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Purification and Characterization of Novel Trypsin-Like Serine Proteases from Mouse Spleen[1]

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Novel trypsin-like serine proteases (mouse trypsin-type serine proteases 1 and 2 [MTSP-1 and -2]) were purified to homogeneity from mouse spleen. Each protease consisted of a single polypeptide with a molecular mass of about 29 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. Both were totally inhibited by diisopropylfluorophosphate, soybean trypsin inhibitor, aprotinin, antipain, and leupeptin and partially inhibited by chymostatin and dithiothreitol, suggesting that they are trypsin-like serine proteases. They hydrolyzed synthetic substrates for trypsin-like proteases but not those for chymotrypsin-like proteases, elastase and kallikrein. MTSP-1 hydrolyzed *tert*-butyloxycarbonyl (Boc)-Asp(OBzl)Pro-Arg-amino-4-methyl-coumaryl-7-amide (MCA) and Boc-Ile-Glu-Gly-Arg-MCA faster than Boc-Phe-Ser-Arg-MCA. On the other hand, MTSP-2 hydrolyzed Boc-Phe-Ser-Arg-MCA most rapidly, with a specific activity 15 times higher than that of MTSP-1. The N-terminal amino acid sequence of MTSP-1 was Ile-Val-Gly-Gly-Tyr-Thr-His-Leu-Asp-Asn-Gln-Val-Pro-Tyr. This sequence was 71% homologous with the N-terminal of bovine trypsin. The Boc-Phe-Ser-Arg-MCA hydrolyzing activity of mouse spleen significantly ($p < 0.01$) increased to about 1.5-fold the basal activity 2 weeks after an injection of Freund's complete adjuvant, suggesting that these proteases are involved in the immune response.

Key words: adjuvant, immune response, mouse spleen, serine protease, trypsin-like.

Serine proteases are present in a variety of cells and their involvement in a diversity of immune responses has been demonstrated in cell killing by cytotoxic T lymphocytes (*1-8*), activation of B and T cells (*9-11*), and cell proliferation (*12-16*). Some serine protease inhibitors have been shown to inhibit several immune responses (*17-19*). Despite these well documented studies, the functions of serine proteases derived from lymphocytes remain poorly characterized.

Recently, we found that the Boc-Phe-Ser-Arg-MCA hydrolyzing activity of mouse spleen was elevated by the injection of Freund's complete adjuvant. In this study, we purified and characterized trypsin-like serine proteases (mouse trypsin-type serine proteases 1 and 2 [MTSP-1 and -2]) from mouse spleen. Their properties show that MTSP-1 and -2 are novel serine proteases. Our results suggest that MTSP-1 and -2 are involved in immune responses.

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Abbreviations: Boc, *tert*-butyloxycarbonyl; DFP, diisopropyl fluorophosphate; HIV, human immunodeficiency virus; IL-1, interleukin-1; MCA, 4-methyl-coumaryl-7-amide; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

MATERIALS AND METHODS

Materials—S-Sepharose and benzamidine-Sepharose 6B were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. The Biofine HIC-PH column was from Japan Spectroscopic, Tokyo. Various synthetic substrates and protease inhibitors were from the Peptide Institute, Osaka. All other reagents were commercial products of the highest grade available.

Methods—Enzyme and inhibitor assays: The Boc-Phe-Ser-Arg-MCA hydrolyzing activity of the protease was measured in 0.2 M Tris-HCl, pH 8.0, at 25°C by the method of Sawada *et al.* (*20*). One unit of activity is defined as the amount that released 1 μmol of 7-amino-4-methyl-coumarin/min/ml at 25°C. Inhibitors and activators, as solutions in H_2O, dimethyl sulfoxide, or ethanol, were preincubated for 5 min with the enzyme, then the reaction was started by adding the substrate. The concentration of dimethyl sulfoxide and ethanol in the reaction mixture did not exceed 1.0%. The residual enzyme activities were compared with that of the control mixtures with dimethyl sulfoxide or ethanol.

