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Cécile Wandersman, Thierry Andro, Yves Bertheau. Extracellular proteases in *Erwinia-chrysanthemi*. Journal of general microbiology, 1986, 132, pp.899-906. hal-02724929

**HAL Id: hal-02724929**

**<https://hal.inrae.fr/hal-02724929>**

Submitted on 2 Jun 2020

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## Extracellular Proteases in *Erwinia chrysanthemi*

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(Received 18 April 1985; revised 1 October 1985)

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Extracellular proteolytic enzyme activity has been detected in cultures of *Erwinia chrysanthemi*. This activity, which appears when the cells are grown in the presence of peptides, is rather unstable. A hyperproteolytic mutant was isolated which produces two proteases of apparent polypeptide molecular mass of 50 and 55 kDa, respectively. The 50 kDa protease, which is produced in the largest amounts, has been purified to near homogeneity. It has the properties of a neutral serine protease. The 50 and 55 kDa proteases are unrelated antigenically. Preliminary evidence suggests that both proteases are also produced by the wild-type strain, but that they are either produced in much smaller quantities or are much less stable.

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### INTRODUCTION

Both Gram-positive and Gram-negative bacteria secrete proteins into the medium (Priest, 1977; Pugsley & Schwartz, 1985). In Gram-positive bacteria, which possess a single membrane, the secretion process has been extensively studied, and mainly appears to be similar to the signal peptide pathway of protein export as originally described for the endoplasmic reticulum in eukaryotic cells (Walter *et al.*, 1984). In Gram-negative bacteria, the export of proteins to the periplasmic space, or to the outer membrane, has also been the subject of numerous studies (Michaelis & Beckwith, 1982; Randall & Hardy, 1984), especially at the genetic level, but comparatively little is known of the secretion, *sensu stricto*, into the medium (Pugsley & Schwartz, 1985). As a model system to approach this latter problem we have chosen to characterize the protease(s) secreted by *Erwinia chrysanthemi*.

*E. chrysanthemi* is a Gram-negative phytopathogenic bacterium responsible for soft rot disease in various plants (Chatterjee & Starr, 1980). Since it is taxonomically rather close to *Escherichia coli*, genetic tools developed for this latter organism can be used to study it (Chatterjee & Starr, 1980; Kotoujansky *et al.*, 1982). *E. chrysanthemi*, as well as other *Erwinia* species, produces several extracellular enzymes. Among them are pectinases and cellulases, which have already been characterized and shown to play a role in the pathogenic properties of the organism (Collmer *et al.*, 1985; Reverchon *et al.*, 1985; Kotoujansky *et al.*, 1985; Keen *et al.*, 1984). *E. chrysanthemi* also secretes one or several proteases, but these have not been characterized, and their role in pathogenicity is unknown (Chatterjee & Starr, 1980; Grimont *et al.*, 1977).

In the present work we describe proteases which are present in a wild-type strain of *E. chrysanthemi*, and in a mutant which produces increased amounts of proteolytic activity.

### METHODS

*Bacterial strains.* Two strains of *Erwinia chrysanthemi* were used: strain 3937JS2, which is a streptomycin-resistant mutant of the wild-type virulent strain 3937J isolated from infected saintpaulia plants (Kotoujansky *et al.*, 1982), and strain HP3, a spontaneous mutant of 3937JS2 which hyperproduces proteolytic activity (this work).

