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Characterization and Origin of Protease Activity in Cultivated Soils

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Abstract

Most organisms, especially non-nitrogen fixers, must decompose to low molecular nitrogenous compounds in order to assimilate nitrogen. Hydrolysis of proteins, like ammonification and nitrification, is an important process in the nitrogen cycle with plays an essential role in soil fertility. Soils have sufficient levels of proteolytic activity to support the nitrogen metabolism in situ in soils. Most soils contained a neutral metalloprotease when protease activity was assayed using N-benzyloxycarbonyl-L-phenylalanyl-L-tyrosyl-L-leucine (Z-Phe-Tyr-Leu) as a substrate. The isoelectric points of the main metalloprotease component in the extract of samples from an Andosol in a tomato field and a Gray Lowland soil in a paddy field were estimated to be 4.9 and 2.9, respectively. The molecular weight of the main metalloprotease component from the Andosol and the Gray Lowland soil was estimated to be 47×10^3 and 37×10^3, respectively. The metalloprotease split preferentially the peptide bonds with hydrophobic amino acid residues. Characteristics of the protease activity of paddy field soil under double cropping conditions (rice-wheat) were intermediate between those of an upland soil and a paddy soil under monoculture of rice. Proteolytic *Bacillus* spp. was a major source of soil protease in water-logged paddy fields.

Discipline: Soils, fertilizers and plant nutrition
Additional key words: metalloendopeptidases, nitrogen transformation, soil enzymes, soil extract

Introduction

It is well-known that when moist soil from a paddy field is air-dried, microbial, plant and animal cells are destroyed and organic nitrogen in remoistened soil sample is more mineralized than when moist soil was not air-dried. Protein-N which is the main form of soil N[10] is mineralized to ammonia via amino acids. Main mineralization process of biomass N is considered to involve amino acid formation from protein and ammonification from amino acids. Hydrolysis of protein is a rate-limiting process in nitrogen mineralization which plays an essential role in soil fertility.

Population of mankind on earth amounts to 5,600 million people and 700 million of them suffer from a shortage of food. There is a growing need for increasing the production of food to match the increase in population while avoiding pollution of various kinds of ecosystems. Sustainable agriculture is required globally but technology to achieve this objective has not yet been developed. Regulation of the metabolism of soil nitrogen is important for efficient supply of nitrogen to crops without load to environment. Knowledge concerning protease activity and its control in soil of agricultural land is necessary for the development of a technology for the promotion of sustainable agriculture in future.

Protease activity in various types of soils in Japan

In order to estimate the soil protease activity, the following method[14] was applied by which the amount of substrate decreased or the products formed during incubation at a fixed temperature were determined. Protease activity in various types of soils from several sites in Japan was estimated using Z-Phe-Leu (N-benzyloxycarbonyl-L-phenylalanyl-L-leucine) as a substrate at pH 8 and 30°C (Fig.1). Fig.1 shows that in a soil with an adequate level of protease activity nitrogen metabolism can operate *in situ*.

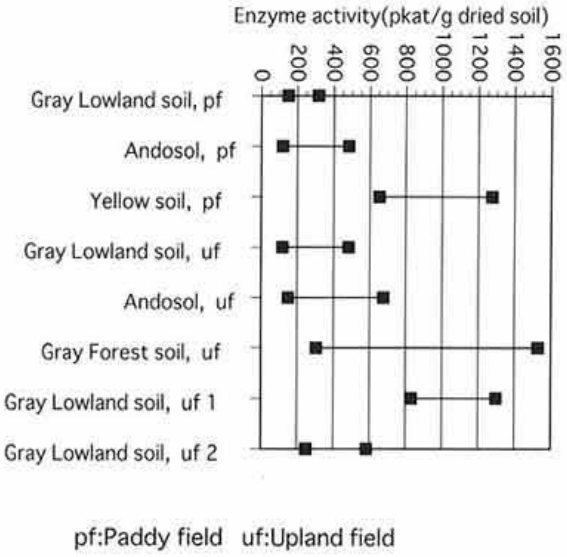

Fig. 1. Comparison of protease activity in various types of soils in Japan[2,15,18,20,28]
uf 1: 2 weeks after manure application,
uf 2: Harvest stage after manure application.

