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Properties of protease extracted from tea-field soil

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Abstract Crude enzyme extract was obtained from a low-pH soil from a tea field by shaking with 0.1 M PO_4^{3-} buffer (pH 7.0). Hydrolytic activity toward benzyloxycarbonyl-*L*-Phe-*L*-Leu (Z-*L*-Phe-*L*-Leu) and Z-*L*-Phe-*L*-Tyr-*L*-Leu showed two pH optima, at about pH 5 and 9, suggesting that the soil contained at least two protease components. The acid-type protease in the extract was assumed to be Ser-carboxypeptidase because phenylmethanesulphonyl fluoride and diisopropylphosphoro fluoridate inhibited its activity. Peptide bonds in the *C*-terminal residues of Leu-enkephalin and angiotensin I were split more by protease than those in the *N*-terminal residue. The apparent molecular weight of the acid-type protease was estimated to be 75 kDa by Sephadex G-100 gel filtration and the isoelectric point 4.4 by isoelectric focusing. A neutral-type protease in the extract was assumed to be a metallocarboxypeptidase because only *o*-phenanthrorine inhibited its activity. Peptide bonds in the *C*-terminal residues of Leu-enkephalin and angiotensin I were hydrolyzed to a greater extent than those in the *N*-terminal residues. The apparent molecular weight of the neutral-type protease was estimated to be 37 kDa and the isoelectric point 5.8, 8.0 and 9.4. The isoelectric point 9.4 fraction showed the highest relative activity.

Key words Tea-field soil · Soil protease · Serine-carboxypeptidase · Metallocarboxypeptidase

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Introduction

Proteases in soil are known to be important agents in promoting the soil N cycle (Ladd and Paul 1973; Nannipieri et al. 1983; Asmar et al. 1994; Watanabe and Hayano 1996). Ladd and Butler (1972) and Ladd (1972) noted that there was a shortage of information about soil enzymes active in the N cycle, despite the many reports on soil activity, and there is still a lack of information on this.

Many types of soil have neutral metalloproteases, e.g. in pastures (Ladd 1972; Watanabe and Hayano 1994), tomato fields (Hayano et al. 1987), and rice paddies (Hayano et al. 1993, 1995; Watanabe and Hayano 1993, 1994, 1996; Takeuchi and Hayano 1994). Ser-type soil proteases have been reported for forest soils (Mayaudon et al. 1975) and paddies (Watanabe and Hayano 1993). The point common to these reports about soil proteases was that the optimum pH of the proteases was neutral or alkaline.

Tea fields in Japan are supplied with large quantities of N fertilizer in order to produce attractively coloured, tasty tea products. The soil pH is generally low owing to the presence of NO_3^-. Considerable amounts of fertilizer N can be assimilated by the soil microbial biomass and is taken up by the tea plants via micobial-biomass N. Tea trees prefer acidic conditions, but a low soil pH is not suitable for common soil proteases whose optimum pHs are neutral or alkaline (Ladd and Butler 1972). In order to deepen our understanding of the soil N cycle of tea fields, we studied properties of protease extracted from tea-field soil, and found soil proteases which had optimum pHs in the acidic region.

Materials and methods

Soil samples

Soil was taken from the surface layer (0–0.1 m) of a tea field (acrisol) at Toyohashi, Aichi, Japan, in August 1995, and immediately

sieved (2-mm mesh) and used in the experiment. The total N content was17 g/kg^{-1} dry soil and the C content 103 g/kg^{-1}. The water content was 0.73 kg/kg^{-1} dry soil and the soil pH was 3.4. The particle-size distribution was as follows: sand 41.8%, silt 21.0% and clay 37.2%.

Enzyme extraction

Soil-enzyme extraction followed the method of Hayano et al. (1987). Soil was extracted by shaking with 0.1 M PO_4^{3-} (pH 7.0) and concentrated by salting out with $(NH_4)_2SO_4$. After the extract was dialyzed, the brown preparation was used as crude enzyme extract.

