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棉花病菌 *Xanthomonas campestris* pv. *malvacearum* 的一种具特异肽键专一性的胞外蛋白酶的纯化与鉴定*

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摘要 棉花病原体 *Xanthomonas campestris* pv. *malvacearum* 在酪蛋白(脱脂奶)存在下生长时产生胞外蛋白酶活性,其中至少包含3种蛋白酶,表观分子量分别为29(蛋白酶-1)、38和43 kD。蛋白酶-1被纯化,其最适pH在5.5~7.5之间。抑制研究表明蛋白酶-1可被Phosphoramidone、EDTA及1,10-邻二氮杂菲抑制,然后用锌离子温育重新激活,说明这是一个金属蛋白酶。发现蛋白酶-1特异地裂解肽链的天冬氨酸残基或半胱氯酸残基的氨基端侧,这种高度的肽键专一性预示这个酶在蛋白质链顺序分析中及由较大蛋白质制备特定多肽方面可能十分有用。

关键词 *Xanthomonas campestris* pv. *malvacearum*,胞外蛋白酶,棉花疫病

PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR PROTEASE WITH UNUSUAL PEPTIDE BOND SPECIFICITY FROM *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

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Abstract The cotton pathogen *Xanthomonas campestris* pv. *malvacearum* produces extracellular protease activity when grown in the presence of casein-proteins (skim-milk). At least three proteases are produced with apparent molecular weights of 29 kD, 38 kD and 43 kD. Protease-1 can be inhibited by phosphoramidone, EDTA and 1,10-phenanthroline and reactivated by incubating with zinc ions, suggesting that protease-1 is a metalloprotease. It was found that protease-1 only cleaved the peptide bond of aspartic acid and cysteic acid from N-terminal side, confirming previous observations. This property could be very useful for protein sequence analysis and preparation of specific peptides from larger proteins.

Key words *Xanthomonas campestris* pv. *malvacearum*, Extracellular protease, Cotton blight

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Xanthomonas campestris pv. *malvacearum* is the causal agent of bacterial blight, a world-wide cotton disease. The detailed biological mechanism of pathogen-host interaction in this disease is still poorly understood. Some evidence suggests that the extracellular bacterial enzymes may play a role in pathogenicity. Venere *et al*[1] reported pectic enzymes preceding necrosis in leaves of susceptible cotton plants, as well as in those resistant to cotton blight. Extracellular proteases are produced by almost all the pathogenic strains of *X. campestris* pv. *malvacearum*[2], whereas the role of extracellular protease in bacterial plant disease has been little investigated by genetic methods. Gholson *et al*[3] reported that protease-deficient mutants of *X. campestris* pv. *malvacearum* show reduced disease symptoms and bacterial populations in the host plant. Some evidence from other *Xanthomonas* pathovars also supports the involvement of extracellular protease. An extracellular protease isolated from *X. campestris* pv. *oryzae* might play an active role in leaf blight of rice[4].

To understand whether the proteases from *X. campestris* pv. *malvacearum* play a role in cotton blight pathogenesis, the extracellular proteases from an Oklahoma field isolate of *X. campestris* pv. *malvacearum* were investigated. This work describes the purification of an unusual protease from a culture supernatant of the bacterium and the characterization of several properties of this enzyme including optimum pH studies, inhibition studies, and peptide bond cleavage specificity.

1 MATERIALS AND METHODS

1.1 Bacteria and culture conditions

The *Xanthomonas campestris* pv. *malvacearum* strain 3631 used was previously described by Essenberg *et al*[5]. Bacteria were amplified in nutrient broth and was shaken in a flask until an optical density of about 0.1 (OD_{600}) was reached. To the resuspended bacteria in MOPS buffer[6,7] L-alanine, L-arginine-HCl, L-asparagine, L-histidine-HCl, L-isoleucine, L-threonine, and L-phenylalanine were added as amino acids and glycerol as the carbon source (Fig. 1). (For routine protease production, the ammonium chloride in this medium was replaced with skim-milk plus L-glutamic acid as the nitrogen source.)