Extraction of protease from soil

Formerly, proteases were classified into peptidase and proteinase but recently, the enzymes have been classified into exopeptidase and endopeptidase depending on the mode of splitting of the peptide bond[17]. Moreover, proteases were classified into 4 types; serine type, cysteine type, aspartic type and metal type, according to the amino acid residues or metal as a prosthetic group at the active site of the enzyme molecules. Specific inhibitors are used to differentiate the proteases. Extraction of enzyme from soil is an essential procedure to characterize proteases. Protease, like other hydrolytic enzymes, can be extracted with phosphate buffer[4], Tris-borate buffer[12] and pyrophosphate buffer[16]. High molecular substrates in soil usually occur in an insoluble form. Digestive enzymes which form complexes with humic materials, are loosely attached to insoluble substrate due to steric hindrance of the enzyme for the substrate. Enzyme which does not interact appreciably with humic materials and clay minerals is likely to contribute to the digestion of insoluble high molecular substrate in soil *in situ*.

Characterization of protease activity in soils

Characteristics of protease activity in soils of Japan, were compared as shown in Table 1. Protease-active extract from an Andosol in a tomato field released mainly Tyr-Leu from Z-Phe-Tyr-Leu (N-benzyloxycarbonyl-L-phenylalanyl-L-tyrosyl-L-leucine). Optimum pH of the activity was about 7, suggesting that the enzyme belonged to the neutral type. Hydrolysis of Z-Phe-Tyr-Leu was inhibited by EDTA but not by the protease inhibitors of serine type (PMSF), cysteine type (PCMB) and aspartic type (pepstatin). The soil extract hydrolyzed angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) at the -Tyr-Ile- and -Pro-Phe-sites and the activity was inhibited by EDTA. After inactivation of the protease activity by EDTA and elimination of EDTA by dialysis, addition of metal ion such as Zn^{2+} and Mn^{2+} to the dialyzed extract, enabled to recovered the activity[14], suggesting that metalloendopeptidase (3.4.24)[17] is the main component

Table 1. Comparison of properties of protease from different soils

Source	Inhibitor	Optimum (pH)	Main product from Z-Phe-Tyr-Leu	Reference
Andosol under tomato field	EDTA	7	Tyr-Leu	Hayano et al. (1987)[4]
Gray Lowland soil under monoculture rice	EDTA, PCMB (at pH5)	8.5	Leu	Takeuchi & Hayano (1994)[22]
Gray Lowland soil under double cropping condition				Hayano et al. (1995)[5]
Rice straw compost plot				
Rhizosphere	EDTA, PCMB	8	Tyr-Leu	
Non-rhizosphere	EDTA, PCMB	8	Tyr-Leu, Leu	
Chemical fertilizer plot				
Rhizosphere	EDTA, PCMB	7–8	Tyr-Leu	
Non-rhizosphere	EDTA, PCMB	8	Tyr-Leu	

of the protease in the soil extract. The apparent isoelectric point of the main metalloendopeptidase component was estimated to be 4.9 by isoelectric focusing, and the apparent molecular weight of the main component was estimated to be 4.7×10^4 by gel filtration[4].

A protease component extracted from a paddy soil under monoculture of rice produced Leu and a small amount of Tyr-Leu from Z-Phe-Tyr-Leu as hydrolyzed products[22]. The apparent isoelectric point and the apparent molecular weight of the protease were 2.8 and 3.7×10^4, respectively. As the metal component in the protease of an Andosol is easily removed by EDTA in neutral pH, it is suggested that the bond between the enzyme protein and metal of the protease extracted from paddy soil under monoculture of rice was more stabilized than that from Andosol in the tomato field.

Protease-active extract from a paddy soil under double cropping of rice and wheat released Tyr-Leu and a small amount of Leu from Z-Phe-Tyr-Leu as reaction products[6]. The soil extracts hydrolyzed angiotensin I at the -Tyr-Ile-, -Pro-Phe- and -Phe -His-sites while EDTA inhibited the hydrolysis of the -Tyr-Ile- and -Pro-Phe-sites but not that of the -Phe-His-site. The protease activity at the -Tyr-Ile- and -Pro-Phe-sites was higher than that in C and N terminals of angiotensin I, suggesting that the main protease component of the soil extracts from the paddy field under double cropping of rice and wheat was a neutral metalloendopeptidase (3.4.24).

Vallee et al. (1960) have reported that the Zn of carboxypeptidase A binds to a cysteine residue of the enzyme[24]. According to their findings most of the protease components in the extracts from paddy field soils can be classified into a neutral metalloprotease which contains an SH group at or near the active site[24]. The results obtained in inhibitor experiments indicate that two types of metalloproteases occur in soils: an Andosol in a tomato field soil contained a metalloprotease without SH group whereas Gray Lowland soils in a paddy field contained a metalloprotease with SH group (Fig. 2).

