Pyrrolidonyl Peptidase. An Enzyme for Selective Removal of Pyrrolidonecarboxylic Acid Residues from Polypeptides*

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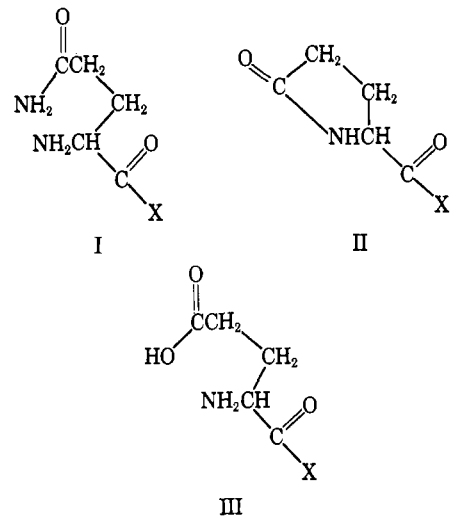
ABSTRACT: A strain of *Pseudomonas fluorescens* has been found to be a rich source of an enzyme which selectively removes pyrrolidonecarboxylic acid residues from polypeptide chains. The enzyme has been partially purified and a preliminary characterization with regard to specificity accomplished. Further purification attempts have been hindered by the instability of the more purified enzyme preparations. The physiological role of the enzyme is unknown.

A large number of naturally occurring peptides and proteins are thought to have pyrrolidonecarboxylic acid (pyroglutamic acid)[1] as their amino-terminal residue (Table I), a situation presumed to arise from the cyclization of terminal glutaminyl (I) or glutamyl (III) residues. In this regard, an enzyme has been found which will cyclize terminal glutamine residues on peptides (Messer and Ottesen, 1964). Pyrrolidonyl peptides (II) also can arise artifactually during the isolation of peptides after proteolysis, glutamine-terminating peptides being especially liable to cyclize (Sanger and Thompson, 1953; Smyth *et al.*, 1962). The absence of an α-amino group in these peptides and proteins has been a major handicap in the characterization of many of these materials, since amino-terminal analyses, including stepwise degradation methods (Edman and Begg, 1967), cannot be carried out.

Our original aim was to look for an enzyme which would open pyrrolidone rings, the hope being that such an enzyme would render terminal amino groups accessible in PCA-terminating peptides. To this end we isolated a microorganism from the soil which will grow on free PCA as the sole source of carbon and nitrogen. At the time we were unaware that Maruyama and Nomura (1956) had previously isolated a similar organism in order to study the nature of PCA degradation. Both organisms have been identified tentatively as strains of *Pseudomonas fluorescens*. The experiments of Maruyama and Nomura (1956), along with thermodynamic considerations of the equilibrium concentrations of glutamic acid and free PCA (Wilson and Cannan, 1937; Akita *et al.*, 1959), suggested that our attempts were not at all likely to succeed. Fortunately, however, we happened to test a crude extract from this organism on a pyrrolidonyl dipeptide (L-pyrrolidonyl-L-alanine) and found that free PCA and alanine were produced. This result was entirely unexpected since no other enzyme has been reported which will hydrolyze pyrrolidonyl dipeptides, and in our own hands ten available proteases had no detectable activity toward the same dipeptide (Table II). Even if completely nonspecific, then, such an enzyme would be useful for identifying pyrrolidonecarboxylic acid in peptides, and thus we turned our efforts to its isolation. As it turns out, the enzyme is highly specific and selectively removes PCA from the amino terminals of polypeptides. We suggest the name pyrrolidonyl peptidase.



It must be emphasized that, although the organism we have used as a source for this enzyme was isolated by the soil-enrichment method on PCA,[2] we can

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[1] Abbreviations used: PCA, free pyrrolidonecarboxylic acid; Pyr, the sequence designation for pyrrolidonyl residues in peptides or proteins, as in Pyr-Ala and Pyr-Val.

[2] Dr. R. Stanier has subsequently found that 166 of 175 strains of *Pseudomonas* in his collection will grow on PCA as the sole source of carbon and nitrogen.

