In Vitro Processing of Pro-subtilisin Produced in Escherichia coli*

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In a previous paper (Ikemura, H., Takagi, H., and Inouye, M. (1987) J. Biol. Chem. 262, 7859–7864), we demonstrated that the pro-sequence consisting of 77 amino acid residues at the amino terminus of subtilisin is essential for the production of active subtilisin. When the aggregates of pro-subtilisin produced in *Escherichia coli* were solubilized in 6 M guanidine hydrochloride and dialyzed against 200 mM sodium phosphate buffer (pH 7.1 or 6.2), pro-subtilisin was efficiently processed to active subtilisin. When more than 14 residues were removed from the amino terminus of the pro-sequence, active subtilisin was no longer produced as in the *in vivo* experiments. Similarly, active subtilisin would not renature under the same conditions once solubilized in guanidine hydrochloride. When the aspartic acid residue at the active site (Asp2) was altered to asparagine, processing of mutant pro-subtilisin was not observed even in the presence of wild-type pro-subtilisin. Inhibitors such as phenylmethanesulfonyl fluoride or *Streptomyces* subtilisin inhibitor did not block the processing of wild-type pro-subtilisin. These facts indicate that processing of pro-subtilisin is carried out by an intramolecular, self-processing mechanism. When the sample was dialyzed against 20 mM sodium phosphate (pH 6.2), no active subtilisin was found, suggesting that the highly charged nature of the pro-sequence plays an important role in the process of refolding of denatured pro-subtilisin.

Subtilisins, alkaline serine proteases produced by bacilli, have been used widely as a model system for protein engineering: creating more stable enzymes (1–3), changing substrate specificities (4–6), and pursuing structural and physicochemical characterization of the enzyme (7–8). These studies have been carried out by taking advantage of x-ray crystallographic data of the enzyme (9–10) and of the fact that the gene for the enzyme has been cloned and that its DNA sequence has been determined (11–15). Subtilisins are produced from pre-pro-subtilisins consisting of the signal sequence (pre-sequence) of 29 residues essential for protein secretion, the pro-sequence of 77 residues, and mature subtilisin of 275 residues (11–15). The pre-sequence functions as the signal peptide required for protein secretion across the membrane. However, the roles of the pre-sequence have been obscured in studies using *Bacillus* expression systems (11, 12, 16–19). Recently, we were able to produce a large amount of pro-subtilisin in *Escherichia coli* using a high expression-secretion vector, pIN-III-OmpA (19). In this study, we have demonstrated that the pre-sequence is required for the production of enzymatically active subtilisin. We propose that the pro-sequence is essential for guiding the appropriate folding of the subtilisin molecule.

In this report, we were able to establish an *in vitro* system for processing of pro-subtilisin produced in *E. coli* to active subtilisin. Using this system, we demonstrate that the pro-sequence is also required for *in vitro* processing. Characterization of the processing reaction revealed that cleavage of the pro-sequence occurs by an intramolecular, self-processing mechanism. We discuss possible roles of the pro-sequence in the production of active subtilisin.

EXPERIMENTAL PROCEDURES

Expression of Subtilisin Gene in *E. coli*—Construction of and the structure of plasmid pH212 were described previously (19). The expression of pre-pro-subtilisin from pH212 was carried out as described previously (19). Pro-subtilisin produced from pH212 (19) consists of the entire pro-sequence of 77 residues plus 6 additional residues at the amino terminus of the pro-sequence derived from the secretion vector pIN-III-OmpA (Ref. 20; see Fig. 1). pH216 (19) is identical to pH212 except that a mutation was introduced in the coding region of subtilisin by oligonucleotide-directed site-specific mutagenesis which resulted in replacement of Asp at position 32 of the mature subtilisin with Asn. Asp71 is known to play an important role in the active site of the protease, and its alternation to Asn has been shown to cause complete loss of enzymatic activity (16). Pro-subsequences of pre-pro-subtilisins from pH1102 and pH1103 (19) are shorter by 14 and 44 residues, respectively, than that of the full-sized pro-sequence (see Fig. 1). pH215 (19) is identical to pH212 except that the full-sized pro-sequence is directly connected to the OmpA signal peptide in pH215. Similarly, pH161 is identical to pH216 except that the full-sized pro-sequence is directly connected to the OmpA signal peptide in pH161 (see Fig. 1). In pH7700, the OmpA signal peptide is directly fused to mature subtilisin (Ref. 19; see Fig. 1). Subtilisin E produced in *E. coli* cells carrying pH212 was purified using a CM52 column as described previously (19). The purified protein showed a single band on SDS-Polyacrylamide gel electrophoresis.

