Chapter 8

An improved micromethod for tyrosine determination and its application for tyrosine analysis in enzyme assay

Introduction

Tyrosine is a non-essential amino acid that is synthesized in the body from phenylalanine and serves as a building block for several important brain chemicals and hormones (1). Because of its physiological importance, a sensitive procedure for the assay of tyrosine in micro-quantities assumes importance (2, 3). The initial iodometric and polarographic methods were changed to spectrophotometric procedures that were based on reduction of phosphomolybdate by tyrosine, in the presence of alkaline copper, to form a blue color complex (3). The first quantitative and reproducible method for colorimetric estimation of tyrosine was described in detail by Folin and Ciocalteu (4), where the application of phenol reagent [also known as Folin-Ceocalteu (FC) reagent] was introduced in turn to overcome the problem of precipitation and turbidities in the method. Anson (5) and Spies (3) subsequently reviewed this method for its use in the estimation of tyrosine for enzyme assays. Barret and Heath (6) and Turk *et al.* (7) have further described the modified Anson's method for its application in tyrosine estimation for proteases assay. However, these methods were less sensitive concerning the quantitation range and not so economic in terms of FC reagent usage.

In the light of the above, an assay procedure that is sensitive and at the same time economize the usage of chemicals would be a method of choice. Hence in the present study, range of FC concentration was used to optimize the conditions that give stable color complex and simultaneously reduce the total assay volume thereby imparting

sensitivity to the procedure. The details of these experiments for developing a sensitive micro-method for tyrosine estimation and its application to determine tyrosine released in enzymatic reactions and in tissue are described below.

Chemicals

L (-) Tyrosine was purchased from E. Merck, Dramstadt, Germany. Hemoglobin was obtained from Sigma Chemical Co., St. Louis, USA. FC reagent and bovine serum albumin (BSA) were purchased from SRL, Mumbai, India. All other chemicals were purchased locally and were of analytical–reagent grade.

Experimental

Reagents

Modified alkaline copper reagent: This is a form of the Lowry reagent (8) containing additional sodium hydroxide to neutralize the acid. Briefly, stock 'a' was prepared by dissolving 1g of trisodium citrate dihydrate and 0.5 g of $CuSO_4.5H_2O$ in 100 ml of water. For Stock 'b' 16 g of NaOH and 50 g of Na_2CO_3 were dissolved in 500 ml of water. For the working solution, 1 ml of stock 'a' was diluted 1:80 i.e. 1 ml of Stock 'a' and 79 ml of distilled water. Then to this solution 20 ml of stock 'b' was introduced to prepare 100 ml of total working solution.

Assay procedures

Conventional procedure

The procedure originally described by Anson (1938) and further revised by Barret and Heath (1977) and Turk *et al.*(1984) was employed as a conventional procedure. Briefly, aliquots of solution containing 5 to 50 µg of tyrosine made in 2.5% trichloroacetic acid (TCA) were taken. The assay volume was made up to 1 ml with 2.5% TCA. To this 2 ml of modified alkaline copper reagent (the working solution) was added followed by 0.6 ml of 1:3 diluted FC reagent. The total volume was 3.6 ml and the absorbance was taken at 750 nm.

Present procedure

Aliquots of solution containing 2 to 16 µg of tyrosine made in 2.5% TCA were taken and the volume was made up to 0.4 ml with 2.5% TCA. To this 1 ml of modified alkaline copper reagent was added. This was followed by 0.1 ml of suitably diluted FC reagent (i.e. 1 ml 2N FC reagent + 0.5 ml water) with final concentration of 0.2 N in 1.5 ml of total assay volume. The variations in the assay procedure for optimization of the color development conditions with respect to normality of FC reagent are detailed in the individual experiments. In separate experiments the time course of color development was determined under optimized assay conditions. All experiments were carried out in a Shimadzu Model UV-160A UV/VIS spectrophotometer.