*Media.* Complex medium was LB (Miller, 1972). Minimal medium was M63 (Hatfield *et al.*, 1969). The carbon source added to M63 was glycerol at a final concentration of 0.4% (w/v). Casein tryptic hydrolysate was prepared essentially as described by Laskowski (1955). A 100 ml volume of a 1% (w/v) casein solution in 10 mM-Tris/HCl buffer (pH 8.0) was heated for 30 min in boiling water to allow complete solubilization and was then cooled to 30 °C. Crystalline trypsin (1 mg) was added and the mixture was incubated overnight at 30 °C. The trypsin was then inactivated by heating the solution for 1 h in boiling water. A protease assay (see below) showed no remaining trypsin activity after this treatment. Casein hydrolysed by the exoproteases of the HP3 strain was prepared in a similar way. A 500 µl volume of 100-fold concentrated HP3 supernatant medium was added to 100 ml 1% casein solution. After overnight incubation at 30 °C, the proteolytic enzymes were inactivated as described above. The two casein hydrolysates were then lyophilized and the dry materials were resuspended in 10 ml distilled water and filtered through Millipore filters (0.45 µm pore size). Casein peptides of molecular mass higher and lower than 1 kDa were separated by filtering the casein hydrolysates, before lyophilization, through an Amicon concentrator with a UM5 filter. The separated fraction was then lyophilized, resuspended and filtered as above. A preparation containing 10% (w/v) skim milk (Difco) and 1.5% (w/v) agar was used to visualize the proteolytic activity of bacterial colonies. A preparation containing 1% skim milk and 1.5% Noble agar, in 100 mM-Tris/HCl buffer (pH 8.0), was used for cup plate protease assays and for protease zymograms (see below).

*Protease assay.* Protease was assayed by the cup plate technique (Andro *et al.*, 1984). Enzyme solutions were introduced into wells on skim milk agar plates. The plates were incubated at 37 °C. Cleared rings corresponding to casein hydrolysis were measured after 3–4 h. Protease was also assayed by following the hydrolysis of azocasein as described by Braun & Schmitz (1980). Enzyme activity was measured by the increase in  $A_{440} \text{ min}^{-1} \text{ ml}^{-1}$ . All the assays were reproduced at least three times. Representative results are shown; the variability was less than 10%.

*Isolation of protease hyperproducing mutants.* Spontaneous hyperproducing mutants were obtained as follows. Samples of independent cultures of strain 3937JS2 were plated on skim milk agar. After 2 d incubation at 30 °C, small papillae growing out of the bacterial lawn were picked and purified twice on the same medium. Clones exhibiting the largest hydrolysis zones around the colonies were purified and assayed for protease activity.

*Localization of protease activity.* Cultures were centrifuged for 20 min at 5000 *g* at 4 °C. The supernatant medium which contained the extracellular enzymes was concentrated by lyophilization, dialysed three times against 1 l 100 mM-Tris/HCl buffer (pH 8.0) and then assayed for protease activity. The pellets were washed once with the Tris/HCl buffer and the cells were disrupted by sonication. Membranes and intact cells were then sedimented by centrifugation for 30 min in an Eppendorf microfuge at 4 °C. Protease was assayed in the soluble supernatant and the membrane fractions.

*Protease purification.* One litre of an overnight culture grown in LB medium was centrifuged for 30 min at 5000 *g* at 4 °C. The supernatant was filtered through an Amicon concentrator with PM10 filters. The concentrated supernatant (100 ml) was dialysed three times against 5 l 10 mM-Tris/HCl buffer (pH 7.5) and then lyophilized and dissolved in 10 ml 100 mM-Tris/HCl buffer (pH 8.0). It was then filtered through a Biogel P100 column (2 × 50 cm) equilibrated with 100 ml Tris/HCl buffer (pH 8.0) at a flow rate of 5 ml h<sup>-1</sup> (2.5 ml per fraction). Fractions containing the protease were pooled and dialysed five times against 2 l 10 mM-sodium phosphate buffer (pH 7.0) and then chromatographed on a DE52 Whatman DEAE-cellulose column (2 × 20 cm). The column was washed with sodium phosphate buffer and 20 fractions of 5 ml were collected. The column was washed with 500 ml 10 mM-sodium phosphate buffer containing 100 mM-NaCl and then with 150 ml of the same buffer containing 500 mM-NaCl. Protease activity was eluted at the beginning of the 100 mM-NaCl step. Fractions containing the activity were pooled, dialysed three times against 1 l water and lyophilized. The dry material was dissolved in 1.5 ml 10 mM-Tris/HCl buffer (pH 7.5). The protein concentration was approximately 1 mg ml<sup>-1</sup>, as determined by the Lowry method.