TABLE I: Some Peptides and Proteins Thought to End with Pyrrolidonyl Residues.

Peptide or Protein	Source	Number of Residues in Chain	Reference
Fastigiatin	Algae	3	Dekker *et al.* (1949)
Elodoisin	Mollusca	11	Anastasi and Erspamer (1963)
Physalaemin	Amphibia	11	Erspamer *et al.* (1964)
Fibrinopeptides B (B chain of fibrinogen)	Mammalia	14–21 (*ca.* 500)	Blombäck *et al.* (1963); Blombäck and Doolittle (1963)
Gastrin	Mammalia	17	Gregory *et al.* (1964)
Immunoglobulin light chains	Mammalia	*Ca.* 200	Hood *et al.* (1966)
Immunoglobulin heavy chains	Mammalia	*Ca.* 450	Press *et al.* (1966)

see no connection between this fact and the possession of pyrrolidonyl peptidase activity. The activity is apparently lacking in three other strains of *Pseudomonas* and one strain of *Escherichia coli* which we have tested, but a similar activity has been found in a strain of *Bacillus subtilis*.[3]

Materials and Methods

L-Pyrrolidonecarboxylic acid (mp 156–157°) was purchased from Aldrich Chemical Company. L-Pyrrolidonyl-L-alanine (mp 205–206°) and L-pyrrolidonyl-L-valine (mp 175–176°) were prepared from the *t*-butyl esters (Cyclo Chemicals) of L-alanine and L-valine according to the procedure described by Press *et al.* (1966). PCA and pyrrolidonyl peptides which do not contain lysine are completely ninhydrin negative. Accordingly they were identified after paper chromatography and paper electrophoresis by the chlorine gas method of Reindel and Hoppe (1954). The most unequivocal identifications of free PCA were made on low-voltage electrophoresis at pH 4.1 (300 V, 2.5 hr). Under these conditions free PCA moves toward the anode about twice as fast as aspartic acid.

Fibrinopeptides B. Descriptions of the artiodactyl fibrinopeptides B used as test substances in this study have appeared elsewhere (Doolittle *et al.*, 1967; Mross and Doolittle, 1967). The amino acid analyses were performed on a modified (long light-path cuvet) Spinco amino acid analyzer (Spackman *et al.*, 1958). Edman degradations (Edman, 1960) were performed as previously described (Doolittle *et al.*, 1967).

Culture Conditions. The organism is maintained on agar slants containing salts and L-PCA. Batches of the bacteria were prepared by rinsing slants into a liquid inoculum containing 0.5% PCA and the usual salts and trace metals. When the inoculum was grown, it was transferred to a large volume of a glucose minimal medium containing ammonium salts. Growth was conducted at 30° with shaking. When the optical density at 600 mμ was greater than 1, the cells were harvested and frozen unwashed.

Assay. Pyrrolidonyl peptidase activity was followed by monitoring the exposure of alanine amino groups by the ninhydrin method of Hirs *et al.* (1956). Usually 25 μl of 0.5% Pyr-Ala was mixed with 50 μl of buffered enzyme solution and incubated in a 3-ml glass-stoppered centrifuge tube. At appropriate times 1.0 ml of absolute ethanol was pipetted into the mixture to poison the enzyme and precipitate the bulk of the protein. After sitting for at least 15 min, tubes were centrifuged at 800*g*, and the supernatant fluids were decanted. Aliquots (0.5 ml) were tested with the ninhydrin reagent.

Buffers. During the early stages of enzyme preparation a 0.05 M phosphate buffer (pH 7.3), containing 0.03 M

TABLE II: Negative Attempts to Hydrolyze L-Pyrrolidonyl-L-alanine with Ten Available Proteases.