Processing of Pro-subtilisin to Active Subtilisin in Vitro—Since pro-subtilisin was produced as aggregates by this method, substantial purification was achieved by low-speed centrifugation of a crude cell extract, as shown previously (19). The pellet prepared from a 30-ml culture was dissolved in 300 ml of 6 M guanidine hydrochloride solution containing 10 mM Tris-HCl (pH 7.0). The solution was then centrifuged at 90,000 rpm for 14 min using a Beckman TLA-100.2 rotor to remove insoluble materials (see Fig. 2). The supernatant (30 ml) was then dialyzed against 25 ml of a buffer solution at 4°C for 2 hr by the drop dialysis technique (21). The dialysate was collected and centrifuged at 90,000 rpm for 14 min using the same rotor described above. One-sixth of the supernatant was used for protease activity assay, as described previously (19). One-third of the same supernatant

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; S-SI, streptococcal subtilisin inhibitor.
was analyzed by SDS-polyacrylamide gel electrophoresis using a 17.5% polyacrylamide gel, as described previously (19).

RESULTS

Processing of Pro-subtilisin in Vitro—When a crude cell extract of pH1215 (Fig. 2, lane 1) was centrifuged at low speed, three major proteins were isolated in the pellet, as can be seen in (Fig. 2, lane 2). Of these three major bands, two at higher molecular masses from top to bottom are 66, 45, 31, and 14 kDa.

Fig. 1. Structure of OmpA-subtilisin from pH1212. The solid box represents mature subtilisin consisting of 275 residues. The hatched box indicates the pro-sequence of 77 residues. The empty box represents an extra sequence consisting of 6 residues between the OmpA signal peptide and the pro-sequence (19). The OmpA signal peptide is shown by a small empty box. The residues are numbered from the amino-terminal residue of mature subtilisin (+1). In pH1215, the OmpA signal peptide is directly connected to the pro-sequence (19). pH1216 and pH1161 are identical to pH1212 and pH1215, respectively, except that the Asp residue at position 32 of subtilisin in pH1215 is replaced with Asn (19).

Fig. 2. SDS-polyacrylamide gel electrophoresis of pro-subtilisin from pH1215. Samples were applied to a 17.5% polyacrylamide gel and after electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, total cell extract; lane 2, low-speed centrifugation pellet; lane 3, insoluble fraction after 6 M guanidine hydrochloride solubilization; lane 4, insoluble fraction after dialysis of the 6 M guanidine hydrochloride supernatant against 10 mM sodium phosphate buffer (pH 7.1) for 90 min; lane 5, soluble fraction after dialysis; lane 6, insoluble fraction after dialysis of the 6 M guanidine hydrochloride supernatant against 200 mM sodium phosphate buffer (pH 7.1) for 90 min; lane 7, soluble fraction after dialysis; lane 8, purified subtilisin E. The solid arrowhead near 45 kDa indicates the position of pro-subtilisin; the open arrowhead indicates the position of mature subtilisin; and the small solid arrowhead at ~14 kDa indicates a new band appearing after dialysis of the 6 M guanidine hydrochloride supernatant against 200 mM sodium phosphate buffer (pH 7.1) (lane 7). Protein standards were applied to the left. The molecular masses from top to bottom are 66, 45, 31, and 14 kDa.

