Detection and Identification of Microorganisms in Soil and Natural Environment

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Abstract

A detection method of soil microorganisms which is related to soil protease production was developed as follows. In paddy fields and several upland fields the major proteolytic soil microorganisms were *Bacillus* spp., which produced extracellular serine protease with similar properties to those of soil protease of these fields. In upland fields amended with a large amount of slurry, however, the major proteolytic soil microorganisms were gram-negative bacteria such as *Serratia marcescens*. This microorganism produced extracellular metalloprotease with similar properties to those of soil protease in these fields. The results obtained suggested that difference in the soil conditions might cause differences in proteolytic bacterial flora which resulted in characteristic difference in the major soil proteases. DNA primers, which were obtained from the N-terminal amino acid sequence of the protease of *S.marcescens* amplified not only the serratial metalloprotease gene but also that of the other gram-negative bacteria isolated from the slurried fields.

However they did not amplify protease genes of proteolytic *Bacillus* spp. By use of the DNA primers and DNA probes which were synthesized by PCR, the homologous metalloprotease gene was detected in DNA directly extracted from soils.

Introduction

The soil microorganisms are considered to be the main source of soil protease. Many investigators have attempted to relate soil protease activity and microbial number with varied results. This may be due to the fact that the enzyme activity is (1) primarily extracellular in nature and not closely associated with the microbial population (2) associated with a large percentage of the microbial population while the media selected only a fraction of the microbial population which was not representative of the whole (3) specific to a small fraction of the microbial population which was not selected for by any of the culture media, and (4) from a non-microbial source, e.g. plant roots or its residues and other soil biota.

In the current report research results on soil microorganisms which are assumed to be the major source of soil protease are outlined.

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Elucidation of origin of soil protease

In these experiments, soil samples from the surface layer of paddy fields (Gray Lowland soil) and upland fields (Andosol) in Kyushu island that were subjected to various treatments were used. Three soil samples were taken from the experimental paddy fields, each with a different fertilizer treatment; yearly application of rice-straw compost and chemical fertilizer (**POM**), yearly application of chemical fertilizer (**PCF**), and no fertilizer application (**PNF**). Two soil samples were taken from the surface layer of upland fields in Nishigoshi; one from a 3-year mono-culture of sweet potato supplied with chemical fertilizer (**UCF**), and the other from a fallow land that had been developed from a grassland in the previous year (**UUF**). Two soil samples amended with slurry, were taken from the surface layer of an upland field in Miyakonojyo, Japan (Andosol); one under a 10-year mono-culture of corn amended with slurry (600 t ha^{-1} $year^{-1}$)(**UMS**), and the other from a 10-year mono-culture of corn amended with slurry (120 t ha^{-1} year ha^{-1})(**USS**).

In order to elucidate the origin of the soil protease, the growth of specific microorganisms in soil was inhibited by treatment with an antibiotic and protease activities of the soil were measured. As the soil z-FLase and caseinase activities of paddy field (**PCF**), and upland field (**UCF, UUF**) soils decreased after chloramphenicol treatment accompanied with the decrease of the total bacterial number, it was assumed that both proteases were of bacterial origin (Table 1)(Watanabe and Hayano, 1994). As the z-FLase activity of soil from slurried fields (**UMS,USS**) decreased by chloramphenicol treatment, it was assumed that the soil z-FLase was mainly of fungal origin. As the caseinase activities of soil from slurried field (**UMS, USS**) did not change by chloramphenicol treatment, but increased by the treatment with cycloheximide, it was assumed that the soil caseinase was mainly of bacterial origin and that fungal contribution was also significant (Table 1).

Flora of proteolytic bacteria in soils

Since soil protease displays specific properties, it is assumed that specific soil microorganisms which produced the extracellular protease with the same properties as those of the soil protease may be the major source of soil protease. In total, 742 proteolytic bacteria (**AD**; azocoll degraders) were isolated from the soils. The proteolytic bacteria with higher extracellular protease activity (**GL**; gelatin liquefiers) were screened from azocoll degraders (Watanabe and Hayano, 1993a; Watanabe *et al.*,1994) and the properties of their extracellular protease were compared with those of soil protease.

