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High-performance liquid chromatographic–fluorimetric assay of chymotrypsin-like esterase activity

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Abstract

A sensitive and reproducible assay for the determination of chymotrypsin-like esterase activity is reported. This method is based on fluorimetric detection of a dansylated amino acid, 5-dimethylaminonaphthalene-1-sulfonyl-L-phenylalanine, enzymatically formed from the substrate 5-dimethylaminonaphthalene-1-sulfonyl-L-phenylalanine ethyl ester, after separation by high-performance liquid chromatography using a C_{18} reversed-phase column and isocratic elution. This method is sensitive enough to measure 5-dimethylaminonaphthalene-1-sulfonyl-L-phenylalanine at concentrations as low as 40 pmol/ml, yields highly reproducible results and requires less than 9.5 min per sample for quantitation. The optimum pH for chymotrypsin-like esterase activity was 7.7–8.3. The K_m and V_{max} values were, respectively 25 μM and 0.241 pmol/μg protein/h with the use of enzyme extract obtained from mouse kidney. The approximate molecular mass of this enzyme was estimated to be 67 000 by gel filtration. Chymotrypsin-like esterase activity was strongly inhibited by *N*-tosyl-L-phenylalaline chloromethyl ketone. Among the mouse organs examined, the highest specific activity of the enzyme was found in lung. This new method would be useful for clarification of the physiological role of this enzyme.

Keywords: Chymotrypsin-like esterase; Enzymes

1. Introduction

Esterases (ester hydrolases) (EC 3.1.1.) are widely distributed in nature and catalyze the hydrolysis of ester bonds [1]. On the basis of oligopeptide subsite mapping studies, chymotrypsin-like esterase shows a preferential substrate specificity for Phe/Tyr in the P1 position [2]. Although chymotrypsin-like esterases have been isolated and characterized in the secretory granules of lymphocytes, mast cells, neutrophils and macrophages in various tissues [3–10], the real physiological functions of these esterases are not clear. Recently, Hanna et al. [11] isolated and purified a chymotrypsin-like esterase from human lymphocyte granules that reacted preferentially with the oligopeptide substrate succinyl–Phe–Leu–Phe–

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thiobenzyl ester. Furthermore, this enzyme has enzymatic characteristics similar to cathepsin A [12], and is identical with the so-called lysosomal protective protein [13–16].

Lysosomal protective protein was initially identified as a glycoprotein regulating the expression of β-galactosidase (EC 3.2.1.23) and neuraminidase (EC 3.2.1.18) through the formation of a high-molecular-mass multienzymic complex in lysosomes. It is synthesized in human fibroblasts as a single precursor and then processed to a mature form, consisting of 32- and 20-kDa peptides linked by disulfide bonds [13]. A deficiency of this protein in humans leads to a genetically determined metabolic storage disorder, galactosialidosis, that can be fatal to infants [17,18].

The most commonly used assay system for detecting and estimating chymotrypsin-like esterase activity is based on the ability of the enzyme preparation to convert synthetic substrates such as benzyloxycarbonyl–L-Phe–*p*-nitrophenyl ester [4] and benzyloxycarbonyl–L-Tyr–*p*-nitrophenyl ester [7] to the corresponding product *p*-nitrophenol. The degree of conversion to product is determined spectrophotometrically at 410 nm. Further, synthetic peptides, have also been prepared and utilized as substrates for chymotrypsin-like esterase activity. These substrates are succinyl–Phe–Leu–Phe–thiobenzyl ester [19] and *N*-acetyl–D,L-Phe–β-naphthyl ester [20], and they were based on derivatization of product formed enzymatically with 4,4-dithiodipyridine and naphthanil diazo Blue B reagent, respectively. A few other assays of this enzyme activity have been reported. These include a continuous spectrophotometric assay using benzoyl–L-Tyr ethyl ester as substrate [21] and a fluorimetric assay using benzyloxycarbonyl–Phe–4-methyl umbelliferyl ester as substrate [22].

In this paper, we describe a new and sensitive assay for chymotrypsin-like esterase activity using 5-dimethylaminonaphthalene-1-sulfonyl-L-phenylalanine ethyl ester (N-DNS–Phe–OEt) as substrate by high-performance liquid chromatography (HPLC) on a reversed-phase column to achieve a rapid and selective separation of substrate and product. This system is suitable for a routine assay of chymotrypsin-like esterase activity.