Enzyme assay

The applicability of the procedure for the determination of tyrosine released in the protease reaction was ascertained for Cathepsin D assay using hemoglobin as the substrate. Male albino rats of Charles-Foster strain (200-250 g) were used. The animals were killed by decapitation and the livers were quickly dissected out and placed in beakers containing chilled (0-4 °C) 0.25 M sucrose. Tissue homogenates (10% w/v) were prepared in 0.25 M sucrose using a Potter-Elvehjem type glass – Teflon homogenizer. The homogenate was centrifuged at 650 × g for 10 min at 4 °C in a Sorvall RC 5B*plus* centrifuge using a SS34 rotor. The pellet containing nuclei and unbroken cell debris was discarded and the supernatant (S_1 fraction) was used within 15 min for the measurement of the 'free' activity. For the measurement of the 'total' activity the S_1 fraction was diluted (1:5) with Tris[tris(hydromethyl)aminoethane]-HCl buffer (10 mM, pH 7.4) containing 0.1% Triton X-100 and subjected to three cycles of freezing and thawing.

The measurement of Cathepsin D activity was carried out as described previously (6, 9, 10). The tyrosine positive materials were estimated by the methods described above. The ratio of Total activity/Free activity is taken as the index of lysosomal membrane integrity (9). The suitability of the present procedure was also checked for free tyrosine pool in rat liver nuclei free homogenate. Protein was estimated according to the method of Lowry *et al.,* (1951) using bovine serum albumin as the standard. Statistical analysis of the data was done by Students' *t*-test.

Results and discussion

Folin and Ceocalteu (1927) have shown that the concentration of FC reagent is important for stable and the quantitative blue color development. Thus, effects of varying concentration of suitably diluted FC reagent on the time course of color development were evaluated using 8 μg tyrosine samples in the first set of experiments. The results are shown in Fig.1. It can be noted that the addition of suitably diluted FC reagent with final concentration of 0.07 N resulted in ill-defined color development. After addition of FC reagent, the color intensity was declined rapidly till 3 h and at the end of 4 h it remained 11-13% of the maximum color intensity for 0.07 N. Subsequently, the concentration of FC reagent was raised to 0.13 N of the final concentration. With this concentration, the color intensity was improved but it followed the same pattern as 0.07 N. Thus, the intensity was declined till 4 to 6 h and at the end of 8 h it remained 60-65% of the maximum color intensity for 0.13 N. In the next set, the FC reagent concentration was taken 0.20 N of the final. At this concentration, the color development was found to be rapid. Thus within 30 min 80-90% of the optimum color developed. By 1 h, 90-94% of the optimum color development had occurred and at the end of 2 h, it was 95-99%. Finally, the FC reagent was used at the concentration of 0.27 N and, as is evident from Fig. 1, the course of color development was rapid similar to that found in 0.2 N and it followed the same pattern.

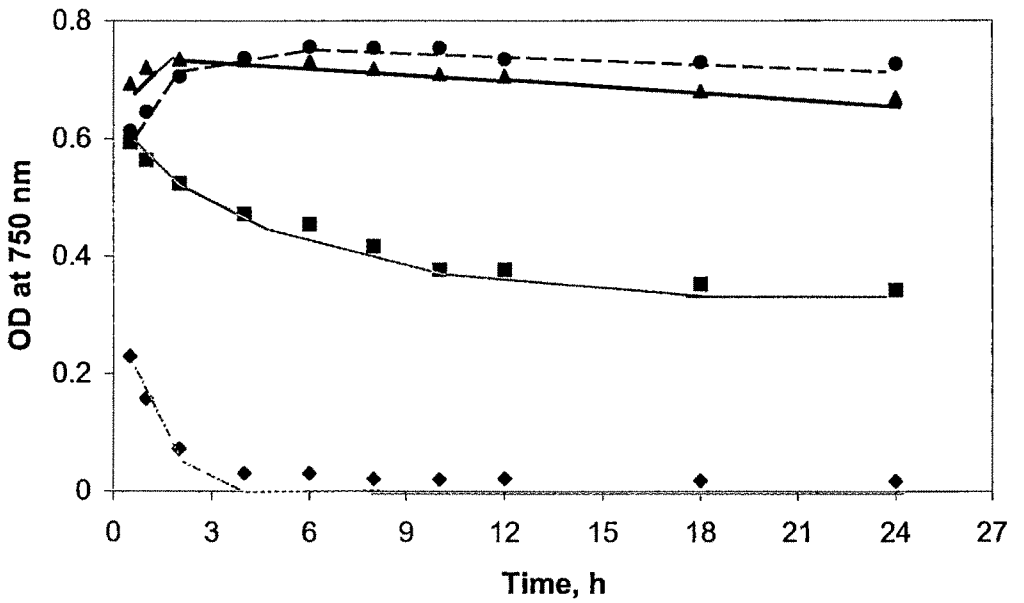

Figure 1. Effect of varying the concentration of FC reagent on the course of color development. In the 1.5 ml assay system the tyrosine concentration was 8 μg. The final concentrations of FC were 0.07 N (·····◆·····); 0.13 N (——■——); 0.20 N (– –●– –·) and 0.27 N (——▲——). The optical density was measured at 750 nm at various time intervals as indicated.