Enzyme substrates

Benzyloxycarbonyl-*L*-Phe-*L*-Leu (Z-Phe-Leu) was purchased from Sigma, Z-*L*-Phe-*L*-Tyr-*L*-Leu (Z-Phe-Tyr-Leu), Z-*L*-Glu-*L*-Tyr (Z-Glu-Tyr), Z-*L*-Gly-*L*-Leu (Z-Gly-Leu), benzoyl-*L*-Arg-amido (Bz-Arg-NH_2.HCl.H_2O), Leu-enkephalin (i.e. Tyr-Gly-Gly-Phe-Leu), and angiotensin I (i.e. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-2AcOH.4H_2O) were purchased from the Peptide Institute (Osaka).

Optimum pH estimation

The protease assays described in this paper followed mostly that described by Hayano et al. (1987). The reaction mixtures, containing 0.02 ml enzyme extract, 0.48 ml of 0.2 M TRIS-malate buffer (pH 4.6–10) or lactate buffer (pH 2.5–5), and 0.5 ml of 2 mM Z-Phe-Leu or Z-Phe-Tyr-Leu were incubated in a test tube at 30 °C for about 14 h. The ninhydrin method (Watanabe and Hayano 1993) was used to estimate the amount of products such as amino acids and peptide produced via protease activity. For each pH, there were three replicatesfor the reaction mixtures and two replicates for the controls, which contained distilled water instead of substrate.

Inhibitor experiment

The reaction mixture, containing 0.02 ml enzyme extract, 0.48 ml of 0.2 M acetate buffer (pH 5.5) or Atkins-Pantin buffer (pH 8.0) and 0.5 ml of 2 mM Z-Phe-Leu with or without inhibitor (see Table 1) were incubated in a test tube at 30 °C for about 15 h. There were three replicates for the reaction mixtures and two or three replicates for the controls, to which 1 ml of 0.3 M NaOH was added before the incubation for each treatment.

Table 1 Effect of inhibitors on the benzyloxycarbonyl-Phe-Leuase (Z-Phe-Leuase) activity of a tea-field soil extract. Values are activities relative to those of controls minus inhibitors

Inihibitors (1 mM)	Relative activity	
	pH 5.5 Mean ± SD	pH 8.0 Mean ± SD
EDTA	82 ± 13	100 ± 9
EDTA (10 mM)	93 ± 19	101 ± 10
o-Phenanthroline	88 ± 7	38 ± 16
p-Chloromercuribenzoic acid	88 ± 12	84 ± 4
Phenylmethylsulphonyl fluoride	36 ± 7	96 ± 5
Diisopropyl-phosphoro fluoridate	29 ± 22	102 ± 7
Pepstatin	106 ± 12	101 ± 4

Substrate specificity experiment

Using Z-Phe-Tyr-Leu, Leu-enkephalin or angiotensin I as a substrate, the reaction mixture, containing 0.05 ml soil extract, 0.1 ml of 0.2 M acetate buffer (pH 5.5) or Atkins-Pantin buffer (pH 8.0), 0.25 ml of 2 mM substrate, and 0.1 ml distilled water was incubated in a test tube at 30 °C for 2–12 h. After incubation, 0.1 M HCl was added to inactivate the protease, and the reaction mixture was filtered through a 0.45-μm filter. The kind and the amount of released amino acid in the reaction mixture was estimated by using an amino acid analyzer (L8500; Hitachi).

Using Z-Glu-Tyr, Z-Gly-Leu or Bz-Arg-NH_2.HCl.H_2O as a substrate, the reaction mixture was mostly the same as that used to determine the optimum pH, except that 0.2 M acetate buffer (pH 5.5) or Atkins-Pantin buffer (pH 8.0) were used.

Molecular weight and isoelectric point estimation

The molecular weight of the main soil protease component was estimated by gel chromatography. The soil extract was fractionated using a Sephadex G-100 column (10 × 900 mm) and 10 mM TRIS-HCl (pH 7.4) with 0.2 M NaCl as the eluting buffer. The protease activity was estimated by incubating0.25 ml protease fraction with the reaction mixture containing 0.25 ml of 0.4 M TRIS-NaOH buffer (pH 5.5 and pH 8.0), and 0.5 ml of 2 mM Z-Phe-Leu. Chymotrypsinogen A, egg albumin and bovine serum albumin calibration proteins (Boehringer Manheim) were used as the standards.

