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Physical Characterization of the Cloned Protease III Gene from *Escherichia coli* K-12*

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Analysis of the cloned protease III gene (*ptr*) from *Escherichia coli* K-12 has demonstrated that in addition to the previously characterized 110,000-M_r protease III protein, a second 50,000-M_r polypeptide (p50) is derived from the amino-terminal end of the coding sequence. The p50 polypeptide is found predominantly in the periplasmic space along with protease III, but does not proteolytically degrade insulin, a substrate for protease III. p50 does not appear to originate from autolysis of the larger protein. Protease III is not essential for normal cell growth since deletion of the structural gene causes no observed alterations in the phenotypic properties of the bacteria. A 30-fold overproduction of protease III does not affect cell viability. A simple new purification method for protease III is described.

Protease III (*ptr*) is a 110,000-M_r Mg^{2+}-dependent endopeptidase which has been shown to have proteolytic activity on a limited number of small polypeptides of less than 6,000 M_r such as insulin, glucagon, and autoclaved β-galactosidase fragments (2). Swamy and Goldberg (14) have demonstrated that the majority of protease III activity (called protease Pi [for periplasmic insulin-degrading] in their nomenclature) is normally located in the periplasmic space. Since the reported protease III mutants have no detectable phenotypic alterations (3), the role of this enzyme in the metabolism of the cell has not yet been identified.

Recently, the structural gene for protease III was cloned as a part of a 19-kilobase *Bam*HI fragment (5). Initial characterization of the fragment showed that *ptr* was physically located between the *recB* and *recC* genes. Preliminary maxicell experiments suggested that the *ptr* structural gene encoded two polypeptides of 110,000 and 50,000 M_r. In this communication, it is shown that the smaller protein is derived from the 110,000-M_r species, but does not possess proteolytic activity on a normal protease III substrate, insulin. In addition, both polypeptides are found in the periplasmic space. Finally, it has been shown that deletion of the *ptr* gene does not cause any demonstrable phenotypic alterations to the bacteria. The amplification of protease III activity has permitted the development of an improved purification procedure.

MATERIALS AND METHODS

Strains and media. The *Escherichia coli* K-12 strains and plasmids used are listed in Table 1. The media employed were Luria broth (10), Luria broth supplemented with 2% agar, and K medium (15). Plasmids and Tn*1000* insertions in the plasmids have been described previously (5).

Enzymes and chemicals. Special reagents were obtained from the following sources: *Staphylococcus aureus* V-8 protease, Miles Laboratories; enzyme-grade sucrose and ammonium sulfate, Schwarz/Mann; β-insulin, chloramphenicol, methionine, and spectinomycin, Sigma Chemical Co.; [^{35}S]methionine, Amersham Co.; and ^{125}I-insulin, New England Nuclear Corp.

Maxicell analysis. The analysis of polypeptides produced by various plasmids followed the maxicell procedure of Sancar et al. (13). To isolate periplasmically located products from the cells, osmotic shockates were made after radioactive labeling for 30 min with [^{35}S]methionine in the normal manner (13). The cells were subsequently washed in an Eppendorf tube with 1 ml of 20% sucrose–50 mM Tris–0.1 mM disodium EDTA (pH 7.5) and suspended in 1 ml of 0.5 mM $MgCl_2$ (12). After incubation at 4°C for 30 min, the cells were collected by centrifugation. The supernatant is called the osmotic shockate. The labeled cells were then suspended in sodium dodecyl sulfate (SDS) sample buffer, boiled, and subjected to electrophoresis (11) on 10% SDS-polyacrylamide gels. The osmotic shockate was precipitated with 5% trichloroacetic acid, washed twice with acetone, dried, and then boiled in SDS sample buffer before SDS-polyacrylamide gel electrophoresis.

Peptide mapping. The similarity of protease III and p50 was analyzed by the method of Cleveland et al. (4), which compares the polypeptide fragments produced by partial proteolysis. Areas on dried gels corresponding to the [^{35}S]methionine-labeled protease III and p50 bands were excised and treated as described for proteins in gel slices (4). The products of proteolysis by *S. aureus* V-8 protease were separated on 12.5% SDS-polyacrylamide gels. The gels were dried and subjected to autoradiography.