The nature of blue color formed in terms of its absorption characteristics was ascertained further for different concentrations of FC reagent. The results are shown in Fig. 2. Since the difference between the pattern of color development and absorption characteristics for 0.20 N and 0.27 N FC reagent was negligible (see Fig. 1, Fig. 2C and 2D), and keeping the advantage of rapid color development with these concentrations, in all the experiments performed subsequently, the final concentration of FC reagent was kept at 0.2 N.

Concurrently, for comparison, the spectra of blue color formed using the conventional and the present procedures were also recorded using the 8 μg of tyrosine for the specified assay systems as above. These spectra are shown in Fig. 3. As it is evident, the color complex formed using the conventional (Fig. 3A) and the present procedures (Fig. 3B) showed absorption maxima at 750 nm. Also the peak height was higher with present procedure than that with the conventional method. The color species produced by the conventional procedure and by the present procedure were stable up to 24 h (Fig. 1).

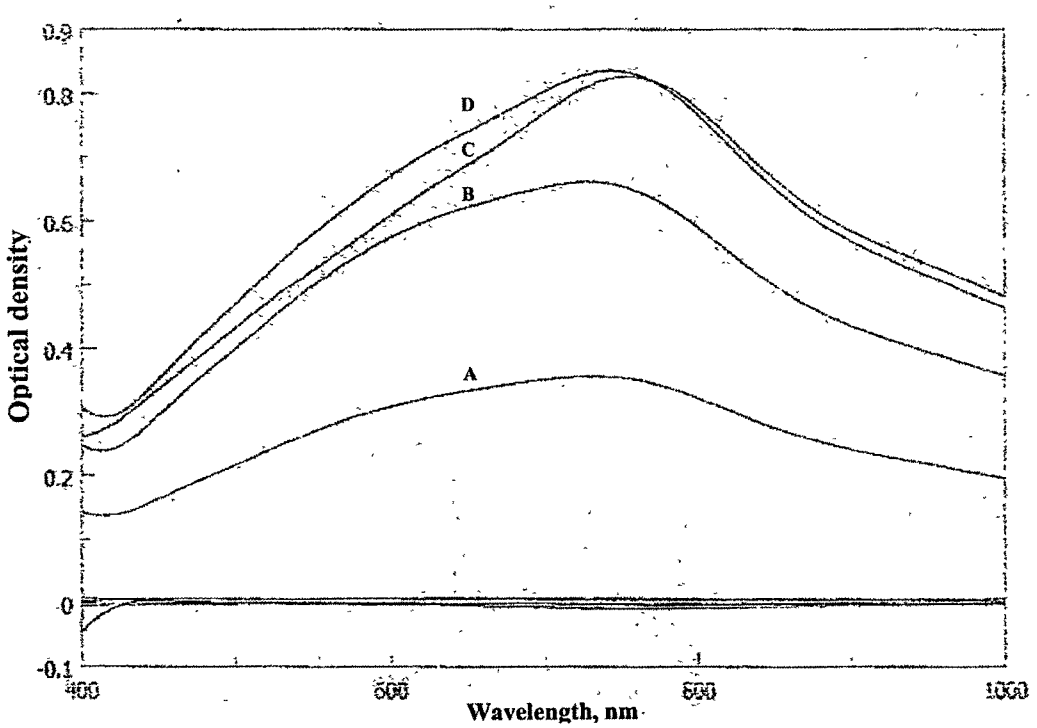

Figure 2. Effect of varying the concentration of FC reagent on the spectra of blue color. In the 1.5 ml assay system the tyrosine concentration was 8 μg. The final concentrations of FC were 0.07 N (A), 0.13 N (B), 0.20 N (C) and 0.27 N (D). The spectra were recorded in Shimadzu UV-160A UV/VIS spectrophotometer over the wavelength span of 400 to 1000 nm. The optical density measurements were carried out at the end of 2 h.