2. Experimental

2.1. Chemicals

Dithiothreitol, ascorbic acid and 1,10-phenanthroline monohydrate were purchased from Wako (Tokyo, Japan). 5-Dimethylaminonaphthalene-1-sulfonyl chloride (N-DNS-chloride), 5-dimethylaminonaphthalene-1-sulfonyl–L-phenylalanine free acid (N-DNS–Phe), L-phenylalanine ethyl ester hydrochloride, *N*-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, *p*-chloromercuriphenylsulfonic acid (PCMS), diisopropylfluorophosphate (DFP), iodoacetic acid (IAA), *N*-tosyl–L-phenylalanine chloromethyl ketone (TPCK), reduced glutathione and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Antipain and leupeptin were from Peptide Institute (Osaka, Japan). 1-Heptanesulfonic acid sodium salt was from Aldrich (Milwaukee, WI, USA). Sephacryl S-200 HR was from Pharmacia (Uppsala, Sweden). Acetonitrile was of chromatographic grade (Wako). Other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of enzyme source

Male ICR mice weighting 20–25 g were purchased from Charles River (Japan) and housed on a 12-h light–dark cycle for at least 1 week before the beginning of all experiments. Food and water were available ad libitum. All operations were carried out at 0–4°C unless stated otherwise. Mice were killed by decapitation. After washing the whole kidney with saline, it was cut into small pieces and homogenized in nine volumes of 0.25 *M* sucrose with a glass–PTFE homogenizer. Homogenates were separated according to the method of Deguchi et al. [23].

Each homogenate was centrifuged at 900 *g* for 10 min to separate a crude nuclear fraction (P_1), at 5000 *g* for 10 min to separate a crude mitochondrial fraction (P_2), at 8000 *g* for 10 min to separate a crude lysosomal fraction (P_3) and, finally, at 105 000 *g* for 60 min to separate a microsomal fraction (P_4) and a supernatant (S; the soluble fraction). The

pellets (P_1, P_2, P_3 or P_4) were resuspended in ice-cold 0.25 *M* sucrose.

In order to determine the localization of enzyme activity, various organs were dissected on ice. Tissues were homogenized in nine volumes of 0.25 *M* and/or 0.32 *M* sucrose with a glass–PTFE homogenizer. The homogenates were used as an enzyme source.

2.3. Peptide synthesis

N-DNS–Phe–OEt was synthesized by the method of Wiedmeier et al. [24] with minor modifications. A 218-μmol amount of L-phenylalanine ethyl ester hydrochloride was dissolved in 4 ml of 100 m*M* sodium carbonate–sodium bicarbonate buffer (pH 9.6). To this solution, 44 μmol of N-DNS–chloride in 6 ml of acetone was added. The reaction mixture was stirred at room temperature overnight, and thereafter the acetone was evaporated under reduced pressure. The synthetic dansylated product was purified by reversed-phase HPLC on a TSK gel ODS-PREP column (300×7.8 mm I.D., particle size, 10 μm, Tosoh) before use. Following purification, the structure was confirmed by nuclear magnetic resonance and infrared spectra.

5-Dimethylaminonaphthalene-1-sulfonyl–L-Phe–L-Leu (N-DNS–Phe–Leu) was also prepared as reported previously [25].

2.4. Assay of chymotrypsin-like esterase activity

The assay of chymotrypsin-like esterase activity is based on the fluorimetric measurement of N-DNS–Phe liberated enzymatically from the substrate, N-DNS–Phe–OEt, after separation by HPLC. The reaction mixture contained 50 m*M* Tris–HCl buffer (pH 7.9), 40 μ*M* N-DNS–Phe–OEt, and enzyme plus water in a total reaction volume of 250 μl. Incubation was carried out at 37°C, and the reaction was terminated by heating at 95°C for 5 min in boiling water. After centrifugation, N-DNS–Phe–Leu was added to clear supernatant as the internal standard, and an aliquot of the mixture obtained was subjected to HPLC analysis. The net peak height of N-DNS–Phe liberated enzymatically from the substrate (experimental incubation) was obtained by subtracting the peak height of N-DNS–Phe formed non-enzymatically (control incubation), and determined the amount from the peak height of N-DNS–Phe–Leu added as an internal standard. The net peak height of N-DNS–Phe of standard incubation was also obtained by subtracting the peak height of N-DNS–Phe formed non-enzymatically (blank incubation). Three blank incubations were performed to obtain the peak height of N-DNS–Phe formed non-enzymatically in each experiment. One unit of enzyme activity is defined as the amount of enzyme necessary for the conversion of 1 pmol of the substrate into the corresponding product in 1 min at 37°C.

