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Spectrophotometric assay of immobilized tannase

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Abstract. A procedure for the assay of immobilized tannase with Polyacrylamide gel, collagen and Duolite-S-762 as matrices is described. It is based on the spectrophotometric determination of gallic acid formed by the enzymatic hydrolysis of tannic acid. The kinetic parameters of the enzymatic reaction have been studied and an assay procedure has been formulated. This method appears to be much more accurate than those reported earlier.

Keywords. Tannase; immobilization; gallic acid; tannic acid; spectrophotometric assay.

Introduction

Tannase (Tannin acyl hydrolase EC.3.1.1.20) is an enzyme which catalyses the hydrolysis of tannic acid (Weetall and Detar, 1974). It also acts on esters of gallic acid commonly found in tea extracts. The hydrolysis of these esters enhances the solubility of tea in cold water (Coggon *et al.,* 1975). Hence soluble tannase has been recommended for use in the preparation of instant tea (Sanderson and Coggon, 1977). Use of immobilized tannase may have advantages in the preparation of instant tea. While the assay of soluble tannase is simple (Iibuchi *et al.,* 1967; Coggon *et al.,* 1975), that of the matrix bound tannase is more complicated. A method described by Weetall and Deter (1974) for the assay of immobilized tannase is not satisfactory because of high error and does not appear to be easy. Therefore, there is a need for an easy and reliable method of assay. The method described below is based on the determination of gallic acid formed from the tannic acid instead of measuring the disappearance of tannic acid, which is the basis of the method of Weetall and Detar (1974).

Materials and methods

Tannic acid was obtained from Bush Co. Ltd., London, UK, and purified according to the procedure of Iibuchi *et al.* (1967). Gallic acid was obtained from the British Drug House, Poole, England and recrystallized from alcohol. Tannase and collagen hydrochloride were gifts from Enzyme Development Corporation, New York, USA, and FMC Corporation, Nutley, New Jersey, USA respectively. Glutaraldehyde was a product of E. Merck, Darmstadt, Germany. Duolite-S-762 was purchased from Diamond Shamrock Corporation, Cleveland, Ohio, USA.

Acrylamide and N-N′-methylene bis-acrylamide were obtained from Koch-Light Laboratories, Colnbrook, England. All other chemicals used were of analytical grade.

Tannase (soluble) had about 1200-1500 units of activity per mg enzyme; one unit is equivalent to that amount of enzyme which causes a change in absorbance of 0.01 at 250 nm per min at 25 ° C. When assayed at 310 nm (disappearance of tannic acid, 0.01 absorbancy change/min at 25°C is equivalent to one unit) using 0.008% tannic acid as substrate, the enzyme had a specific activity of 1200-1800.

Enzyme immobilization

(i) *Entrapment in Polyacrylamide gel:* Tannase (100 mg) was dissolved in 20 ml of 50 mM sodium acetate buffer (pH 4.7) and entrapped in Polyacrylamide gel according to the procedure of Chibata *et al.* (1976). The weight of the Polyacrylamide gel containing tannase was about 26 g (drained weight). The washings contained 28% and the gel retained about 72% of the original activity.

(ii) *Complexing with collagen:* Tannase (100 mg) was mixed with 50 ml of 1% collagen hydrochloride in 50 mM sodium acetate buffer (pH 4.7) and cross-linked with glutaraldehyde according to the procedure of Vieth *et al.* (1973). The dry weight of the enzyme-bound collagen membrane was about 620 mg with about 90% of the original activity.

(iii) *Tannase on Duolite-S-762:* Tannase (100 mg) in 10 ml of 50 mM sodium acetate buffer (pH 4.7) was adsorbed onto 10g Duolite-S-762 and cross-linked with glutaraldehyde according to the procedure of Olson and Stanley (1973). Drained weight of enzyme-bound Duolite-S-762 was about 12.5g and washings collected from it had about 22% of the original activity: Duolite-S-762 retained about 78% of the original activity.