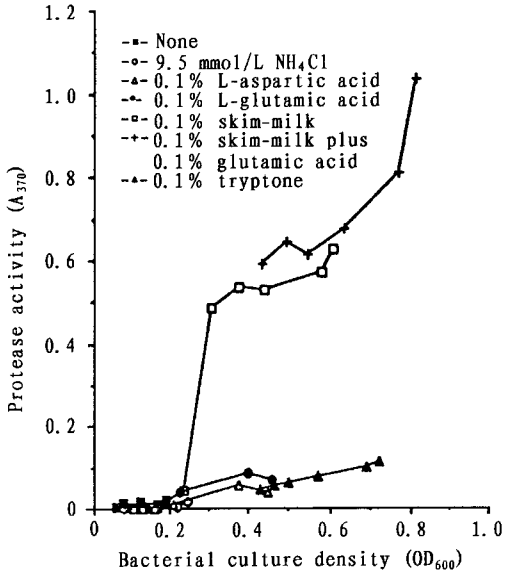

Fig. 1 Protease production as a function of growth on various nitrogen sources

1.2 Purification of protease

Cultures were grown to an apparent optical density (OD_{600}) of 0.5 to 0.6. The remaining steps were carried out at 4 ℃. The supernatant obtained by centrifugation was mixed with Whatman Carboxymethyl Cellulose CM-52 and stirred overnight. This resulted in the disappearance of detectable protease activity from the supernatant. The CM-52 cellulose column material with adsorbed protease was subjected to chromatography eluting with 0.01 mol/L Tris-HCl (pH 7.5), until the absorbance of the effluent at 280 nm was the same as the buffer. Protease activity was then eluted from the column with a linear gradient of 0 to 1.0 mol/L NaCl in buffer. Fractions were collected and assayed for protease activity and protein concentration (Fig. 2). The active fractions with $A_{370} > 0.2$ from the column were combined and loaded on a DEAE Cellulose column at pH 9.0. All flow-

through fractions with protease activity were adjusted back to pH 7.5. Then, protease fractions from the DEAE column were concentrated using a stirred ultrafiltration cell. The concentrated solution (4.0 mol/L NaCl in buffer) was applied directly to a Octyl-Sepharose CL-4B Column. The proteases were eluted using a step gradient of decreasing NaCl concentrations (Fig.3). Active fractions were individually dialyzed against distilled water, lyophilized, and dissolved in H_2O for gel electrophoresis (SDS-PAGE) and other studies.

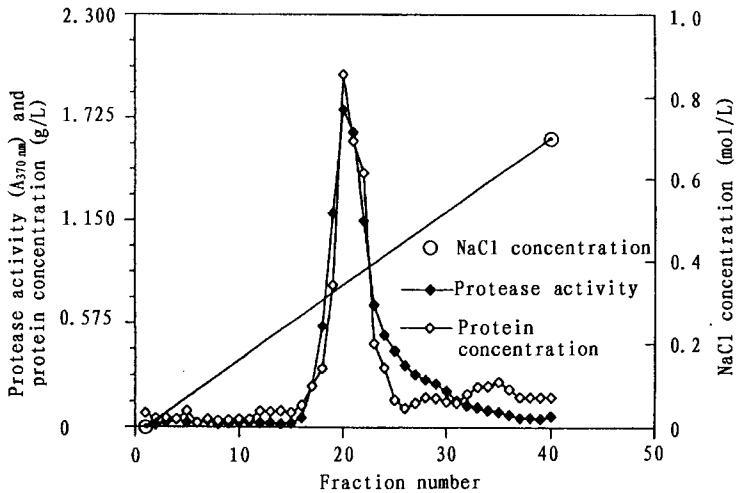

Fig.2 Cation exchange chromatography on CM-52 cellulose of the *Xanthomonas campestris* pv. *malvacearum* filtrate by a batch process eluted with a linear gradient of 0.0 to 1.0 mol/L NaCl in Tris-HCl (pH 7.5) buffer

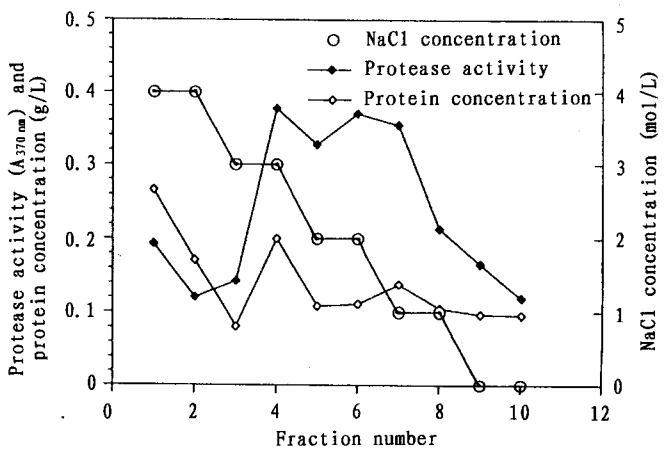

Fig.3 Purification of protease-1 by reverse phase chromatography on Octyl-Sepharose CL-4B Column

1.3 Enzyme assays

Protease activity was measured using 1% azocasein. One unit of activity was defined as the amount of enzyme needed to produce an increase in absorbance at 370 nm of 1.0 in 15 min[8].

