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Assay of leucine aminopeptidase activity in vitro using large-pore reversed-phase chromatography with fluorescence detection

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Abstract

A chromatographic method for determination of leucine aminopeptidase (LAP) activity in complex matrices is described. L-Leucine-β-naphthylamide was used as the substrate and its hydrolytic product, β-naphthylamine, was monitored by fluorescence at 280 nm excitation and 400 nm emission wavelengths. Under optimized conditions, the components in the incubation mixture were baseline separated and eluted out of a large-pore (300 Å) reversed-phase C_4 column (RPC_4) within 15 min with a non-linear gradient elution of methanol (0.05% (v/v) trifluoroacetic acid additive). The detection limit of the hydrolytic product reached 0.35 pmol at three time signal-to-noise (S/N) ratio with 5 μl sample injection. The method showed a wide dynamic range for quantitation of both the hydrolytic product (10 ng/ml to 80 μg/ml) and LAP (0.1–46.0 μg/ml) with correlation coefficient larger than 0.998 and reproducibility <3 and 10% R.S.D. ($n = 3$), respectively. A fairly broad range of incubation time could be selected within 1 h. The LAP activities and concentrations in rabbit serum, tears, and mouse lens homogenates were determined to be 41.8 (0.3 mg/ml), 2.8 (40.0 μg/ml), and 1.6 pmol/(μl min) (17.5 μg/ml), respectively, with reproducibility of 2–9% R.S.D. ($n = 3$) and intra- and inter-day variation for the retention time of the hydrolytic product being <1% R.S.D. ($n = 3$). The results indicate that the present method is rapid and sensitive as compared to the conventional one.

Keywords: Enzymes; Leucine aminopeptidase

1. Introduction

Leucine aminopeptidase is a zinc metalloenzyme, which exists in either monomers or assemblies of a relatively high-mass (54 kDa) subunit in subcellular organelles, in cytoplasm, or as membrane components of cells in plants [1], bacteria [2], and animals [3]. By virtue of removing N-terminal amino acids of polypeptides, it is proposed to modulate the hydrolysis of proteins that are involved in the ubiquitin-dependent pathway [4]. Moreover, it is also assumed to regulate

Abbreviations: LAP, leucine aminopeptidase; L-Leu-β-NapNH, L-leucine-β-naphthylamide; β-NapNH, β-naphthylamine; RPC_4, reversed-phase C_4 chromatography

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the activity of oligopeptides in the process of signal transduction/neurotransmission [5] in target tissues and maturation of proteins. However, its precise cellular functions, physiological and pathological roles, as well as mechanisms of regulation, are not well understood up to now [6]. Alteration of its activity has been reported to indicate acute or chronic diseases [7] and to associate with corneal wound-healing process [8]. Determination of its activity in cell extracts and in body fluids has significant utilities in investigation of cellular functions, clinical diagnosis of diseases, and monitoring of drug treatment on concerned tissues [9].

Assay of LAP activity in vitro was generally carried out by monitoring the hydrolytic products either spectrophotometrically [10–13] or fluorimetrically using various substrates [14–16]. The application of those methods has been demonstrated in assay of LAP activity in human serum [14] and urine [17], tissue extracts of bovine lenses [18] and human brain [19], as well as cell lysates of neurons and erythrocytes. Due to complexity of the matrix components in biological samples, direct measurement of absorbance and fluorescence of the hydrolytic products is not accurate and reproducible. Careful selection of the detection conditions [20,21], transformation of the hydrolytic product into a diazo complex [17] via a multi-step reaction, and even precipitation of the interfering proteins by ethanol [9,14] before quantitation of the hydrolytic product were often used to alleviate the problem. Since much manual work is required in those experiments, the conventional methods are tedious and difficult to achieve high-throughput of sample analysis. To reduce the total analysis time and minimize the interfering effects of the matrix components on the measurement of the fluorescence, a capillary–electrophoresis-based method achieved in a microfabricated quartz-chip with two-photon excited fluorescence detection has been reported recently [22]. The method is proved to solve the problem in the two aspects of assay of LAP activity in vitro, but the technical limitations caused by the microchip make further improvement needed before it can be used as a routine method in clinical analysis.