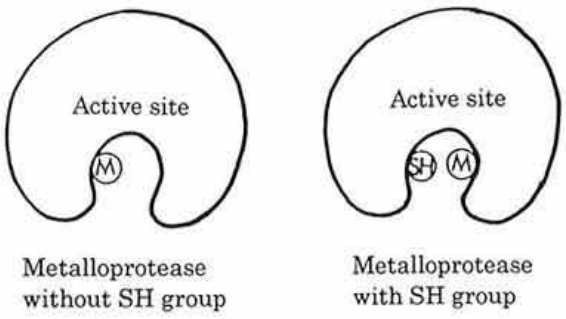

Fig. 2. A concept of protease molecules based on inhibitor experiments

Table 1 shows that the protease activity of all the soil samples was optimal at neutral pH and sensitive to metal chelator, suggesting that the soils commonly contain a neutral metalloprotease. Some differences were observed among the soil proteases. Protease activity of the extracts from paddy field soils was inhibited by EDTA and PCMB, whereas, the activity of the extract from a tomato field soil was inhibited by EDTA but not by PCMB. The protease of the soil under monoculture of rice produced mainly Leu from Z-Phe-Tyr-Leu, whereas the protease from the other two soils produced mainly Tyr-Leu from Z-Phe-Tyr-Leu. The results suggest that the paddy field under monoculture contains a carboxypeptidase as main protease component whereas the other two soils mainly contain an endopeptidase, and that the characteristics of the protease activity of the soils of the paddy field under double cropping were intermediate between those of the upland soil and paddy field soil with monoculture.

Electrostatic bond, hydrogen bond and Van der Waals bond are known to be involved in the interaction between enzyme and substrate. Analysis of site specificity of soil metalloprotease was carried out according to the method of Ichishima et al. (1983). Relationship between hydrophobicity of amino acid residue in angiotensin I and splitting site with the protease is shown in Fig. 3. Assuming that the binding site of the enzyme is S and of the substrate is P, soil metalloendopeptidase[4,6] recognized the hydrophobic amino acid residues adjacent to the bonds to be split in the substrate as well as the interaction brought about between S_1-P_1 and S_1'-P_1' and then the bond in P_1-P_1' was hydrolyzed. In the case of serine endopeptidase of *Aspergillus sojae*, S_2 and S_2' were attached to P_2 and P_2' which are amino acid residues with a hydrophobic side-chain[8]. Serine-type protease activity was detected in the extracts from soils and bacteria when casein was used as a substrate instead of Z-Phe-Tyr-Leu[27].

Non-denatured proteins with a tertiary structure such as collagen and keratin occur in the soil ecosystem. Splitting sites of the substrate in non-denatured state are reported to be different from those in denatured state[7,9,11]. Changes in initial cleavage site between native and denatured substrates may occur in the hydrolysis of proteins in the soil

