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An Ultramicro Assay for Leucine Aminopeptidase Activity in Biological Materials by Phosphorimetry

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A highly sensitive method for the phosphorimetric assay of leucine aminopeptidase (EC 3.4.11.1) in biological samples is described. *p*-Nitroaniline, formed enzymatically from L-leucyl-*p*-nitroanilide (incubation time, 15 min), is extracted with ether and determined phosphorimetrically in a mixture of ether and ethanol. The method is precise and very sensitive, requiring as little as 0.1 μl of human serum or 0.01—0.1 μg of protein of rat tissue. The limit of detection for *p*-nitroaniline formed is 10 pmol.

Keywords——leucine aminopeptidase; phosphorimetry; ultramicro assay; human serum; rat tissue; L-leucyl-*p*-nitroanilide; *p*-nitroaniline

Leucine aminopeptidase (LAP; L-leucyl-peptide hydrolase, EC 3.4.11.1) in human serum is increased in patients with diseases of the liver[1] and bile ducts.[2] Assay of this enzyme activity is especially useful in the diagnosis of such diseases and in the assessment of placental function for evaluating the status of pregnancy.[3]

LAP has usually been assayed by colorimetric and fluorimetric methods. In the colorimetric methods, L-leucyl-*p*-nitroanilide[4] and L-leucyl-β-naphthylamide[5] are widely used as substrates. Fluorimetric methods using L-leucyl-β-naphthylamide[6] and 7-L-leucyl-4-methylcoumarylamide[7] as substrates are more sensitive.

Recently, it was found that *p*-nitroaniline phosphoresces very intensely in a mixture of ether–ethanol (7 : 2, v/v) at 77 °K, and this finding was successfully applied to an ultramicro assay for γ-glutamyl transpeptidase in biological materials using γ-L-glutamyl-*p*-nitroanilide as the substrate.[8] A highly sensitive method for the assay of LAP in biological samples has now been developed, based on the phosphorimetric determination of *p*-nitroaniline formed from L-leucyl-*p*-nitroanilide. Human serum and rat tissue homogenates were employed to establish the assay procedure.

Experimental

Reagents——All chemicals and solvents were of reagent grade, unless otherwise stated. Double-distilled water was used. *p*-Nitroaniline (Wako) and L-leucyl-*p*-nitroanilide (Sigma) were purified by recrystallization.

Apparatus——The phosphorescence spectra and intensities were measured for sample solutions in ether–ethanol in the form of glassy solids at liquid nitrogen temperature (77 °K), using a Hitachi MPF-3 spectrofluorometer equipped with a Hitachi phosphoroscope attachment and quartz sample tubes (4.0 mm i.d., 5.0 mm o.d., 200 mm long; sample volume, *ca.* 150 μl). The spectral band widths of the excitation and emission monochromators were both 10 nm. The phosphorescence spectra are uncorrected. The phosphorescence lifetimes were measured on a Hitachi V-550 synchroscope. pH was measured with a Hitachi–Horiba M-7 pH meter at 25 °C.

Biological Sample Solutions——Normal sera were obtained from healthy volunteers in this laboratory. Pathological sera were supplied by Kyushu University Hospital. These sera were diluted 50 times with normal saline before use. These tissue homogenates were prepared as follows. Male Donryu rats were killed by decapitation, and portions (30—50 mg) of liver, spleen, lung, adrenal gland and kidney were rapidly removed, blotted and placed in

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4.0—5.0 ml of ice-cold 0.25 M sucrose. Each tissue was homogenized in a Potter–Elvehjem homogenizer and centrifuged at 30000 *g* for 20 min. The supernatant liquid was diluted with 0.25 M sucrose to contain 0.01—0.1 μg of protein for kidney and 0.1—1.0 μg of protein for liver, spleen, lung and adrenal gland in 5 μl. Protein concentration was determined by the method of Lowry *et al.*[9]

Procedure——The substrate solution was 0.05 M phosphate buffer (pH 7.2) containing 1.8 mM L-leucyl-*p*-nitroanilide and 4% (v/v) ethanol. The solution (50 μl) was preincubated at 37 °C for *ca.* 2 min, then incubated for exactly 15 min after addition of 5 μl of sample solution at 37 °C. The reaction was stopped by the addition of 5 μl of 3.0 M trichloroacetic acid, and the mixture was cooled in ice-water. The *p*-nitroaniline produced was extracted into 1.0 ml of ether by shaking for about 5 min. After a brief centrifugation, 0.7 ml of the ether phase was diluted with 0.2 ml of ethanol.