The purpose of this work is to develop an in vitro assay method to determine the activity of the title-mentioned enzyme using high-performance liquid chromatography with fluorescence detection. By separating the substrate, the hydrolytic product, and the proteins or lipids involved in the incubation mixture with a large pore (300 Å) reversed-phase C_4 column (RPC_4), it is hoped that the elimination of the matrix effects on the measurement of fluorescence of the enzymatic reaction and the determination of the activity of LAP could be performed on-column simultaneously. As a result, the operation in assay of LAP activity could be significantly simplified, and the developed method could be transferred to a routine laboratory for clinical assay of LAP activity in ocular tissues and biological fluids.

2. Experimental

2.1. Chemicals and standard solutions

Organic solvents were HPLC grade obtained from Fisher Scientific (Leicestershire, UK). Other chemicals were purchased from Sigma (St. Louis, MO, USA). Leucine aminopeptidase (ES 3.4.11.1) was stored at 4 °C prior to use. The stock solutions of L-leucine-β-naphthylamide hydrochloride (L-Leu-β-NapNH) and β-naphthylamine (β-NapNH) were prepared in methanol and stored at 0 °C prior to use. The stock solution of EDTA (disodium ethylenediaminetetraacetate dihydrate, >99.0%, ACS reagent) was prepared in Millipore water, which was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All buffer solutions were sterilized and filtered through a 0.22 μm membrane filter before use. Spectrophotometric grade (>99%) trifluoroacetic acid (TFA) was used as the additive of the mobile phase in chromatography. A leucine aminopeptidase kit from Sigma Diagnostics (Sigma, St. Louis, MO, USA) was used to determine the LAP activity colorimetrically.

2.2. Apparatus

Chromatographic analysis was carried out on a Delta PAK C_4 column (Waters, Milford, MA, USA), 150 mm × 3.9 mm i.d., 300 Å, 5 μm, which was connected to two Waters pumps (Model 515), a photo-diode array (PDA) detector (Model 996), a scanning fluorescence detector (Model 474), and an injector (Model 7725). The control of the whole system and integration of the eluted peaks were performed by Millennium version 3.0501 (Waters, Milford, MA, USA). The pH values of the buffer solutions were adjusted using InoLab pH meter (WTW

GmbH, Weiheim, Germany) with uncertainty being ±0.01 pH. Sonication of the tissues was performed on a Microson Ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA). Centrifugation of all samples was performed on a Microfuge® R centrifuge (Beckman Coulter, Fullerton, CA, USA). Incubation of all enzymatic reactions was carried out in a waterbath (PolyScience, Niles, IL, USA) set at 35.0 ± 0.2 °C. The spectrophotometric measurement of the hydrolytic product was carried out on DU®640B spectrophotometer (Beckman, Fullerton, CA, USA).

2.3. Determination of protein concentrations

Protein concentration was determined by the Bradford method, using bovine serum albumin as the standard reference, following the protocol described in the instructions of Coomassie® plus protein assay reagent kit (Pierce, Rockford, IL, USA). The absorbance of the protein–dye complex was measured at 595 nm.

2.4. Calibration of the quantitation methods

2.4.1. Fluorescent method

Calibration of the quantitation method, using the hydrolytic product (β-NapNH), was carried out on the chromatographic system coupled with a fluorescence detector as described above. A series of NapNH standard solutions were prepared in a formulated incubation mixture, which contained 0.35 mg/ml of the L-Leu-β-NapNH substrate, 50 mmol/l EDTA, and 50 mmol/l phosphate buffer (pH 7.4) solution. Five microliter of the mixture was injected into the C_4 column, which was eluted by a non-linear gradient of the water (solvent A):methanol (solvent B) (v/v) mobile phase (containing 0.05% TFA additive). The gradient started at 40% solvent B and maintained for 5 min, followed by increasing to 75% solvent B at 5.50 min and further increased to 90% solvent B at 6.00 min. The mobile phase was finally increased to 100% solvent B at 6.50 min. After all the components were eluted out, the column was re-equilibrated for 15 min before the next injection. The hydrolytic product was monitored at 280 nm excitation (λ_{Ex}) and 400 nm emission (λ_{Em}) wavelengths against the control solution that contained no β-NapNH. Peak area of the hydrolytic product was used to establish the calibration line in the β-NapNH concentration range given in Section 3.

