CHAPTER 6

APPLICATION OF TANNASE FROM Aspergillus foetidus
IN HYDROLYSIS OF TANNIC ACID AND
SOLUBILIZATION OF TEA CREAM

6.1 INTRODUCTION

Tannases are industrially important enzymes produced by plants, fungi, bacteria and yeast. Tannase is utilized in a number of industrial applications including manufacture of instant tea, wine and gallic acid (Ascension et al 2003) and solubilization of tea cream in instant tea processing (Nagalakshmi et al 1985).

One of the applications of tannase is the hydrolysis of tannins to gallic acid, a key intermediate required for the synthesis of an antibiotic drug, trimethoprim (Sabu et al 2005a). Gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant (Aguilar et al 2007). It is also used in the manufacture of printing inks, as an ingredient of photographic developers and for preparation of dyestuffs (Hadi et al 1994).

Tannase hydrolyses the polyphenols present in the tea infusions thereby reducing the cream formation and there is a correlation between cream formation and hydrolysis of galloyl esters of tea polyphenols by tannase (Nagalakshmi et al 1985). Using tannase, the effect of gallated polyphenols on loss of solids in instant tea processing has been studied (Thomas and Murtagh 1985). Turbidity reduction of tea extract using encapsulated form of tannase is reported by Boadi and Neufeld (2001).
The amount of tea cream formed in tea infusion depends on its chemical composition; gallated catechins have stronger creaming ability than un-gallated ones (Lu et al 2009).

The objective of this study involves application of tannase obtained by partial purification of tannase from *A. foetidus* in the hydrolysis of tannic acid to gallic acid as well as solubilization of tea cream by the conventional optimization of one factor at a time method.

6.2 MATERIALS AND METHODS

6.2.1 Source of Tannase

Tannase produced by *Aspergillus foetidus* MTCC 6322 by submerged culture fermentation was partially purified by ATPE and used in this study. Tannase activity of the partially purified preparation was 58 U/ml/min.

6.2.2 Hydrolysis of Tannic Acid

Before standardizing parameters for hydrolysis of tannic acid using tannase from *A. foetidus*, qualitative analysis by TLC was carried out to observe whether gallic acid was formed (Mahendran et al 2006). The reaction mixture containing 10 ml of 1% tannic acid in 20mM acetate buffer, pH 5.0 and 1.0 ml of tannase (40U) was kept for incubation at 30°C for 1 h. After spotting, plates were kept in solvent system comprising ethyl acetate, chloroform and formic acid (4:4:1) and after drying, the plates were developed by spraying a solution of FeCl₃.

The following parameters viz. pH, temperature, tannase concentration, time and tannic acid concentration were taken up to study the hydrolysis of tannic acid using one factor at a time method. Tannic acid
(0.2-1.4%) was incubated with tannase (10-50U) at pH 3.0-9.0 at 30-70 ºC for 15-90 min and gallic acid was estimated according to Sharma et al (2000).

6.2.3 Solubilization of Tea Cream

The protocol for tea cream solubilization was based on an earlier report by Nagalakshmi et al (1985) with suitable modifications.

8.0 g of dry tea leaves (Brooke bond-red label) were mixed with 100 ml 0.02 M acetate buffer, pH 6.0 and kept for low boiling at 90 ºC for 6 min. The liquor was then filtered through a muslin cloth. The total solid content was calculated by evaporating 10.0 ml of the tea brew in a china dish and then the dish was transferred to a dessicator and cooled. The weight of solids in 10 ml of liquid was referred as [A].

10.0 ml of tea brew was treated with tannase at the required temperature for the given incubation time. It was then kept overnight at 4 ºC for the cream formation to occur after which the sample was centrifuged at 10,000 rpm at 4 ºC for 20 min to separate the cream. The supernatant obtained was analyzed for solid content as mentioned above. The solid content thus obtained was referred to as [B]. The procedure given above was followed for tea brew treated with inactivated tannase and the solid content thus obtained was referred to as [C].

The weight of cream solids was obtained by the difference between the two estimates [A - C]. The weight of the solubilized cream was obtained by the difference between the two estimates [B - C]. The percentage of cream solubilization was calculated as follows:

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\text{Percentage solubilization of cream} = \frac{\text{Solubilized cream solids (B - C)}}{\text{Cream solids (A - C)}} \times 100
\]
6.2.4 Optimization of Conditions for Solubilization of Tea Cream

The optimization of process parameters such as tea extract (5-15 ml), tannase (50-200U), temperature (20-50°C), pH 4.0-7.0 and time (1-4 h) was taken up by one factor at a time method and the percentage cream solubilization was calculated as described above.