Time-course labeling of polypeptides. To determine the origin of p50, maxicells containing either pCDK35 or pBR325 were labeled for 20 min with [^{35}S]methionine in a way similar to that reported by Jackson and Summers (8). Spectinomycin (100 μg/ml) and unlabeled methionine (30 μM) were then added to the medium, and at the time points of 0, 10, 20, 30, 45, 60, and 90 min after the addition of [^{35}S]methionine, 0.5-ml samples were removed to 0.5 ml of spectinomycin (100 μg/ml) and processed to produce periplasmic extracts as well as shocked-cell extracts (membrane and cytoplasmic components). The extracts were concentrated and subjected to SDS-polyacrylamide electrophoresis as described above. The gels were then fixed, stained, dried, and autoradiographed.

Enzyme activity. Protease III activity on insulin was measured by the method of Goldberg et al. (6). The reaction mixture (500 μl) contained 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 10 μg of β-insulin, and enough ^{125}I-insulin to give

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TABLE 1. Bacterial strains used

Strain	Characteristics		Source or derivation
	ptr	Plasmid	
AC42	Δ[a]		G. Smith
SK1592	+		S. Kushner
SK5103	Δ	pCDK3::Tn*1000*-36	AC42 transformant
SK5110	Δ	pCDK3::Tn*1000*-73	AC42 transformant
SK5111	Δ	pCDK3	AC42 transformant
SK5112	Δ	pBR325	AC42 transformant
SK5116	+	pBR325	SK1592 transformant
SK5117	+	pCDK3	SK1592 transformant
SK5118	+	pCDK35	SK1592 transformant
SK5119	+	pCDK3::Tn*1000*-24	SK1592 transformant
SK5121	+	pCDK3::Tn*1000*-73	SK1592 transformant

[a] Δ indicates that the gene is deleted (see Fig. 1).

15,000 cpm. After 1 h at 37°C, the reaction was terminated by the addition of 40 μl of 30 mg of bovine serum albumin per ml and 120 μl of 50% trichloroacetic acid. After 30 min at 4°C, the samples were centrifuged at 15,000 × *g* for 10 min, and the ^{125}I remaining in the supernatant was quantitated by scintillation spectrometry. One unit of protease III activity is defined as that amount of protein degrading 1 μg of β-insulin in 1 h at 37°C. Periplasmic extracts from cultures grown in K medium were used to analyze protease activity in various genetic backgrounds. Protein determinations were by the method of Bradford (1).

RESULTS

Relationship of protease III and p50. The isolation of the Tn*1000* insertions shown in Fig. 1 has been described previously (5). In this study these insertions were used to analyze the relationship between protease III (p110) and the 50,000-M_r (p50) protein which appeared to map in the same place. Both proteins were originally identified in the analysis of the *recB* and *recC* genes which closely flank this region. In addition, Fig. 1 also shows the extent of the deletion in this region of the *E. coli* chromosome in strain AC42 as determined by Southern analysis (data not shown). The viability of this strain indicates that *ptr* is not an essential gene.

Figure 2 shows maxicell analysis of strains carrying various Tn*1000* insertions in the *ptr* locus of pCDK3 as well as a subclone (pCDK35) which carries only the *ptr* coding sequences. Osmotic shockates (12), which represent periplasmically located proteins, and the shocked-cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis. Since β-lactamase (*bla*) is transported to the periplasm (9) and chloramphenicol acetyl transferase (*cat*) and thymidylate synthetase (*thyA*) are located in the cytoplasm, these three plasmid-specified gene products served as internal controls for the two cell fractions.