2.4.2. Spectrophotometric method (Sigma Diagnostics method)

The hydrolytic product of the enzymatic reaction was converted to a diazo complex, and the blue color complex was measured at 565 nm on a spectrophotometer. The calibration procedure was followed precisely as described in the Sigma Diagnostics instruction except that the total volume of the solution was reduced to 2.5 ml, i.e. the volume of all the solutions was reduced by two-fold to minimize the consumption of the reagents in the kit. The absorbance of the β-NapNH standards in 2 mol/l HCl solution was measured in a 1 cm cuvette against a pure water blank at the given wavelength.

2.5. Assay procedure of the enzyme activity

Before initiation of the reaction, the enzyme solution and the incubation mixture, which contained known amount of the substrate in a given buffer solution at a certain pH value, was thermally equilibrated at 35.0 °C for 10 min. The enzymatic reaction was initiated by adding 5 μl of the enzyme/sample solution into the incubation mixture. The total volume of the reaction mixture, unless otherwise stated, was controlled to be 100 μl. After incubation for 30 min, triplicates of 5 μl of the reaction mixture were removed from the incubation solution and equal volume of 100 mmol/l EDTA was added into the withdrawn solutions to stop the reaction. Once mixing completely, the entire mixture was injected into the C_4 column, which was eluted using the optimized conditions described above. The peak area of the hydrolytic product was estimated automatically and the activity of the LAP enzyme in the samples was worked out, which was expressed in pmol/(μl min) unit.

Procedures for assay of the enzyme activity by Sigma Diagnostics method is briefed in the footnote of Table 2.

2.6. Collection and pretreatment of samples

Lenses were obtained from mice (balb/c strain, 2-month-old female, ca. 14.5 g). Tears and blood samples were obtained from New Zealand white rabbits (6–month-old female, ca. 2.0 kg). Handling of the animal is in compliance with the Research Statement for the use of animal in Ophthalmology and Vision

Science. The mouse lens (wet weight, ca. 6.2 mg), which was cut into small pieces using a surgical blade, was suspended in 500 μl of 100 mmol/l Tris–HCl buffer (pH 7.6) at 4 °C. The lens was homogenized by using a sonicator operated in pulse mode at 10% power output for five cycles, each cycle lasted for 2 s. The tissue homogenate was then centrifuged at 1000 × *g* for 10 min at 4 °C to remove cell debris and nucleus. The supernatant of the tissue homogenate was further centrifuged at 20,000 × *g* for 30 min. The supernatant was subject to protein quantitation and activity assay.

Tears were collected from the lower meniscus of the rabbit eyes with 10 μl capillary tubes [23]. About 10 μl of the tear fluids was collected for each eye within 10–15 min. The newly collected tears were then blown to a 0.5 ml vial using a 1 ml pipette fitted with a clean tip and centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant of the tear sample was subject to subsequent assay of LAP activity.

Blood was withdrawn from the ear of the rabbit, which was then centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant (serum) was used in the activity assay.