Protease	Test Substrate	Pyr-Ala
Pronase	Sheep fibrinopeptide A	No reaction
Nagarse	Sheep fibrinopeptide A	No reaction
Papain	Sheep fibrinopeptide A	No reaction
Pepsin	Sheep fibrinopeptide A	No reaction
Thermolysin	Sheep fibrinopeptide A	No reaction
Chymotrypsin	Sheep fibrinopeptide A	No reaction
Trypsin	Sheep fibrinopeptide B	No reaction
Carboxypeptidase A	Gly-Leu-Tyr	No reaction
Carboxypeptidase B	Pyr-Phe-Ala-Arg	No reaction
Leucine aminopeptidase	Gly-Leu-Tyr	No reaction

[3] The *B. subtilis* is strain 23WT and was provided by Dr. M. Simon.

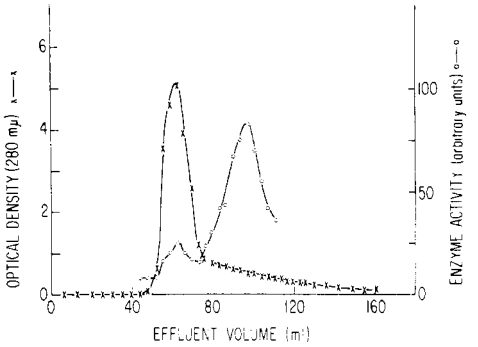

FIGURE 1. Gel filtration on Sephadex G-200 (2.5 × 32 cm) of 42% saturated ammonium sulfate cut from crude extract. The small peak of apparent enzyme activity under the main protein peak has been shown, in separate experiments, to be due to a nonspecific ninhydrin reaction with unprecipitated protein. Ammonium ions, which also interfere with the assay, begin to elute at about 120 ml.

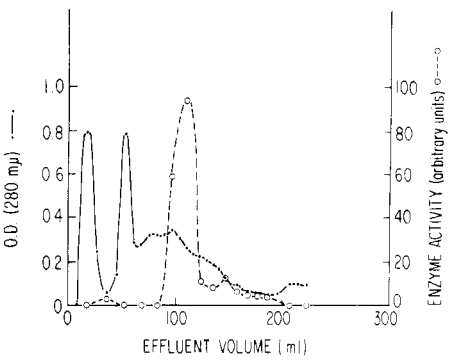

FIGURE 2: Chromatography on DEAE-Sephadex of active fractions from G-200. Elution effected with an NaCl gradient. See text for details.

mercaptoethanol and 0.001 M EDTA, was used. Throughout this paper it is referred to simply as the "phosphate buffer." For chromatography on DEAE Sephadex, Tris buffers were employed. The starting buffer, "Tris buffer A," was 0.05 M Tris, adjusted to pH 8.0 with HCl and containing 0.03 M mercaptoethanol and 0.001 M EDTA. The gradient elution was carried out by adding a second buffer, "Tris buffer B," which was identical with the starting Tris buffer except that it contained 3% NaCl. Glass-distilled water was used throughout.

Results

Preparation of the Enzyme. Purification steps were assayed using Pyr-Ala as the test substrate. Usually about 25 g wet wt of cells was suspended in 60 ml of phosphate buffer and sonicated in a glass flow-around cell at 0–6°. The sonified material was centrifuged at 30,000*g* for 60 min at 0°. The supernatant fluid, designated the crude extract, usually had an OD_{280} of about 50. The enzyme activity was precipitated by the cautious addition of saturated ammonium sulfate (enzyme grade) to 42% saturation. After standing at 0° for 30 min, the preparation was centrifuged at 30,000*g* for 20 min at 0°. The supernatant fluid was discarded and the precipitate was dissolved in 10 ml of cold phosphate buffer. Half of the dissolved material was applied to each of two Sephadex G-200 columns (2.5 × 32 cm). It is important that this operation be handled speedily; the initial flow rate should be about 35 ml/hr. In order to maintain these fast flow rates it was necessary for us to repour columns after every usage and to use new G-200 after only a few passages. The enzyme activity is retarded on G-200 and comes well behind the major protein fraction (Figure 1). The activity is well ahead of the ammonium sulfate peak, however, which would obviously interfere with the ninhydrin assay. The active fractions were pooled and reprecipitated by the addition of solid ammonium sulfate to about 60% saturation. These slurries could be kept at 0° for periods up to 1 month without significant loss of activity. When the enzyme is to be used, about 5 ml of slurry is centrifuged, dissolved in 1 ml of phosphate buffer, and dialyzed *vs.* three batches (two changes) of the same phosphate buffer for 1 hr each. At this stage of purification the enzyme is 30–40-times purified relative to the crude extract and is referred to as "the G-200 enzyme."