Protease III (p110), p50, and β-lactamase were seen in the osmotic shockate fraction when pCDK3- or pCDK35-containing maxicells were labeled with [^{35}S]methionine (Fig. 2, lanes 6 and 7). The next three lanes show the osmotic shockate fractions pf pCDK3 that contain the three different Tn*1000* insertions located in the protease III gene (Fig. 2; 5). In Fig. 2, lane 8, a truncated polypeptide of 62,000 M_r is seen as well as the 50,000-M_r polypeptide. The size of this polypeptide is in agreement with the physical location of the Tn*1000*-71 insertion in Fig. 1. Lane 9 shows a truncated polypeptide of 74,000 and the 50,000-M_r polypeptide, since the Tn*1000*-73 insertion is located at the carboxyl end of the protease III gene. The third Tn*1000* insertion (Tn*1000*-24) causes the production of a truncated polypeptide of 32,000 M_r which is masked by the *thyA* gene product of 33,000 M_r in whole-cell extracts (Fig. 2, lane 5), but is easily seen in the

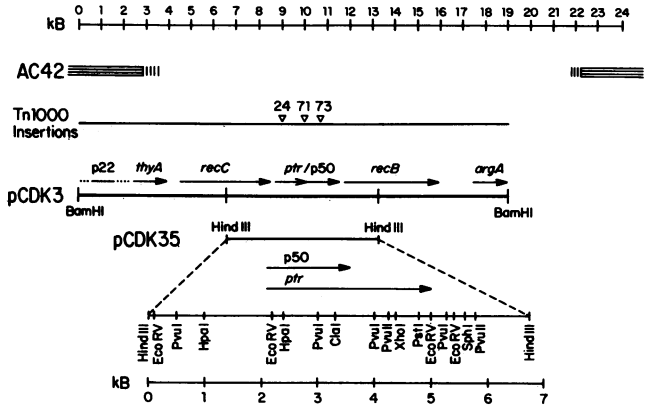

FIG. 1. Physical analysis of the *ptr* region of the *E. coli* chromosome. The extent of the deletion in AC42 is indicated at the top. The location of the Tn*1000* insertions in *ptr* are indicated. The location of *ptr*/p50 on pCDK35 is shown.

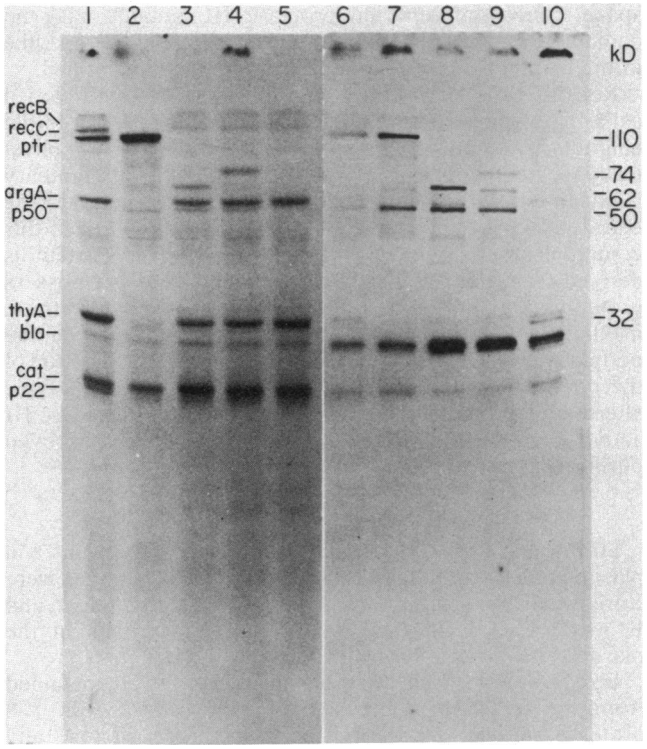

FIG. 2. Maxicell analysis of the products of the protease III gene. Lanes 1 through 5 are cytoplasmic extracts. Lanes 6 through 10 are periplasmic extracts. Lanes 1 and 6, pCDK3; lanes 2 and 7, pCDK35; lanes 3 and 8, pCDK3::Tn*1000*-71; lanes 4 and 9, pCDK3::Tn*1000*-73; lanes 5 and 10, pCDK3::Tn*1000*-24. The cytoplasmic extract lanes were exposed for 2 days. The periplasmic extracts were exposed for 3 weeks. Longer exposure times were required for the periplasmic extracts because CSR603 does not respond well to osmotic shock.