The isoelectric point (PIapp) was estimated by isoelectric focusing (Rotofor; Bio-Rad). Fractionation was done using 2.5 ml enzyme extract in about 50 ml of 2% ampholytes (Bio-Lyte pH 3–10; Bio-Rad). Samples were run at 12 W for 4 h with cooling. The protease activity of each fraction was estimated by incubating 0.1 ml protease fraction with the reaction mixture containing 0.15 ml of 0.4 M TRIS-NaOH buffer (pH 5.5 and pH 8.0), and 0.25 ml of 2 mM Z-Phe-Leu.

The two trials of each experiment gave mostly same results, thus only the results from one trial of each are shown in this paper.

Results

Optimum pH

The pH curve of Z-Phe-Leuase activity of the tea-field soil extract showed two peaks at about pH 5 and 9. The optimum pH for Z-Phe-Tyr-Leu hydrolysis was 9.5, and a shoulder appeared in the curve at about pH 6 (Fig. 1).

Properties of protease

In the inhibitor experiment, Z-Phe-Leuase activity at pH 5.5 was inhibited by both phenylmethanesulphonyl fluoride (PMSF) and diisopropylphosphoro fluoridate (DFP). EDTA, *o*-phenanthroline, and *p*-chloromercuri benzoate (PCMB) showed little inhibition of Z-Phe-Leuase activity and with pepstatin there was none (Table 1). At pH 8.0, *o*-phenanthroline showed the only effective inhibition of Z-Phe-Leuase activity.

Using Z-Phe-Tyr-Leu as a substrate, the protease mainly released Leu, and released Tyr-Leu slightly at pH 5.5 and 8.0 (Fig. 2). Using Leu-enkephalin or angio-

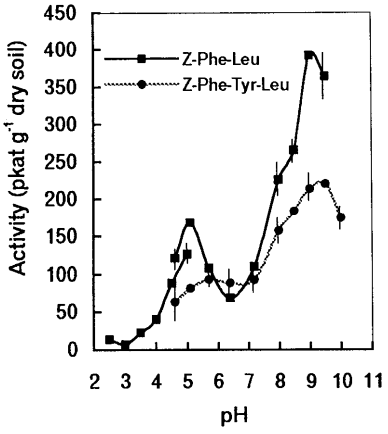

Fig. 1 Effect of pH on the hydrolytic activity of the tea-field soil extract with respect to Z-Phe-Leu and Z-Phe-Tyr-Leu. The *vertical bars* indicate SDs

tensin I as a substrate, the peptide bond in the *C*-terminal residue was split to a greater extent than that in the *N*-terminal residue, both at pH 5.5 and 8.0.

When comparing soil-extract substrate specificity for the *C*-terminal residue of substrates at pH 5.5 and 8.0, Z-Phe-Leu was the most affected, followed by Z-Phe-Tyr-Leu and Leu-enkephalin. Z-Gly-Leu, Z-Glu-Tyr, and Bz-Arg-NH_2 were hydrolyzed slightly (Table 2).

Apparent molecular weight and PIapp estimation

The apparent molecular weight of the main component of extractable protease in the soil was estimated by gel filtration in a protease-activity assay at pH 5.5 and pH 8.0, with Z-Phe-Leu as a substrate. At pH 5.5, a peak appeared for fraction no.17, and at pH 8.0, for fraction no. 24 (Fig. 3). From the results of eluting chymotrypsinogen A, egg albumin, and bovine serum albumin as the standard, the molecular weight of the acid-type protease was estimated to be 75 kDa, and that of the neutral-type protease about 37 kDa.

The PIapp of the main protease components of the soil were estimated by electric focusing using soil extracts. For the Z-Phe-Leuase activity of the fraction at pH 5.5, the PIapp was estimated to be 4.5, and at pH 8.0 was 5.8, 8.0, and 9.7 (Fig. 4). A fraction with PIapp 9.7 showed the highest relative activity.

Discussion

The tea-field soil extract probably contained at least two soil protease components, because the pH curves of Z-Phe-Leu and Z-Phe-Tyr-Leu hydrolysis showed two peaks at about pH 5 and 9. The estimated molecular weight of the protease indicated at pH 5.5 was 75 kDa and the PIapp 4.5, and at pH 8.0 the molecular weight was 37 kDa and the PIapps 5.8, 8.0, or 9.4.

Z-Phe-Leu was the most effective substrate for the soil extract among Z-dipeptide and bovine serum albumin. This result agreed with that reported by Ladd and Butler (1972).