2.5. Chromatographic conditions

Analysis of the product was performed using a Shimadzu (Kyoto, Japan) HPLC system consisting of a LC-10AD pump, RF-10A fluorescence detector, CTO-10A column oven, DGU-12A degasser, SCL-10A system controller and C-R6A Chromatopac. The system was operated at 35°C at a flow-rate of 0.9 ml/min employing a TSK-gel ODS-80TM (particle size, 5 μm) reversed-phase column (150×4.6 mm I.D.) fitted with a TSK guard gel ODS-80TM (15×3.2 mm I.D., particle size, 5 μm). The mobile phase consisted of 10 m*M* sodium acetate buffer containing 0.1% 1-heptanesulfonic acid sodium salt (pH 3.5)–acetonitrile (35:65, v/v). The fluorescence was monitored with excitation at 330 nm and emission at 458 nm.

2.6. Determination of molecular mass

All of the following procedures was carried out at 0–4°C. Mouse kidney (1.03 g) was homogenized in nine volumes of 0.25 *M* sucrose with a glass–PTFE homogenizer. The homogenate was centrifuged at 105 000 *g* for 80 min and the resulting supernatant was subjected to size-exclusion chromatography on a Sephacryl S-200 HR column (2.6×65 cm) equilibrated with 0.2 *M* Tris–HCl buffer (pH 7.5), and eluted with the same buffer at a flow-rate of 0.35 ml/min. The UV absorbance of the eluate was monitored at 280 nm, and 100 μl aliquoted from the column fractions (2.5 ml/tube) was assayed for

chymotrypsin-like esterase activity. The following proteins were used as molecular mass markers: BSA (dimer 130 000, monomer 67 000), ovalbumin (43 000), soybean trypsin inhibitor (20 100), myoglobin (18 800) and cytochrome C (12 500).

2.7. Protein determination

Protein concentration was measured by the modified Lowry method [26] using BSA as a standard protein.

2.8. Validation parameters

Recovery was assessed by spiking known amounts of N-DNS–Phe into the reaction mixture. The intra- and inter-assay precision and accuracy were evaluated to ensure reproducibility before actual assay. The intra-assay precision was assessed by analyzing six samples at three concentrations (0.2, 2.0 and 20.0 nmol/ml) for 1 day. The inter-assay precision was assessed by using the same method on 5 different days. The intra- and inter-assay precision of non-enzymatically formed N-DNS–Phe were also assessed by analyzing six samples for each of the periods described above.

3. Results

Subcellular distribution of mouse kidney revealed that approximately 17 and 61% of the total chymotrypsin-like esterase activity could be recovered in the P_4 and the supernatant fraction, respectively. Therefore, the supernatant fraction was used as a source of chymotrypsin-like esterase activity in further experiments.

This HPLC–fluorimetric detection system for the measurement of N-DNS–Phe–OEt and N-DNS–Phe was found to be very sensitive. The calibration graphs for N-DNS–Phe and N-DNS–Phe–Leu injected both showed good linearities with correlation coefficients of 0.9996 and 0.9993, respectively, within 40 pmol/ml and 40 nmol/ml. The recovery and precision data for N-DNS–Phe spiked into the reaction mixture are shown in Table 1. The recoveries (mean±SD, $n=6$) over three different concentrations were more than 98.8%.The intra- and inter-assay precision expressed as the relative standard deviations were $<2.0\%$. The intra- and inter-assay precision of non-enzymatically formed N-DNS–Phe expressed as RSD values were 1.93 and 1.90%, respectively. The statistical validation indicate satisfactory performance for the assay method. Fig. 1 shows the chromatographic patterns of the reaction mixture after 2 h incubation with 6.77 μg of protein prepared from mouse kidney supernatant. The blank incubation (Fig. 1A) contained N-DNS–Phe–OEt and N-DNS–Phe–Leu, and the standard incubation contained exogenous *N*-DNS–Phe in addition to N-DNS–Phe–OEt and N-DNS–Phe–Leu (Fig. 1B). The retention times for N-DNS–Phe, N-DNS–Phe–Leu and N-DNS–Phe–OEt were 3.3, 4.2 and 8.8 min, respectively (Fig. 1A and B). As shown in the blank incubation (Fig. 1A), a small peak of N-DNS–Phe, formed non-enzymatically from the substrate during incubation, was found at 3.3 min. The experimental incubation under the standard assay conditions (Fig. 1C) produced a significant amount of N-DNS–Phe in 3.3 min, whereas the

