presence of L-ornithine monohydrochloride, but maximum enzyme production was noted in the presence of DL-alanine. This result is likely to be due to the fact that ornithine is a non-protein amino acid, but its stimulation in cell division is notable.

This is probably the first report where the stimulation of tannase production by amino acids and their analogues has been shown. Further study is necessary to understand the mechanism of enzyme stimulation in microorganisms by different amino acids and their analogues.

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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. Hadi TA, Banerjee R, Bhattacharya BC. Optimization of tannase biosynthesis by a newly isolated *Rhizopus oryzae*. Bioprocess Engg. 1994;11:239-43.
3. Mukherjee G, Banerjee R. Production of gallic acid biotechnological routes (Part 1). Chim Oggi/Chem Today. 2003;21: 59-62.
4. Gaathon A, Gross Z, Rozhanski M. Propyl gallate: enzymatic synthesis in a reverse micelle system. Enzyme Microb Technol. 1989;11:604-9.
5. Zhang Q, Tsukagoshi N, Miyashiro S, Udaka S. Increased production of α-amylase by *Bacillus amyloliquifaciens* in the presence of glycine. Appl Environ Microbiol. 1983;46: 293-5.
6. Ikura Y, Horikoshi K. Stimulatory effect of certain amino acids on xylanase production by alkalophilic *Bacillus* sp.

Agric Biol Chem. 1987;51:3143-5.
7. Balakrishnan H, Srinivasam MC, Rele MV. Extracellular protease activities in relation to xylanase secretion in an alkalophilic *Bacillus* sp. Biotechnol Lett. 1997;19: 559-601.
8. Gupta S, Bhushan B, Hoondal GS. Enhanced production of xylanase from *Staphylococcus* sp. SG-13 using amino acids. World J Microbiol Biotechnol. 1999;15:511-2.
9. Mondal KC, Banerjee D, Jana M, Pati BR. Colorimetric assay method for determination of the tannin acyl hydrolase (EC 3.1.1.20) activity. Anal Biochem. 2001;295: 168-71.