In paddy fields (**POM, PCF, PNF**), *Bacillus* spp. were the major proteolytic bacterial group of **AD** and **GL** (Table 2). *B.subtilis* (6 isolates), *B.cereus* (4 isolates), and *B.mycoides* (3 isolates) produced a large amount of extracellular z-FLase which showed similar properties to those of the extracted soil z-FLase in some paddy fields. *B.subtilis* (8 isolates) produced a large amount of extracellular serine caseinase, which

showed similar properties to those of the extracted soil caseinase in paddy fields (Watanabe and Hayano, 1993b).

Most of the proteolytic bacteria in upland fields (**UCF, UUF**) belonged to be *Bacillus* spp. (Table 2)(Watanabe and Hayano, 1994). *B.amyloliquefaciens* (U80) produced a large amount of extracellular serine caseinase, which showed the same properties as those of the overall soil caseinase in the upland field. On the other hand, the proportion of proteolytic *Bacillus* spp. to the total proteolytic bacteria (**GL**) was lower in 120 t and 600 t slurried fields (**USS**) compared to the upland fields (Table 2). Two isolates with high extracellular z-FLase activity and six isolates with high extracellular caseinase activity were gram-negative bacteria. Strains s-131 and s-132 with high extracellular caseinase were identified as *Serratia marcescens*, which produced extracellular metalloprotease with similar properties to those of the overall soil caseinase in slurried fields (**UMS, USS**) but also to those in paddy fields and cultivated upland field (Table 3). These gram-negative bacteria could be a major source of the overall soil caseinase in many type of soils.

Production mechanisms of soil protease in paddy fields.

In the paddy fields (**POM, PCF, PNF**)under water-logged condition, extracted soil z-FLase and caseinase activities increased after irrigation (July 21) then decreased to undetectable level (Fig.1, 2)(Watanabe and Hayano, 1996a). The overall soil z-FLase and caseinase activities in these paddy fields maintained a high level during rice cultivation (z FLase, 191 pKat to 702 pKat g^{-1} dry soil; Caseinase, 220 pKat to 758 pKat g^{-1} dry soil). The number of azocoll degraders was found to be correlated with the total soil z-FLase activities ($P<0.05$)(Watanabe and Hayano, 1995). The number of spores of *Bacillus* spp., counted on peptone-polymyxin agar plate after pre-heating at 80℃ for 20 min (PP(80)) showed a correlation with the overall soil caseinase activity ($P<0.1$)(Fig. 3)(Watanabe and Hayano, 1995).

By the inoculation of ON-023, a *B.subtilis* with a high caseinase-producing activity, to the sterilized soil, the overall soil caseinase activity increased while that of the soil z-FLase decreased (Watanabe and Hayano ,1996b). Significant correlations between the spore number and the overall soil caseinase activity ($P<0.05$) and between the vegetative cell number, counted on BTV medium (Akiba and Katoh, 1986), and the overall soil z-FLase activities were observed (Watanabe and Hayano, 1996b). It was noted that the production of z-FLase in soil was related to the growth of *Bacillus* spp. and that of caseinase in soil might be related to the sporulation of *Bacillus* spp.

Development of selective detection method for soil proteolytic bacteria

Extracellular caseinase from *B.subtilis* (NN-212) from paddy fields (**PNF**) and caseinase from *B.amyloliquefaciens* (U-80) from upland fields (**UCF**) were isolated using CM-Sephadex C-50, and Sephadex G-100 column chromatography and purified by

SDS-PAGE. They were serine proteases (28,000 M.W.) with optimum pH value of 10 and optimum temperature of 60℃, as in the case of subtilisin produced by *Bacillus* spp.(Takami *et al.* 1990). NH_2terminal amino acid sequence of the protease isolated from U-80 was Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly, which was identical with that of subtilisin BPN' (Fig.4)(Vasantha *et al.*, 1984; Takami *et al.*, 1990), while that from NN-212 was identical with that of subtilisin Amylosacchariticus (Fig.4)(Kurihara *et al.*, 1972).