(iv) *Tannase co-valently attached to cynuric chloride activated cellulose:* An amino chloro-S-triazinyl derivative of cellulose was prepared by reacting cellulose (20 g) with 2-amino-4,6, dichloro-S-triazine (2 g, prepared from cynuric chloride. Tannase (200 mg in 20 ml of 0.05 M sodium acetate buffer, pH 4.7) was co-valently attached to the amino chloro-S-triazinyl derivative of cellulose according to the procedure of Lilly (1976). This immobilized enzyme preparation was found to be very active intially but lost its activity within 50 hours.

Enzyme assays

(i) *Assay for soluble tannase:* The assay procedure for soluble tannase was modified to suit our experimental conditions and to compare its activity with that of immobilized tannase. In this assay procedure, the product (gallic acid) was determined at 250 nm instead of measuring the disappearance of tannic acid at 310 nm.

The enzyme (2-5 μg/ml) and the substrate (0.008%) were prepared in 50 mM sodium acetate buffer (pH 4.7). The enzyme and substrate were replaced by buffer in both control (without enzyme) and blank (without enzyme and substrate). The incubation mixtures in a total volume of 10 ml containing 9.8 ml substrate and

0.2 ml enzyme were incubated for 20 min at 25°C. The reaction was stopped by heating the tubes in a boiling water bath. After cooling them to room temperature (25°C),the unhydrolyzedtannicacid was removed by adsorption on Polyacrylamide gel (1 g), by shaking in a gyratary shaker for 1 h. The reaction, mixtures were filtered and the filtrates read at 250 nm against a blank, in a Beckman spectrophotometer (Model 26). The absorption at 250 nm for control was negligible, and the difference in absorbance at 250 nm between test and control was taken as a measure of soluble tannase activity.

(ii) *Immobilized tannase assay:* The assay of tannase entrapped in polyacrylamide gel is described in the following procedure. Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added separately to the enzyme-free and enzyme-entrapped Polyacrylamide gel (1 g each) and incubated in a gyratary shaker (120 rpm) for 20 min at 25°C. The absorbance of the filtrates was determined at 250 nm against a blank of 50 mM sodium acetate buffer (pH 4.7).
The difference in absorbance (Δ A_{250})obtained between the reaction mixtures containing the enzyme and that without enzyme was essentially due to gallic acid released by the action of the enzyme and was taken as a measure of immobilized tannase activity. A unit of activity is defined as the amount of enzyme which causes a change in absorbance at 250 nm of 0.01 per min at 25°C. Other systems such as tannase immobilized on collagen membrane and Duolite-S-762 were also assayed similarly.

Results and Discussion

The absorption spectra of tannic and gallic acids, the substrate and the product of the reaction catalysed by tannase are shown in figure 1. The ϵ_m (molar extinction coefficient) of tannic acid (molecular weight 1700-1800) and gallic acid (molecular weight, 170) are 27,200 and 4910 respectively at 250 nm and 32,400 and 555

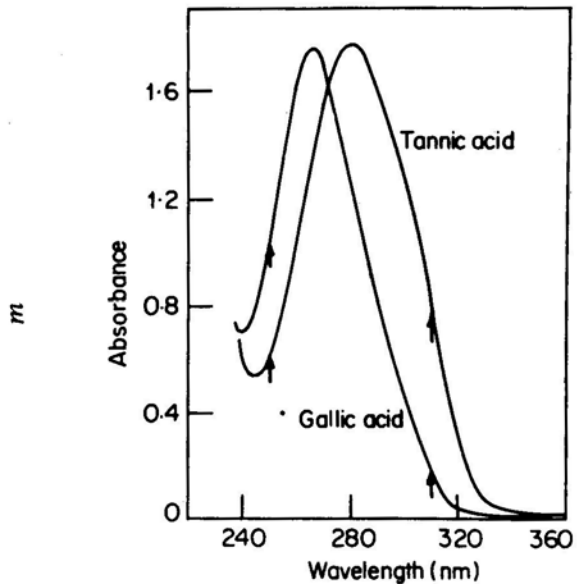

Figure 1. Absorption spectrum of tannic acid (40 µg/ml) and gallic acid (40 µg/ml) in 50 mM sodium acetate buffer (pH 4.7).

respectively at 310 nm. Hence either the disappearance of tannic acid or the formation of gallic acid can be used as a measure of tannase activity by measuring the changes in absorbance at 310 nm (for tannic acid) or at 250 nm (for gallic acid). Assay of soluble tannase by measuring the disappearance of tannic acid at 310 nm is quite satisfactory (Iibuchi *et al.,* 1967; Coggon *et al.,* 1975). Soluble tannase has also been assayed using methylgallate as substrate (Yamada *et al.,* 1968). However these methods have not been found suitable for the assay of matrix-bound tannase due to small difference in UV absorption of methylgallate and gallic acid, and absorption of tannic acid.