Table 1
Statistical validation for the determination of N-DNS–Phe in reaction mixture

Theoretical concentration (nmol/ml)	Intraassay ($n=6$)			Interassay ($n=6$)		
	Measured concentration mean±SD (nmol/ml)	RSD[a] (%)	Recovery (%)	Measured concentration mean±SD (nmol/ml)	RSD[a] (%)	Recovery (%)
0.2	0.198±0.004	2.0	99.0	0.199±0.002	1.0	99.6
2.0	1.990±0.026	1.3	99.5	1.980±0.015	0.8	98.8
20.0	20.120±0.186	0.9	100.6	20.020±0.362	1.8	100.1

[a] RSD, relative standard deviation.

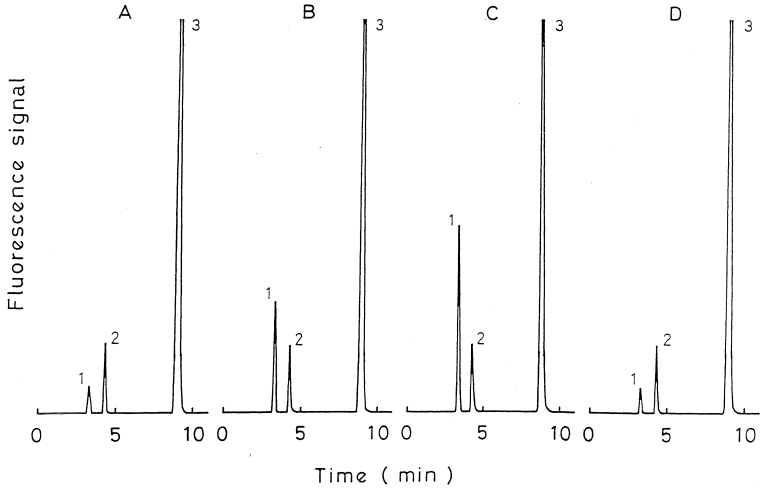

Fig. 1. HPLC elution patterns of chymotrypsin-like esterase activity determined using enzyme in mouse kidney. Conditions are described in Experimental. Peaks: 1=N-DNS–Phe; 2=N-DNS–Phe–Leu; 3=N-DNS–Phe–OEt. A 500-pmol amount of N-DNS–Phe–Leu (I.S.) was added to each sample after incubation. (A) Blank incubation: N-DNS–Phe–OEt was incubated without enzyme at 37°C for 2 h. (B) Standard incubation: 250 pmol of N-DNS–Phe was added to the sample tube before incubation as a standard sample. The two peak heights of N-DNS–Phe and N-DNS–Phe–Leu correspond 25 and 50 pmol, respectively. (C) Experimental incubation: N-DNS–Phe–OEt was incubated with 6.77 μg of protein in mouse kidney extract at 37°C for 2 h. (D) Control incubation: a control tube without the enzyme was incubated, the same amount of active enzyme was added, and the resulting tube was kept in an ice bath before heating at 95°C for 5 min.

control incubation produced a small amount of N-DNS–Phe (Fig. 1D). This small peak of N-DNS–Phe was also produced non-enzymatically during incubation.

The enzyme reaction was found to be linear with time at 37°C at least for about 6 h (data not shown).

The pH dependence of enzyme activity was investigated in 50 m*M* sodium phosphate buffer (pH 6.0–7.8), 50 m*M* EPPS–NaOH buffer (pH 7.5–8.4), 50 m*M* Tris–HCl buffer (pH 7.5–8.5) and 50 m*M* borate–KCl buffer (pH 8.0–8.8). The catalytic activity of the enzyme was greatest at a pH of ~7.7–8.3, with very little activity below pH 6.0 and above pH 8.5 (Fig. 2).

Chymotrypsin-like esterase activity was investigated as a function of the amount of enzyme extract obtained from mouse kidney. Perfect linearity was observed for plots of the amount of N-DNS–Phe, at least from 0.30 to 5.88 units, formed enzymatically from N-DNS–Phe–OEt against those of enzyme (data not shown).