Extracellular caseinase from *S.marcescens* (s-132) from slurried field (**USS**) was isolated using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography and purified by SDS-PAGE and the enzymes from *S.marcescens* (s-131:**USS**) and gram-negative bacteria (m-110; **UMS**) were also purified by the same way. They were metalloprotease (50,000 M.W.) with optimum pH 9.5 and optimum temperature of 40℃, as in the case of serratial protease produced by *S. marcescens* (Aiyappa and Harris, 1976). NH_2-terminal amino acid sequence of the purified protease from s-132 was identical with that of serratial protease (Fig. 4)(Nakahama *et al.*, 1986; Braunagel and Benedik, 1990).

Several combinations of DNA primers (20 mer) synthesized on the basis of the reported DNA sequence of serratial metalloprotease gene (Nakahama *et al.*, 1986), amplified genomic DNA of the proteolytic *S.marcescens* (s-132) to give a 1.68 Kb band on gel electrophoresis. DNA sequences of the amplified 1.68 Kb gene (294 and 275 bps of 3' and 5' terminal sequences) were found to be identical with those of the previously reported protease gene of serratial metalloprotease (Nakahama *et al.*, 1986)(Fig. 5). These primers amplified those of the other proteolytic *S.marcescens* (s-112, s-131) and type strains of *Serratia* spp. but did not amplify those of the other strains including proteolytic *Bacillus* spp.(Watanabe *et al.*, 1995). Therefore these DNA primers were found to amplify selectively the metalloprotease gene homologous to the serratial metalloprotease gene.

The metalloprotease gene was amplified in the DNA extracted from a German Phaeozem soil. Bacteria or fungi with the homologous metalloprotease gene are estimated to amount to about 10^6 cells/g (wet soil).

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Table 1 Soil protease activities and microbial counts after antibiotic treatment (ratio to control)

Soil	Treatment	Microbial counts[a]			Protease activities[b]	
		Fungi	Bacteria	Bacillus	z-FLase	Caseinase
		$\times 10^4$	$\times 10^7$	$\times 10^6$		
Fallow land Andosol[c]	Control	(2.2±1.4)	(46.9±21.7)	(63.6±13.3)	(103.4±5.0)	(41.1±7.8)
	Chloramphenicol	610.5**	0.032**	0**	0.425**	0**
	Cycloheximide	0.09**	1.243	1.091	0.850	2.776**
Cultivated Andosol[c]	Control	(11.1±4.6)	(65.1±8.4)	(50.4±10.6)	(326.7±63.0)	(222.4±29.4)
	Chloramphenicol	742.3**	0.048**	0.069**	0.352**	0.038**
	Cycloheximide	0.21**	0.857**	1.256**	1.030	1.282**
Gray Lowland soil[c]	Control	(54.7±7.3)	(27.5±11.2)	(48.4±10.4)	(213.6±15.1)	(1033.1 ±89.8)
	Chloramphenicol	16.2**	0.084**	0.023**	0.779**	0.132**
	Cycloheximide	1.09	2.647**	0.442**	0.712**	1.244**
Slurried (600 t)	Control	(14.8±4.3)	(20.5±9.0)	(46.2±7.5)	(227.5±96.4)	(873.9 ±384.3)
	Chloramphenicol	57.0**	0.254**	1.487**	1.062	0.886
	Cycloheximide	0**	0.888	1.762**	0.891	1.501**
Slurried (120 t)	Control	(0.7±0.8)	(29.2±8.4)	(64.9±15.4)	(214.4±14.5)	(296.6±78.7)
	Chloramphenicol	435.7**	0.051**	0.376**	0.660**	1.085
	Cycloheximide	0.14	0.668**	1.336**	0.278**	1.675**

**Siginificantly different from control at 1% level.

[a]CFU g^{-1} dry soil ±95% confidence interval.

[b]pKat leucine equivalent liberated g^{-1} dry soil ±95% confidence interval.

[c]Soils were sampled on June 3, 1992.