periplasmic fraction (lane 10). It is important to note that all of the polypeptides that appear to be related to protease III are found in the periplasm. Even the smallest polypeptide (32,000 M_r), which results from a Tn*1000* insertion (Tn*1000*-24), is located periplasmically. The *recB*, *recC*, *argA*, *thyA*, and p22 gene products were not seen in the periplasmic extract in any appreciable amounts.

To see whether the p50 polypeptide is encoded in the same reading frame as the protease III gene product, the *S. aureus* V-8 protease (7) digests of each protein were compared by polyacrylamide gel electrophoresis (4). Almost all of the bands of the digested p50 protein had a corresponding band in the p110 digest (data not shown).

Periplasmic extracts were incubated at 37°C in protease III assay buffer for various time periods, and the relative amounts of p50 versus p110 were measured electrophoretically. No change was seen in the amounts of either protein, indicating the p50 did not arise from the autolysis of p110 in vitro (data not shown). Subsequent results suggested either autolysis or processing of p110 to yield p50 in vivo.

To determine whether the p50 product resulted from either an early transcriptional or translational termination or was processed from the completed p110, a pulse-chase experiment was performed. At the first time point of 10 min, p110 labeled well but no p50 was evident (Fig. 3). The chase period began after 20 min of labeling with the addition of unlabeled methionine as well as spectinomycin to inhibit further protein synthesis. As the chase continued for 90 min, p50 accumulated while the amount of p110 in the periplasm did not increase, as determined by densitometric tracings of the autoradiogram.

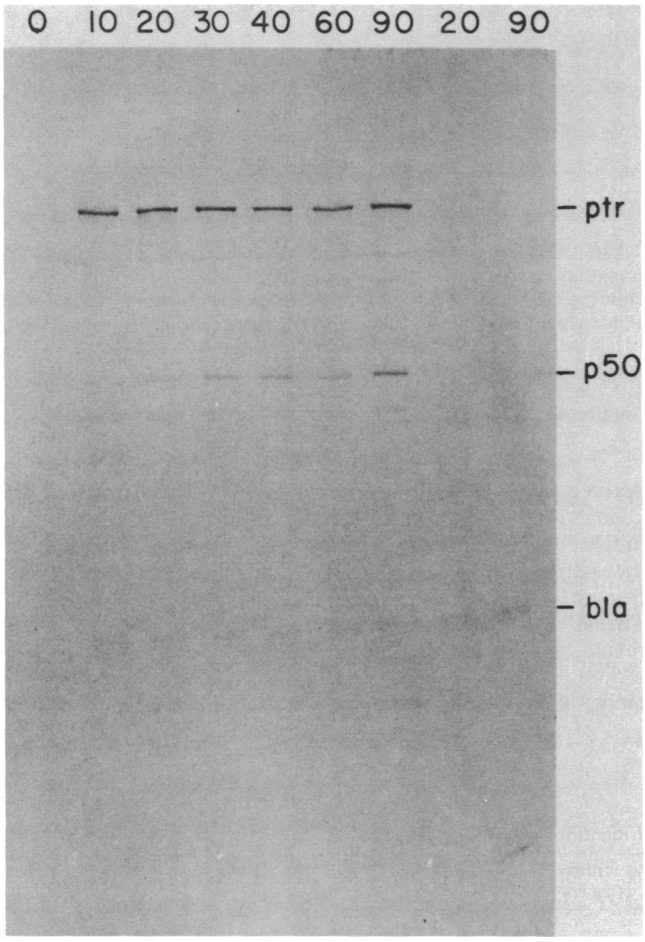

FIG. 3. Pulse-chase labeling of pCDK35. The first seven lanes are periplasmic extracts from cells containing pCDK35 that were labeled with [^{35}S]methionine for the indicated times. The last two lanes show periplasmic extracts of pBR325 labeled for the indicated times.