3. Results and discussion

3.1. Factors influencing the enzymatic reactivity

To find out the optimal compositions of the incubation mixture, kinetics of the enzymatic reaction was primarily investigated at different concentrations of the substrate and LAP, as well as buffer types. After initiation of the enzymatic reaction, 5 μl of the incubation mixture was removed at 20 min intervals and mixed with equal volume of 100 mmol/l EDTA to stop the reaction. This EDTA-stopped solution was then injected onto the HPLC system without separating column. The injector and the detectors were connected by a stainless steel union. About 20% of the L-Leu-β-NapNH substrate could be hydrolyzed within 2 h as shown in Fig. 1(A), where the infinity of the reaction was estimated on the basis of the fluorescence observed after overnight incubation of the remaining solution. Changes in the initial rates of the enzymatic reaction at different conditions was systematically studied as shown in Fig. 1(B)–(D), where initial rates of the reaction were obtained by plotting of peak heights versus times. From Fig. 1(B), we see that the initial rate increases with increase of the substrate concentration when it is <0.20 mg/ml. Further increasing of the substrate concentration results in decrease of the initial rate owing to re-absorption of the emitted light by the excessive substrate in the incubation mixture. Although there is a slight fluctuation of the initial rates at the concentrations between 0.3 and 0.5 mg/ml, the overall trend of change in the initial rates is decreasing. To ensure the reaction to be carried out at a pseudo-first order condition, the substrate concentration should be maintained at concentrations of about 10 times larger than the Michaelis–Menten constant (K_m). Only under such conditions is the enzymatic activity linearly correlated with the LAP concentration in the incubation mixture, otherwise the activity is the function of both the substrate and the LAP concentrations. Using the Lineweaver–Burk plot based on the data shown in Fig. 1(B) at the concentration <0.2 mg/ml, we worked out the K_m-value for the enzymatic reaction. It was about 0.060 ± 0.007 mg/ml (0.20 ± 0.02 mmol/l). The substrate concentration at 10 time larger than the K_m-value was used in the formulated incubation mixture.

In contrary, no such a quenching effect was observed for LAP on the reactivity as shown in Fig. 1(C), where the initial rate increases with the increase of LAP concentration from 0.2 to 35 μg/ml, although the linearity is not maintained throughout the whole range. There is an inflection point at the LAP concentration of about 24 μg/ml, indicating that the reaction order was slightly different at the LAP concentrations below and above the inflection point. This is due to the fact that the pseudo-first order condition was deviated from at higher LAP concentrations under the given substrate concentrations. The LAP effect on the initial rates was not further studied beyond the range indicated in Fig. 1(C), because the enzymatic reaction was very slow at LAP concentration <0.2 μg/ml and the fluorescence detector approached saturation at LAP concentration larger than 35 μg/ml.

The influence of buffer types and concentrations on the initial rates is shown in Fig. 1(D). No significant difference in the initial rates was observed in phosphate buffers at two concentrations (50 and 100 mmol/l) at pH 7.4 as compared to that in Tris–HCl buffers. As a result, 100 mmol/l phosphate buffer at pH 7.4 was used in the following experiments.