Assay of the effects of Freund's complete adjuvant on Boc-Phe-Ser-Arg-MCA hydrolyzing activities of mouse spleens: An equal volume of Freund's complete adjuvant was mixed with saline to form a stable emulsion and injected at a dose of 0.1 ml/pad into all the foot pads of 7-week-old BALB/c mice weighing 25-30 g. The mice were

given laboratory chow and water *ad libitum*. After the indicated period, the mice were killed and the spleens were homogenized in 10 volume of 50 mM sodium acetate buffer, pH 5.0, containing 0.3 M NaCl, in a Potter-Elevehjem glass homogenizer on ice. The enzyme activities were measured in the homogenates as described above.

Determination of kinetic parameters: The steady-state parameters K_m and k_{cat} were determined from initial velocity measurements at various substrate concentrations. The K_m values were calculated from double-reciprocal plots using a linear-regression program. The molecular masses of MTSP-1 and -2 were assumed to be 28.5 and 29 kDa, respectively, with one catalytic site per enzyme molecule.

Electrophoresis: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% gels containing 0.1% SDS at room temperature by the method of Laemmli (*21*). After electrophoresis, the proteins were stained with silver. The molecular markers were phosphorylase *b* (92 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Amino acid sequencing: MTSP-1 was bound to a PVDF membrane by centrifugation at 5,000×*g* with a ProSpin cartridge (Applied Biosystems, USA), washed with 20% methanol to remove the salt and glycerol and subjected to automated Edman degradation in an Applied Biosystems 492 pulsed-liquid sequencer. Phenylthiohydantoin (PTH)-amino acids from each cycle were analyzed on line using an Applied Biosystems 140C PTH-amino acid analyzer.

Protein determination: Protein was determined by the method of Lowry *et al.* (*22*), with bovine serum albumin as the standard.

RESULTS

Purification of Trypsin-Type Serine Proteases from Mouse Spleens—We purified two mouse trypsin-like serine proteases (MTSP-1 and -2) from mouse spleens. All procedures were carried out at 0-4°C and the enzyme activities were determined as described in "MATERIALS AND METHODS."

Fifty mouse spleens were homogenized in 10 volumes of 50 mM sodium acetate buffer, pH 5.0, containing 0.3 M NaCl and sonicated for 10 seconds on ice. After centrifugation at 10,000×*g* for 20 min, the active material was precipitated with 70% saturated ammonium sulfate and dissolved in a minimum volume of 20 mM sodium acetate buffer, pH 5.0, then eluted through a Sephadex G-25 column with 20 mM sodium acetate buffer, pH 5.0. The eluate was directly applied to a S-Sepharose column (5.0× 5.0 cm), equilibrated with the same buffer. The column was washed with the same solution until the absorbance of the eluate at 280 nm returned to the base line, then the material was eluted with 200 ml of a linear gradient of 0–0.8 M NaCl in the same buffer. Fractions of 4.0 ml were collected and the enzyme was eluted in a single peak at about 0.2 M NaCl, pooled, precipitated with 75% saturated ammonium sulfate, dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.0, containing 0.5 M NaCl and 10% (v/v) glycerol, then applied to a benzamidine-Sepharose 6B column (4.0 ml) equilibrated with the same solution. The column was washed until the absorbance at 280 nm returned to the base line, then the active material was eluted with 1 mM HCl, pH 3.0, containing 0.5 M NaCl and 10% (v/v) glycerol, and fractions of 7.0 ml were collected (Fig. 1). After concentration using a Diaflo YM-10 membrane (Amicon), the enzyme was fractionated by high performance liquid chromatography with a Biofine HIC-PH column (7.5×75 mm), equilibrated with 100 mM sodium acetate buffer, pH 5.0, containing 1.0 M ammonium sulfate and 10% (v/v) glycerol. The column was washed with the same solution and the enzyme was eluted with a linear gradient of 1.0–0 M ammonium sulfate in the same solution at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. At this step, the enzyme activities were detected in two peaks. These, termed MTSP-1 and MTSP-2, were eluted with 0.2 and 0 M ammonium sulfate respectively (Fig. 2). The purified enzymes were collected, concentrated by ultrafiltration and stored at −80°C until use.