TABLE 2. Protease III activity in the periplasm of various *E. coli* strains

Strain	*ptr*[a]	Plasmid	Activity (U/mg)[b]
SK5103	Δ	pCDK3::Tn*1000*-36	27.5
SK5111	Δ	pCDK3	23.5
SK5112	Δ	pBR325	0
SK5110	Δ	pCDK3::Tn*1000*-73	0
SK5116	+	pBR325	5.1
SK5117	+	pCDK3	36.5
SK5118	+	pCDK35	66.1
SK5119	+	pCDK3::Tn*1000*-24	6.5

[a] Δ indicates complete deletion of chromosomal *ptr* sequence.
[b] One unit equals the degradation of 1 μg of β-insulin in 1 h at 37°C. Enzyme activity was assayed as described in the text.

Enzyme activity. Since protease III is the only periplasmic enzyme in *E. coli* known to degrade insulin, the amount of activity encoded by the various plasmids was tested. Periplasmic extracts from strains containing either pCDK35 or pCDK3 had an approximately 7- to 13-fold increase in insulin-degrading activity as compared with strains with only pBR325 (Table 2). In contrast, strains carrying Tn*1000*-insertions in the *ptr* gene did not overproduce insulin-degrading activity (Table 2). These results indicate that p50 does not possess insulin-degrading activity. To confirm this hypothesis, AC42 Δ*ptr* was transformed with pCDK3, pBR325, and pCDK3::Tn*1000*-73. No protease activity was detected except in the presence of an intact *ptr* gene (Table 2).

Purification. Since the protease III activity was amplified in cells containing pCDK35 and secreted to the periplasm, a rapid purification procedure was developed.

Cell growth. Strain SK5118(pCDK35) was grown in 2 liters of Luria broth to Klett (no. 42 green filter). This resulted in approximately 10 g of cells (wet weight). Cells were harvested by centrifugation (10,000 × *g*, 20 min) and suspended in 200 ml of 50 mM Tris (pH 7.5)–0.1 mM disodium EDTA–20% sucrose at 4°C. The cells were again collected by centrifugation and suspended in 200 ml of 0.5 mM $MgCl_2$. After 30 min at 4°C with occasional mixing, the cells were again centrifuged. The supernatant was designated the periplasmic fraction (fraction I).

Ammonium sulfate precipitation. Fraction I was precipitated by the addition of 0.565 g of ammonium sulfate (80% saturation) per ml with stirring. One hour after the ammonium sulfate was dissolved, the precipitate was collected by centrifugation. The ammonium sulfate pellet was suspended in 250 ml of buffer A (50 mM Tris, pH 7.5, 10 mM $MgCl_2$) and dialyzed extensively against buffer A (fraction II).

DEAE-Sephadex chromatography. Fraction II was diluted to a conductivity of 5.3 (mohm) and applied to a DEAE-Sephadex column (2.0-cm diameter, 10-ml bed volume) that had been equilibrated with buffer A. Protease III activity appeared in the wash. The active fractions were dialyzed against buffer B (20 mM potassium phosphate, pH 7.0) (fraction III).