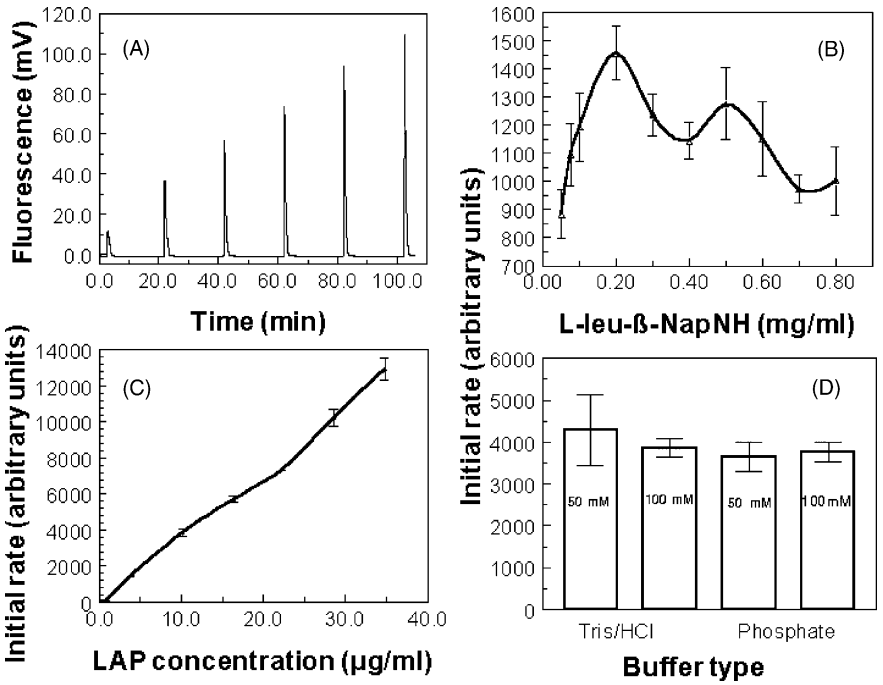

Fig. 1. Kinetic results of the enzymatic reaction at different conditions. (A) Changes in fluorescence of the enzymatic reaction within 2 h hydrolysis at 35.0 °C. Conditions: Tris–HCl buffer at pH 7.4, 100 mmol/l; L-Leu-β-NapNH, 0.70 mg/ml; LAP, 4.1 μg/ml; total volume, 0.5 ml. (B) Effect of the substrate concentrations (0.05–0.8 mg/ml) on the initial rate carried out at the same conditions as given above. (C) Effect of LAP concentrations (0.2–34.8 μg/ml) on the initial rate carried out in the same buffer as given above at 0.2 mg/ml of the substrate concentration. (D) Effect of buffer types (Tris–HCl and phosphate) and salt concentrations (50 and 100 mmol/l) on the initial rate at LAP concentration of 10 μg/ml; other conditions are the same as in Fig. 1(C).

3.2. Chromatographic analysis and evaluation of the quantification method

Although LAP showed no apparent quenching effect on the fluorescence of the reaction in comparison with the substrate, the quenching effect brought about by the complex matrix in biological samples was severe [20]. Therefore, all the components in the incubation mixture should be separated from the hydrolytic product for accurate measurement of the fluorescence. Under optimal conditions as described in Section 2, the components in a formulated incubation mixture were baseline separated and eluted out of the column within 15 min as shown in Fig. 2. For simplicity, leucine was not included in the formulated mixture, but it was well separated from β-NapNH with retention time at 3.0 min when injected onto the C_4 column individually (data not shown). By spiking known amount of β-NapNH in the formulated mixture and injecting 5 μl of the solution into the C_4 column, the detection limit was found to be 0.35 pmol (injection amount) at three times S/N ratio. The quantitation showed a dynamic range from 10 ng/ml to 80 μg/ml with $R^2 = 0.9999$ and reproducibility <3% R.S.D. for triplicate injections. Beyond the upper quantitation limit, the fluorescence detector approached saturation. For comparison, the quantitation results using spectrophotometry (Sigma Diagnostic method) are given in Table 1. With regard to the limit of detection, dynamic range, and total analysis time, the present method is more sensitive and convenient than the classic Sigma Diagnostic method.

To select a proper incubation time that allows the hydrolytic product to be detectable at short period of time, we compared the change of peak area of the hydrolytic product at two incubation times and at different LAP concentrations. As can be seen in Fig. 3, the peak area of the hydrolytic product increases linearly