1.4 Other methods

SDS-PAGE of the enzyme fractions was carried out as described by Weber *et al*[9]. Protein concentration was determined with the BCA assay[10]. The peptides produced by incubation of protease from *X. campestris* pv. *malvacearum* with substrates (insulin A chain and B chain, glucagon, Thymopoietin Ⅱ Fragment 32-36) were separated by HPLC using C-18 (5 μm) reversed phase col-

PMSF and 3, 4-dichloroisocoumarin, but are inhibited by EDTA, phosphoramidone and 1, 10-phenanthroline with remained activity of 53%, 3%, and 7%, respectively. Protease-1 activity was enhanced by $ZnCl_2$; presumably the purified proteases did not have enough Zn^{2+} to give full activity due to loss of Zn^{2+} through the 10 000 MW cutoff membrane during the concentration step. Thermolysin was severely inhibited by the last four reagents, while trypsin, was not. This result indicates that protease-1 is metalloproteases and Zn^{2+} ions are required for its activity.

2.4 Peptide bond specificity

The peptide bond cleavage specificity of *X. campestris* pv. *malvacearum* protease-1 was investigated using oxidized insulin A chain, oxidized insulin B chain, glucagon and Thymopoietin Ⅱ Fragment 32-36. The complete sequences of these peptides are shown in Fig.5. The peptide fragments generated by protease cleavage were separated on HPLC and then were subjected to mass spectrometry for peptide MW analysis. The substrates (20 μL of 1 mg/0.15 mL 0.01 mol/L NH_4OH and 20 L of 0.01 mol/L Tris-HCl buffer, pH 7.5) were digested with the purified protease-1 (30 μL of 0.1 mg/mL) from *X. campestris* pv. *malvacearum* for one hour at 30 ℃.

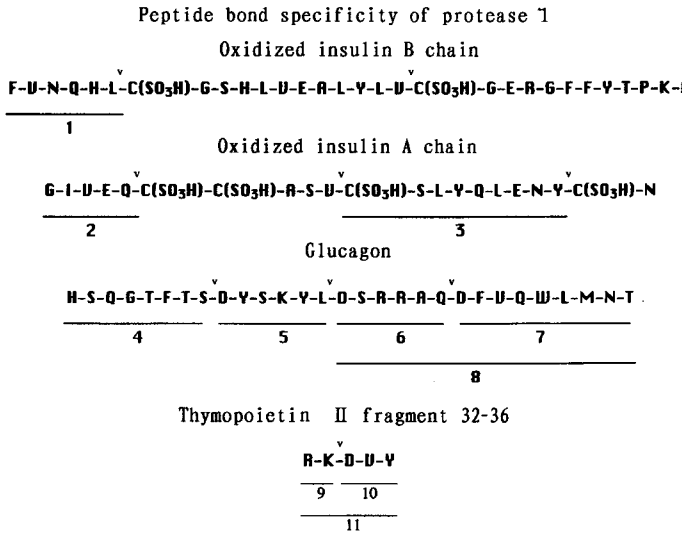

Fig.5　Peptide bond specificity of protease-1 was tested with oxidized insulin B chain, oxidized insulin A chain, glucagon and Thymopoietin Ⅱ Fragment 32-36
The under line sequences show the peptides identified by LSIMS or ESIMS.

The protease-1 digested oxidized insulin B chain gave four peaks (peptide fragments) on HPLC. The amino acid compositions of these peptides indicated that oxidized insulin B chain was cleaved by protease-1 only on the N-terminal side of the two cysteic acid residues as indicated in Fig.5. The LSIMS spectrum of this digestion is shown in Table 1. A mass of 757 is consistent with the molecular weight plus a proton [M + H^+] of the peptide F-V-N-Q-H-L, which was also confirmed by collecting this fragmemt from HPLC and identified by LSIMS. Masses of 779 and 801 are consistent with [M + Na^+] and [M + $2Na^+$] of the above peptide, respectively. Since F-V-N-Q-H-L is the only peptide with a mass of 757 that can be generated from oxidized insulin B chain, finding its mass by LSIMS confirms the predicted cleavage site.