Typical purifications of the proteases from mouse spleens are summarized in Table I. Starting with 5.0 g of spleens, 5.6 μg of MTSP-1 and 0.7 μg of MTSP-2 were obtained in 3.0 and 5.6% yield, respectively. The specific activity of purified MTSP-2 was 15 times higher than that of MTSP-1.

The final MTSP-1 and -2 preparations had the same

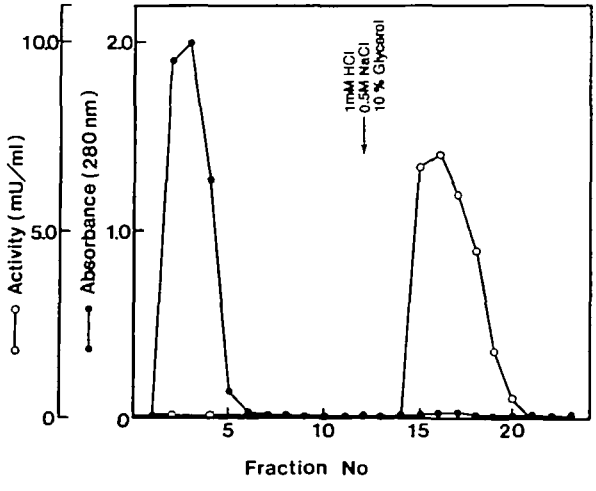

Fig. 1. **Chromatography of MTSP-1 and -2 on benzamidine-Sepharose 6B.** ●, absorbance at 280 nm; ○, Boc-Phe-Ser-Arg-MCA hydrolyzing activity.

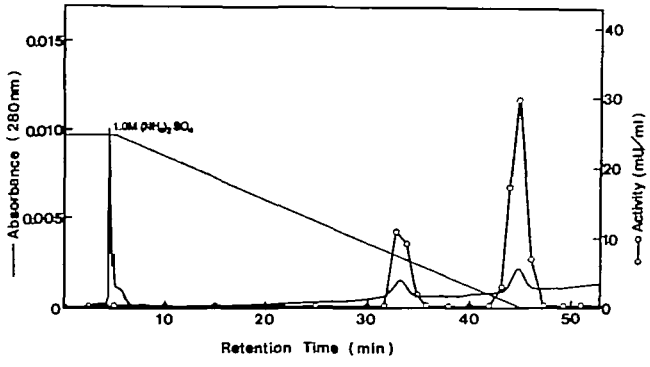

Fig. 2. **High performance liquid chromatography of MTSP-1 and -2 on Biofine HIC-PH.** —, absorbance at 280 nm; ○, Boc-Phe-Ser-Arg-MCA hydrolyzing activity.

molecular mass of about 24 kDa, by Sephadex G-75 gel filtration chromatography (data not shown). In addition, the enzymes gave single bands on SDS-PAGE under reducing conditions, with molecular mass of 28.5 and 29 kDa, respectively (Fig. 3). These results indicate that MTSP-1 and -2 consist of single polypeptides.

The Effects of pH on the Boc-Phe-Ser-Arg-MCA Hydrolyzing Activities—Figure 4 shows the effects of pH on the activities of the two enzymes using Boc-Phe-Ser-Arg-MCA as the substrate. Both enzymes had maximal activities at

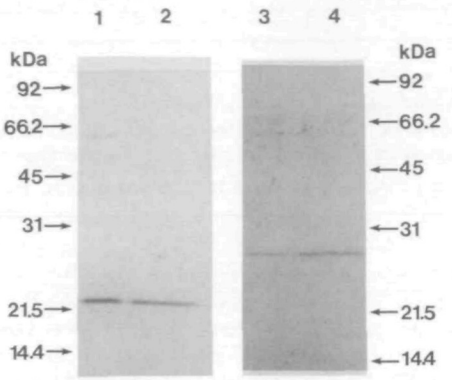

Fig. 3 **SDS-PAGE of purified MTSP-1 and -2.** Electrophoresis was conducted in 12.5% gel as described under the "MATERIALS AND METHODS." Lanes 1 and 3, 3.2 μg of purified MTSP-1 under nonreducing and reducing conditions, lanes 2 and 4, 2.0 μg of purified MTSP-2 under nonreducing and reducing conditions. Molecular mass of marker proteins are shown on the left and right.

