protein prevailing on its degradation. A similar situation might occur in hamsters maintained under the present experimental conditions. It is known that both the liver and the intestine contribute to the formation of apo AI, but attributing the rise in plasma HDL observed in cold-adapted hamsters to one or the other of these two tissues is premature.

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A thiol protease of peritoneal macrophages in the guinea pig[1]

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Summary. Proteolytic enzymes of the guinea pig peritoneal exudate macrophages were investigated using synthetic fluorogenic peptide substrates. Among several enzymes, t-butyloxycarbonyl-phenylalanyl-seryl-arginine 4-methylcoumaryl-7-amide cleaving enzymes had the highest activity, and the activity in exudate macrophages was about 3 times stronger than that in resident macrophages. The molecular weight of the enzyme was around 35,000 and optimal pH around 6.5–7.0. It was inhibited by thiol-blocking reagents, suggesting a thiol protease.
Key words. Protease; amidase; macrophage; guinea pig.

The role of macrophages in cellular immune reactions and inflammations is the focus of much current interest. However, the underlying biochemical reactions are still poorly understood. In a series of experiments designed to explore the chemical mediation of the delayed hypersensitivity reaction (DHR)[3], one of the authors (T.K.) reported the presence of at least three chemotactic factors for macrophages at DHR skin sites (MCFS-1, 2, and 3) in the guinea pig and suggested that these factors participate in mononuclear cell accumulation in their lesions[4–7]. The strongest one (MCFS-1) was a protein of molecular weight (MW) 150,000, and was found to be generated from a precursor protein in the plasma by limited proteolysis by an endogenous trypsin-like protease which hydrolyzed t-butyloxycarbonyl-phenylalanyl-seryl-arginine 4-methylcoumaryl-7-amide[7]. A similar protease was found in the DHR skin sites but the MW was around 600,000; it consisted of a complex of MW 31,000 protease with acidic carrier molecules[8]. Since extractable protease activity in DHR skin sites was chronologically paralleled with the macrophage infiltration, it is, therefore, possible to estimate that the trypsin-like protease in DHR skin sites would derive from emigrated macrogphages in vivo.

In the present experiments, we examined the neutral proteases in macrophages (oil-induced peritoneal macrophages) in guinea pigs to examine whether macrophages had a trypsin-like protease(s), and found a thiol protease.

Materials and methods. Animals. Hartley guinea pigs, 300–500 g, of both sexes, were injected intraperitoneally with 20 ml of sterile liquid paraffin. Four days later, the animals were exsanguinated under ether anesthesia, and their peritoneal cavities were washed three times with 20 ml Hanks balanced salt solution (HBSS) containing 10 IU/ml of heparin. After centrifugation, the cells (1×10^7 cells/ml in HBSS) were homogenized by ultrasonication using a Cell Disruptor, Model W-225R, Heat Systems-Ultrasonics, Inc., New York, U.S.A., and the clear supernatants were recovered by centrifugation for 20 min at 15,000 rpm at 4 °C and used as the extracts.

Determination of protease activities. The protease activities were measured by amidolytic activities with the use of recently developed synthetic fluorogenic peptide substrates which had high specificity and sensitivity; t-butyloxycarbonyl-phenylalanyl-seryl-arginine 4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA); succinyl-glycyl-prolyl-leucyl-glycyl-proline 4-methylcoumaryl-7-amide (Suc-Gly-Pro-Leu-Gly-Pro-MCA); carbobenzoxy-phenylalanyl-arginine 4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA); succinyl-glycyl-proline 4-methylcoumaryl-7-amide (Suc-Gly-Pro-MCA); succinyl-alanyl-alanyl-prolyl-phenylalanine 4-methylcoumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA), and succinyl-alanyl-prolyl-alanine 4-methylcoumaryl-7-amide (Suc-Ala-Pro-Ala-MCA) (Protein Research Foundation, Osaka, Japan). For the study, 10 µl of substrate stock solution (5mM) in dimethylsulfoxide (DMSO) or water were mixed with 440 µl of assay buffer (final concentration of substrate during reaction, 100 µM) and preincubated for 5 min at 37 °C. Then 50 µl of enzyme solution was added and incubated for 10 min at 37 °C, and the reaction was terminated by the addition of 8 M guanidine. A concentration of 50 mM Tris-HCl