Strong protease activity was recovered in the soluble fraction when dialyzed against 200 mM sodium phosphate buffer, whereas dialysis against 10 mM buffer gave no activity. As shown in Fig. 2, SDS-polyacrylamide gel electrophoresis also revealed the production of mature subtilisin only when dialysis was carried out under high salt renaturing conditions (lane 7). By contrast, under low salt conditions, no processing was observed, and only unprocessed pro-subtilisin was found in both the insoluble and soluble fractions (lanes 4 and 5, respectively). It is interesting to note that an additional band appeared at approximately 13 kDa, which may be a degradation product of pro-subtilisin or the pro-sequence cleaved from pro-subtilisin (see lane 7, Fig. 2). Exactly the same results were obtained with pH1212, which has 6 extra residues between the OmpA signal peptide and the pro-sequence, as described in Fig. 1 (data not shown).

In order to eliminate the possibility that active subtilisin was derived from pre-existing subtilisin contamination in the pellet fraction, mature active subtilisin purified from cells carrying pH1212 was treated under the same conditions as the pellet fraction. It was found that no protease activity could be recovered at all when subtilisin was dissolved in 6 M guanidine hydrochloride solution, followed by dialysis against 200 mM sodium phosphate buffer (data not shown). This result also indicates that processing of pro-subtilisin to active subtilisin was not caused at the outset by pre-existing active subtilisin. Furthermore, this result is consistent with previous data showing that inactive mature-sized subtilisin produced by pH7700 is not renatured to active subtilisin (19). A mutant pro-subtilisin (the product of pH1161) in which the active site Asp32 was replaced by Asn32 was treated in exactly the same manner as described for pH1215 (Fig. 2). No subtilisin activity, nor a band at the position of mature subtilisin, was detected, as shown in Fig. 3. Instead, pro-subtilisin was seen unprocessed in both the insoluble (lane 6) and soluble (lane 7) fractions after dialysis of the 6 M guanidine hydrochloride fraction against 200 mM sodium phosphate buffer (pH 7.1) at 4 °C for 90 min. When dialysis was carried out under low salt conditions, no processing of pH1161 pro-subtilisin was observed (lanes 4 and 5). These results are consistent with the previous hypothesis that the pro-sequence is essential for guiding the proper folding of subtilisin into the active conformation (19).

When the same renaturation conditions were applied to the products of pH1102 and pH1103 (19), which contain 63 and 33 residues of the 77 residues of the full-sized pro-sequence at the amino terminus of the mature subtilisin, respectively, they could not be processed to the active mature form. Thus, as demonstrated in vivo previously (19), one can conclude that most of the pro-sequence is required for the refolding of pro-subtilisin that is capable of being processed to the enzymatically active form.

Optimal Conditions for in Vitro Processing—As discussed above, the salt concentrations of the buffer used for dialysis were critical for in vitro processing. The subtilisin activity increased 2-fold as the sodium phosphate concentration increased from 100 to 200 mM, indicating that higher ionic strength is required for the refolding of pro-subtilisin.
In Vitro Processing of Pro-subtilisin

FIG. 3. SDS-polyacrylamide gel electrophoresis of pro-subtilisin from pH161. Samples were prepared and applied to a 17.5% polyacrylamide gel as described for Fig. 2. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, total cell extract; lane 2, low-speed centrifugation pellet; lane 3, insoluble fraction after 6 M guanidine hydrochloride solubilization; lane 4, insoluble fraction after dialysis of the 6 M guanidine hydrochloride supernatant against 10 mM sodium phosphate buffer (pH 7.1) for 90 min; lane 5, soluble fraction after dialysis; lane 6, insoluble fraction after dialysis of the 6 M guanidine hydrochloride supernatant against 200 mM sodium phosphate buffer (pH 7.1) for 90 min; lane 7, soluble fraction after dialysis; lane 8, purified subtilisin E. The solid and open arrowheads and the protein standards are the same as described for Fig. 2.

When different salts were used at the same concentration (200 mM) and the same pH (7.1), sodium chloride, sodium acetate, and ammonium sulfate were found to be as effective as sodium phosphate (data not shown). However, when Tris-HCl or guanidine hydrochloride was used, no active subtilisin was produced, leaving most of the pro-subtilisin in an insoluble form. These results suggest that "soft" cations such as Tris and guanidium which have low charge density are not suitable for the refolding of pro-subtilisin.