Further purification of the enzyme could be accomplished on DEAE Sephadex (A-25). Approximately 60 ml of ammonium sulfate slurries from G-200 columns were centrifuged and dissolved in 10 ml of Tris buffer A and dialyzed *vs.* two changes of that same buffer for 1 hr each. The material was applied to a 1.2 × 15 cm column and elution effected with a NaCl gradient (100 ml of Tris buffer A and 100 ml of Tris buffer B). The elution profile is depicted in Figure 2. The peak tubes had specific activities more than 100 times greater than the crude extract. At this stage ("the A-25 enzyme") the enzyme activity is highly unstable, however, and attempts at storage under ammonium sulfate or by freezing have been unsuccessful so far. For this reason most of the experiments to be described in this report were conducted on the "G-200 enzyme."

Inhibition by Sulfhydryl-Inactivating Reagents. The enzyme activity is very sensitive to substances which interact with sulfhydryl groups, such as *p*-mercuriphenylsulfonate and iodoacetamide. Incubations (5 min) of either crude extracts or the G-200 enzyme with concentrations of *p*-mercuriphenylsulfonate in the 10^{-4} M range resulted in complete inactivation.

Specificity of the Enzyme. Characterization of the enzyme specificity has two aspects. In the first instance we want to establish whether or not it will attack all pyrrolidonyl peptides, no matter what the nearest neighboring amino acids. Secondly we would like to know if the enzyme is specific for the peptide bond linking pyrrolidonyl groups to other amino acids or

TABLE III: Proposed Amino Acid Sequences of Fibrinopeptides B from a Variety of Artiodactyl Species.[a]

Pig	Ala-Ile-Asp-Tyr-Asp-Glu-Asp-Glu-Asp-Gly-Arg-Pro-Lys-Val-His-Val-Asp-Ala-Arg
Camel	Ala-Thr-Asp-Tyr-Asp-Glu-Glu-Glu-Asp-Asp-Arg-Val-Lys-Val-Arg-Leu-Asp-Ala-Arg
Elk	Pyr(His,Ser,Thr)(Asp,Tyr,Asp,Glu,Glu,Glu,Glu,Asp)Arg-Ala-Lys(Leu,His,Leu,Asp,Ala)Arg
Muntjac	Pyr(His,Ser,Thr)(Asp,Tyr,Asp,Glu,Val,Glu,Asp,Asp)Arg-Ala-Lys(Leu,His,Leu,Asp,Ala)Arg
Reindeer	Pyr(His)Leu-Ala-Asp-Tyr-Asp-Glu-Val(Glu,Asp,Asp)Arg-Ala-Lys-Leu-His-Leu-Asp-Ala-Arg
Mule deer	Pyr-His-Leu(Ala,Asp,Tyr,Asp,Glu,Val)Asp-Asp-Asp-Arg-Ala-Lys(Leu,His,Leu)Asp-Ala-Arg
Pronghorn	Pyr(Pro,Ser)(Tyr,Asp,Tyr,Asp,Glu,Glu,Glu,Asp,Asp)Arg-Ala-Lys-Leu-Arg(Leu,Asp,Ala)Arg
Sheep, Goat	Gly-Tyr-Leu-Asp-Tyr-Asp-Glu-Val-Asp-Asp-Asn-Arg-Ala-Lys-Leu-Pro-Leu-Asp-Ala-Arg
Ox	Pyr-Phe-Pro-Thr-Asp-Tyr-Asp-Glu-Gly-Gln-Asp-Asp-Arg-Pro-Lys-Val-Gly-Leu-Gly-Ala-Arg
Water buffalo	Pyr(Phe,Pro,Thr,Asp,Tyr,Asp,Glu,Gly,Gln,Asp,Asp,Arg,Pro,Lys)Leu(Gly,Leu,Gly,Ala)Arg
Cape buffalo	Pyr(Phe,Pro,Thr,Asp,Tyr,Asp,Glu,Gly,Gln,Asp,Asp,Arg,Pro,Lys)Ser(Gly,Leu,Gly,Ala)Arg

[a] Adapted from Mross and Doolittle (1967).

whether this activity is only part of a much broader proteolytic specificity.