*Electrophoresis.* Proteins were analysed by SDS-PAGE following the technique of Laemmli (1970) as modified by Anderson *et al.* (1973).

*Detection of proteolytic enzyme activity on polyacrylamide gels (protease zymogram).* Proteins were separated by SDS-PAGE as described above. The proteolytic activity which is retained under these conditions was detected by placing the polyacrylamide gels on skim milk agar. Bands of clearing corresponding to casein hydrolysis appeared after 3 h incubation at 37 °C.

*Titration curves.* (pH titration in gels: pH range 3–10.) The pH gradient was developed at 30 W (1 h) and the sample (70 µl) was electrophoretically separated, for the second dimension, at 800 V (20 min) as described by Bertheau *et al.* (1984).

The general techniques of preincubation, enzymic visualization and blotting, as applied to pectinases and cellulases, were as described by Bertheau *et al.* (1984); for proteases, the buffer was 50 mM-Tris/HCl (pH 7.5), and the substrate was skim milk (1%, w/v). After incubation, polyacrylamide and agar gels were separated. Proteolytic enzymes appeared as translucent areas in the buffered substrate-containing agar gels, which were coated on agarose gel-bond plastic sheets. To obtain the best visualization, agar gels were submerged in 10% (w/v) trichloroacetic acid.

Table 1. Induction of extracellular protease activity in strains 3937JS2 and HP3

Except for the last line the cells were grown in minimal M63 glycerol medium. The compounds listed were tested at a final concentration of 1% (w/v). Overnight cultures of strains 3937JS2 and HP3 grown in the indicated media were sedimented by centrifugation, and the supernatants were assayed for protease activity (azocasein hydrolysis). The specific activity is expressed as the increase in  $A_{440}$   $\text{ml}^{-1} \text{min}^{-1}$  for a culture density corresponding to an  $\text{OD}_{600}$  of 1, and multiplied by  $10^5$ .

Inducer	Protease specific activity produced by:	
	Strain 3937JS2	Strain HP3
None	<1	<1
Casein	<1	<1
Mixture of the 20 amino acids	<1	<1
Tryptone	18	120
Casein hydrolysed with trypsin	11	72
Casein hydrolysed with the strain HP3 exoproteases	16	100
Casein peptides of molecular mass >1 kDa	27	87
LB medium	22	100

*Immunoblotting.* Proteins were separated by SDS-PAGE and then transferred from the gel to nitrocellulose sheets by the Western blot technique (Bowen *et al.*, 1980) as modified by Briat *et al.* (1984). The antiserum against 50 kDa protease was raised in a rabbit.

## RESULTS

### *An inducible extracellular proteolytic activity in E. chrysanthemi*

Proteolytic activity, as measured by the hydrolysis of azocasein, was sought in the culture medium of strain 3937JS2 (Table 1). No such activity was present when the bacteria were grown in minimal glycerol medium, but it appeared during growth on complete LB medium. Constituents that, when added to the synthetic medium, induced the appearance of proteolytic activity were: tryptone, casein hydrolysed with trypsin, casein hydrolysed by the expoproteases of the hyperproteolytic mutant described later in this paper, and casein peptides, even when of a molecular mass greater than 1 kDa. In contrast, neither casein itself nor an artificial mixture of all 20 amino acids were inducers.

The proteolytic activity in the culture supernatant of strain 3937JS2 was very unstable. In particular, all attempts to concentrate the enzyme(s) led to a considerable decrease in activity. For this reason we decided to look for mutants exhibiting a higher proteolytic activity and which might produce more of the protease(s) or more stable forms of the enzyme(s).