For the blank, the same procedure was carried out, except that the sample solution was replaced with water. To prepare a standard curve, the substrate solution was replaced with *p*-nitroaniline (0.001—50 nmol/50 μl) dissolved in the substrate solution. Phosphorescence intensities were measured at 510 nm with an excitation wavelength of 380 nm.

Results and Discussion

LAP in human serum, and rat liver, spleen, lung, adrenal gland and kidney homogenates was most active at pH 7.2—7.5 in 0.05 M phosphate buffer. Phosphate gave a maximum and constant activity at concentrations of 0.03—0.2 M. Therefore, 0.05 M phosphate buffer of pH 7.2 was used in the procedure, as in the colorimetric method.[5] A maximum and constant activity was obtained in the presence of 1.1—3.0 mM L-leucyl-*p*-nitroanilide with an observed K_m value of 0.301 mM; 1.8 mM L-leucyl-*p*-nitroanilide was used as a saturating concentration for the enzyme reaction. L-Leucyl-*p*-nitroanilide is only slightly soluble in the phosphate buffer. Therefore, the substrate was first dissolved in ethanol, and then diluted with the phosphate buffer to prepare the solution. Ethanol in the substrate solution afforded a clear solution at concentrations of 3% or greater and gave a maximum and constant activity of the enzyme at 7% or less; 4% was employed as the optimum.

The reaction rate was linear with time up to at least 60 min on incubation at 37 °C (Fig. 1). The amount of *p*-nitroaniline formed at an incubation time of 15 min was proportional to the human serum sample-size up to at least 40 μl, and to the amount of protein in rat liver, spleen, lung or adrenal gland homogenate up to at least 50 μg and in rat kidney homogenate up to at least 15 μg.

The *p*-nitroaniline was effectively extracted from the acidified incubation mixture with ether. The extract (0.7 ml) readily formed a clear solid at 77 °K after being mixed with 0.1—

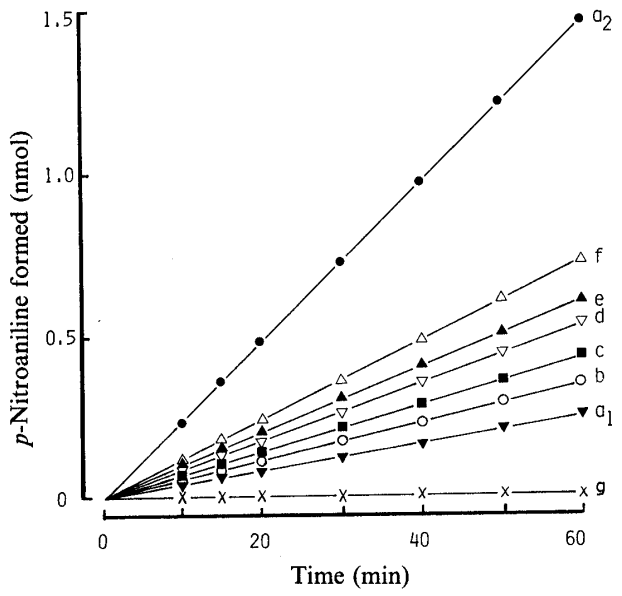

Fig. 1. Effect of Incubation Time on the Enzyme Activity at 37 °C

Portions (5 μl) of sample solution were treated as in the standard procedure for various incubation times.
a_1 and a_2: 50-fold saline-diluted human sera (LAP activities, 40.5 and 240.8 μmol·min^{-1}·l^{-1}, respectively).
b: rat adrenal gland homogenate (0.43 μg of protein; LAP activity, 13.2 pmol·min^{-1}·μg^{-1} protein).
c: rat lung homogenate (0.49 μg of protein; LAP activity, 14.8 pmol·min^{-1}·μg^{-1} protein).
d: rat liver homogenate (0.43 μg of protein; LAP activity, 20.8 pmol·min^{-1}·μg^{-1} protein).
e: rat kidney homogenate (0.08 μg of protein; LAP activity, 125.0 pmol·min^{-1}·μg^{-1} protein).
f: rat spleen homogenate (0.62 μg of protein; LAP activity, 19.2 pmol·min^{-1}·μg^{-1} protein).
g: blank corresponding to a—f.