Ascorbate and reduced glutathione, were examined for their ability to stimulate the chymotrypsin-like esterase activity. Two cofactors tested had no stimulating effect on the chymotrypsin-like esterase activity (data not shown).

A Lineweaver–Burk plot was obtained from the effect of the concentration of N-DNS–Phe–OEt on the rate of formation of N-DNS–Phe by chymotrypsin-like esterase. The Michaelis constant (K_m) and the maximum velocity (V_{max}) toward the N-DNS–Phe–OEt were calculated to be 25 μ*M* and 0.241 pmol/μg protein/h, respectively.

We applied this standard assay method for the distribution of chymotrypsin-like esterase activity in various mouse organs (Table 2). The enzyme activity was determined using the homogenates obtained from spleen, brain, kidney, liver, lung and testis of a 6-week-old mouse. It was found that the chymotrypsin-like esterase activity was distributed unevenly in mouse organs. Among the organs examined, the highest specific activity of the enzyme was found in the lung, and the lowest in the brain. High levels of activity were also observed in the kidney and liver. Moderate levels of activity were seen in the spleen and testis.

In addition, some enzymatic and physicochemical properties of chymotrypsin-like esterase in mouse

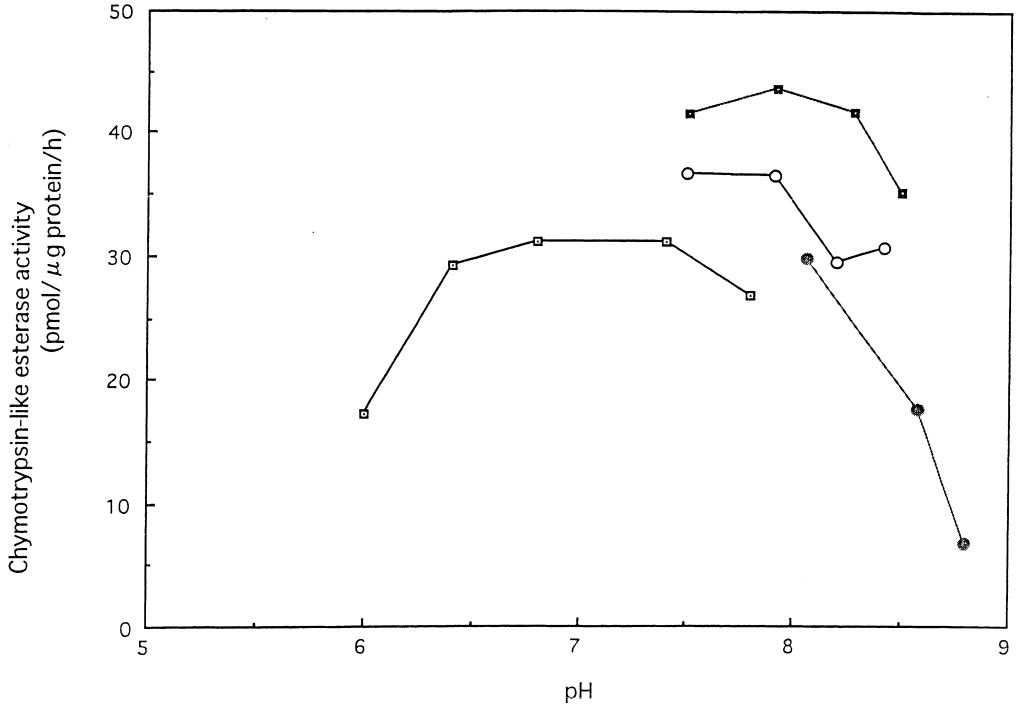

Fig. 2. Effects of pH on chymotrypsin-like esterase activity in mouse kidney: 50 m*M* sodium phosphate buffer (pH 6.0–7.8) (– □ –), 50 m*M* EPPS–NaOH buffer (pH 7.5–8.4) (– ○ –), 50 m*M* Tris–HCl buffer (pH 7.5–8.5) (– ■ –) and 50 m*M* borate–KCl buffer (pH 8.0–8.8) (– ● –) were used. Incubation was carried out at 37°C for 2 h.

kidney were studied by this assay. An estimation of the molecular mass of the enzyme was made by the gel filtration method using a Sephacryl S-200 HR column. The void volume of the column was determined with Blue Dextran 2000. From a plot of log M_r versus K_{av} for protein standards, the molecular mass of the enzyme was estimated to be approximately 67 000 (Fig. 3).