buffer (pH 7.0) was usually used as the assay buffer, and 200 mM acetate buffer (pH 3–6), 100 mM phosphate buffer (pH 6–8), and 200 mM Tris-HCl buffer (pH 8–10) were used for studying the pH dependence of the enzymatic activities. The amount of aminomethylcoumarine (AMC) released by the amidolytic activity was fluorometrically measured by a fluorescence spectrophotometer (Model 650-40, Hitachi) with excitation at 380 nm and emission at 440 nm, according to Morita et al.[9–11] as described previously[12].

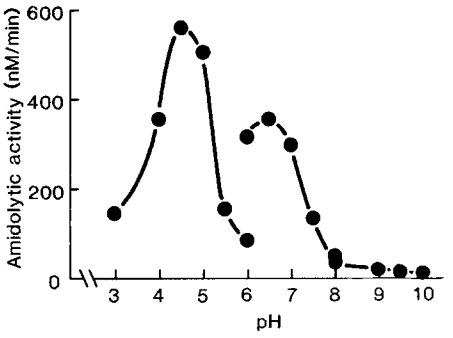
High performance liquid chromatography. The enzymatic products of Boc-Phe-Ser-Arg-MCA were analyzed by reverse-phase high-performance liquid chromatography (HPLC). The HPLC analysis was performed on a Waters liquid chromatograph (compact type) equipped with Model 1660 and 6000 A solvent delivery system, a Model U6K universal sample injector and Model 441 (214 nm) UV detector (Waters Assoc., Milford, Massachusetts, USA). The chromatographic separation was achieved under the following condition: on a NOVA-Pak C_{18} cartilage column (particle size 5 μm, 100 × 8 mm I.D.) with a Waters RCM 100 radial compression module, eluted with 0.1% trifluoroacetic acid containing 5 and 60% of acetonitril. The concentration of acetonitril was increased linearly from 5 to 60% over 50 min. The solvent flow-rate was set at 0.8 ml/min.

Results and discussion. The total number of resident cells in a normal guinea pig was $1.0 \pm 0.4 \times 10^7$ (76.2% macrophages, 6.6% lymphocytes, and 17.7% polymorphonuclear leukocytes). The number of oil-induced peritoneal exudate cells at 4 days was $5.1 \pm 1.8 \times 10^7$ cells and more than 90% of the cells were macrophages as determined by Giemsa and non-specific esterase staining. Viability was more than 90%, judged by trypan blue dye exclusion.

Table 1 shows the amidolytic activities of the extracts from resident and oil-induced peritoneal macrophages at 4 days on several common substrates for serine proteases. Boc-Phe-Ser-Arg-MCA cleaving activity was the highest among them at physiological neutral pH. It was of interest to note that the activity was 3.1-fold increased in the oil-induced macrophage extract over than in the resident macrophage extract.

The hydrolysate of Boc-Phe-Ser-Arg-MCA with the enzyme (37 °C, 10 min) was analyzed by HPLC (data not shown). The products, AMC and Boc-Phe-Ser-Arg released from the substrate, were chromatographed at the retention times of 26.4 and 34 min, respectively. The other products could not be detected on the chromatogram. On the basis of the results, the Boc-Phe-Ser-Arg-MCA cleaving enzyme hydrolyzed only the argininyl-MCA bond of the substrate.

The pH dependency profile of the amidolytic activity showed that the enzyme had biphasic distribution of optimal pH around 4.5 and 6.5 (fig.). The molecular weight (MW) of the enzyme was estimated by Cellulofine GC-700 column chromatography, and was estimated to be approximately 35,000.



pH curve of the Boc-Phe-Ser-Arg-MCA cleaving activities in the extracts of oil-induced peritoneal exudate cells in guinea pigs.

