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Identification of cleavage sites involved in proteolytic processing of *Pseudomonas aeruginosa* preproelastase

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The extracellular elastase (33 kDa) of *Pseudomonas aeruginosa* is synthesized as a 53.6 kDa preproenzyme containing a long, N-terminal propeptide. The free propeptide and the elastase precursor generated upon propeptide removal were isolated from *P. aeruginosa* cells and subjected to N-terminal amino acid sequence analysis. The results identified Ala^{-174} and Ala^{+1} as the amino terminal residues of the propeptide and the elastase precursor, respectively, indicating that: (1) the signal peptide consists of 23 amino acid residues and its molecular weight is 2.4 kDa, (2) the propeptide contains 174 amino acid residues and is of 18.1 kDa molecular weight, and (3) no additional N-terminal proteolytic cleavage is required for elastase maturation.

Proteolytic processing; Preproelastase processing; Elastase; *Pseudomonas aeruginosa*

1. INTRODUCTION

The extracellular elastase of *Pseudomonas aeruginosa* is a 33 kDa neutral metalloprotease, thought to play a role in pathogenesis of *Pseudomonas* infections [1]. We have recently reported that elastase is made by the cells as a preproenzyme of about 60 kDa molecular weight containing a signal sequence and a propeptide with approximate molecular weights of 4 and 20 kDa, respectively [2]. We found that preproelastase as well as the proelastase generated from it upon removal of the signal peptide (proelastase I) are both short-lived. In addition, we demonstrated that, in the periplasm, the propeptide is cleaved en bloc to form a non-covalent complex with the elastase portion of the molecule, which we termed proelastase II. Since the latter protein appeared to be about 500 Da larger than mature elastase, we proposed that an additional proteolytic cleavage was required for elastase maturation [2,3]. Cloning [4,5] and sequencing [6,7] of the elastase structural gene, *lasB*, also indicated that the initial gene product is a preproenzyme and placed the propeptide between the signal sequence and the N-terminal end of mature elastase. These studies established that mature elastase contains 301 amino acids and that preproelastase is a 53.6 kDa protein, consisting of 498 amino acid residues. In addition, four potential leader peptidase cleavage sites were revealed [6–8]. Accurate sizes of the signal peptide and the propeptide were, however, not determined. In this study, by N-terminal sequencing of elastase precursors, we defined the cleavage sites involved in proteolytic processing of preproelastase and established correct sizes for the signal peptide and the propeptide.

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2. MATERIALS AND METHODS

2.1. *Bacterial strain, culture conditions, and cell extract preparation*

Pseudomonas aeruginosa Habs serotype I was grown with shaking at 37°C in tryptic soy broth without dextrose (Difco laboratories). Cells from a 1.6 liter culture were harvested at the end of the logarithmic growth phase (A_{660}~1.8), washed with saline, suspended in 15 ml 0.02 M Tris-HCl, 0.5 mM $CaCl_2$, pH 7.5, and broken by sonication (10 × 15 s pulses using a W-225 Heat Systems Ultrasonics, Inc. sonifier). Cell debris and membranes were removed by ultracentrifugation (100,000 × *g*, 1 h, 4°C) and the clear supernatant (cell extract) dialyzed against phosphate buffered saline containing 0.85 M NaCl, 20 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride and 0.1% Brij 35.

2.2. *Purification of periplasmic elastase (proelastase II) and propeptide*

The propeptide-proelastase II complex was purified from the cell extract by immunoaffinity chromatography on a column of Sepharose coupled to antielastase antibodies as described [3]. Fractions containing the purified proteins were pooled and, to avoid non-specific proteolytic cleavages, concentrated immediately by trichloroacetic acid (10%) precipitation. The precipitate was washed with acetone, air dried, dissolved and heat denatured in SDS sample buffer [10] and subjected to SDS-polyacrylamide gel electrophoresis in a 12% gel [9,10]. The individual proteins were recovered from the gel by electroelution [10,11], followed by exhaustive dialysis against water and lyophilization.

2.3. *Amino acid sequence analysis*

The N-terminal amino acid sequence of proteins was determined with an ABI model 475A gas-phase protein sequencer (Applied Bio-

systems, Foster City, CA). The amounts of propeptide and proelastase II analyzed were 0.5 and 1.7 nmol, respectively.

3. RESULTS AND DISCUSSION

The sequence of the first five amino acid residues of the elastase propeptide isolated from *P. aeruginosa* cells is presented in Fig. 1A. As seen, this sequence is found between positions −174 and −170 of the preproelastase amino acid sequence deduced from the nucleotide sequence of *lasB* [6,7]. It overlaps the second of the four possible signal peptidase cleavage sites noted previously [6,8], identifying the peptide bond between Ala^{-175} and Ala^{-174} as the physiological signal peptidase cleavage site. As the entire 'prepro' domain contains 197 amino acid residues [7], it is apparent that the signal peptide consists of 23 amino acids. Based on the respective amino acid composition, the molecular weight of the signal peptide is 2,408 Dal, i.e. well within the range found for most bacterial signal peptides [12]. Similarly, the sequence Ala-Phe-Ala-Ala (−177 to −174), constituting the junction between the 'pre' and the 'pro' domains, and the preceding proline residue in position −179 represent a typical signal peptidase recognition site [12]. This site was favored by Fukushima et al. [7] as the signal peptidase cleavage site used in vivo.