### *Isolation of a hyperproteolytic mutant*

Spontaneous mutants exhibiting a higher proteolytic activity were selected by growth on skim milk agar, as described in Methods. One such mutant, hereafter called strain HP3, was studied further. It produced five times more proteolytic activity than the parental strain, but the induction pattern was the same (Table 1). In contrast it produced normal amounts of pectinase and cellulase activities (not shown). The supernatant from a culture of strain HP3 could be concentrated without significant loss of the proteolytic activity. This activity was already produced during the exponential phase of growth but the highest specific activities were only reached during early stationary phase (Fig. 1). Overnight cultures of strain HP3 grown in LB (induced) or M63 glycerol (uninduced) media were used to prepare soluble intracellular and membrane fractions as described in Methods. Protease activity could not be detected in soluble cell extracts or in membrane fractions of uninduced or induced cultures. This lack of activity was apparently not caused by the presence of an inhibitor since no decrease in activity was observed when the 10-fold concentrated subcellular fractions were added to the active culture supernatant. Therefore the proteolytic activity appears to be truly exocellular.

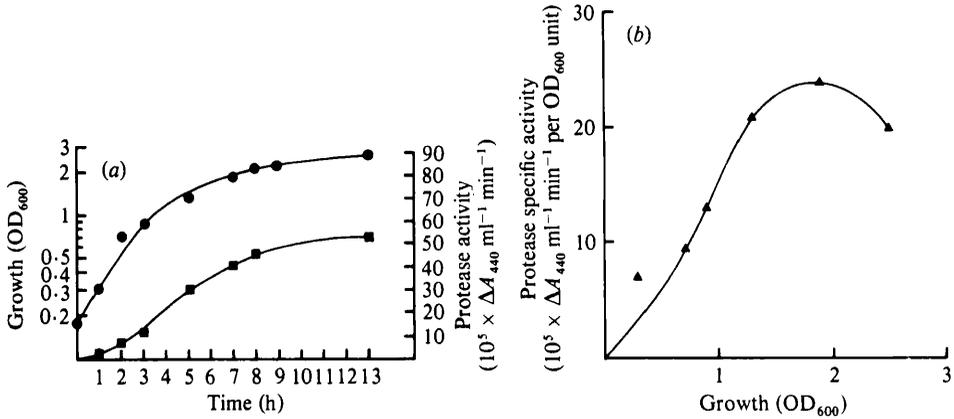


Fig. 1. Production of exoprotease activity during bacterial growth. Cells were grown at 30 °C in LB medium. At the times indicated, 20 ml portions were removed and the supernatant was recovered and concentrated 15-fold as described in Methods. (a) ●, Culture density measured as OD<sub>600</sub>; ■, protease activity in the supernatant. (b) Increase in protease activity related to the cell mass.