Table 1. Enzyme specificity with various peptide-methylcoumaryl amide substrates at physiological pH 7.0

Substrates (final concentration, 100 μM)	Amidolytic activity[a] (nM/min) Oil-induced[b]	Resident[c]	Ratio[d] (O/R)
Boc-Phe-Ser-Arg-MCA (trypsin)	475	153	3.10
Suc-Gly-Pro-Leu-Gly-Pro-MCA (collagenase-like peptidase)	351	303	1.16
Z-Phe-Arg-MCA (plasma kallikrein and cathepsin B)	345	180	1.92
Suc-Gly-Pro-MCA (post proline cleav. enzyme)	170	173	0.98
Suc-Ala-Ala-Pro-Phe-MCA (chymotrypsin)	108	104	1.05
Suc-Ala-Pro-Ala-MCA (elastase)	20	37	

[a] The amidolytic activities were expressed as a velocity of aminomethylcoumarin release (nM/min/2×10^5 cells). [b] Oil-induced peritoneal macrophages at 4 days. [c] Resident macrophages. [d] O/R, oil-induced/resident.

Table 2. Susceptibility of the Boc-Phe-Ser-Arg-MCA cleaving enzyme to various protease inhibitors at pH 7.0

Protease inhibitor	Final concentration (mM)	Remaining activity[a] (%)
None		100
Diisopropylfluorophosphate	1	85
Phenylmethylsulfonylfluoride	1	126
Soybean trypsin inhibitor	0.1	118
N-α-p-tosyl-L-lysine chloromethylketone-HCl	1	10
L-1-tosylamide-2-phenylethyl-chloromethylketone	0.1	8
Chymostatin	0.1	13
Antipain	0.1	5
Leupeptin	0.1	5
Elastatinal	0.1	9
Pepstatin	0.1	113
p-Chloromercuribenzoate	1	2
N-Ethylmaleimide	1	8
Dithiothreitol	1	212
Disodium ethylenediaminetetraacetic acid	1	209

[a] The enzyme was incubated with the compounds indicated at the final concentration designated for 20 min at 37 °C before the substrate was added. Data are given as relative activities, taking the respective activities in the absence of inhibitors as 100%.

As shown in table 2, the enzyme was completely inhibited by 1 mM p-chloromercuribenzoate (PCMB), 1 mM N-ethylmaleimide (NEM), 0.1 mM leupeptin, 0.1 mM antipain, 0.1 mM elastatinal, 0.1 mM chymostatin, 1 mM tosyllysine chloromethylketone (TLCK), 0.1 mM tosylamide phenylethylchloromethylketone (TPCK), but not by 1 mM diisopropylfluorophosphate (DFP), 1 mM phenylmethylsulfonylfluoride (PMSF), or 0.1 mM soybean trypsin inhibitor (SBTI) It was activated by 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA). It is, therefore, suggested that the enzyme might be a thiol protease, not a serine, trypsin-like or chymotrypsin-like protease.

The biological significance of cellular and tissue proteases is receiving increasing attention. Among the endogenous neutral proteases in the macrophages, plasminogen activator[13], collagenase[14] and elastase[15] have been most widely studied and are supposed to be candidates for inflammatory proteases. The present protease is different from these serine proteases and from the Boc-Phe-Ser-Arg-MCA cleaving trypsin-like protease in DHR skin[8], because the latter enzyme was of MW 600,000 and 31,000 with optimal pH 10, and was inhibited by DFP, PMSF, SBTI and PCMB, but not inhibited by NEM or TLCK. Kato et al.[16] reported a chymotrypsin-like protease of macro-

phages with optimal pH 7.0, which was inhibited by chymostatin. However, it is distinct from the present protease because it is not inhibited by leupeptin or antipain and not activated by EDTA or cysteine, and the present protease had no chymotryptic activity under the experimental conditions used.