The peptide fragments generated by protease-1 digestion of oxidized insulin A chain were separated and analyzed by HPLC and LSIMS. As shown by the results summarized in Fig.5, oxidized insulin A chain was only cleaved on the N-terminal side of cysteic acid residues at position 6, 11 and 20, and it did not occur between the adjacent cysteic acid residues at position 6 and 7. Two

peptide fragments were identified by LSIMS (Table 1). Masses of 545, 567, 589, and 611 were from peptide C-Y-V-E-Q, and masses of 1203, 1225, and 1247 were from peptide C(SO_3H)-S-L-Y-Q-L-E-N-Y. Since cysteic acid with its strong negative charge is difficult to ionize, some peptides with multiple cysteic acids did not show up in the spectrum of LSIMS.

Table 1 Lsims or esims pseudomolecular ions of the peptides obtained by *Xanthomonas campestris* pv. *Malvacearum* protease-Ⅰ cleavage of these substrates

Pseudomolecular ions (m/z)	Fragment of Insulin B	Fragments of Insulin A		Fragments of Glucagon					Fragments of Thymopoietin		32-36 Ⅱ
	1	2	3	4	5	6	7	8	9	10	11
$[M+H^+]$	757	545		864	788	732	1 153	1 868	303	396	680
$[M+Na^+]$	779	567	1 203	886	810	754	1 175	1 890		418	702
$[M+2Na^+]$	801	589	1 225		832		1 197	1 912		440	
$[M+3Na^+]$		611	1 247				1 219	1 933		462	
$[M+2NH_4^+]$											716
$[M+2NH_4+Na^+]$											738
$[M+2NH_4+2Na^+]$											760

Since cysteic acid is not a normal constituent of proteins, it was assumed that the natural cleavage site of *X. campestris* pv. *malvacearum* protease-1 is next to an amino acid residue with size and charge characteristics similar to cysteic acid. Aspartic acid, an amino acid which is not present in either the insulin A chain or B chain, resembles cysteic acid both in size and charge. Therefore glucagon, a 29-amino-acid peptide containing three aspartic acid residues and Thymopoietin Ⅱ Fragment 32-36, a five amino acid peptide containing one aspartic acid residue were tested. In both cases, the fragments separated by HPLC and the identification with LSIMS for glucagon and electrospray MS for the Thymopoietin Ⅱ Fragment 32-36 indicated that these peptides were cleaved only on the N-terminal side of aspartic acid residue.

Protease-1 has three cleavage sites on glucagon based on amino acid analysis results (Fig. 5). Five peptide fragments were generated by protease-1 digestion of glucagon, and were identified by LSIMS. Peptide D-S-R-R-A-Q was shown in LSIMS spectrum as 732 $[M+H^+]$ and 754 $[M+Na^+]$, peptide D-Y-S-K-Y-L as 788 $[M+H^+]$, 810 $[M+Na^+]$, and 832 $[M+2Na^+]$, peptide H-S-Q-G-T-F-T-S as 864 $[M+H^+]$ and 886 $[M+Na^+]$, peptide D-F-V-Q-W-L-M-N-T as 1154 $[M+H^+]$, 1175 $[M+Na^+]$, 1198 $[M+2Na^+]$, and 1220 $[M+3Na^+]$; peptide D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T as 1868 $[M+H^+]$, 1890 $[M+Na^+]$ and 1912 $[M+2Na^+]$ (Table 1).

Two peptide fragments generated by protease-1 digestion of Thymopoietin Ⅱ Fragment 32-36 were separated on HPLC, and identified to be the peptide R-K and D-V-Y by ESIMS with pseudomolecular ions of 303 and 396, 418, 440, 462, respectively (Table 1).

The above results indicated that both oxidized insulin A chain and B chain were cleaved by protease-1 only on the N-terminal side of cysteic acid residue and both glucagon and Thymopoietin Ⅱ Fragment 32-36 were cleaved by protease-1 only on the N-terminal side of aspartic acid residue. The peptide bond specificity of protease-1 was also tested by different incubation times (30 min, 1 h and 4 h) with insulin B chain (data not shown), the specificity of protease-1 had not changed. Therefore, it is summarized that protease-1 only cleaves the N-terminal side of aspartic acid.

