An Improved Micromethod for Tyrosine Estimation

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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 the assay system, the suitably diluted FC reagent at the final concentration of 0.2 N gave a rapid optimum color development with an absorption maximum at 750 nm. Color development showed a linear relationship in the range of 2 to 16 μg tyrosine for a described assay system under optimized conditions. Thus, the method is 3-fold more sensitive in terms of its estimation range than a 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 by comparison to the conventional procedure. Under both systems the results obtained by the micromethod were identical to those obtained by the conventional method. In general the method that produces quantitatively a blue color, not only is rapid and economical in terms of chemical usage but also has application for routine biochemical analysis.

Key words: Tyrosine Estimation, Folin-Ciocalteu Reagent, Micromethod

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 (Mathews *et al.*, 2003). Because of its physiological importance, a sensitive procedure for the assay of tyrosine in micro-quantities assumes importance (Spies and Chambers, 1951; Spies, 1957). 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 colored complex (Spies, 1957). The first quantitative and reproducible method for colorimetric estimation of tyrosine was described in detail by Folin and Ciocalteu (1927), where the application of phenol reagent [also known as Folin-Ciocalteu (FC) reagent] was introduced in turn to overcome the problem of precipitation and turbidities in the method. Anson (1938) and Spies (1957) subsequently reviewed this method for its use to estimate tyrosine for enzyme assays. Barret and Heath (1977) and Turk *et al.* (1984) have further described the modified Anson's method for its application in tyrosine determination 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 economizes the usage of chemicals, would be a method of choice. Hence in the present study, a range of FC concentrations was used to optimize the conditions that give a 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 micromethod for tyrosine estimation with an improved range and its application to determine tyrosine released in enzymatic reactions and in tissue are described below.

Materials and Methods

Chemicals and reagents

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. Modified alkaline copper reagent was a form of the Lowry reagent (Lowry *et al.*, 1951) containing additional sodium hydrox-

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ide to neutralize the acid (Barret and Heath, 1977). Briefly, stock 'a' contained 1 g of trisodium citrate dihydrate with 0.5 g of $CuSO_4 \cdot 5\ H_2O$ in 100 ml of water. Stock 'b' containing 16 g of NaOH and 50 g of Na_2CO_3 was dissolved in 500 ml of water. For the working solution, 1 ml of stock 'a' was diluted to 80 ml with water before 20 ml of stock 'b' was introduced.

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 per total assay volume made in 2.5% trichloroacetic acid (TCA) were taken. The volume was made up to 1 ml with 2.5% TCA. To this 2 ml of modified alkaline copper reagent 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: Aliquotes of solution containing 2 to 16 μg of tyrosine per total assay volume 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 followed by 0.1 ml of suitably diluted FC reagent with a final concentration of 0.2 N in 1.5 ml total assay volume. The variations in the assay procedure for optimization of the color development conditions with respect to normality of FC reagent is documented in the individual experiments. In separate experiments the time course of color development was determined under optimized assay conditions. All experiments were carried out with a Shimadzu UV-160A UV/VIS spectrophotometer.

Enzyme assay

The applicability of the procedure for the determination of tyrosine released in the protease reaction was ascertained using hemoglobin as the substrate for cathepsin D assay. Male albino rats of Charles-Foster strain (200–250 g) were used. The animals were killed by decapitation and the livers were quickly dissected 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)aminomethane]-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 (Barret and Heath, 1977; Nerurkar *et al.*, 1988; Khandkar *et al.*, 1996; Acharya *et al.*, 2004). The tyrosine-positive materials were determined by the methods described above. The ratio of total activity/free activity is taken as the index of lysosomal membrane integrity (Nerurkar *et al.*, 1988; Khandkar *et al.*, 1996). 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 Ciocalteu (1927) have shown that the concentration of FC reagent is important for stable and quantitative blue color development. Thus, effects of varying concentrations 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. It was observed that addition of suitably diluted FC reagent with a final concentration of 0.07 N resulted in ill-defined color development. After addition of FC reagent, the color intensity declined rapidly for 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 final concentration. With this concentration, the color intensity was improved but it followed the same pattern as 0.07 N. Thus, the color intensity declines by 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 final concentration of FC reagent was taken as 0.2 N. At this concentration, the color development was found to be rapid, 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 the color developed as found in 0.2 N and it followed the same pattern.