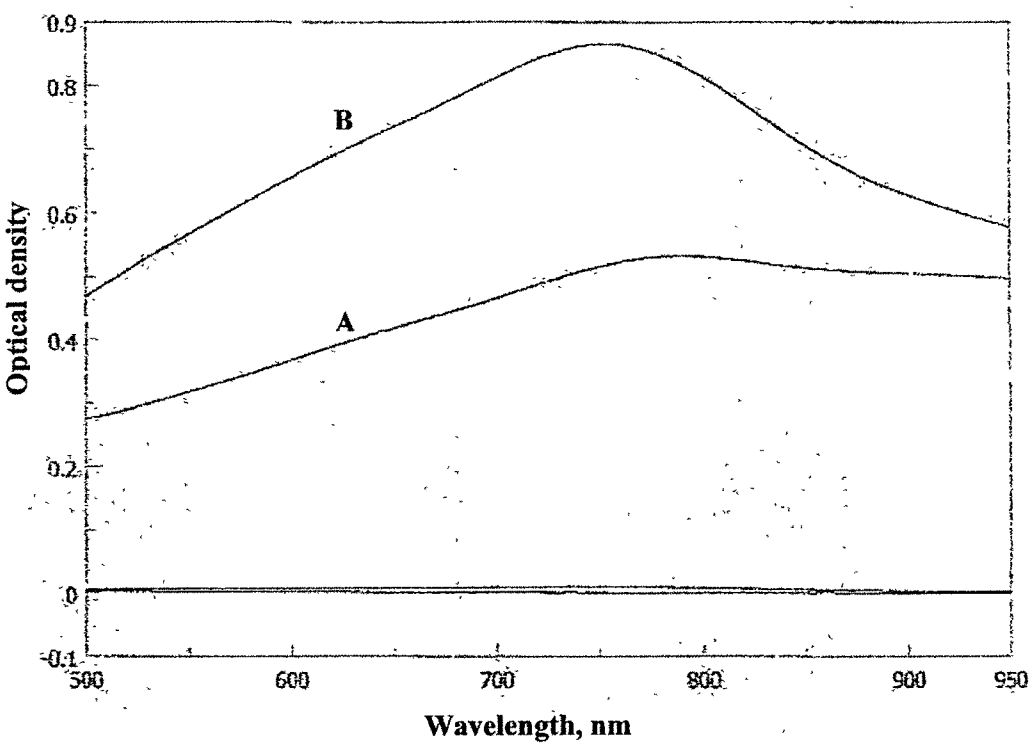

Figure 3. Spectra of blue color produced by the conventional (A) and the present (B) methods. Procedures for color development are detailed in text. The spectra were recorded in Shimadzu UV-160A UV/VIS spectrophotometer over the wavelength span of 500 to 950 nm. The optical density measurements were carried out at the end of 2 h.

In the next set of experiments, the standard curve for tyrosine estimation obtained under optimized conditions by following the present procedure (Fig. 4). As can be noted, not only is the color development showed linear relationship with the concentration of tyrosine but also that the sensitivity in terms of its detection limit improved by 3 fold providing the range of 2-16 µg of tyrosine per prescribed assay system.