The first aspect of the characterization was studied in two ways. First, another dipeptide, L-pyrrolidonyl-L-valine, was synthesized and tested. It was found to be hydrolyzed more slowly than Pyr-Ala (Figure 3), but still at a substantial rate. Secondly, small amounts of a variety of fibrinopeptides B were available from other studies being conducted in our laboratory. Many of these were thought to end with pyrrolidone residues in that they had no available α-amino groups and yielded ninhydrin-negative peptides after digestion with a battery of proteolytic enzymes. Furthermore, the ninhydrin-negative peptides invariably yielded free glutamic acid, among other amino acids, after total acid hydrolysis. Previously one of these fibrinopeptides B (ox) had been characterized extensively in this regard and deduced to be a pyrrolidonyl peptide (Blombäck and Doolittle, 1963). The fibrinopeptides are extremely variable in their amino acid sequences and the peptides available presented several different nearest neighbor amino acids for attack. Incubation of about 50 nmoles of each of these fibrinopeptides (or fragments from them) with 25 μl of G-200 enzyme solution for 16 hr at room temperature released free PCA in the cases of fibrinopeptides B from elk, muntjac, mule deer, pronghorn, ox, water buffalo, and cape buffalo (Table III). These included terminal sequences involving Pyr-His, Pyr-Ser or Pyr-Pro, and Pyr-Phe, suggesting the nature of the amino acid in the adjacent position to the pyrrolidonyl residue is not likely to be a critical factor in the action of the enzyme pyrrolidonyl peptidase. In none of these studies was an attempt made to determine whether other peptide bonds were also split; digests were simply electrophoresed at pH 4.1 and tested for free PCA. In some cases the entire fibrinopeptides (21 residues) were digested; in others only the amino-terminal tri- or tetrapeptides were studied.

In order to establish whether or not the enzyme was specific for pyrrolidonyl peptide bonds, a substan-

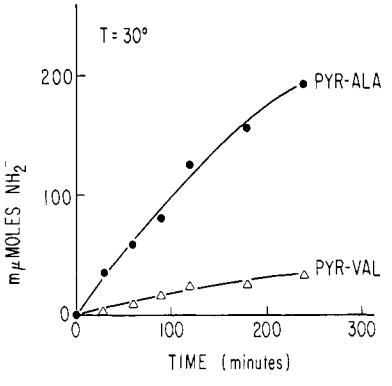

FIGURE 3: Comparison of the susceptibility of two pyrrolidonyl dipeptides to attack by the enzyme. Pyr-Ala, L-pyrrolidone-L-alanine; Pyr-Val, L-pyrrolidone-L-valine.

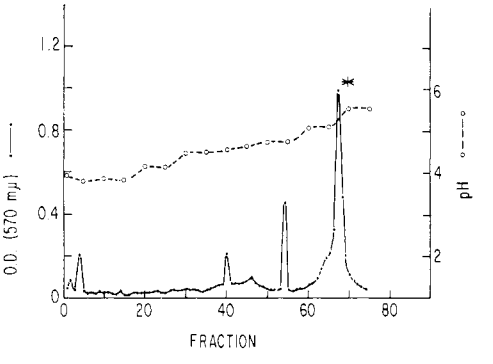

FIGURE 4: Ion-exchange chromatography on Dowex 50X-2 of bovine fibrinopeptide B (1.2 × 15 cm) treated with pyrrolidonyl peptidase. The peak marked with an asterick was pooled and subjected to amino acid analysis and Edman stepwise degradation. (○---○) pH; (●——●) ninhydrin analysis after alkaline hydrolysis.