The effects of various metal ions and inhibitors on chymotrypsin-like esterase activity in mouse kidney extract were examined at a final concentration of 1 and 0.1 m*M*. As shown in Table 3, metals such as Hg^{2+}, Zn^{2+}, Fe^{3+} and Co^{2+} partially inhibited the enzyme activity. DFP (serine protease inhibitor) was also found to be inhibitory to the enzyme. Furthermore, TPCK, a typical chymotrypsin-like serine protease inhibitor, inhibited completely the enzyme activity at a final concentration of 0.1 m*M*. However, the enzyme activity was not affected by other reagents (dithiothreitol, 1,10-phenanthrolin, EGTA, PMSF, IAA, NEM, PCMS and pepstatin A). Further, the chymotrypsin-like esterase activity was not affected by antipain and leupeptin.

Table 2
Localization of chymotrypsin-like esterase activity in mouse organs[a]

Organ	Chymotrypsin-like esterase activity (pmol/μg protein/h)
Spleen	4.87±0.41
Brain	1.81±0.10
Kidney	13.43±0.50
Liver	12.73±0.69
Lung	17.62±1.10
Testis	5.79±0.37

[a] Data are mean±S.E.M. values from six animals.

4. Discussion

As pointed out in other HPLC enzymatic assays, the direct analysis of the product of enzyme action,

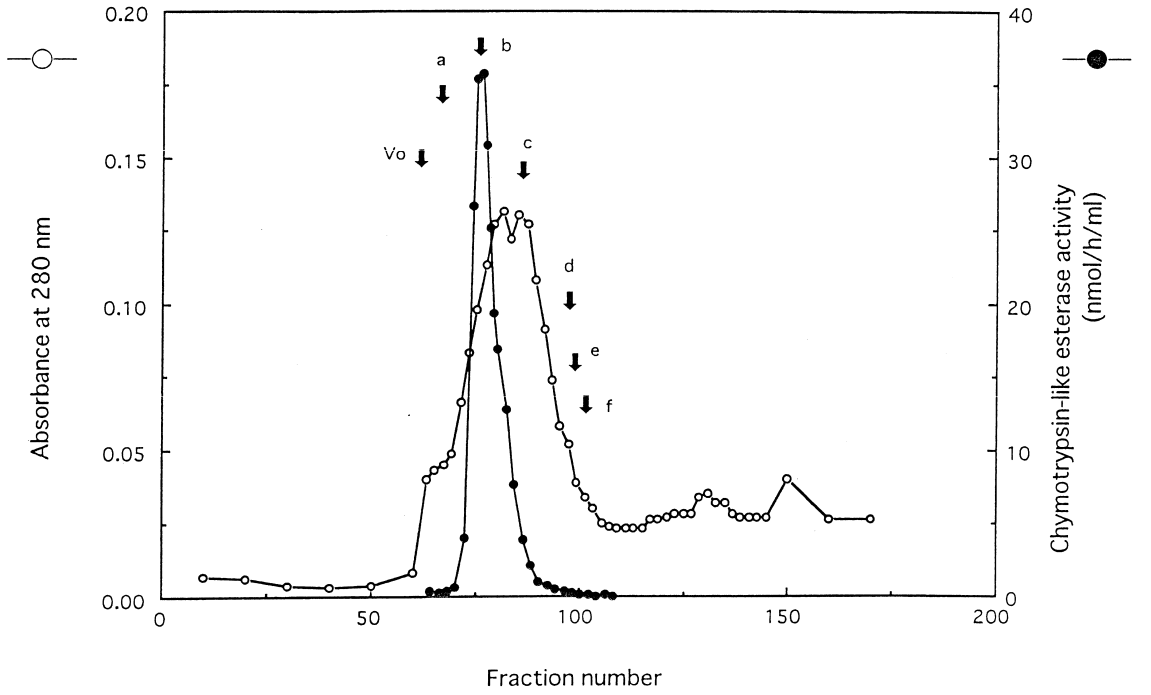

Fig. 3. Sephacryl S-200 HR column chromatography of the supernatant from mouse kidney. Chymotrypsin-like esterase activity (– ● –) and absorbance at 280 nm (– ○ –) were measured. Each arrow indicates: V_o, void volume; (a) BSA dimer (130 000); (b) BSA (67 000); (c) ovalbumin (43 000); (d) soybean trypsin inhibitor (20 100); (e) myoglobin (18 800) and (f) cytochrome C (12 500). Experimental details are described under Experimental.

separated from the substrate and other interfering substances, offers several advantages over the previous spectrophotometric methods.