Table 2 Characterization of the isolated azocoll degraders (AD) and gelatin liquefiers (GL)

	Andosol				Gray Lowland soil				Slurry-amended soil			
	Fallow land		Cultivated		Upland		Water-logged		600t		120t	
	AD	GL	AD	GL	AD	GL	AD	GL	AD	GL	AD	GL
Gram +, rod, spore	100%	100%	96.4%	97.1%	70.8%	84.0%	58.9%	95.2%	58.1%	68.4%	81.0%	44.4%
Gram +, rod			1.2%		2.1%		2.9%	0.8%				
Gram +, cocci							5.8%		4.8%		1.7%	
Gram -, rod			2.4%	2.9%	27.1%	16.0%	27.3%	4.0%	37.1%	31.6%	17.2%	55.6%
Gram -, cocci							5.1%					
Number of isolates	80	77	83	69	48	25	411	124	62	19	58	9

Table 3 Effect of inhibitors on overall (2.5 mmole L^{-1} for the soil suspension) and extracted soil (0.5 mmole L^{-1} for the soil extract) caseinase and bacterial caseinase activities

	inhibitor[a]							
	EDTA	1.10P	pCMB	IAA	PMSF	DFP	PPA	Peps
Tne overall protease								
Gray Lowland soil								
OM(8/16)[b]	+	+	++	-	-	-	-	-
CF(8/16)[b]	+	+	++	-	-	+	-	-
NF(8/16)[b]	++	+	+	-	-	+	-	-
Andosol								
Cultivated	-	+	+	-	-	-	nd	nd
Fallow land	-	-	-	+	+	+	nd	nd
Slurry-amended Andosol								
120 t	+	+	+	-	-	-	nd	nd
600 t	+	++	-	-	-	-	nd	nd
Extracted soil protease								
Gray Lowland soil by 1.0 mole L^{-1} PB								
OM(6/9)[b]	+	+++	+	+	+	-	-	-
CF(6/9)[b]	+	++++	+	+	-	-	-	+
Gray Lowland soil by 0.1 mole L^{-1} PB								
OM(7/22)[c]	-	+	-	-	++	++	-	-
CF(7/22)[c]	-	-	-	+	-	-	-	-
NF(7/22)[c]	+	++	-	+	-	-	-	-
Gram negative bacteria (0.5 mmole L^{-1})								
s-131	+	++++	+	-	-	-	nd	nd
s-132	++	++++	+++	-	+	+	nd	nd
m-110	++	++++	++	-	-	-	nd	nd
B.subtilis (5.0 mmole L^{-1})								
ON-023[c]	++	-	-	-	++++	++	-	-
NN-207[c]	++	-	-	-	++++	++	-	-
NS-201[c]	++	-	-	-	++++	++	-	-
NN-205[c]	+	-	-	-	++++	++	-	-
NN-211[c]	+	-	-	+	++++	++	-	-
NN-212[c]	++	-	-	-	++++	++	-	-

Note: ++++ indicates 0～19% of control; +++, 20～39%; ++, 40～59%; +, 60～79%; -, 80%～. nd indicates not testd. The capital letters of soil and bacteria indicate the fertilizer managements; OM, CF, and NF refer to organic manure, chemical fertilizer, and without fertilizer application. Figures in parentheses indicate the date of isolation. The first number of the strain numbers, indicates the date of isolation; 1 stands for July 21, 2-August 11, and 3-October 16.

[a]EDTA, ethylenediaminetetraacetic acid; 1, 10P, 1, 10-phenanthroline; pCMB, p-chloromercuribenzoic acid; IAA, iodoacetic acid; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; PPA, 3-phenylpropionic acid; Peps, pepstatin.

[b]Cited from Watanabe and Hayano (1996a).

[c]Cited from Watanabe and Hayano (1993b).