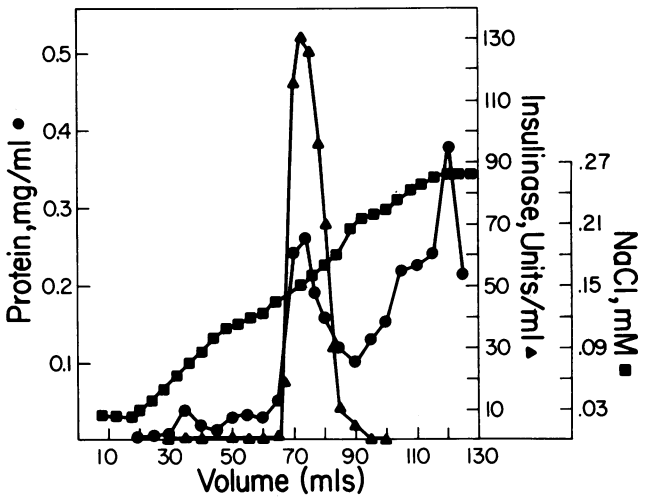

FIG. 4. DEAE-Sephadex profile of protease III. A 20-ml DEAE-Sephadex column equilibrated with buffer A (20 mM Tris, pH 7.5, 10 mM $MgCl_2$) was used. Protease III elutes at around 0.15 M NaCl. Symbols: ●, protein; ▲, protease III activity on insulin; ■, NaCl concentration.

Hydroxyapatite chromatography. Fraction III was applied to a hydroxyapatite column (2.0-cm diameter, 10-ml bed volume) equilibrated with buffer B. The column was washed with 100 ml of buffer B. A gradient of 0.02 to 0.2 M potassium phosphate (pH 7.0) was applied to the column. Protease III eluted midway through the gradient. The appropriate fractions were collected and dialyzed into buffer A (fraction IV).

DEAE-Sephadex chromatography. Fraction IV was applied to a DEAE-Sephadex column (2.0-cm diameter, 20-ml bed volume) that had been equilibrated with buffer A. The column was washed with 100 ml of buffer A. The absorbed proteins were eluted with 150 ml of 0 to 0.25 M NaCl in buffer A. Protease III eluted at approximately 0.15 M NaCl (see Fig. 4). The active fractions were pooled and dialyzed against buffer A that was 50% in glycerol, and these fractions were kept at −20°C (fraction V). A profile of this column is shown in Fig. 4. The procedure resulted in a 7.1% recovery (Table 3) of enzyme greater than 95% pure (Fig. 5). This method is a rapid and much simpler protocol for the purification of protease III, allowing the isolation of 1 mg of enzyme from 2 liters of cells without cell lysis.

DISCUSSION

The data presented here demonstrates that the protease III gene product of 110,000 M_r is cleaved into a smaller molecular weight form of 50,000 M_r. The 50,000-M_r protein (p50) is derived from the amino-terminal portion of the protease III gene as determined by maxicell analysis of Tn*1000* insertions in the protease III gene (Fig. 2). The p50 product is found in the periplasm, as is protease III, and surprisingly, the truncated polypeptides produced as a result of various Tn*1000* insertions are also periplasmically located. This result suggests that secretion requires only the amino-terminal portion of the protein, in contrast to the requirement of a central region for β-lactamase (9). Limited