Herein we report a new assay of chymotrypsin-like esterase activity by the HPLC–fluorimetric detection system using N-DNS–Phe–OEt as substrate. The amino acid of substrate, Phe, was selected because it has been used by several laboratories for detecting chymotrypsin-like esterase activity [3,4,7,20,22]. The proposed sensitive assay of chymotrypsin-like esterase activity has some advantages. First, it is very sensitive, because the detection limit was about 40 pmol/ml of N-DNS–Phe formed enzymatically. Second, more accurate quantitation of the product and better reproducibility were guaranteed in this method by the employment of an internal standard (N-DNS–Phe–Leu). Another fluorimetric assay using benzyloxycarbonyl–Phe–4-methyl umbelliferyl ester as substrate also possesses some advantages such as high sensitivity, rapidity and reproducibility [22].

We also describe some physicochemical properties of chymotrypsin-like esterase in mouse kidney. The approximate molecular mass of this enzyme is estimated to be 67 000 by gel filtration, and its optimum pH is around 8.0. The effects of various chemical reagents and protease inhibitors on chymotrypsin-like esterase activity are also investigated (Table 3). The chymotrypsin-like esterase activity in mouse kidney is completely inhibited by typical chymotrypsin-like serine protease inhibitor (TPCK) at a final concentration of 0.1 m*M*. Further, the enzyme activity was partially inhibited by serine protease inhibitor (DFP), indicating that this enzyme is a chymotrypsin-like serine protease. These properties are very similar to those of the enzymes from other sources [3–5,7,9,11].

Kase et al. [27] demonstrated simultaneous deficiency of chymotrypsin-like esterase, carboxy-terminal deamidase, and acid carboxypeptidase (cathepsin A) activities together with the protective activity against lysosomal/β-galactosidase and neur-

Table 3
Effects of various metal ions and inhibitors on activity of chymotrypsin-like esterase from mouse kidney[a]

Reagent	Final concentration (m*M*)	Chymotrypsin-like esterase activity (% of control)
None	–	100
$MgCl_2$	1	123.2
$MgCl_2$	0.1	114.3
$CaCl_2$	1	120.3
$CaCl_2$	0.1	116.5
$CuSO_4$	1	69.1
$CuSO_4$	0.1	106.6
$HgCl_2$	1	56.8
$HgCl_2$	0.1	64.3
$BaCl_2$	1	82.5
$BaCl_2$	0.1	111.0
$MnCl_2$	1	75.8
$MnCl_2$	0.1	97.9
$ZnCl_2$	1	40.1
$ZnCl_2$	0.1	61.3
$FeCl_3$	1	66.9
$FeCl_3$	0.1	84.5
$CoCl_2$	1	64.0
$CoCl_2$	0.1	77.7
Dithiothreitol	1	116.5
Dithiothreitol	0.1	115.0
1,10-Phenanthroline	1	86.1
1,10-Phenanthroline	0.1	88.1
Diisopropylfluorophosphate	1	31.3
Diisopropylfluorophosphate	0.1	41.0
Phenylmethylsulfonyl fluoride	1	83.9
Phenylmethylsulfonyl fluoride	0.1	96.6
Iodoacetic acid	1	109.7
Iodoacetic acid	0.1	109.8
N-Ethylmaleimide	1	104.5
N-Ethylmaleimide	0.1	105.5
EGTA	1	63.7
EGTA	0.1	104.4
p-Chloromercuriphenyl-sulfonic acid	1	110.0
p-Chloromercuriphenyl-sulfonic acid	0.1	110.2
N-Tosyl-L-phenylalanine chloromethyl ketone	1	0
N-Tosyl-L-phenylalanine chloromethyl ketone	0.1	0
Pepstatin A	20 μg/ml	122.8
Antipain	20 μg/ml	92.0
Leupeptin	20 μg/ml	91.7

[a] Note: the values shown are the means from two experiments.

aminidase in fibroblasts derived from patients with galactosialidosis. If chymotrypsin-like esterase is identical with the multifunctional enzyme protein (lysosomal protective protein) as reported previously [28], further considerations should be given to clarifying the relations between its functional aspects and enzymatic characterizations.

In conclusion, our developed sensitive, accurate and reliable assay of chymotrypsin-like esterase activity is considered to be useful for the above mentioned purposes.

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