TABLE II **Activities of MTSP-1 and -2 on synthetic substrates.**

Substrates (100 μM)	Relative activity[a] (%)	
	MTSP-1	MTSP-2
Boc-Phe-Ser-Arg-MCA (trypsin)[b]	100.0	100.0
Boc-Val-Pro-Arg-MCA (α-thrombin)	17.5	71.4
Boc-Asp(OBzl)Pro-Arg-MCA (α-thrombin)	297.4	49.1
Boc-Ile-Glu-Gly-Arg-MCA (factor Xa)	207.9	55.4
Pro-Phe-Arg-MCA (pancreatic, urinary kallikrein)	39.5	12.5
z-Phe-Arg-MCA (plasma kallikrein, cathepsin B)	13.2	0.0
Boc-Val-Leu-Lys-MCA (plasmin)	94.7	2.7
Glt-Gly-Arg-MCA (urokinase)	42.1	8.9
Suc-Leu-Leu-Val-Tyr-MCA (chymotrypsin)	0.0	0.0
Suc-Ala-Pro-Ala-MCA (elastase)	0.0	0.0

[a]Relative activities of MTSP-1 and -2 are percentages of activity toward Boc-Phe-Ser-Arg-MCA. [b]The enzymes for which the compounds are substrates are shown in parentheses.

TABLE III **Kinetic constants of MTSP-1 and -2.**

	K_m (μM)		k_{cat}[a] (s^{-1})		k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	
	MTSP-1	MTSP-2	MTSP-1	MTSP-2	MTSP-1	MTSP-2
Boc-Phe-Ser-Arg-MCA	10.87	1.12	0.51	7.40	0.05	6.61
Boc-Val-Pro-Arg-MCA	5.00	3.53	0.11	3.93	0.02	1.11
Boc-Asp(OBzl)-Pro-Arg-MCA	13.70	1.70	1.63	5.08	0.12	2.99

[a]k_{cat} is expressed in moles of product formed per mole of enzyme per second.

TABLE I. **Purification of MTSP-1 and -2 from mouse spleens.**

Step	Total volume (ml)	Total protein (mg)	Total activity (mU)[a]	Specific activity (mU/mg)	Recovery (%)
Extract	62.0	248.62	203.11	0.82	100.0
70% $(NH_4)_2SO_4$ ppt fraction	60.0	121.59	224.64	1.85	110.6
S-Sepharose	7.2	20.23	188.70	9.33	92.9
Benzamidine-Sepharose 6B	1.8	0.06	66.27	1,142.50	32.6
Biofine HIC-PH, HPLC					
MTSP-1	4.0	0.0056	5.99	1,069.64	3.0
MTSP-2	4.0	0.0007	10.48	14,971.43	5.2

[a]Units were determined as Boc-Phe-Ser-Arg-MCA hydrolyzing activity.