Tannic acid is absorbed by many compounds particularly charged molecules such as proteins, polyamines, etc. (Hurst and Turner, 1962; Haurowitz, 1950). Adsorption of tannic acid during the assay, either by soluble compounds or by matrices used for binding can lead to erratic results, when tannic acid disappearance is used as a measure of enzyme activity. This may have been one of the reasons for the rather large errors in the assay procedures of Weetal and Detar (1974). Different matrices with and without tannase were tested with a view to determine whether they adsorbed tannic acid, and if so, to what extent. The results show that tannic acid is adsorbed by the matrices used, and even after 30 min incubation, the absorbance at 310 nm is almost minimal and adsorption of tannic acid occurs to nearly 90% (table 1). In the presence of the enzyme, the high initial absorbance at 250 nm due to tannic acid, decreases at first presumably due to adsorption of tannic acid, and then absorption at 250 nm increases apparently due to release of gallic acid.

Table 1. Hydrolysis and adsorption of tannic acid on matrices with and without tannase.

Period of contact	Enzyme-free matrices						Enzyme-immobilized matrices					
	Poly-acrylamide gel[a]		Duolite S-762[b]		Collagen		Poly-acrylamide gel[a]		Duolite-S-762[b]		Collagen	
(min)	Absorbance (nm)						Absorbance (nm)					
	250	310	250	310	250	310	250	310	250	310	250	310
0	0.94	1.05	1.19	1.29	1.22	1.32	1.15	1.14	1.55	0.62	1.61	1.60
10	0.40	0.32	0.28	0.16	0.57	0.51	0.60	0.36	0.78	0.16	1.41	0.77
20	0.20	0.19	0.28	0.13	0.42	0.42	0.76	0.28	0.92	0.16	1.66	0.41
30	0.19	0.15	0.25	0.10	0.34	0.30	1.16	0.28	0.98	0.17	1.64	0.35
60	0.18	0.15	0.25	0.10	0.17	0.11	1.29	0.26	1.17	0.12	1.84	0.24

The reaction mixtures were incubated in a gyratary shaker (120 rpm) at 25^{0}C for various time periods. Then they were filtered and absorbance of the filtrates were measured at 250 and 310 nm.

[a] One g Polyacrylamide gel (enzyme-free and enzyme-entrapped) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

[b] One g Duolite-S-762 (enzyme-free and enzyme-immobilized) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

c One hundred mg collagen (enzyme-free and enzyme-complexed) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

In view of the above results, experiments were conducted to ascertain whether gallic acid is also adsorbed by the matrices. The results (table 2) indicate that gallic acid recovery is almost 100% unlike in the case of tannic acid (table 1) at all the concentrations tested. It is therefore quite clear that gallic acid is not adsorbed by the Polyacrylamide gel. Similar results were obtained with Duolite-S-762 and collagen membrane. Therefore the release of gallic acid by the enzymatic hydrolysis of tannic acid can be used as a satisfactory measure of immobilized tannase activity.

Table 2. Behaviour of gallic acid on Polyacrylamide gel

Concentration of gallic acid in 50 mM sodium acetate buffer (pH 4.7) %	Absorbance of filtrates at 250 nm			
	Control[a]	Gallic acid	Gallic acid with enzyme entrapped polyacrylamide gel	Gallic acid with enzyme free polyacrylamide gel
0.002	0.56	0.61	0.60	0.61
0.004	1.08	1.18	1.06	1.17
0.006	1.60	1.75	1.57	1.68
0.008	2.13	2.34	2.09	2.12

[a] One ml of buffer is added to 10 ml gallic acid to maintain volume constant.