The time course of in vitro processing and the appearance of active subtilisin is shown in Fig. 4. The activity suddenly increased at 60 min when the 6 M guanidine hydrochloride supernatant was dialyzed against 200 mM sodium phosphate buffer (pH 7.1). The activity gradually increased until 90 min, and a slight drop was observed after 120 min of dialysis. This drop in activity may be due to autodegradation of the processed subtilisin.

The effect of pH on in vitro processing is shown in Fig. 5. The samples were dialyzed against 200 mM sodium phosphate buffer at different pH values at 4 °C for 2 h. One can see that the optimal pH values lie between 5.5 and 7.1 and that the highest activity is obtained at pH 6.2. These samples were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6). Processed mature subtilisin was the major product in the soluble fractions of the samples dialyzed at pH 5.5 (lane 4), pH 6.2 (lane 6) and pH 7.1 (lane 8). In contrast, when pro-

FIG. 4. Time course of in vitro processing of pro-subtilisin. Samples were dialyzed against 200 mM sodium phosphate buffer (pH 7.1) at 4 °C. After incubation for the times indicated, dialyzed samples were centrifuged, and the subtilisin activity of the supernatant fractions was measured as described under "Experimental Procedures." The relative activities to that of the sample dialyzed for 120 min are plotted.

FIG. 5. Effect of pH on in vitro processing of pro-subtilisin. Samples were dialyzed against 200 mM sodium phosphate buffers at different pH values at 4 °C for 2 h. The subtilisin activity was measured as described under "Experimental Procedures." The relative activities to that of the sample dialyzed at pH 7.1 are plotted.

FIG. 6. SDS-polyacrylamide gel electrophoresis of pro-subtilisin dialyzed at different pH values. The samples obtained in Fig. 4 were centrifuged, and the insoluble (lanes 1, 3, 5, and 7) and soluble (lanes 2, 4, 6, and 8) fractions were then applied to a 17.5% polyacrylamide gel. The sample at pH 4.5 was applied to lanes 1 and 2, the sample at pH 5.5 to lanes 3 and 4, the sample at pH 6.2 to lanes 5 and 6, and the sample at pH 7.1 to lanes 7 and 8. The solid arrowhead indicates the position of pro-subtilisin, and the open arrowhead indicates the position of mature subtilisin. The molecular mass standards to the left are the same as described for Fig. 2.
active subtilisin (indicated by an open arrowhead) was observed either in the insoluble (lane 1) or soluble (lane 2) fractions. This is consistent with the result in Fig. 5 and the fact that mature active subtilisin is irreversibly denatured below pH 5.0 (22, 23).

**Self-processing of Pro-subtilisin**—As described above, active subtilisin was irreversibly denatured by 6 M guanidine hydrochloride. In addition, inactive subtilisin from pHT700 which was produced without the pro-sequence could not be refolded to active subtilisin, as in the case of pro-subtilisins produced from pH1212 and pH1215. These results indicate that processing of pro-subtilisin from pH1212 and pH1215 to mature active subtilisin (see Fig. 2) cannot be carried out by active subtilisin which might contaminate the pro-subtilisin aggregate fraction since the aggregates must be dissolved in 6 M guanidine hydrochloride in order for the pro-subtilisin molecule to refold correctly. Thus, the processing of pro-subtilisin to active subtilisin by removal of the pro-sequence of 77 residues is likely to be an intramolecular self-processing reaction.