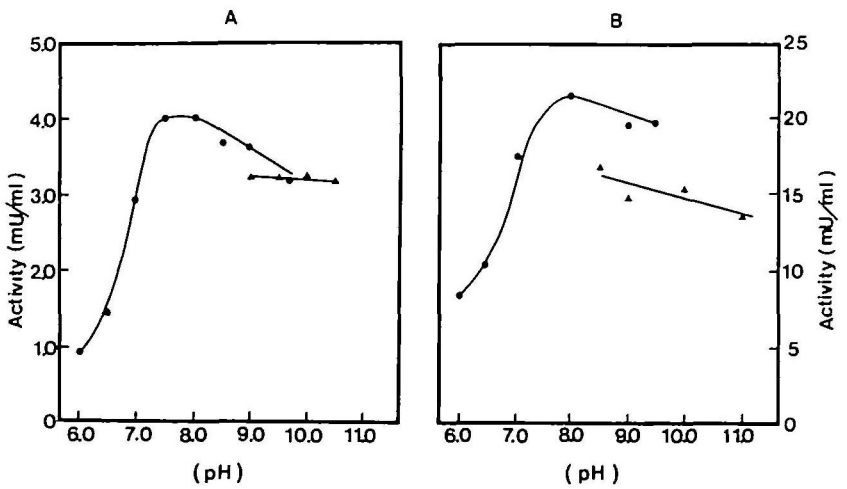

Fig. 4. **The effects of pH on the activities of MTSP-1 and -2.** The protease activities of 3.8 μg of MTSP-1 (A) and 1.2 μg of MTSP-2 (B) were measured using Boc-Phe-Ser-Arg-MCA as substrate. The buffers used at a final concentration of 0.2 M were Tris-HCl buffer between pH 6.0 and 9.5 (●) and glycine-NaOH between pH 8.5 and 11.0 (▲).

TABLE IV. **The effects of inhibitors and other compounds on the activities of MTSP-1 and -2.**

Compounds (10 μM)	Relative activity (%)[a]	
	MTSP-1	MTSP-2
None	100.0	100.0
DFP (500 μM)	37.9	25.3
(1 mM)	12.9	2.5
TPCK	100.0	114.7
TLCK	83.6	88.2
Soybean trypsin inhibitor	0.0	0.0
Aprotinin	0.0	0.0
Antipain	6.6	1.9
Leupeptin	1.0	3.0
Chymostatin	43.1	37.0
Elastatinal	94.1	103.7
E-64	66.7	111.1
Pepstatin A	92.1	103.7
Amastatin	94.8	85.0
Bestatin	90.2	96.3
Arphamenine A	92.1	85.2
Arphamenine B	92.1	96.3
Phosphoramidon	95.1	107.5
DTT (10 mM)	8.3	65.0
EDTA (10 mM)	107.3	90.0
EGTA (10 mM)	87.8	80.0
$CaCl_2$ (10 mM)	90.2	105.0

[a]Values are remaining activities of MTSP-1 and -2 as percentages of the activities on Boc-Phe-Ser-Arg-MCA without compounds, measured as described in "MATERIALS AND METHODS."

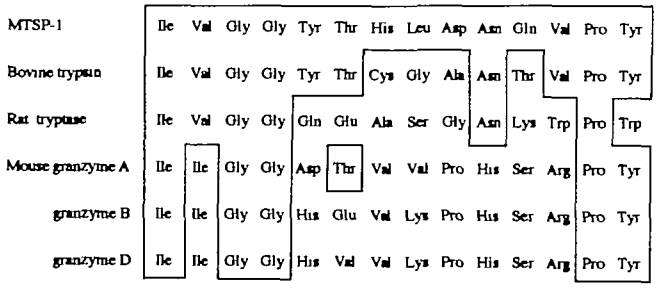

Fig. 5. **N-terminal sequence analysis of MTSP-1.** The first fourteen amino acid residues of MTSP-1 were determined on a pulsed-liquid sequencer. The N-terminal sequences of bovine trypsin (*23*), rat tryptase (*35, 36*), and mouse granzymes (*26*) are shown for comparison.

pH 8.0 and retained high activities between pH 7.0 and 11.0, suggesting that they are neutral and alkaline proteases.

Substrate Specificities—The activities of the purified enzymes for various synthetic peptide substrates are shown in Table II. MTSP-1 and -2 hydrolyzed the trypsin substrates rapidly but they did not hydrolyze those of chymotrypsin, elastase, kallikrein, and urokinase. MTSP-1 hydrolyzed Boc-Asp(OBzl)Pro-Arg-MCA and Boc-Ile-Glu-Gly-Arg-MCA, 3- and 2-fold faster respectively, than Boc-Phe-Ser-Arg-MCA, whereas MTSP-2 preferentially hydrolyzed Boc-Phe-Ser-Arg-MCA. The kinetic constants of MTSPs towards three typical synthetic peptide substrates are shown in Table III. The specificity constant (k_{cat}/K_m) values of MTSP-2 towards all the substrates examined were about 25–130 times higher than those of MTSP-1. The values also indicated that Boc-Asp(OBzl)Pro-Arg-MCA and Boc-Phe-Ser-Arg-MCA were the best substrates for MTSP-1 and MTSP-2, respectively.