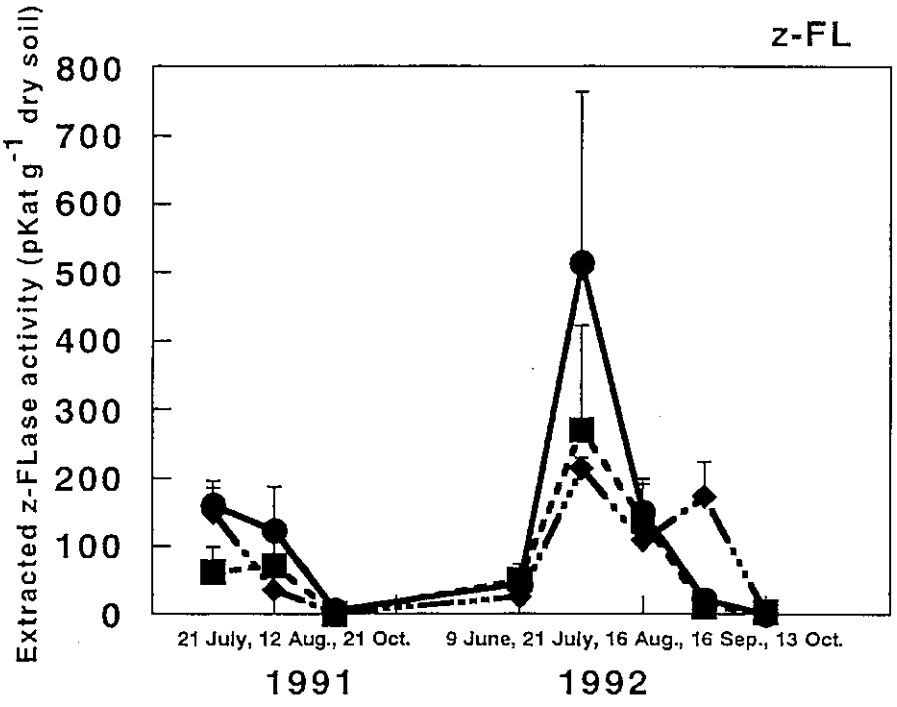

Fig.1 Seasonal variation of soil z-Flase activity extracted with 0.1 mole L^{-1} PB, pH 7.0 in paddy fields (organic manure, ●; chemical fertilizer, ■; and field without fertilizer application, ◆). Bar indicates 95% confidence interval.

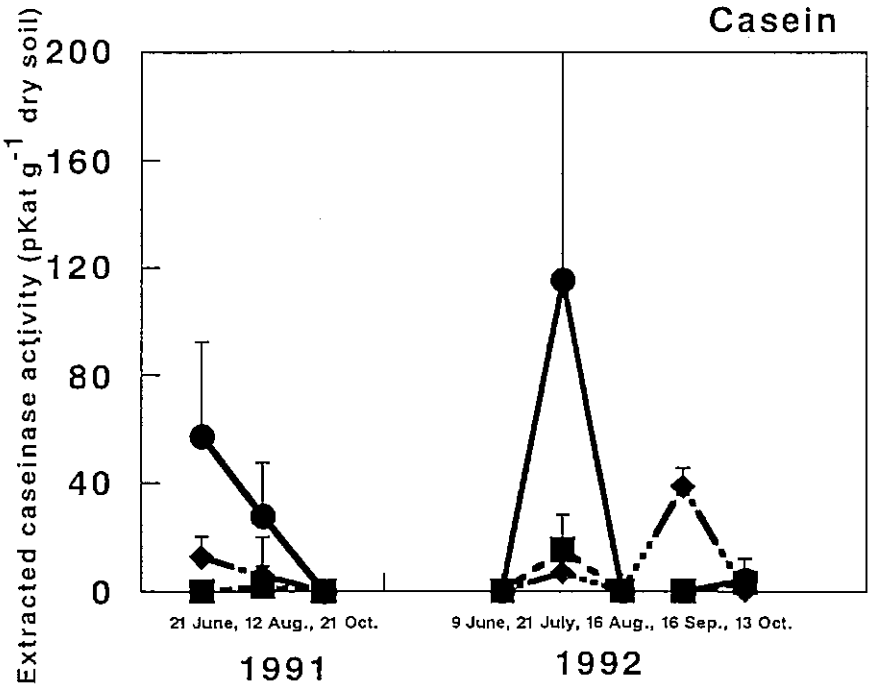

Fig.2 Seasonal variation of soil caseinase activity extracted with 0.1 mole L^{-1} PB, pH 7.0 in paddy fields (organic manure, ●; chemical fertilizer, ■; and fields without fertilizer application, ◆). Bar indicates 95% confidence interval.