In order to test this possibility, we examined whether pro-subtilisin from pH1216 in which Asp" could be activated (or processed) by exogenous active subtilisin. For this purpose pH1216 pro-subtilisin labeled with ["S]methionine was mixed with unlabeled pH1212 pro-subtilisin in different ratios. The mixtures in 6 M guanidine hydrochloride were then dialyzed against 200 mM sodium phosphate buffer (pH 6.2) at 4 °C for 90 min for renaturation. If pH1216 pro-subtilisin refolded by dialysis could be processed by active subtilisin generated by pH1212 pro-subtilisin, one could expect the appearance of a new radioactive band at the position of mature subtilisin. As shown in Fig. 7, when pH1212 pro-subtilisin was added at a concentration of one-tenth of the amount of pH1216 pro-subtilisin labeled with ["S]methionine, no significant reduction of the pro-subtilisin band was observed (lane 2). The cleaner background in lane 2 in comparison with the control lane without the addition of pH1216 pro-subtilisin (lane 1) is probably due to degradation of contaminating proteins by active subtilisin generated from pH1216 pro-subtilisin. Even at the 9 times higher concentration of pH1212 pro-subtilisin, no band appeared at the position of mature subtilisin (indicated by an open arrowhead; see lane 3). In this case, the pro-subtilisin band also disappeared, most likely due to degradation of pH1216 pro-subtilisin by active subtilisin generated from pH1212 pro-subtilisin. When pH1212 pro-subtilisin labeled with ["S]methionine was treated in the same way as described above, a new band appeared at the position of mature subtilisin (lane 4). These results demonstrate that mutant pro-subtilisin cannot be processed by coexisting active subtilisin. In another experiment, active mature subtilisin E was added to the pH1216 pro-subtilisin solution after dialysis under optimum conditions to examine if pH1216 pro-subtilisin can be processed. No processing was observed (data not shown). Since both the products from pH1212 and pH1216 have 6 extra residues between the OmpA signal peptide and the pro-sequence, the same experiment was carried out with pH1215 (for the wild type) and pH1161 (for the Asn" mutant) pro-subtilisins, which have no extra residues at their amino termini. Identical results were obtained as those with pH1212 and pH1216 (data not shown).

**Effects of Subtilisin Inhibitors on Self-processing**—In order to characterize further the mechanism of the self-processing reaction, the effects of two subtilisin inhibitors, phenylmethanesulfonyl fluoride (PMSF) (22) and streptococcal subtilisin inhibitor (S-SI) (24-26), on the processing of pro-subtilisin were examined. Even in the presence of PMSF (an inhibitor of serine proteases) added to the protein solution at a final concentration of 2 mM as well as to the dialyzing buffer at a final concentration of 1 mM, a band was detected at the position of mature subtilisin when analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). In this case, however, no subtilisin activity was detected. Similarly, when S-SI was added at a final concentration of 90 nM to the pro-subtilisin solution prior to dialysis, no activity was detected after dialysis, but the mature subtilisin band appeared on SDS-polyacrylamide gel electrophoresis (data not shown). These results show that neither of these inhibitors could block the processing of pro-subtilisin, although both completely inhibited the activity of mature subtilisin. These results indicate that the self-processing reaction of pro-subtilisin is somewhat different from the subtilisin enzymatic reaction, as will be discussed below.

Fig. 7. Mixing experiments with pro-subtilisin from pH1216 and pH1212. A 10-ml culture of cells carrying pH1216 was labeled at 37 °C for 2 h with 100 μCi of ["S]methionine after the addition of isopropyl-1-thio-β-D-galactopyranoside, and a pro-subtilisin solution in 6 M guanidine hydrochloride was prepared as described under "Experimental Procedures." The solution of nonradioactive pH1212 pro-subtilisin in 6 M guanidine hydrochloride was also prepared as described under "Experimental Procedures." Lane 1, 3 μl of the solution of pH1216 pro-subtilisin labeled with ["S]methionine was mixed with 27 μl of nonradioactive pH1216 pro-subtilisin solution. The mixture was dialyzed against 200 mM sodium phosphate buffer (pH 6.2) at 4 °C for 90 min. After dialysis, insoluble materials were removed by centrifugation, and the proteins in the soluble fraction were precipitated with 10% trichloroacetic acid. The pellet thus obtained was dissolved and subjected to SDS-polyacrylamide gel electrophoresis as previously described (19). Lane 2, the radioactive pH1216 pro-subtilisin solution (3 μl) was mixed with 12 and 15 μl of the nonradioactive pH1216 and pH1212 pro-subtilisin solutions, respectively; lane 3, the radioactive pH1216 pro-subtilisin solution (3 μl) was mixed with 27 μl of the nonradioactive pH1212 pro-subtilisin solution; lane 4, the radioactive pH1212 pro-subtilisin solution was prepared as described for radioactive pH1216 pro-subtilisin as described above. This solution (3 μl) was mixed with the nonradioactive pH1212 pro-subtilisin solution (27 μl). Samples in lanes 2-4 were treated as described for the sample in lane 1. After SDS gel electrophoresis using a 17.5% acrylamide gel, the gel was dried and subjected to autoradiography. Molecular mass standards are the same as described for Fig. 2.
DISCUSSION