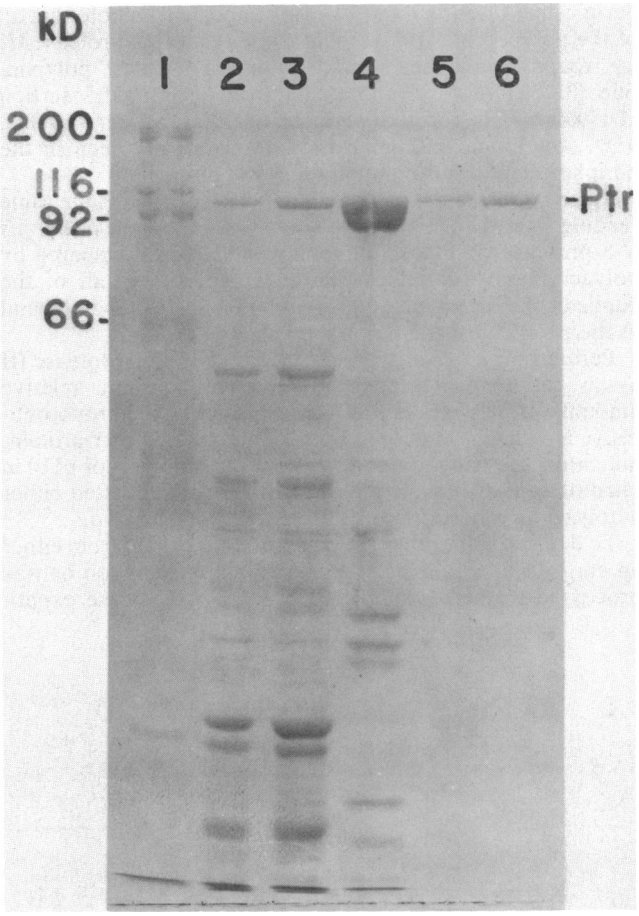

FIG. 5. SDS-polyacrylamide gel electrophoresis of protease III preparations. Protease III fractions were applied to a 8% polyacrylamide gel containing 0.1% SDS. After electrophoresis, the gel was stained with Coomassie blue. The molecular weight standards are indicated in the left margin. Lane 1, High-molecular-weight standards; lane 2, 10 μg of fraction II; lane 3, 15 μg of fraction III; lane 4, 15 μg of fraction IV; lane 5, 1.1 μg of fraction V; lane 6, 3 μg of fraction V.

TABLE 3. Summary of the purification of protease III

Fraction	Vol (ml)	Total activity (u × 10^3)[a]	Protein (mg/ml)	Sp act (U/mg)	% Recovery	Purification (fold)
I. Shockate	200	90	5.15	8.8		
II. 80% ammonium sulfate	53	39	2.05	36	43	4.11
III. DEAE-Sephadex	50	59	0.77	154	66	
IV. Hydroxyapatite	15	50	1.3	583	55	66.5
V. DEAE-Sephadex	5	6	0.11	11,400	7.1	1,299

[a] One unit equals the degradation of 1 μg of β-insulin in 1 h.

proteolysis of ^{35}S-labeled protease III and p50 proteins by *S. aureus* V-8 protease has suggested that p50 has homology with protease III, as would be expected if the two proteins are derived from the same reading frame. However, this relationship must be confirmed by amino acid sequencing of the NH_2-terminal ends of the two polypeptides. Only the complete protease III gene has enzymatic activity against insulin (Table 2).

The pulse-chase maxicell experiment demonstrated that although protease III is made and transported to the periplasm quite rapidly, the p50 product appears in the periplasm at a slower rate. Even after 90 min of labeling, there was 50% less of the p50 product than of the protease III product, as determined by densitometric analysis of the autoradiographs. Since new protein synthesis was inhibited after 20 min of labeling with [^{35}S]methionine, it was expected that the amount of protease III observed would decrease as the amount of p50 increased. Surprisingly, only the level of p50 increased with time. Since little p50 was seen from whole-cell extracts that had been osmotically shocked (Fig. 2, lanes 1 through 5), we believe that p50 is predominantly produced in the periplasm. However, the poor response of UV-irradiated CRS603 cells to osmotic shock precludes any definite conclusion at this time. The role of this protein has not been identified. It is possible that p50 is merely a stable specific breakdown product of protease III. This would explain its increase with time except for the fact that the amount of protease III does not appear to decrease coordinately. It is possible that there is only room in the periplasmic space for a certain amount of protease III, and after that point, p50 begins to accumulate. Degradation of periplasmically located protease III would permit the export of additional p110 protein from the cytoplasm, resulting in the apparent constant levels seen in Fig. 3.

The protease III purification procedure presented here is much simpler than any other previously described method. The methods presented by Goldberg et al. (6) and Cheng and Zipser (2) both require additional chromatography steps with considerably lower yields. In addition, the use of an osmotic shock retains most of the cytoplasmic proteins in the first step.

Since the protease III structural gene can be deleted from *E. coli* chromosome without any apparent detrimental effects, its activity cannot be essential for normal metabolism in the cell. Conversely, overexpression of protease III upon cloning the gene on a multicopy plasmid also does not appear to have any effects on cell viability. This raises several questions on the regulation of expression of protease III in the cell. Assuming that protease III is involved in the degradation of unwanted proteins, it would need to be available at only certain times and places. It is possible that protease III is functionally compartmentalized in some fashion. The presence of an inhibitor of the enzyme could also be a factor in its regulation.

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