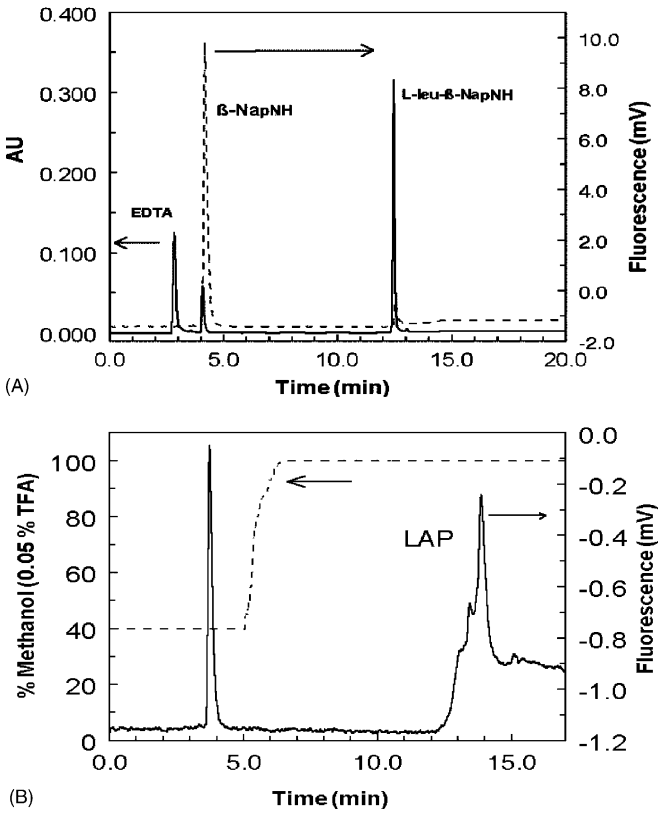

Fig. 2. Chromatograms of the formulated incubation mixture and the LAP standard separated on the C_4 column under the elution conditions as given in Section 2. (A) Chromatogram of the formulated incubation mixture containing 7.0 μmol/l β-NapNH. Injection, 5 μl; flowrate, 0.5 ml/min. Detection: UV (solid line), $\lambda = 280$ nm; fluorescence (dashed line), $\lambda_{Ex} = 280$ nm, $\lambda_{Em} = 400$ nm. (B) Chromatogram of the LAP standard solution obtained under the same elution profile (dashed line) as in Fig. 2(A). LAP concentration, 0.4 mg/ml; injection, 5 μl.

Table 1
Comparison of the quantitation results obtained by the RPC_4/fluorescence and Sigma Diagnostics of LAP

	Present method	Sigma Diagnostic method[a]
Detection conditions	$\lambda_{Ex} = 280$ nm; $\lambda_{Em} = 400$ nm	$\lambda = 565$ nm
Limit of detection[b]	10 ng/ml	30 ng/ml
Dynamic range	10 ng/ml to 80 μg/ml	30 ng/ml to 50 μg/ml
Linearity	0.9999	0.9996
Total analysis time[c]	<15 min	>50 min

[a] The reproducibility for triplicate measurement is <6%.
[b] Detection limit was estimated at three times S/N ratio.
[c] The total analysis time is estimated for single run measurement.

with increase of LAP concentrations up to 45 μg/ml with $R^2 > 0.998$ for 30 min incubation time. On the other hand, this linearity is only maintained at LAP concentrations <35 μg/ml for 60 min incubation time, indicating that the pseuo-first order condition could not be maintained at higher LAP concentrations. Although 60 min incubation time showed a bit narrower dynamic range for LAP quantitation, the method allows a wide selection of incubation time within 1 h and quantitation of LAP in the range indicated in Fig. 3.

3.3. LAP activities in ocular samples

As LAP mRNAs are widely expressed, it may play an important role in the cellular functions of many

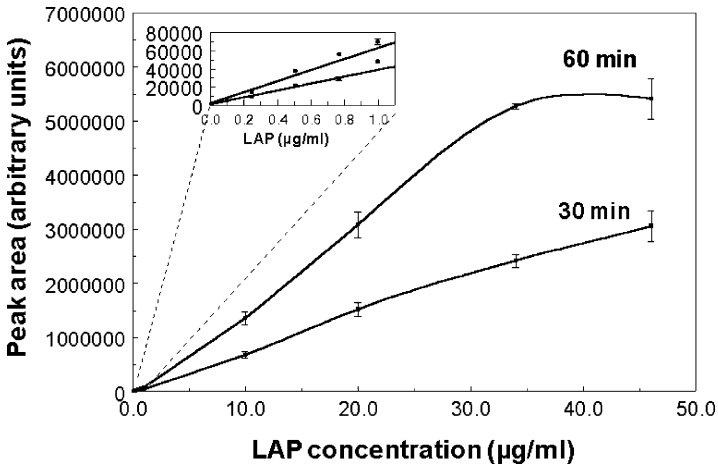

Fig. 3. Changes in fluorescence of the hydrolytic product at different LAP concentrations for 30 and 60 min incubation at 35.0 °C. Conditions: the substrate, 2.4 mmol/l (0.70 mg/ml); buffer, 100 mmol/l phosphate at pH 7.4; total volume, 100 μl; detection, $\lambda_{Ex} = 280$ nm, $\lambda_{Em} = 400$ nm; LAP concentrations (0.1–46.0 μg/ml); injection, 5 μl incubation mixture + 5 μl of 100 mmol/l EDTA. R^2 of the linear lines are 0.9983 and 0.9739, respectively, for 30 and 60 min incubation times in the whole range of the LAP concentrations. Chromatographic conditions are the same as in Fig. 2.