Gallic acid (10 ml) was kept in contact with 1.0 g enzyme-free and enzyme (tannase) entrapped Polyacrylamide gel for 1 h on a gyratary shaker (120 rpm), and filtered. The absorbance of the filtrates was measured at 250 nm against 50 mM sodium acetate buffer (Ph 4.7).

Kinetic parameters essential for a satisfactory enzymatic reaction (assay) have been determined. The time course of the reaction catalysed by the immobilized tannase was studied employing Polyacrylamide gel, Duolite-S-762 and collagen membrane. The results graphically represented in figure 2 show that under the conditions used, the reaction rate is linear from 10 min to at least up to 40 min in all the cases. When the substrate concentration was increased, the rate of reaction increased proportionately at least up to 0.008% tannic acid in the case of collagen-bound enzyme and to 0.01% in the other two cases (figure 3). Higher concentrations of tannic acid are probably necessary to saturate the enzyme or for substrate to efficiently permeate the matrices, but lead to much higher blanks at either $A_{310\ nm}$ or $A_{250\ nm}$. The effect of immobilized tannase concentration on the reaction rate was studied by varying the concentration of matrix bound tannase and lead to a proportionate increase in the reaction rate with 0.008% tannic acid (figure 4). In most of these cases (figures 2, 3 and 4), the plots do not pass through the origin. This may be because there was a difference in $A_{250\ nm}$ between experimental sample and the control at zero min. This was also noticed in the experiment on the effect of immobilized enzyme concentration on activity by Weetal and Detar (1974). However it does not vitiate the assay since the differences In A_{250} between 30 min and 10 min are used for calculation of activity.

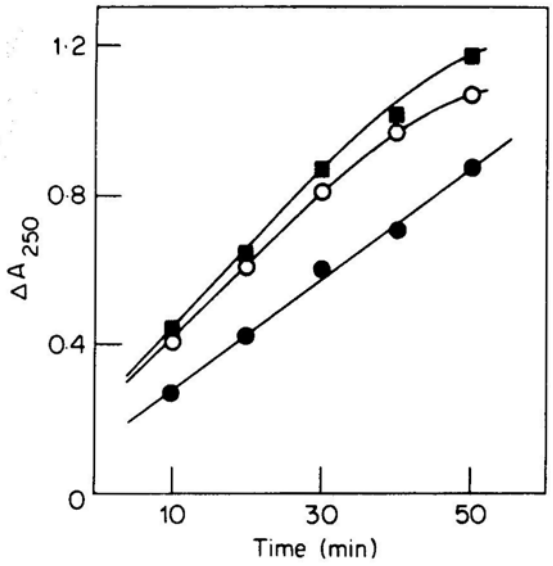

Figure 2. Time course of the reaction catalysed by immobilized tannase. Collagen-enzyme (10mg, ■); Duolite-enzyme (200mg,O); and polyacrylamide-enzyme (100mg,•. Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added at 25^0C. Blank contained adsorbant, without the enzyme.

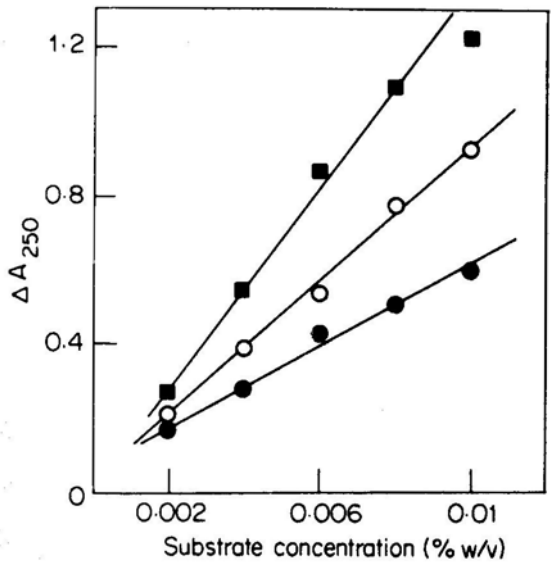

Figure 3. Effect of substrate concentration. Ten ml of substrate tannic acid in 50 mM sodium acetate buffer (pH 4.7) of various concentrations (0.002% to 0.01%) was added to tannase immobilized on collagen (50 mg. ■); Duolite (1 g, O) and Polyacrylamide gel (1 g, •). Incubation was for 20 min at 250C.