TABLE V. **The effects of the injection of Freund's complete adjuvant on the activities of trypsin-like serine protease in mouse spleens.** Freund's complete adjuvant was injected into the foot pads. At the indicated times, the mice were bled and the Boc-Phe-Ser-Arg-MCA hydrolyzing activities of the homogenates of the mouse spleens were measured as described in "MATERIALS AND METHODS." Values are means ± standard deviations ($n=7$).

Weeks after injection	Specific activity (μU/mg)
0	297.3 ± 56.7
1	206.1 ± 19.6
2	422.2 ± 85.8[a]
3	471.3 ± 145.4[a]

[a]Significant difference from the control value ($p<0.01$).

The Effects of Inhibitors on Boc-Phe-Ser-Arg-MCA Hydrolyzing Activities—Table IV shows the effects of various inhibitors and several compounds on the Boc-Phe-Ser-Arg-MCA hydrolyzing activities of the purified proteases. DFP inhibited both MTSP-1 and -2, suggesting that they are serine proteases. Natural and microbial peptide inhibitors of trypsin, soybean trypsin inhibitor, such as aprotinin, antipain and leupeptin, at a concentration of 10 μM almost completely inhibited both proteases. Elastatinal, an inhibitor of elastase had no effect. E-64, a thiol protease inhibitor partially inhibited MTSP-1, but did not affect MTSP-2. Inhibitors of aminopeptidases and metalloendopeptidases had no effect on either protease. Dithiothreitol at a concentration of 10 mM totally inhibited MTSP-1, but partially inhibited MTSP-2.

The N-Terminal Amino Acid Sequence of MTSP-1—The N-terminal amino acid sequence of MTSP-1 was determined (Fig. 5). MTSP-1 did not start with a common Ile-Ile-Gly-Gly sequence of granzymes, but with Ile-Val-Gly-Gly sequence as same as bovine trypsin and rat tryptase and showed the highest homology (71%) with trypsin.

The Effects of Freund's Complete Adjuvant on the Boc-Phe-Ser-Arg-MCA Hydrolyzing Activities in the Mouse Spleens—The Boc-Phe-Ser-Arg-MCA hydrolyzing activities of the mouse spleens were measured at the indicated intervals after the injection of Freund's complete adjuvant into the foot pads (Table V). The specific activity significantly increased about 1.5-fold two weeks after the injection ($p<0.01$).

DISCUSSION

We purified two novel trypsin-like serine proteases (MTSP-1 and -2) from normal mouse spleens to homogeneity. MTSP-1 and -2 had similar molecular mass and the same optimal pH. They hydrolyzed the substrates of trypsin-like serine proteases and were inhibited by trypsin inhibitors such as DFP, soybean trypsin inhibitor, aprotinin, leupeptin, and antipain. In addition, these trypsin-like properties of MTSP-1 were supported by the fact that the N-terminal sequence showed high homology (71%) with bovine trypsin (*23*). MTSP-2 hydrolyzed Boc-Phe-Ser-Arg-MCA preferentially with a k_{cat}/K_m value toward the substrate that was 130-fold higher than that of MTSP-1. The different substrate specificities and different susceptibilities of MTSP-1 and -2 to dithiothreitol suggest that

MTSP-1 and MTSP-2 are distinct enzymes. However, we cannot exclude the possibility that one protease is derived from the other as a result of modification during the purification. We are currently studying the relation between MTSP-1 and -2 by analyzing the N-terminal sequences.

A serine protease of B lymphocytes is reportedly induced by anti-immunoglobulin and is secreted during the mitogenesis-linked process (*24*). Biró *et al.* also reported that a trypsin-like serine protease which is expressed at high levels on the cell surface of activated B cell lines might be implicated in C3 activation on B lymphocytes and play a role in facilitating cell-cell interactions in the same way as the serine proteases which are expressed on the surface of T lymphocytes and mature macrophages (*25*). These trypsin-like serine proteases, however, have not yet been purified or characterized. The B cell proteases should be further characterized and the reported B cells clones analyzed using antibodies against MTSP-1 and -2 to clarify the relationship between the MTSPs and B cell proteases.