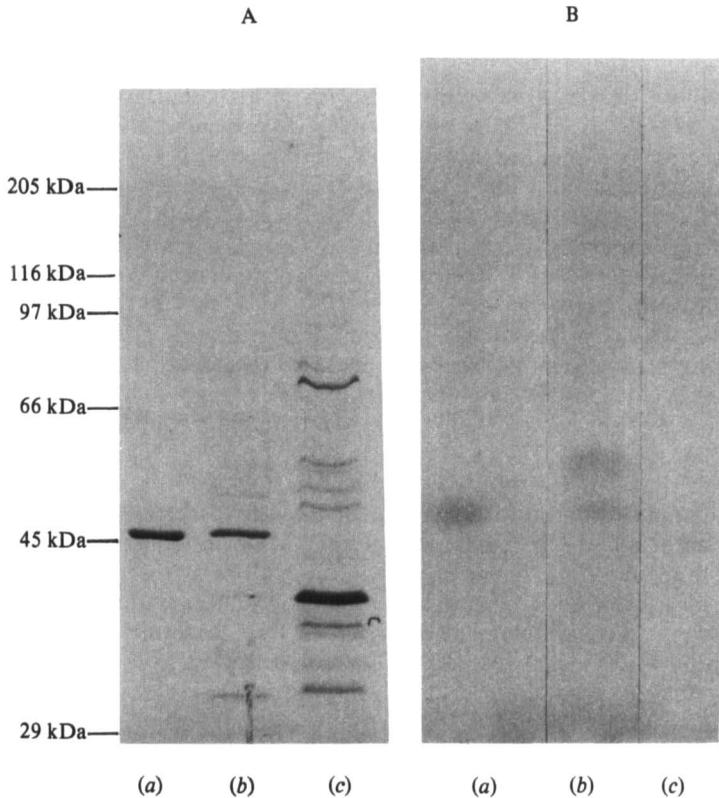


Fig. 2. Exoproteases produced by mutant HP3. Cultures (100 ml) of strain HP3, grown overnight in the media indicated, were sedimented by centrifugation. The supernatants were concentrated 100-fold and subjected to SDS-PAGE. (a) purified 50 kDa exoprotease; (b) supernatant of strain HP3 grown in LB medium; (c) supernatant of strain HP3 grown in M63 glycerol; A, Coomassie blue staining; B, detection of proteolytic activity on SDS-PAGE. A and B correspond to identical halves of the same gel.

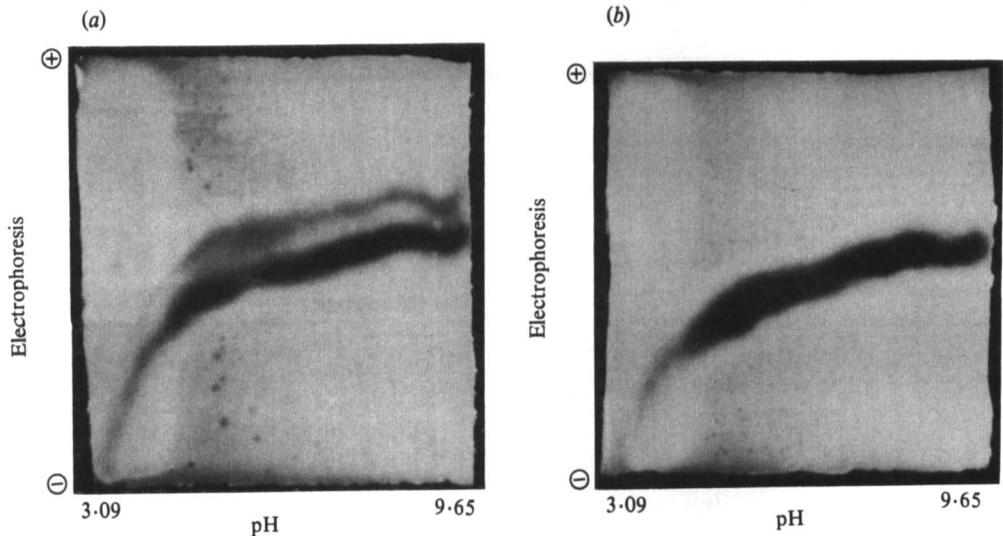


Fig. 3. Titration curves of strain HP3 exoproteases. Cultures (100 ml) of strain HB3 grown in LB medium were sedimented by centrifugation and concentrated 100-fold. (a) Concentrated HP3 supernatant; (b) purified 50 kDa protease.