tissues [3,4]. In ophthalmology, LAP has been described as the predominant protease in lens metabolism [18]. However, a clear view of its role in ocular tissues is still obscure. Due to the fact that LAP can cleave several peptide hormones such as oxytocin, vasopressin, and angiotensins [19], it is probably involved in the regulation of the vasoconstrictive activities of the ocular vessels or of tear flow. Knowledge of its activity is of great help for surgeons to manage the post-operative symptoms in ocular surface surgery. Using the present method, we determined the LAP activities in mouse lens extract, rabbit serum and tears as given in Table 2. As the complex matrices of the samples were well separated from β-NapNH and eluted out of the column as broad peaks at 12–18 min (data not shown). The column was regenerated for next injection with intra- and inter-day variation in retention times of the β-NapNH peaks being <1% R.S.D. ($n = 3$). Furthermore, The LAP amounts in the samples were approximately estimated using curves in Fig. 3. They were 0.3 mg/ml (<9% R.S.D.) in rabbit serum, 40.0 μg/ml (<11% R.S.D.) in rabbit tears, and 17.5 μg/ml (<11% R.S.D.) in mouse lens extracts, respectively, which account for

Table 2
LAP activities in rabbit serum, tears, and mouse lens homogenate obtained by the present and Sigma Diagnostic methods

Sample	LAP activity[a] (pmol/(μl min))			
	Fluorescence method	R.S.D. (%)	Sigma Diagnostic method[b]	R.S.D. (%)
Rabbit serum[c]	41.8 ± 0.5	<2	43.5 ± 2.7	<6
Rabbit tears[c]	2.8 ± 0.2	<7	2.7 ± 0.3	<10
Lens (mouse)[c]	1.6 ± 0.2	<9	2.0 ± 0.3	<13

[a] All measurements were repeated for at least three times.

[b] Ten microliter of the samples were diluted to 250 μl with Millipore water, which were then mixed with 250 μl of the substrate (reagent in Sigma Diagnostics kit). After incubation for 30 min at 35.0 °C, 250 μl of 2 mol/l HCl (reagent in the kit) was added to stop the reaction. The assay of the LAP activity was performed against the diluted sample blanks following the given instruction.

[c] The total protein concentration in the samples was determined using Bradford method as described in Section 2. They are 53.0 mg/ml (<9% R.S.D.) for serum, 7.1 mg/ml (<3% R.S.D.) for tears, and 2.0 mg/ml for the lens homogenate (<13% R.S.D.) with triplicate measurements.

about 0.6% of the total protein in serum and tears, and 0.9% of the total protein in the lens homogenate. Nevertheless, the substrate used in the present study could also be hydrolyzed by aminopeptidase N or membrane alanyl aminopeptidase (EC 3.4.11.2) and cystinyl aminopeptidase (EC 3.4.11.3) [24], the LAP concentration estimated here would be higher than it is if those aminopeptidases were co-existed in the assayed samples. In addition, several unidentified fractions in tissue/cell extracts and serum have been reported to hydrolyze the substrate [25], the protein amount estimated using the present method should be interpreted as the total proteins that could hydrolyze the substrate in such samples.

The present method provides a practical alternative to estimate the LAP activity and its amount presented in the samples simultaneously by only measurement of the fluorescence of the hydrolytic product. In comparison, the LAP activities in the same samples were also determined using the Sigma Diagnostic method as given in Table 2. The results obtained by the present method are in good agreement with that observed by the conventional one.

As the background matrices were removed on-column and no conversion of the hydrolytic product into a diazo complex was needed, the present method is convenient and reproducible. It has been proved to be sensitive and accurate for rapid assessment of LAP activity in tissue samples and biological fluids. The method can be utilized over a fairly broad range of incubation times and enzyme concentrations at different situations to allow further characterization of LAP related cellular functions and diseases diagnosis. It can be used as routine assay of LAP activity in clinical samples.

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