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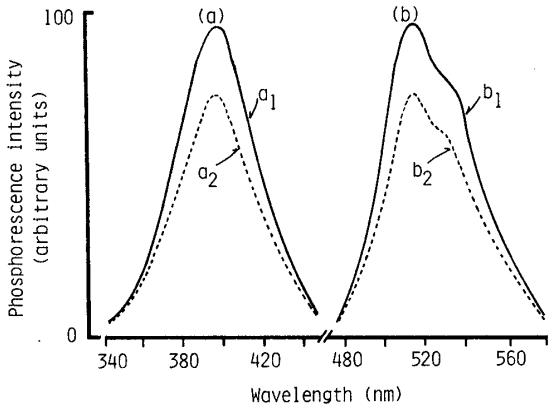

Fig. 2. Excitation and Emission Spectra of the Phosphorescence of the Final Solution in the Standard Procedure and of *p*-Nitroaniline

a: excitation spectra; b, emission spectra.
a_1 and b_1: portions (5 μl) of 50-fold saline-diluted human serum with a LAP activity of 40.0 μmol·min^{-1}·l^{-1} were treated according to the standard procedure.
a_2 and b_2: *p*-nitroaniline (30 pmol/ml) was dissolved in a mixture of ether–ethanol (7:2, v/v).

0.3 ml of ethanol; 0.2 ml of ethanol was routinely used. The standard curve was linear up to *p*-nitroaniline concentrations exceeding 50 nmol.

The phosphorescence excitation (maximum, 380 nm) and emission (maximum, 510 nm) spectra, and lifetime (0.47 s) for the final solution were identical with those of *p*-nitroaniline dissolved in the same solvent (Fig. 2).

The limit of detection for *p*-nitroaniline was 10 pmol, which gave a phosphorescence intensity of twice the blank. The detection limit is much better than those of fluorimetric methods,[6,7] and may permit the assay of LAP in only 0.05 μl of normal serum, 0.005 μg of protein in rat kidney homogenate or 0.02 μg of protein in liver, spleen, lung and adrenal gland homogenates.

Comparison with a colorimetric method,[5] also based on *p*-nitroaniline formation (but requiring larger samples) was made for normal and pathological sera. The correlation coefficient was 0.982 ($n=25$), and the regression equation for the present method (x) against the colorimetric method (y) was $y=1.05x+0.10$. The within-day precision of the present method was examined using sera with mean LAP activities of 44.5 and 508.6 μmol·min^{-1}·l^{-1}. The coefficients of variation were 3.2 and 3.8% ($n=15$), respectively.

LAP activity in normal serum assayed by the present method was 40.8 ± 16.1 μmol·min^{-1}·l^{-1} (mean $\pm$ S.D., $n=14$). The activity in sera of patients with liver diseases was 520.6 ± 243.7 μmol·min^{-1}·l^{-1} (mean $\pm$ S.D., $n=23$). The values are in agreement with the data obtained by other workers.[4-7] LAP activities found in rat (Donryu, male, 7 weeks of age) tissue homogenates were 21.2 (liver), 19.2 (spleen), 16.6 (lung), 119.8 (kidney) and 14.2 (adrenal gland) pmol·min^{-1}·μg^{-1} protein. These results are similar to those obtained by a fluorimetric method based on L-leucyl-β-naphthylamide as the substrate.[10]

The present method is not affected by bilirubin present in the biological sample, though the colorimetric and fluorimetric methods[4,6,7] using synthetic L-leucyl compounds as substrates do suffer bilirubin interference as a result of light absorption at the same wavelength as *p*-nitroaniline.

This study provides the first phosphorimetric method for the assay of LAP. Although L-leucyl-β-naphthylamide and 7-L-leucyl-4-methylcoumarylamide are not readily obtainable, L-leucyl-*p*-nitroanilide is commercially available. The method is precise, and should be useful for biological investigations where only an extremely small amount of serum or tissue (such as a biopsy sample) is obtainable.

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