Literature on the distribution of the neutral thiol protease in macrophages is scarce. Hayashi et al.[17,18] extracted and purified 2 neutral SH-dependent proteases from Arthus skin sites and found them to be derived from perivascular histiocytes and polymorphonuclear leukocytes of rabbits. These hydrolyzed casein optimally at pH 7.1 and hemoglobin at pH 6–7, and had MW of 200,000 and 14,000. These proteases seem to be different from the present protease, because the MW of the latter is 35,000. Next, Cathepsin B, H and L[19,20] are thiol proteases and are widely distributed in mammalian tissues and cells. Cathepsin B is of MW 27,500 with an optimal pH around 6.0, and has peptidyldipeptidase activity. Cathepsin H has a molecular weight of 28,000 with optimal pH 6.8, and has low leupeptin sensitivity. Cathepsin L is of MW 21,000–24,000 with optimal pH 5.5, and hydrolyzed preferentially Z-Phe-Arg-MCA. The present protease has a MW of 35,000 with optimal pH 6.5–7.0 and, hydrolyzed preferentially Boc-Phe-Ser-Arg-MCA more rapidly than Z-Phe-Arg-MCA, and has high leupeptin sensitivity and no peptidyldipeptidase activity under the experimental conditions used. On the basis of MW, optimal pH and some other characters, the present protease would appear to differ from cathepsins B, H, and L. However, in the absence of satisfactorily purified preparations, it is not possible to say anything beyond this description. Further study will clarify the answer to these questions and the biological role of the present enzyme.

The Z-Phe-Arg-MCA cleaving activity in the present macrophage extract has a similar optimal pH and inhibitor profile to cathepsin L except that its MW is 35,000. The exact difference or identity between these two enzymes needs further investigation. The Suc-Gly-Pro-Leu-Gly-Pro-MCA cleaving enzyme has a MW of more than 67,000 with optimal pH 7, and is inhibited by DFP and PCMB and slightly inhibited by leupeptin, chymostatin, antipain, elastatinal and pepstatin, which suggests that the enzyme may be a post-proline cleaving enzyme[21].

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Reduction of high affinity glutamate uptake in rat hippocampus by two polyamine-like toxins isolated from the venom of the predatory wasp *Philanthus triangulum* F.

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Summary. Two components of the venom of the predatory wasp *Philanthus triangulum* F. significantly reduce – to a greater or less extent – the high affinity uptake of glutamate in rat hippocampus. A concentration of 10 μM δ-PTX caused a reduction of 74%, while the other component, β-PTX, at the same concentration, caused a reduction of 18%. Hence the effect of δ-PTX on high affinity glutamate uptake in the hippocampus is comparable with its effect on high affinity glutamate uptake in insect neuromuscular junctions. Contrary to our previous findings that β-PTX has no effect on high affinity glutamate uptake in insect glutamatergic terminal axons, however, β-PTX significantly reduces high affinity glutamate uptake in the hippocampus, albeit less effectively than δ-PTX.

Key words. Glutamate; high affinity uptake; hippocampus; insect toxins; β-PTX; δ-PTX; polyamines.

The β and δ polyamine components of the venom of the wasp *Philanthus triangulum* F. (β-PTX, mol. wt 243 and δ-PTX, mol. wt 435) have been described as regards their effects on glutamatergic insect neuromuscular junctions[1,2]. One of the effects of δ-PTX on these junctions is the inhibition of high affinity glutamate uptake[2]. Glutamate, an excitatory amino acid, seems to be an important transmitter in vertebrate central nervous systems[3–6]. This induced us to investigate the effects of the two toxins on a vertebrate glutamatergic system.

Below a description is presented concerning the results of an investigation of the effects of β and δ-PTX on the high affinity glutamate uptake in the glutamatergic system of the hippocampus.

Material and methods. Hippocampal slices (200 μm) taken from rats (200 g) were prepared and incubated under high affinity uptake conditions[4,7–9]. To establish the effects of β-PTX and δ-PTX on the glutamate uptake in rat hippocampal slices, the latter (6 in 500 μl incubation medium) were preincubated in a