6.3 RESULTS AND DISCUSSION

6.3.1 Hydrolysis of Tannic Acid

TLC confirmed the formation of gallic acid on hydrolysis of tannic acid (Figure 6.1).

![Figure 6.1 Analysis of tannic acid hydrolysis by thin layer chromatography](image)

Lane 1: Hydrolysate
Lane 2: Gallic acid standard

GA : Gallic acid
6.3.2 Effect of pH on the Hydrolysis of Tannic Acid

Tannic acid (0.2%) at different pH (3.0-9.0) was used as substrate with tannase (20U) in the reaction mixture and the mixture was incubated at 30 ºC for 30 min. At pH 6.0, the gallic acid yield of 89.6 µmol/min was obtained (Figure 6.2).

6.3.3 Effect of Temperature on the Hydrolysis of Tannic Acid

Tannic acid (0.2%) at pH 6.0 was used as substrate with tannase (20U) in the reaction mixture and the incubation was at different temperatures (30 ºC - 70 ºC) for 30 min. An optimum temperature of 40 ºC was shown to yield gallic acid of 133.0 µmol/min (Figure 6.3).

6.3.4 Effect of Tannase Concentration on the Hydrolysis of Tannic Acid

Tannase (10–50 U) was used for hydrolysis of 0.2% tannic acid and the incubation was at pH 6.0 and at 40 ºC for 30 min. A gallic acid yield of 376 µmol/min was observed when 40U tannase was used (Figure 6.4).

6.3.5 Effect of Reaction Time on the Hydrolysis of Tannic Acid

Different reaction times (15 – 90 min) were tried for the hydrolysis of 0.2% tannic acid using 40 U of tannase. The mixture was incubated at pH 6.0 and at a temperature of 40 ºC. Gallic acid yield of 518 µmol/min was obtained at a reaction time of 60 min (Figure 6.5).
Figure 6.2 Effect of pH on hydrolysis of tannic acid

Temperature- 30°C; Tannase- 20U; Time-30 min; Tannic acid- 0.2%

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
Figure 6.3 Effect of temperature on hydrolysis of tannic acid

pH- 6.0; Time- 30 min; Tannic acid- 0.2%; Tannase-20U

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
Figure 6.4  Effect of tannase concentration on hydrolysis of tannic acid

pH- 6.0; Temperature- 40°C; Time- 30 min; Tannic acid- 0.2%

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.05.
Figure 6.5  Effect of reaction time on hydrolysis of tannic acid

pH- 6.0; Temperature- 40°C; Tannase- 40U; Tannic acid- 0.2%

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.05.
6.3.6 The Effect of Tannic Acid Concentration on the Hydrolysis of Tannic Acid

Different tannic acid concentrations (0.2% - 1.4%) were tried using 40 U of tannase and the incubation mixture was kept at pH 6.0 and at 40°C for 60 min. An optimal yield of 745 µmol/min of gallic acid was obtained using 1.0% tannic acid (Figure 6.6).

Thus, for hydrolysis reaction, tannic acid-1%, tannase - 40U, pH 6.0, temperature of 40°C and a reaction time of 1h were found to be optimal with a gallic acid yield of 745 µmol/min (Figure 6.2 to 6.6). While the hydrolysis of tannic acid to gallic acid was confirmed qualitatively by Mahendran et al (2006), it was shown quantitatively in this study.

6.3.7 Optimization of Conditions for Solubilization of Tea Cream

Studies on tea cream solubilization were carried out by one factor at a time method.

6.3.8 Effect of Tea Extract Concentration on Solubilization of Tea Cream

Tea extract (5.0 – 20.0 ml) at pH 5.0 was mixed with 100 U of tannase and the mixture was incubated for 2 h at 30°C. Using 10 ml of tea extract, 42% cream solubilization was observed (Figure 6.7).