The acid-type-protease activity was strongly inhibited by PMSF and DFP, but not by EDTA, *o*-phenanthroline, PCMB, or pepstatin, suggesting that the protease component was a Ser type. Although Ser proteases may be endo- or exo-types, including carboxy-

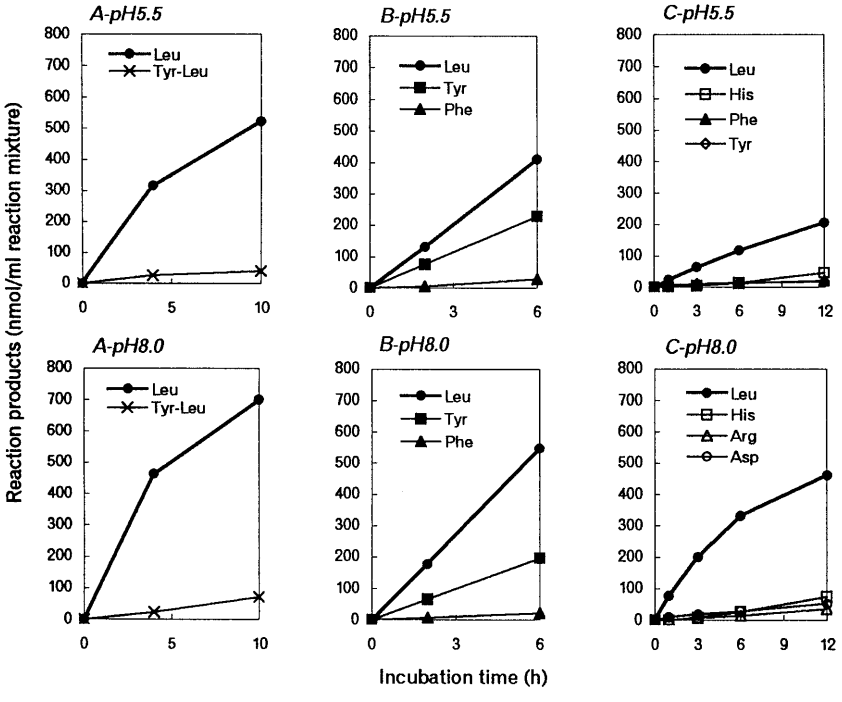

Fig. 2 Time-course for the release of reaction products from benzyloxycarbonyl-Phe-Tyr-Leu (Z-Phe-Tyr-Leu; ***A***), Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin; ***B***), and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-2AcOH.4H_2O (angiotensin I; ***C***)

Table 2 Tea-field soil extract specificity for the *C*-terminal residues of substrates at pH 5.5 and pH 8.0. The activities were calculated relative to that for the substrate Z-Phe-Leu. The data are means ± SDs. *Bz* Benzoyl

Substrate (1 mM)	pH 5.5	pH 8.0
Z-Phe-Leu	100 ± 20	100 ± 8
Z-Phe-Tyr-Leu	89	61
Leu-enkephalin	165	63
Angiotensin I	37	22
Z-Gly-Leu	0 ± 23	11 ± 6
Z-Glu-Tyr	21 ± 29	9 ± 5
Bz-Arg-NH_2	10 ± 25	3 ± 5

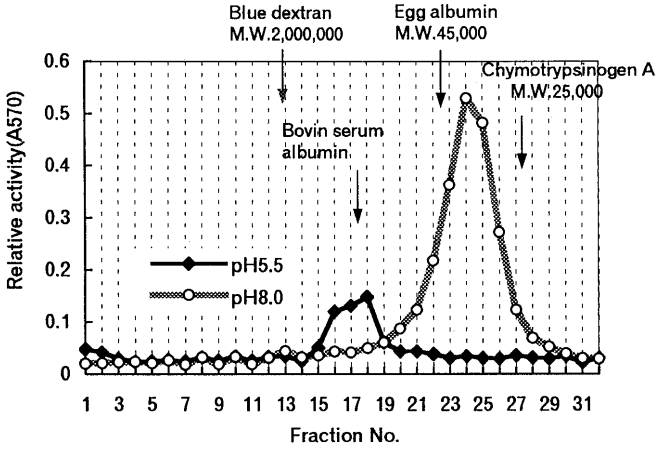

Fig. 3 Gel filtration of tea-field soil extract on Sephadex G-100. Z-Phe-Leuase activity of each fraction was estimated at pH 5.5 and pH 8.0