tial amount (*ca.* 1 μmole) of ox (bovine) fibrinopeptide B was digested for 12 hr at room temperature with 0.5 ml of G-200 enzyme. High-voltage electrophoresis at pH 2 of an aliquot of the reaction mixture indicated one major band which was distinctly more basic than a sample of undigested bovine B run along side. The reaction mixture was freeze dried, redissolved in 0.1 M ammonium acetate (pH 3.8), and chromatographed on Dowex 50X-2 (Figure 4). The main peak, accounting for more than 75% of the starting material, eluted from the column at pH 5.2. Ordinarily the ox fibrinopeptide B elutes from Dowex 50 under these conditions at about pH 4.7, and indeed a minor peak appeared at that position. An aliquot of the major peak was subjected to total acid hydrolysis and its amino acid composition compared with a companion sample of undigested ox peptide B (Table IV). The treated B material was lacking only one residue of glutamic acid. Finally, the remainder of the preparation (*ca.* 0.5 μmole) was taken through five cycles of the Edman stepwise degradation method and the following sequence was determined: Phe-Pro-Thr-Asp-Tyr. Thus, by all criteria employed the enzyme preparation had liberated the PCA without significantly breaking any of the other 19 peptide bonds in the substrate molecule.

TABLE IV: Amino Acid Analyses[a] of Bovine Fibrinopeptide B before and after Treatment with Pyrrolidonyl Peptidase.

Amino Acid	Residues/mole	
	Untreated	Treated
Lysine	1.0	1.1
Arginine	2.1	1.9
Aspartic acid	4.1	4.2
Threonine	0.9	0.9
Glutamic acid	3.1	2.2
Proline	1.9	1.7
Glycine	3.0	3.0
Alanine	1.0	1.0
Valine	1.0	0.9
Leucine	1.0	1.1
Tyrosine	0.9	1.1
Phenylalanine	1.0	0.9
Total residues	21	20

[a] Total acid hydrolysis accomplished with 5.7 N HCl, 107°, 20 hr, evacuated, sealed tubes.

Discussion

The availability of an enzyme which selectively removes pyrrolidone residues from polypeptide chains should be a distinct advantage in the characterization of many proteins. This advantage will be increased if the enzyme can be further purified and stabilized. Already it has proved useful in positively identifying PCA as the terminal residue of a variety of fibrinopeptides B.

Apart from its usefulness in amino acid sequence work, however, the question of the physiological role of the enzyme remains unanswered. The fact that no activity could be demonstrated in three other strains of *Pseudomonas*, even though two of these would grow on PCA as the sole source of carbon and nitrogen, coupled with the observation of a similar pyrrolidone splitting activity in a strain of *B. subtilis*, which will *not* grow on free PCA as a source of carbon and nitrogen, would seem to rule out any connection between the pyrrolidonyl peptidase activity and the method used to isolate the organism which we have used as a primary source. It is our intention to undertake a larger survey, including plant and animal tissues, as part of a broad effort to understand the function of the enzyme.

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References

Akita, S., Tanaka, K., and Kinoshita, S. (1959), *Biochem. Biophys. Res. Commun. 1*, 179.

Anastasi, A., and Erspamer, V. (1963), *Arch. Biochem. Biophys. 101*, 56.

Blombäck, B., Blombäck, M., and Edman, P. (1963), *Acta Chem. Scand. 17*, 1184.

Blombäck, B., and Doolittle, R. F. (1963), *Acta Chem. Scand. 17*, 1816.

Dekker, C. A., Stone, D., and Fruton, J. S. (1949), *J. Biol. Chem. 181*, 719.

Doolittle, R. F., Schubert, D., and Schwartz, S. A. (1967), *Arch. Biochem. Biophys. 118*, 456.

Edman, P. (1960), *Ann. N. Y. Acad. Sci. 88*, 602.

Edman, P., and Begg, G. (1967), *European J. Biochem. 1*, 80.

Erspamer, V., Anastasi, A., Bertaccini, C., and Cei, J. M. (1964), *Experientia 20*, 489.

Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W., and Shepherd, R. C. (1964), *Nature 204*, 931.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.

Hood, L., Gray, W. R., and Dreyer, W. J. (1966), *J. Mol. Biol. 22*, 179.