6.3.9 Effect of Tannase Concentration on Solubilization of Tea Cream

10 ml of tea extract was treated with tannase (50-200U) at pH 5.0 and at 30°C for 2h. Tannase concentration of 150 U was found to be optimum with 48% tea cream solubilization (Figure 6.8).
Figure 6.6 Effect of tannic acid concentration on hydrolysis

pH - 6.0; Temperature - 40°C; Tannase - 40U; Time - 60 min

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.05.
Figure 6.7  Effect of tea extract concentration on solubilization of tea cream

Tannase -100U; pH- 6.0; Temperature- 30°C; Time- 2 h

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
Figure 6.8 Effect of tannase concentration on solubilization of tea cream

Tea extract- 10 ml; pH- 6.0, Temperature- 30°C; Time- 2 h

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
6.3.10 Effect of Temperature on Solubilization of Tea Cream

Different temperatures (20°C - 50°C) were tried using a tea extract concentration of 10.0ml with 150U of tannase at pH 5.0 for 2h. An optimum tea cream solubilization of 54% was observed at 40°C. When tannase treatment was conducted at 20 °C, tea cream solubilization to the extent of 21.0 % was observed (Figure 6.9).

![Figure 6.9 Effect of temperature on solubilization of tea cream](image)

Tea extract- 10 ml; pH- 6.0, Tannase- 150 U; Time- 2 h

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
6.3.11 Effect of pH on Solubilization of tea Cream

10 ml of tea extract was used for solubilisation of tea cream using 150 U tannase at different pH (4.0 - 7.0). The mixture was incubated at 40°C for 2h. At pH 6.0, the maximum tea cream solubilization (64%) was obtained (Figure 6.10).

![Figure 6.10: Effect of pH on solubilization of tea cream](image)

Tea extract- 10 ml; Tannase- 150U; Temperature- 40°C; time- 2 h

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
6.3.12 Effect of Incubation Time on Solubilization of Tea Cream

10 ml of tea extract was treated with 150 U of tannase at pH 6.0 and at 40°C for different time periods. Highest yield of 64% tea cream solubilization was obtained at 2h (Figure 6.11).

![Figure 6.11 Effect of incubation time on solubilization of tea cream](image)

Tea extract- 10 ml; Tannase- 150 U; pH- 6.0; Temperature- 40°C

Means with the same superscript were not significantly different. Means bearing different superscripts differed significantly by Duncan’s multiple range analysis at p<0.001.
The parameters after optimization by one factor a time method were: 10.0 ml of tea extract (8%), pH 6.0, 150U of tannase and incubation time of 2.0 h at 40°C with 64.0% solubilization of tea cream. In this study, 64.0% solubilization of tea cream was observed at 40°C whereas Nagalakshmi et al (1985) reported 93.75% at 45°C.

A process for producing a tea extract which formed little or no haze using a mixture of oxidase, tannase and tea extract was reported earlier (Francis and Spradlin 1985). Certain processes involving tannases, e.g. the manufacture of cold soluble tea, should be conducted at low temperature to avoid negative changes in sensory characteristics of the product (Boadi and Neufeld 2001). In an earlier report, strain *Verticillium* sp. P9 was shown to hydrolyse tea tannins at low temperature (Monika et al 2007).

In the conventional process of tea cream solubilization, the tea decoction was chilled to 5°C and centrifuged. The pellet (the cream fraction) obtained was subjected to alkali treatment by adding aqueous 20% sodium hydroxide and the temperature was increased to 90 °C with simultaneous aeration for 60 min. Then, neutillization was carried out by using mild acid (Sheetal et al 2001). Thus, it could be observed that the chemical process necessarly needed high inputs of temperature and alkali. Environmental wise, the COD of the effluent coming out from the chemical process was bound to be high due to the use of alkali and acid. Comparatively, the enzymatic process was safe and costwise also, this enzymatic process could be made comparatively cheaper.

Thus, partially purified fungal tannase was found to be useful in the hydrolysis of tannic acid and also in the solubilization of tea cream.
6.4 CONCLUSION

Studies were taken up for the application of tannase from *A. foetidus* for the hydrolysis of tannic acid to gallic acid and also for solubilization of tea cream. The optimal conditions for hydrolysis of tannic acid to gallic acid were: tannic acid -1%, tannase – 40 U, pH 6.0, temperature of 40°C and a reaction time of 60min with a gallic acid yield of 745µmol/min. Conventional method of optimization for tea cream solubilization was carried out. The results showed that 64% solubilization of tea cream was achieved using 8.0% tea extract (10ml) with a tannase concentration of 150U, incubation temperature of 40°C, pH 6.0 and a treatment time of 2 h. Application studies showed that the fungal tannase had good potential for use in the hydrolysis of tannic acid and solubilization of tea cream.