#### *The hyperproteolytic mutant produces two different proteases*

Concentrated supernatant of cultures grown in M63 glycerol (uninduced) and LB (induced) media were subjected to SDS-PAGE. The protein patterns shown in Fig. 2 indicate several differences between the uninduced and induced samples. Many protein bands were only present in the M63 glycerol sample. This may be explained by a repression of protein synthesis by the complex rich LB medium. Nevertheless, two protein bands, corresponding to apparent molecular masses of 50 kDa and 55 kDa, were present only in the sample from induced cultures. These two proteins, despite the fact that they had been denatured by electrophoresis, had proteolytic activity, as shown by blotting the gel into skim milk agar (Fig. 2). The existence of two different proteases was also demonstrated by pH titration in gels (titration curves) (Fig. 3). The major one, which corresponded to the 50 kDa protein, had a pI of 5.8, whereas the minor one (55 kDa) had a pI of 4.6. The 50 kDa protease was purified as described in Methods (Figs 2 and 3). In contrast, the 55 kDa protease could not be recovered from any of the fractions from the various purification steps. The pH optimum for activity of the 50 kDa protease, as measured by hydrolysis of azocasein, was between 6.0 and 8.0 (data not shown). The enzyme was not inactivated by 10 mM-EDTA, but it was totally inactivated by 10 mM-phenylmethylsulphonyl fluoride; therefore the 50 kDa enzyme is a neutral serine protease (Walsh & Wilcox, 1970).

#### *Immunochemical comparison of the 50 kDa and 55 kDa proteases*

The fact that the 55 kDa protein disappeared during purification suggested that it might be converted, perhaps by autodigestion, into the 50 kDa protein. If this were so, one might have expected the two proteins to cross-react immunologically. To test this hypothesis, the proteins of a concentrated HP3 supernatant were separated by SDS-PAGE, and then transferred to nitrocellulose sheets for immunodetection with antibodies against 50 kDa protein antibodies (Fig. 4). This immunoblotting revealed two protein bands. One corresponded to the 50 kDa protease, and the other to a protein of lower molecular mass (approximately 30 kDa), which lacked proteolytic activity. Pulse-chase experiments followed by immunoprecipitation suggested that the latter band corresponds to a degradation product of the 50 kDa protein (data not shown). No protein of molecular mass higher than 50 kDa was detected by the immunoblotting. This result indicates that the 50 and 55 kDa proteins are not closely related.

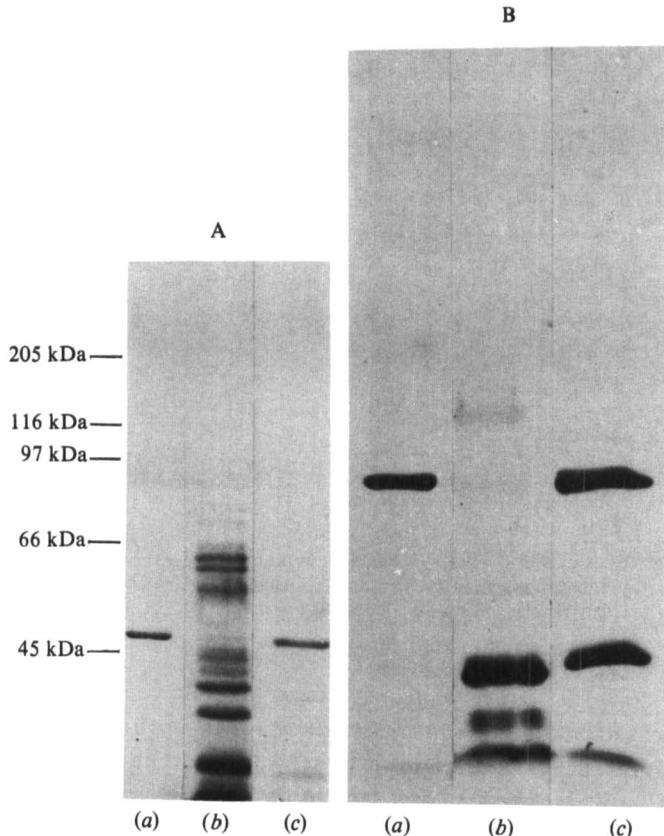


Fig. 4. Analysis of wild-type and HP3 mutant supernatants with anti-50 kDa protease antibodies. The 100-fold concentrated supernatant of strain HP3 grown in LB medium was the same as in the experiment shown in Fig. 2. The 1000-fold concentrated supernatant of strain 3937JS2 grown in LB was used. (a) Purified 50 kDa protease; (b) 3937JS2 supernatant; (c) HP3 supernatant; A, Coomassie blue staining; B, immunoblot. A and B correspond to two identical halves of the same gel. Note that the two photographs A and B are not on the same scale.