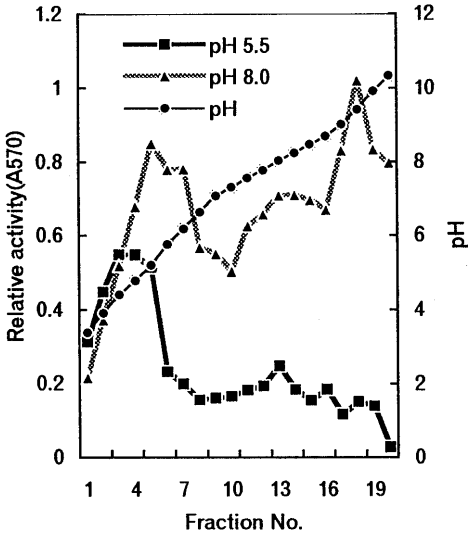

Fig. 4 Isoelectric focusing of tea-field soil extract. Z-Phe-Leuase activity of each fraction was estimated at pH 5.5 and pH 8.0

peptidase and amidopeptidase, the main component of Z-Phe-Leuase in the extract appeared to be exo-carboxypeptidase, because protease from the tea-field soil mainly led to the release of Leu from Z-Phe-Tyr-Leu, which is used as the smallest useful substrate to distinguish between endo- and exo-types of protease (Hayano et al. 1995). The soil extract hydrolyzed the C terminal residue of Leu-enkephalin and angiotensin I rather than the *N*-terminal residue.

Other properties were similar to those described in previous reports. For Ser protease, the optimum endo-type pH reportedly ranges from neutral to alkaline, and the optimum pH of exo-carboxypeptidase from 5 to 6 (Enzyme Nomenclature 1992; Tsuru and Funatsu 1993), while that of the acid-type protease in our study was estimated to be 5.5. The molecular weight of the endo-type is about 20–50 kDa and that of the exo-type about 50–150 kDa (Tsuru and Funatsu 1993), while the molecular weight of the acid-type protease in the tea-field soil was estimated to be about 75 kDa. The reported PIapps of Ser-carboxypeptidases of plants and microorganisms range from 3.6 to 4.6 (Tsuru and Funatsu 1993), whilst that of the acid-type protease from the tea field was 4.4. *Saccharomyces cervisiae* (Felix and Brouillet 1966), *Aspergillus oryzae* (Nakadai et al. 1972), *Aspergillus saitoi* (Ichishima 1972), and *Penicillimu janthinellum* (Yokoyama et al. 1975) produce Ser-carboxypeptidases.

The main component of the neutral-type protease fraction from the tea-field soil was metallocarboxypeptidase, as indicated by the inhibition of Z-Phe-Leuase activity in the presence of *o*-phenanthroline. The preferential cleavage sites of Z-Phe-Tyr-Leu and the amino acid released from Leu-enkephalin and angiotensin I were similar to those of the acid-type protease in the tea-field soil, so it was thought to be an exo-carboxypeptidase. The molecular weight of the neutral-type protease from the tea field was 37 kDa and the PIapp was 5.8, 8.0, or 9.4. In a previous study the molecular weight of metallocarboxypeptidases ranged from 21 to 40 kDa and the PIapp from 4.7 to 9.7 (Tsuru and Funatsu 1993). The insensitivity of this neutral-type protease to EDTA was similar to that found for a metallocarboxypeptidase from *Streptomyces griseus* (Seber et al. 1976) and *Micromonospora melanosporea* (Muro et al. 1990).

Hayano et al. (1995) suggested that most soils harbour neutral metalloproteases, which are known to include both exo- and endo-types. An endo-type metalloprotease was the major component of the protease fraction in a tomato-field soil (Hayano et al.1987) and rice-wheat-rotation fields (Hayano 1993; Hayano et al. 1995). Pasture soil (Ladd 1972) and monoculture paddies (Takeuchi and Hayano 1994) had mainly exo-type metalloproteases, as did the tea-field soil we studied.

Although the largest peaks of the pH curve for the hydrolysis of Z-Phe-Leu and Z-Phe-Tyr-Leu with the tea-field soil extract were in the alkaline region, the soil pH of this field was very low (3.4). This indicated that the acid-type soil protease probably plays a more significant role than the neutral-type protease in N cycling in this soil.

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