In this study, we were able to demonstrate in vitro processing of pro-subtilisin to active subtilisin. We showed that this process, which requires proteolytic removal of the aminoterminal pro-sequence of 77 residues, is an autocatalytic and intramolecular reaction and does not require the action of active subtilisin. It has been proposed that maturation of prepro-subtilisin is mediated by autoproteolysis that involves trace amounts of active subtilisin (16). In this work, however, we excluded the possibility that maturation of pro-subtilisin is initiated by trace amounts of active mature subtilisin contaminant in the preparation from the following facts. (a) When active subtilisin was denatured by 6 M guanidine hydrochloride, the enzyme was irreversibly denatured, and we were unable to renature it under the conditions used for renaturation of pro-subtilisin. (b) Subtilisin inhibitors such as PMSF and S-SI could not block the processing of pro-subtilisin to subtilisin. (c) Intermolecular processing of pH1216 (inactive) pro-subtilisin (Asp22 at the active site was not replaced by Asn) by pH1212 (active) pro-subtilisin was not observed.

Recently, pre-pro-subtilisins in which a catalytic group of the enzyme was mutated were shown to be processed by mixing cell cultures producing mutant pre-pro-subtilisins with a helper culture producing wild-type pre-pro-subtilisin (6). It is possible that such intermolecular processing the pro-sequence may have to be linked to pro-subtilisin, or the pro-sequence may be cleaved at a different site. It is possible that prepro-subtilisin can be processed by both intra- and intermolecular mechanisms.

It should be noted, however, that the wild-type pro-subtilisin resulting from the removal of the pro-sequence has an intrinsic property to process itself autocatalytically to cleave its own pro-sequence. This autocatalysis still requires the function of the aspartic acid residue at position 32 which is a part of the catalytic center of subtilisin (9) since pH1216 (Asp22 → Asn) pro-subtilisin was unable to cleave the pro-sequence. The autocatalytic reaction was, however, not inhibited by subtilisin-specific inhibitors, S-SI and PMSF. If the same active site of subtilisin is involved in the autocatalytic reaction, the active site is probably occupied by the pro-sequence or a part of the pro-subtilisin structure so that the inhibitors cannot interact with the active site. Alternatively, pro-subtilisin is folded in such a way that the tertiary structure of the subtilisin part of pro-subtilisin is different from that of the mature subtilisin molecule. As a result, the subtilisin inhibitors may not be able to bind pro-subtilisin unless the pro-sequence is removed.

The tertiary structure of subtilisin determined by x-ray crystallography reveals that the charged amino acid residues are not evenly distributed on its surface (9, 10). There is a relatively large surface area adjacent to the substrate-binding site where no charged amino acid residues are located at all. It is therefore reasonable to speculate that this rather hydrophobic surface area is originally covered by the highly charged pro-sequence. This structure then prevents PMSF and S-SI from binding to pro-subtilisin. The fact that S-SI binds to the uncharged area (27) and that S-SI cannot inhibit self-processing of pro-subtilisin is consistent with the hypothesis mentioned above.

The in vitro processing experiments in this work are consistent with previous in vitro experiments in which we concluded that the full-sized pro-sequence is essential to guide the proper folding of the subtilisin structure (19). This system using pIN-III-OmpA vectors allows us to prepare large amounts of various pro-subtilisins. Purification of these pro-subtilisins is now in progress in our laboratory, and characterization of these proteins will shed light on the roles of the pro-sequence in the protein's folding as well as the autocatalytic reaction.

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