Among the serine proteases of T lymphocytes, granzymes are fairly well characterized enzymes. Although granzymes A, B, D, F, and H have been labeled with [^{3}H]-DFP and have serine residues at the active sites (*26*), only granzymes A, B, and F were found to have enzymatic activities toward the synthetic substrates (*27, 28*). No substrates have not been found for murine granzymes C, D, and E. Granzyme A hydrolyzes several Arg- or Lys-containing thiobenzyl esters, suggesting that it is a trypsin-like protease. However, granzyme A has a relative molecular mass of 35/60 kDa (reduced/unreduced). Granzyme B does not hydrolyze substrates containing Lys or Arg at P_1 site but effectively hydrolyzes Boc-Ala-Ala-Asp-SBzl. Granzyme F has a chymotrypsin-like activity toward Suc-Phe-Leu-Phe-SBzl, when the enzyme is assayed at a high concentration. Granzyme D has been reported to be a trypsin-like protease which hydrolyzed Suc-Ala-Phe-Lys-AMC and Pro-Phe-Arg-AMC at pH 5.0 (*29*). Odake *et al.* concluded that the trypsin-like activities in the fractions of granzymes D, E, and F were due to another unidentified tryptase or contaminating granzyme A (*28*). In addition, the granzymes are expressed in only cytotoxic T lymphocytes, as shown by RNA blot hybridization (*30*). However, we detected higher Boc-Phe-Ser-Arg-MCA hydrolyzing activity in BALB/c nu-/- mouse than that in the normal mouse spleens, suggesting that the trypsin-like protease is present in cells other than mature T lymphocytes (data not shown). The N-terminal sequence of MTSP-1, Ile-Val-Gly-Gly, reveals that MTSP-1 does not belong to the granzyme family, which start with a common Ile-Ile-Gly-Gly sequence. These differences in enzymatic properties between MTSPs and granzymes suggest that MTSPs are different from the granzymes which have been characterized so far.

A plasminogen activator (PA), which is a trypsin-like serine protease, is produced and secreted from macrophages stimulated by thioglycollate (*31*). It is involved in the release of IL-1 from activated monocytes (*32, 33*). Differences in the molecular mass and the effects of aprotinin and detergents, such as SDS and Triton X-100, on their activities distinguish MTSPs from PA (*34*).

A trypsin-like serine protease, named tryptase TL2, which is immunologically cross-reactive with tryptase from rat mast cells (*35, 36*) and supposed to be involved in the infection of CD4$^+$ T lymphocytes with HIV (*37*), has been purified from a human T4$^+$ lymphocyte clone (*38*). However, MTSP-1 and -2 were not immunochemically cross-reactive with the antibody against this rat mast cell tryptase (data not shown) and the N-terminal sequence of MTSP-1 showed only 43% homology with rat tryptase, which showed a high degree of homology with other tryptases purified from different species and organs. In addition, the inhibitor susceptibilities to aprotinin and dithiothreitol and the molecular mass of MTSPs are different from those of tryptase TL2.

We found that the Boc-Phe-Ser-Arg-MCA hydrolyzing activity in the mouse spleens increased about 1.5-fold two weeks after the injection of Freund's complete adjuvant, which enhances the production of antibodies by activating B and T lymphocytes *in vivo*. We also purified the same enzymes from the spleens of mice that received Freund's complete adjuvant by injection into their foot pads as from normal spleens, and no other enzyme peak was evident through all the purification steps (data not shown). These results suggest that the trypsin-like serine proteases MTSP-1 and -2 are induced in spleen cells concomitantly with the activation of B and T lymphocytes by the injection of Freund's complete adjuvant.

Although a study into the cells in which MTSPs are present is now in progress, the results presented here support the evidence that serine proteases in cells involved in the immune response are implicated in cell functions and development. MTSPs may also play some roles in the immune response. To elucidate the mechanism and the physiological functions of MTSPs in the immune system, further studies into the cell distribution, the subcellular localization and the effects of MTSPs on the immune systems *in vitro* and *in vivo* are also now underway.

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