The nature of the blue color formed in terms of its absorption characteristics was ascertained further for different concentrations of FC reagent (Fig. 1, upper part, A–D). Since the difference between the pattern of color development and absorption characteristics for 0.2 N and 0.27 N FC reagent was negligible, 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.

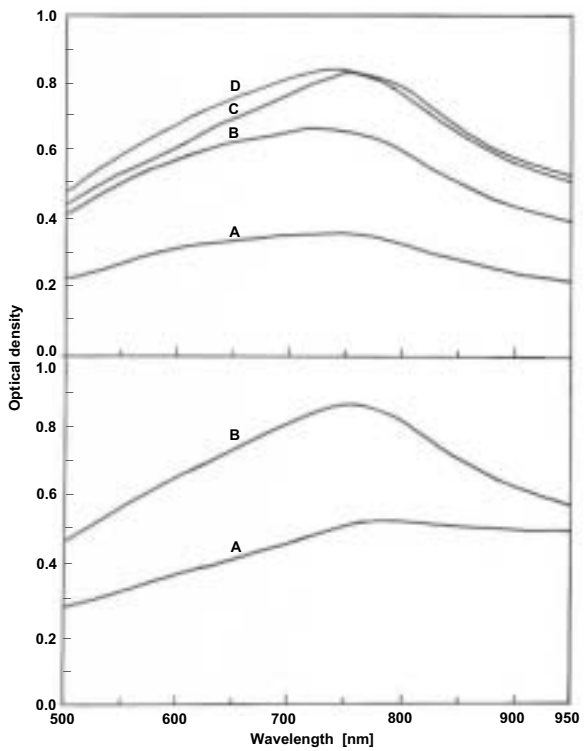

Fig. 1. The spectra of the blue color complex. The spectra were recorded with a Shimadzu UV-160A UV/VIS spectrophotometer over the wavelength span of 500 to 950 nm. After addition of FC reagent, the optical density measurements were carried out at the end of 2 h. Upper part: Effect of varying the concentration of FC reagent on the spectra of blue color: In 1.5 ml assay system 8 μg of tyrosine was taken. The final concentrations of FC were 0.07 N (A), 0.13 N (B), 0.20 N (C) and 0.27 N (D). Lower part: The spectra of blue color produced by the conventional and the present methods: A and B respectively. The details of the assay system are as given in the text.

Table I. 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 10 min per mg protein.

Assay procedure	Free activity	Total activity	Total activity: 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 ± SE of 12 independent observations.

Concurrently, for comparison, the spectra of blue color formed by the conventional and the present procedures were also recorded using the 8 μg of tyrosine for the specified assay systems as above (Fig. 1, lower part, A–B). As it is evident, the color complex formed using the conventional (Fig. 1A) and the present (Fig. 1B) procedures showed absorption maxima at 750 nm. Also, the peak height was higher with the present procedure than with the conventional method. The color species produced by the conventional procedure and by the present procedure were stable up to 24 h (data not shown).

In the next set of experiments, the standard curve for tyrosine estimation was obtained under optimized conditions by following the present procedure. It was observed that, not only the color development showed linear relationship with the concentration of tyrosine, but also the sensitivity in terms of its detection limit improved by 3-fold providing the range of 2–16 μg tyrosine per described assay system.

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 micromethod were compared (Table I). As

Table II. 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.

can be noted from data in Table I, the 'free' and 'total' activities for cathepsin D and the ratio of total activity/free activity were in the expected range (Nerurkar *et al.*, 1988; Khandkar *et al.*, 1996; Acharya *et al.*, 2004) 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 II), the values were in excellent agreement for both the methods.

Thus, the procedure described presents an improved micromethod for determination of tyrosine released in enzymatic reactions and in biological materials. Because of its increased sensitivity it offers an additional advantage in terms of reducing the volume of enzyme assay by a factor of 4–5, thereby economizing the input of materials and sample. In conclusion, the micromethod described here is a convenient procedure for tyrosine estimation for routine biochemical analysis.

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