3 DISCUSSION

The production of this extracellular protease by *X. campestris* pv. *malvacearum* appears to be regulated by nutritional factors. Appreciable protease activity is produced by *X. campestris* pv. *malvacearum* cultivation only when protein is present in the growth media (Fig. 1). It is possible that the protease inducer is a peptide(s) with N-terminal aspartate which would be formed by a low constitutive level of *X. campestris* pv. *malvacearum* protease. The requirement of protein in the

growth medium for protease secretion by *X. campestris* pv. *malvacearum* may also be related to the fact that this organism is a pathogen[12]. It is not known whether the proteases characterized here are also the major extracellular proteases expressed by *X. campestris* pv. *malvacearum* in *planta*.

The peptide bond specificity of protease-1 was studied using oxidized insulin A chain, oxidized insulin B chain, glucagon, and the Thymopoietin Ⅱ Fragment 32-36. After the treatment, the digested peptides were subjected to HPLC and identified by either LSIMS or electrospray mass spectrometry. Results obtained from HPLC and mass spectrometry revealed that glucagon and Thymopoietin Ⅱ Fragment 32-36 were cleaved on the N-terminal side of aspartic acid residue, and insulin A chain and insulin B chain were cleaved on the N-terminal side of the oxidized cysteine, i.e. cysteic acid (no aspartic acid residue is present in insulin A or B chain). The cleavage at cysteic acid by this protease is due to the similarity between aspartic acid and cysteic acid both in size and in charge. Since cysteic acid is not a normal constituent of proteins, it is apparent that the natural cleavage site of protease-1 is on the N-terminal side of aspartic acid residues.

Of the many proteases which have been characterized, only a very few are as specific as protease-1 in that they cleave peptide bonds adjacent to only one amino acid (22 to 29). It appears that the *X. campestris* pv. *malvacearum* protease-1 is the only protease so far isolated from a wild-type organism which specifically cleaves N-terminal to aspartic acid. Protease-1 with its high peptide bond specificity could be very useful for protein sequence analysis and the production of specific peptides from larger proteins.

REFERENCES

1 Venere R J, Brinkerhoff L A, Gholson R K. Pectic enzyme: An elicitor of necrosis in cotton inoculated with bacteria. *Proc Okla Acad Sci*, 1984. **64**:1 ~ 7

2 Verma J P. Bacterial Blight of Cotton. Florida: CRC Press, 1986. 93 ~ 135

3 Gholson R K, Rodgers C, Pierce M. Extracellular proteases of *Xanthomonas campestris* pv. *malvacearum*. *Phytopathology*, 1989. **79**:1199

4 Xu G W, Gonzalez C F. Evaluation of TN 4431-induced protease mutants of *Xanthomonas campestris* pv. *oryzae* for growth in plants and pathogenicity. *Phytopathology*, 1989. **79**:1210

5 Essenberg M, Doherty M d'A, Hamilton B K *et al*, Identification and effects on *Xanthomonas campestris* pv. *malvacearum* of two phytoalexins from leaves and cotyledons of resistant cotton. *Phytopathology*, 1982. **72**:1349 ~ 1356

6 Neidhardt F C, Bloch P C, Smith D F. Culture medium for enterobacteria. *J Bacteriol*, 1974. **119**:736 ~ 747

7 McNally K, Gabriel D, Essenberg M. Useful minimal media for *Xanthomonas campestris* pv. *malvacearum*. *Phytopathology*, 1984. **74**: 875

8 Jensen S E, Phillippe L, Teng Tseng J *et al*. Purification and characterization of exocellular protease produced by a clinical isolated and a laboratory strain of *Pseudomonas aeruginosa*. *Can J Microbiol*, 1980. **26**:77 ~ 86

9 Weber K, Pringle J R, Osbirne M. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. *Methods Enzymol*, 1972. **26**:3 ~ 27

10 Smith P K, Krohn R I, Hermanson G T *et al*. Measurement of protein using bicinchoninic acid. *Anal Biochem*, 1985. **150**:76 ~ 85

11 Fenn J B, Mann M, Meng C K *et al*. Electrospray ionization-principles and practice. *Mass Spectrometry Rev*, 1990. **9**:37 ~ 66

12 Gholson R K, Essenberg M. Role of a neutral protease in phytopathogenicity of *Xanthomonas campestris* pv. *malvacearum*. *Federation Proc*, 1987. **46**:2212