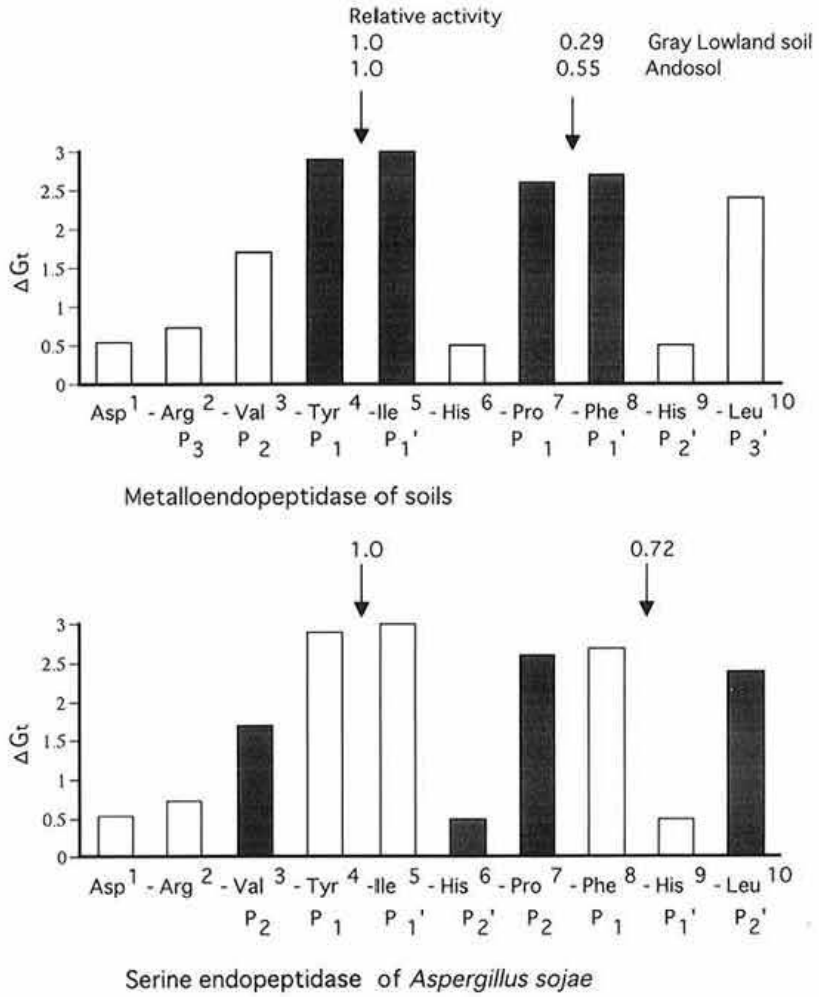

Fig. 3. Relationship between splitting site of the substrate and hydrophobicity of side-chains of amino acid residues adjacent to those involved in the bond to be split by soil metalloendopeptidase
Arrows indicate the position of the splitting site.
The value above the arrows shows the relative hydrolytic activity.
ΔGt in ordinate indicates the hydrophobicity of side-chains of amino acid residues (kcal/mol)[23].
Data of serine endopeptidase of *Aspergillus sojae* were cited from Ichishima et al.[8].
P and P′ indicate binding sites of substrate side according to Shechter & Berger[21].

ecosystem.

Origin of soil protease

Proteases are widely distributed in microbes, plants and animals. Reviews by Ladd (1978) indicate that enzymes in soils originated from animals, plants and microorganisms, while the presence in soils of enzymes derived directly and specifically from animal and plant sources, has not yet been demonstrated conclusively; a widespread, often tacit assumption is that soil enzymes are derived primarily from microorganisms[13]. Rempe and Kaltagova (1965) observed that the root of oat plant cultivated aseptically excreted invertase and peroxidase but not protease in the medium[19]. Hayano et al. (1983) observed that the proportion of protease activity per root dry matter in potting medium with tomato plant was lower than that of other enzymes such as phosphatase and β-glucosidase[3]. These observations suggest that the contribution of protease derived directly from plant was probably negligible or much less significant

Table 2. Protease activity and viable counts of microorganisms in antibiotic-treated soil samples

Treatment	Viable counts (cfu g^{-1} dried soil)			B/F[a]	Protease activities[b]	
	Bacteria	Actinomycetes	Fungi		Tyr-Leu liberation	Leu liberation
No treatment	2.4×10^8	6.5×10^8	4.0×10^5	600	85	93
Chloramphenicol	1.7×10^6	1.5×10^5	4.0×10^5	4	0	140
Cycloheximide	2.3×10^8	2.7×10^7	7.3×10^3	31500	132	122

a): Ratio of bacteria to fungi.
b): Z-Phe-Tyr-Leu was used as substrate and activity was expressed as pmoles of reaction product liberated $s^{-1}g^{-1}$ dried soil.

than that of other soil enzymes.

Hayano (1993)[6] observed that the selective inhibition of bacterial and actimomycetes growth in remoistened, oven-dried and inoculated soil indicated that Leu was the only reaction product when the soil was incubated with Z-Phe-Tyr-Leu, and that selective inhibition of fungal growth in the soil showed that Tyr-Leu and Leu were liberated from Z-Phe-Tyr-Leu (Table 2). These findings suggest that the soil did not contain any fungal proteases.

Proteolytic bacteria and actinomycetes were isolated from soils of a paddy field under double cropping conditions (rice and wheat) using azocoll-nutrient agar medium[1]. Average protease activity of the bacteria isolated from azocoll-nutrient agar was 33.7 times higher that of the bacteria isolated from nutrient agar and 6.7 times higher that of actinomycetes from albumin agar[25].

Watanabe and Hayano (1993) have characterized proteolytic bacteria in paddy field soils under rice cultivation and enumerated them using azocoll agar plates[26]. *Bacillus* spp. were the proteolytic bacteria most frequently observed, accounting for 59% of the isolates. Of the 411 proteolytic bacteria isolated, 124 isolates showed a higher proteolytic activity than others based on gelatin liquefaction tests and most of them consisted of *Bacillus* spp. Of this group, *Bacillus subtilis* and *Bacillus cereus* were the main species. Characteristics of the proteases from *Bacillus* spp. were similar to those of soil protease in terms of the response to protease inhibitor and optimum pH of the activity for the hydrolysis of Z-Phe-Tyr-Leu and Z-Phe-Leu[27]. These observations indicate that proteolytic *Bacillus* spp. is a major source of soil protease in water-logged paddy fields.

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