The N-terminal amino acid sequence of mature elastase was determined by Bever and Iglewski [6] and by Fukushima et al. [7]. The junction between the 'pro' and mature sequences, shown in Fig. 1B, was thereby identified and the peptide bond His–Ala in positions 197–198 of preproelastase proposed as that cleaved upon proteolytic processing of the propeptide. These residues were renumbered as −1 and +1, the latter representing the first amino acid of mature elastase [6,7]. Although we too found evidence for an en bloc removal of the propeptide, our observations suggested that the elastase moiety generated at this step is larger than the mature enzyme. We hypothesized that the periplasmic form of elastase, proelastase II, contains a short, presumably N-terminal, extra sequence that is cleaved before secretion [2,3]. To examine whether this is indeed the case, we determined the N-terminal amino acid sequence of the first 15 amino acids of periplasmic elastase (proelastase II) from *P. aeruginosa* by Edman degradation, and located it within the preproelastase sequence. Fig. 1B shows that the N-terminal amino acid sequence found for the periplasmic form of elastase is identical with that of the mature enzyme. This result is against the possibility that periplasmic elastase contains an extra sequence at its N-terminus. It is consistent though with an earlier report showing that the N-terminal residue of cell-associated elastase in *P. aeruginosa* is alanine [13], and with the recent demonstration that the N-terminal amino acid sequence of processed elastase, isolated from *E. coli* cells expressing the *lasB* gene, is identical with that of mature elastase [14].

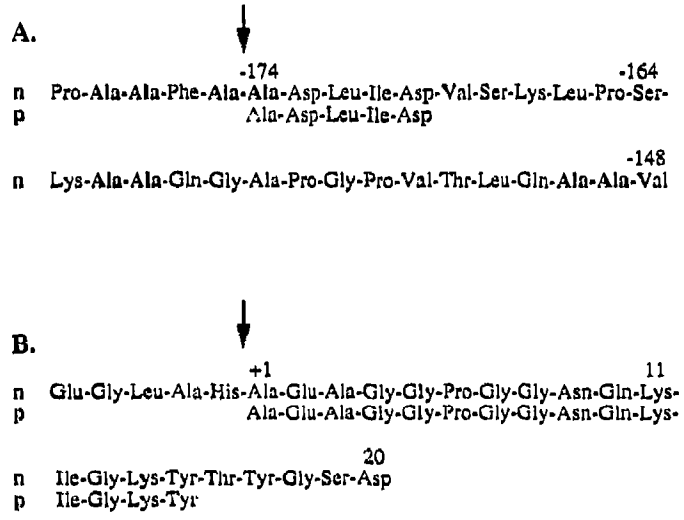

Fig. 1. N-Terminal amino acid sequences of *Pseudomonas aeruginosa* elastase propeptide (A) and of the periplasmic elastase precursor, proelastase II (B). n and p, amino acid sequences deduced from the nucleotide sequence of *lasB* [6,7] and those obtained by Edman degradation of the proteins, respectively. Numbers are in reference to the first amino acid residue of mature elastase (Ala^{+1}) and the bold letters within sequence n in (A) designate potential signal peptidase cleavage sites [6,8]. Arrows, peptide bonds cleaved upon removal of the signal peptide (A) and the propeptide (B).

Furthermore, this result confirms the conclusion of Bever and Iglewski [6] and Fukushima et al. [7], based on amino acid sequencing of mature elastase, that the His–Ala bond shown in Fig. 1B is cleaved upon proteolytic processing of the propeptide. The identification of the two physiological proteolytic processing sites within the amino terminal domain of preproelastase allowed calculation of the correct size of the propeptide, indicating that it is 174 amino acids long and having a molecular weight of 18,159 Da.

In view of the present demonstration the proelastase II is not extended at its amino-terminal end, the question of whether or not it undergoes an additional proteolytic cleavage prior to secretion remains open. The possibility that proelastase II is extended at its C terminus is not very likely because all but the last two carboxy-terminal residues (Ala^{300} and Leu^{301}) predicted from the nucleotide sequence of *lasB*, were shown to be present in a tryptic C-terminal peptide derived from mature elastase [7]. The expected loss in molecular mass due to removal of the C-terminal dipeptide Ala–Leu, even if it does occur, is not sufficient to account for the different electrophoretic mobilities we observed for proelastase II and elastase. The apparent size difference between the two proteins could reflect a major conformational change which might take place upon elastase maturation. The suspected size difference could also arise from chemical modifications of proelastase II due to a relatively long exposure to urea during its purification [3]. If any of the latter two possibilities

proves correct it would imply that the primary structures of both the processed periplasmic elastase and the mature enzyme are identical. Experiments to resolve these questions are under way.

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