Maruyama, Y., and Nomura, M. (1956), *J. Biochem. (Japan) 43*, 327.

Messer, M., and Ottesen, M. (1964), *Biochim. Biophys. Acta 92*, 409.

Mross, G., and Doolittle, R. F. (1967), *Arch. Biochem. Biophys.* (in press).
Press, E. M., Piggot, P. J., and Porter, R. R. (1966), *Biochem. J. 99*, 356.
Reindel, F., and Hoppe, W. (1954), *Chem. Ber. 87*, 1103.
Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 366.
Smyth, D. G., Stein, W. H., and Moore, S. (1962), *J. Biol. Chem. 237*, 1845.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Wilson, H., and Cannan, R. K. (1937), *J. Biol. Chem. 119*, 309.

Soybean Inhibitors. I. Separation and Some Properties of Three Inhibitors from Commercial Crude Soybean Trypsin Inhibitor*

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ABSTRACT: Commercial crude soybean trypsin inhibitor (STI) has been separated into a number of components by diethylaminoethylcellulose chromatography. In addition to the well-known soybean trypsin inhibitor of Kunitz (Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291) (referred to as "component F_2" in this paper when obtained in a chromatographically pure state), two other homogeneous protein inhibitors (F_1 and F_3) have been isolated and partially characterized. Unlike STI, neither F_1 nor F_3 is a stoichiometric inhibitor of tryptic activity. Also, F_1, like STI, is a moderate inhibitor of chymotrypsin, whereas F_3 has a marginal effect on this enzyme. F_1, F_2, and F_3 have different electrophoretic mobilities on acrylamide gel and different molecular weights within the range 18,000–24,000.

All three proteins contain tryptophan; however, F_3, unlike F_1 and F_2, is devoid of tyrosine. The pH profiles of the intensity of tryptophan fluorescence for F_1 and F_2 are similar, while that for F_3 differs from the other two in the pH range ~1–9. Of the estimated five tyrosine residues in F_1, four are readily iodinated under conditions where only two of the four residues in F_2 (STI) react to form diiodotyrosine.

A number of trypsin and chymotrypsin inhibitors have been isolated from soybean protein. The first to be crystallized and studied in detail was the classical soybean trypsin inhibitor of Kunitz (1945–1947), STI.[1] Although other inhibitors had been reported to be present in soybeans (Laskowski and Laskowski, 1954; Lipke *et al.*, 1954), only within the last few years have several of these been purified and characterized (Rackis *et al.*, 1959, 1962; Rackis and Anderson, 1964; Birk, 1961; Birk *et al.*, 1963; Yamamoto and Ikenaka, 1967).

In addition to the soybean inhibitors, a number of other trypsin and chymotrypsin inhibitors have been isolated in highly purified form from both plant and animal sources. Some examples include the four distinct lima bean inhibitors (Jones *et al.*, 1963), a pancreatic trypsin inhibitor (Kassell *et al.*, 1963), and chicken ovomucoid (Rhodes *et al.*, 1958). At least two chymotrypsin-specific inhibitors have been reported in the literature, namely, the crystalline *Ascaris* inhibitor which is specific for both α-chymotrypsin and chymotrypsin B (Peanasky and Laskowski, 1960) and an inhibitor isolated from potatoes (Ryan and Balls, 1962). This investigation reports the isolation of two additional inhibitors from commercial crude soybean trypsin inhibitor which, apparently, are quite distinct in properties from all other soy inhibitors.

Materials

All enzymes, enzyme substrates, and protein inhibitors were purchased from the Worthington Biochemical Corp., Freehold, N. J. Crude soybean trypsin inhibitor

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[1] Abbreviations used: STI, crystalline soybean trypsin inhibitor of Kunitz (1945–1947); crude STI, crude Kunitz inhibitor obtained from Worthington Biochemical Corp.; $SBTIA_1$ and $SBTIA_2$, soybean trypsin inhibitors A_1 and A_2 described by Rackis *et al.* (1962); TAME, *p*-toluenesulfonyl-L-arginine methyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester.