A New Method for the Determination of Leucine Aminopeptidase in Serum (use of p-dimethylaminobenzaldehyde for coloration)

Morito TAKENAKA
Department of Clinical Pathology, Yamaguchi University, School of Medicine, Ube
Present address: Yamaguchi Prefectural Central Hospital, Hofu
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Leucine aminopeptidase (LAP) is an exopeptidase belonging to protease, which was described and named by Smith[1] in 1951. This enzyme shows remarkable increase in serum in hepato-biliary disorders and cancer of the head of the pancreas.

Folk and his associates[2] devised a procedure for the determination of leucine aminopeptidase activity, in which l-leucyl-β-naphthylamide hydrochloride was used as chromogenic substrate and the β-naphthylamine liberated enzymatically was colorized by diazo B red reaction.

It was Green and his group[3] who employed Bratton-Marshall's reagent for the first time to determine the released β-naphthylamine, and their method was modiffied by Goldbarg et al[4] in 1958. Goldbarg's modification has since been favored widely as the best method for LAP determination.

However, it was felt in our laboratory that Goldbarg's procedure was somewhat complicated for routine clinical chemistry on account of the step of deproteinization which was necessary for the as a preliminary treatment complete development of the coloration of β-naphthylamine.

Accordingly, an attempt was made to search for a new method dispensing with deproteinization, and alcoholic solution of p-dimethylaminobenzaldhyde was obtained as a coloring reagent suitable for such purpose. This enabled us to perform LAP determination in two steps, namely, incubation for enzymatic decomposition directly followed by coloration constitutes the whole procedure. This paper aims to describe the new method.

METHOD

Reagents

1) Substrate: 40 mg of l-leucyl-β-naphthylamide hydrochloride is dissolved in water and made to a volume of 100 ml. (This is stable for about 3 months in a refrigerator).

2) 0.1 M phosphate buffer (pH 7.0): 0.1 M solution of disodium hydrogen phosphate (Na_2HPO_4) and that of sodium dihydrogen phosphate (NaH_2PO_4) are mixed in proportion of 61 ml to 39 ml.

3) 0.2 N-Hydrochloric acid.

4) Colorizing regent: Four Gm. of p-dimethylaminobenzaldehyde (p-DAB) is dissolved in 100 ml. of 98 per cent ethanol.

5) Standard stock solution: An amount of 30.1 mg of β-naphthylamine hydrochloride is dissolved in 100 ml of distilled water. The solution is equivalent to 24 μg/ml of β-naphthylamine.

Procedure

(1) Enzymatic reaction: phosphate buffer (0.5 ml) and sample serum (0.02 ml) are introduced into test tubes A and B (blank), respectively. Hydrochloric acid of 0.2 N (0.2 ml) is added to B and then substrate solution (0.5 ml) is added to both A and B. Immediately, the tubes are incubated in a water bath (37°C) for 30 minutes precisely. At the end of the time hydrochloric acid solution of 0.2 N (2.0 ml) is added and mixed to stop enzyme activity.

(2) Coloration: Solution of p-DAB (2.0 ml) is added to both A and B, and mixed. A yellow color develops instantly.

(3) Colorimetry: After 10 minutes, optical densities E_A and E_B for A and B, respectively, are measured in a Klett-Summerson electrophotometer with a blue filter.

(4) Calculation: The activity (units) of the enzyme which is expressed in terms of the amount (mg) of β-naphthylamine liberated by 100 ml of serum (at 37°C for one hour) is read by applying the difference of optical densities ($E_A - E_B$) to the calibration curve.

If the liberated β-naphthylamine is more than 10 μg, repeat the determination with serum half as much as the amount specified above (namely 0.01 ml is used).

Calibration curve

Preparation of standard solutions: Standard solutions of β-naphthylamine (6, 12, and 20 μg/ml in concentration) are made by diluting the stock standard solution (24 μg/ml) with distilled water. Aliquots of 0.5 ml of distilled water and the standard solutions are introduced in four test tubes (0), (6), (12), and (20), respectively.

Coloration: phosphate buffer (0.5 ml), 0.2 N-hydrochloric acid (2.0 ml) and p-DAB solution (2.0 ml) are added to all the tubes and mixed.

Colorimetry: The optical densities of the solution of the tubes are measured in a Klett-Summerson electrophotometer in the same way as described above using the solution in tube (0) as blank for adjusting the reading of optical den-

sity to zero.

Construction of calibration curve: The optical densities of the solutions of tubes (6), (12) and (20) are collated with the activities of LAP, namely 30, 60, and 100 units, to construct a calibration curve, which makes a straight line passing through the origin.

RESULT AND DICUSSION

The coloration of β-naphthylamine with p-dimethylaminobenzaldehyde is extremely stable. It develops almost fully five minutes after addition of reagent and remains constant for a long time after 10 minutes. The coloration follows Beer's law. The intensity of color is not affected by the ambient temperature over the range between 15°C and 35°C. Light has no influence on the coloration: the color is produced under direct sunshine as satisfactorily as in the dark.

The absorption maximum of the colored solution lies at 465 mμ (Fig. 1), and the sample and the standard show the same absorption curve. The development of the color depends to a certain extent on the concentration of p-DAB and hydrochloric acid: coloration is unsatisfactory with a p-DAB reagent of too low concentration. The amount of hydrochloric acid specified in this procedure is optimum.

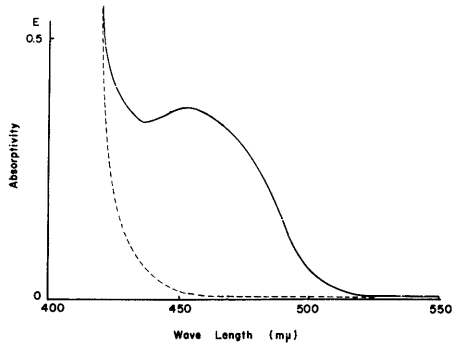

Fig. 1. Absorption spectrum of β-naphthylamine colorized with p-dimethylaminobenzaldehyde. Solid line: sample solution, colorized after incubation to demonstrate the enzyme activity. Broken line: blank solution. The serum, buffered substrate and color reagent are mixed in the same way as in the case of sample solution, but incubation for enzymatic process was omitted. Maximum difference of optical densities is obtained at 465 mμ.

The p-DAB coloration of β-naphthylamine is very sensitive. One μg. of β-naphthylamine (in 5 ml) gives an optical density of 38 (Klett reading), which is several times as large as that obtained with Bratton-Marshall's reagent. This contributes to the successful shortening of incubation time to 30 minutes even with a serum sample as little as 0.02 ml.

Simplicity is the most salient feature of the present method: The color is developed with a single addition of the mixture of hydrochloric acid and p-DAB solution to the samples which have been incubated. However, it may be preferred to add hydrochloric acid and p-DAB solution separately, since their mixture stains yellowish red in several hours when it is allowed to stand at room

temperatures.

The coloration remains clear even when the serum in the reaction mixture is increased (from 0.02 ml) to 0.05 ml, and Beer's law is still followed under such a condition. Deproteinization is therefore entirely unnecessary for coloration as a preliminary treatment. Nevertheless, a blank tube is needed for each serum sample to eliminate the slight light absorption exerted by the serum protein around 465 mμ. The blank tube is particularly required when sulfonamide which reacts with p-DAB is present in the serum.[5] The enzyme kinetics of LAP was discussed early by Green[3] and Goldbarg.[4]

In our experiment maximum activity of LAP for abnormal as well as normal sera was obtained when 1-leucyl-β-naphthylamide in substrate buffer is maintained at a concentration between 190 and 195 mg/dl. This is consistent with the results obtained by Green.[3] However, the concentration is twice as high as that specified by Goldbarg.[4]

Higher activity is obtained with phosphate buffer than with veronal buffer. Optimum pH of phosphate buffer is between 6.7 and 7.0. As to the concentration of phosphate buffer 0.05 M was preferred for the detection of varied activity of LAP in pathologic sera, although normal sera usually showed maximum activity with 0.1 M phosphate buffer. Variation in LAP in pathologic sera was more sensitively estimated with 0.05 M buffer than with 0.1 M buffer.

It was also noted that higher activity was obtained when 0.02 ml of serum was directly mixed with the substrate-buffer (1 ml) than when 1.0 ml of diluted serum equivalent to 0.02 ml was used.

In the present procedure, the activity of LAP (or the amount of liberated β-naphthylamine) increases in linear proportion with the prolongation of incubation time from 30 minutes to 60 minutes.

A comparative study with 25 sera as material demonstrated that the new method and Goldbarg's procedure were in good agreement (coefficient of corre-

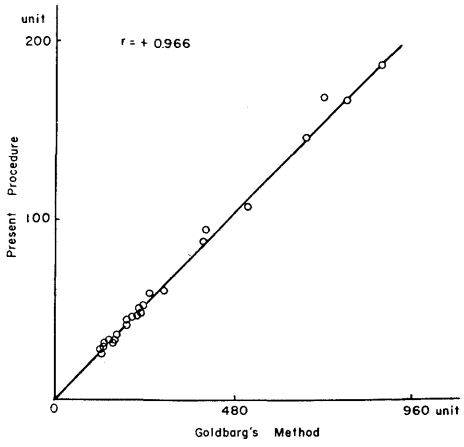

Fig. 2. Comparison of the present procedure with Goldbarg's method on normal and pathological sera.

lation is 0.966) (Fig. 2). The average normal LAP activities by the new method for male and female (25 persons for each group) were 38.5 and 34.8 units, respectively. However, there was no significant difference between two sexes when examined by statistical analysis (level of significance: 1%). The normal serum LAP activity was from 15 units to 60 units (rejection limit, $\alpha=0.05$).

CONCLUSION

A simple rapid method for the determination of leucine aminopeptidase activity with p-dimethylaminobenzaldehyde as color developing reagent was invented.

Blood serum (0.02 ml) was mixed with phosphate buffer of pH 7.0 (0.5 ml) and substrate (1-leucyl-β-naphthylamide) solution (0.5 ml) to be incubated at 37°C for 30 minutes. The enzymatic activity was stopped by adding 0.2 N hydrochloric acid (2.0 ml), and the β-naphthylamine liberated enzymatically during the incubation was colorized with p-dimethylaminobenzaldehyde reagent. The yellow color thus developed was subjected to photoelectric colorimetry.*

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* This procedure can be applied to the determination of urinary LAP activity. Urine is collected for twenty-four hours in a container kept in a refrigerator. The total volume (Vml) is measured. About 5 ml of the urine is introduced in a Visking tube and tightly sealed to be dialyzed against a large amount of water for about 15 hours. The dialyzed urine (0.2 ml instead of 0.02 ml) is used for LAP determination (L units) in the same way as in the case of serum. Daily output of LAP in urine (output) is calculated by the following equation.

$$\text{Output} = LV - 1000 \text{ units per day.}$$