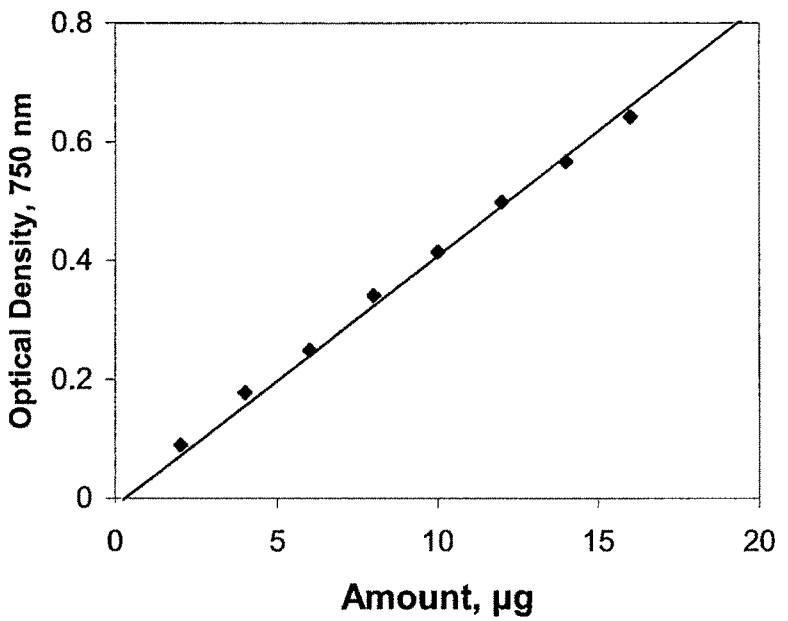

Figure 4. Standard curve for tyrosine determination by the present procedure. In 1.5 ml assay system the concentration of tyrosine was varied from 2 to 16 µg. Other experimental conditions were the same as described in text. The measurement of optical density was carried out at 750 nm at the end of 2 h.

The applicability of the present procedure for enzymatic analysis was then evaluated for lysosomal protease – cathepsin D using hemoglobin as the substrate. The 'free' and 'total' activities of the enzyme following the conventional and the modified micro-method were compared (Table 1). As can be noted from data in Table 1, the 'free' and 'total' activities for cathepsin D and ratio of Total activity/Free activity were in the expected range (9, 10, 11) indicating lysosomal membrane integrity, and the results by the two methods were identical. When the applicability of the present method for free tyrosine pool of the tissue was checked (Table 2), the values were in excellent agreement for both the methods.

Table 1. Activity of rat liver lysosomal cathepsin D. Experimental details are as given in the text. The cathepsin D activity is expressed as μg tyrosine positive materials per 10min per mg protein.

Assay procedure	Free activity	Total activity	$\frac{\text{Total activity}}{\text{Free activity}}$
Conventional	15.12 ± 1.04	83.26 ± 3.97	5.79 ± 0.40
Present	17.36 ± 0.90	90.05 ± 3.30	5.25 ± 0.32

The results are given as mean ± SEM of 12 independent observations.

Table 2. Free tyrosine pool of rat liver. Experimental details are as given in the text.

Assay procedure	Free tyrosine pool (μg tyrosine/g tissue)
Conventional	210.39 ± 10.27
Present	209.31 ± 13.05

The results are given as mean ± SE of six independent observations.

Thus the micromethod that has been developed seems to be 3 fold more sensitive in terms of concentration range for tyrosine analysis (Fig. 4) as compared to that of the conventional procedure. The present procedure produced a stable blue color species by employing 0.2 N FC reagent at the final concentration of the total assay volume (1.5 ml). Thereby it minimizes the excess usage of the reagent. The color development followed a rapid course and about 95% of the optimum color development was seen within 1 to 2 h. Also, the color was stable up to 24 h that gives flexibility in making optical density measurements in assays where large numbers of samples are to be handled. Because of its increased sensitivity it offers an additional advantage in terms of reduction in the volume of enzyme assay by a factor of 4 to 5, thereby reducing the input of materials and sample.

Thus, the procedure described presents an improved micromethod for determination of tyrosine released in enzymatic reactions and in the biological materials. In conclusion, the micromethod described here is a convenient procedure for tyrosine estimation for routine biochemical analysis.

Publication:

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Reference

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Summary

A modified and improved micromethod for tyrosine determination has been developed. The method is sensitive, economic and applicable for estimation of tyrosine released in enzymatic reactions and in tissue. A range of Folin Ciocalteu (FC) reagent was used to optimize the conditions for the development of blue color. Thus in 1.5 ml of assay system, suitably diluted FC reagent at the final concentration of 0.2 N gave a rapid optimum color development with absorption maximum at 750 nm. Color development showed a linear relationship up to 16 µg tyrosine concentration. Thus the method is 3 folds more sensitive than that of conventional method. The blue color formed was stable up to 24 h. The applicability of the method for tyrosine determination in the assay of lysosomal cathepsin D and in tissue was checked in comparison to the conventional procedure. Under both the systems the results obtained by the micromethod were identical to those obtained by the conventional method. In general the method which produces quantitatively blue color not only is rapid and economical in terms of chemical usage but also has application for routine biochemical analysis.