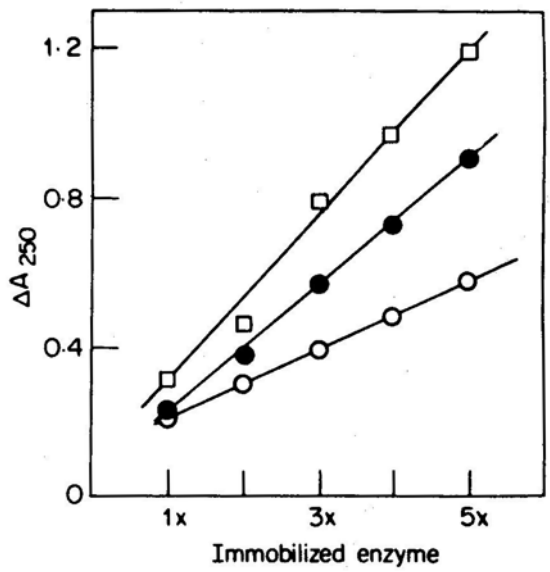

Figure 4. Effect of immobilized tannase at different concentrations. Collagen-enzyme (5 to 25 mg, □), Duolite-enzyme (20 to 100 mg, O) and polacrylamide gel enzyme (50 to 250 mg, •), Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added. Incubation for 20 min at 25°C.

In the above cases, gallic acid was detectable as a product of hydrolysis by paper chromatography (figure 5) according to the procedure of Iibuchi *et al.* (1972) and the product had a UV absorption spectrum similar to that of gallic acid.

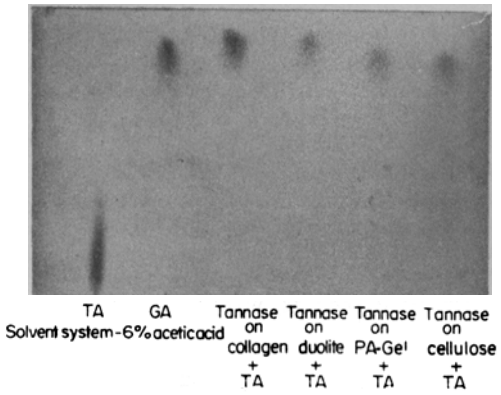

Figure 5. Paper chromatography of tannic acid and its hydrolyzed product by the action of immobilized tannase with standard gallic acid.

Ten ml of tannic acid (0.4%) in 50 mM sodium acetate buffer (pH 4.7) was added to tannase immobilized on collagen (200mg), Duolite (2 g) and Polyacrylamide gel (2g.), incubated for 4 h at 250C on a gyratary shaker (120 rpm), and filtered. The filtrates (20 µl) were spotted on Whatman (3 mm) chromatographic paper along with standard gallic (0.4%) and tannic acid (0.4%). Six % acetic acid was used as solvent system and 2% ferric chloride as the spraying agent.

Conclusions

On the basis of the above data, the following method of determination of immobilized tannase activity has been proposed. Enzyme bound matrix (50 mg collagen or 1 g Duolite or 1 g Polyacrylamide gel; quantity can be reduced depending on the activity units retained in the matrix) and the same quantity of similarly treated enzyme free matrix are added separately to two 25 ml Erlenmeyer flasks containing 10 ml tannic acid (upto 0.01%) in 50 mM sodium acetate buffer (pH 4.7). The reaction mixtures are incubated for 20 min at 25° in a gyratary shaker (120 rpm). The filtrates obtained from the reaction mixture are read at 250 nm against the buffer.The ΔA_{250} is taken as a measure of immobilized tannase activity. A unit of activity has been defined previously. With chitin, 20-30% adsorption of product (gallic acid) was observed and hence this method is not likely to be satisfactory for the chitin-tannase complex. Tea tannins are also efficiently hydrolyzed by the action of tannase immobilized on Polyacrylamide gel, Duolite-S-762 and collagen membrane. Gallic acid was identified as a product of hydrolysis by paper chromatography.

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