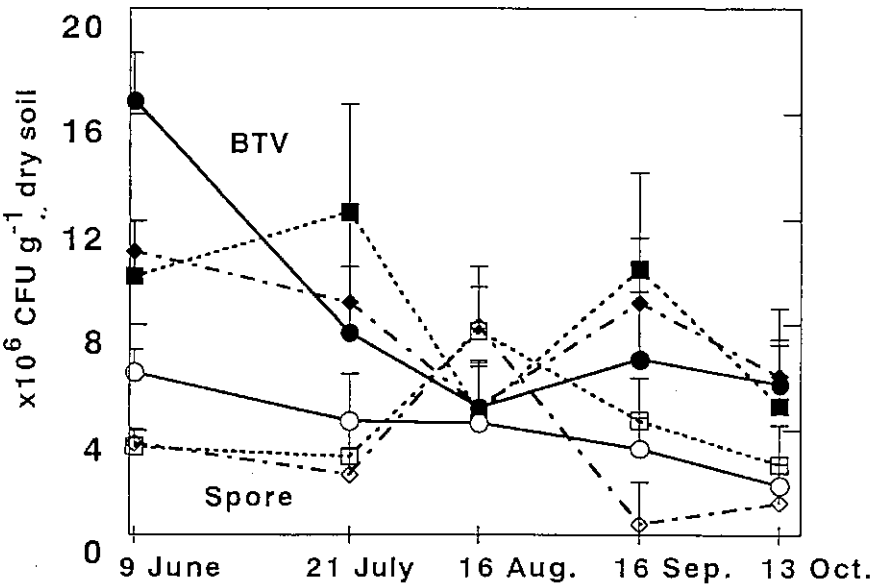

Fig.3 Seasonal variation of number of vegetative cells (BTV; closed) and spores (PP (80); open) of *Bacillus* spp. in paddy fields (organic manure, ●, ○; chemical fertilizer, ■, □; and field without fertilizer application, ◆, ◇). Bar indicates 95% confidence interval.

U-80	Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly
N-212	Ala-Gln-Ser-Val-Pro-Tyr-Gly-Ile-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly
Subtilisin BPN'	Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly
Subtilisin Amylosacchariticus	Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Ser-Gln-Ile-Lys-Ala-Pro-Ala-Leu-His-Ser-Gln-Gly
s-132	Ala-Ala-Thr-Thr-Gly-Tyr-Asp-Ala-Val-Asp-Asp-Leu-Leu-His-Tyr-His-Glu-Arg-Gly-Asn
Serratial protease	Ala-Ala-Thr-Thr-Gly-Tyr-Asp-Ala-Val-Asp-Asp-Leu-Leu-His-Tyr-His-Glu-Arg-Gly-Asn

Fig.4 N-terminal amino acid sequence of subtilisin (subtilisin BPN' (Vasantha *et al.*, 1984) and subtilish Amylosacchariticus (Kurihara *et al.*, 1972)) and serratial metalloprotease.

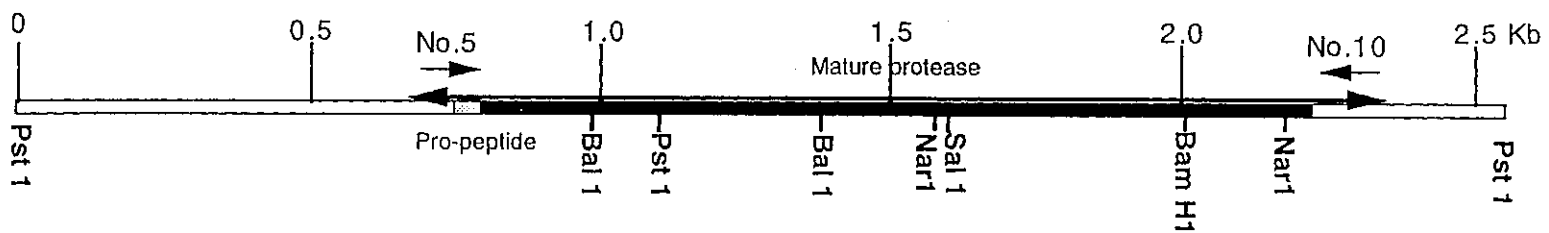

Fig.5 Restriction map of serratial metalloprotease gene (Nakahama *et al.*, 1986. Nucleic Acids Res. 14: 5843-5855). Arrow indicates amplified region of *S. marcescens* (s-132).