#### *Comparison of the proteases synthesized by the wild-type strain and the hyperproteolytic mutant*

The proteins of concentrated supernatant from a culture of the wild-type strain (3937JS2) were separated by SDS-PAGE and stained with Coomassie blue. Since the proteolytic activity in the culture supernatant of strain 3937JS2 was very unstable (see above), we used a 1000-fold concentrated supernatant which was only concentrated by filtration without lyophilization in order to decrease the protease inactivation. This highly concentrated supernatant sample exhibited several protein bands but none corresponding to the 50 kDa and 55 kDa proteins (Fig. 4). However, a faint proteolytic activity could be seen in the 55 kDa region, indicating that the minor protease found in the mutant may also be produced by the wild-type strain (data not shown). Immunoblotting of the gel failed to reveal the presence of intact 50 kDa polypeptide. However, antibodies against the 50 kDa protease antibodies did react with two polypeptides of lower molecular mass, one of which had the same migration rate as the inactive 30 kDa polypeptide in the supernatant of strain HP3 (Fig. 4).

#### DISCUSSION

Extracellular proteolytic enzyme activity has been found in cultures of *Erwinia chrysanthemi*. This activity appeared when the cells were grown in the presence of peptides, but not in the presence of a mixture of amino acids, indicating that intact peptide bonds are involved in the

induction process, as already reported for proteolytic enzymes produced by *Serratia marcescens* (Braun & Schmitz, 1980; Bromke & Hammel, 1979; Castaneda-Agullo, 1956) and various *Vibrio* species (Dreisbach & Merkel, 1978; Keil-Dlouha *et al.*, 1976). The fact that casein itself is not an inducer, even though casein peptides do induce, indicates that the basal level of protease is insufficient to release inducing peptides from the protein.

Due to its great instability, the proteolytic activity produced by the wild-type strain proved difficult to study. Instead we chose to study the protease(s) produced by a hyperproteolytic mutant, which was shown to produce two proteases of apparent polypeptide molecular mass of 50 and 55 kDa. The 50 kDa protease, which was produced in the largest amounts, was purified to near homogeneity. The two proteases were antigenically unrelated. Our evidence suggests that the wild-type strain also produces the 55 kDa protease, albeit in low amounts, and possibly the 50 kDa protease, which seems to be rapidly converted into inactive but immunologically cross-reacting degradation products. In view of these results the nature of the mutation responsible for protease hyperproduction is not clear. It may correspond to increased synthesis of the two proteases, but could also be a structural mutation which might render the 50 kDa protease less active, such that the autodigestion of this protein, as well as the digestion of the 55 kDa protease would be reduced.

The 50 and 55 kDa proteases appear to be truly exocellular. No activity could be detected in cell extracts, and the release of the proteases into the growth medium was not associated with detectable cell lysis. It is interesting that recently isolated pleiotropic mutants of *E. chrysanthemi*, which failed to secrete cellulases and pectinases, were unimpaired in their extracellular proteolytic activity (Andro *et al.*, 1984). This suggests the existence of at least two pathways for protein secretion in this bacterium. Studies on the mechanisms whereby the proteases are secreted will require a characterization of their structural genes. This work is now in progress, using a recently isolated cosmid library (Reverchon *et al.*, 1985). It should also shed light on the possible relationship between the two proteases, and on the nature of the hyperproteolytic mutant.

We thank Maxime Schwartz, Tony Pugsley, Catherine Desaynard and Alain Kotoujansky for very useful discussions and help in preparing the manuscript. This work was supported by grants from the Centre National de la Recherche Scientifique (UA 04 11 49) by the Ministère de la Recherche et de la Technologie (82 V 1279 and ATP Microbiologie CP 96.0031).

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