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SPECIFIC CLEAVAGE OF REDUCED AND *S*-CARBOXAMIDOMETHYLATED NEUROPHYSIN II BY THE COLLAGENASE OF *CLOSTRIDIUM HISTOLYTICUM*

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Summary

Purified collagenase of *Clostridium histolyticum* was shown to cleave reduced and *S*-carboxamidomethylated bovine neurophysin between Cys-13 and Gly-14. The scission resulted in formation of two separable fragments: a smaller peptide arising from residues 1 through 13, and a larger peptide comprising the remainder of the residues of the protein. By dansylation procedures, the smaller peptide was shown to have amino-terminal alanine as expected from the sequence of neurophysin II, and the larger peptide had amino-terminal glycine as anticipated. These results show that collagenase indeed cleaves bovine neurophysin II in accord with the specificity postulated for that enzyme, i.e., scission between -X-Gly- in a sequence of -Pro-X-Gly-Pro-Y-. This result, obtained with a non-collagenous protein substrate, is further confirmation of the specificity of collagenase as established by its action on collagens and on synthetic oligopeptides.

Introduction

Bovine neurophysin II (also known as bovine MSEL-neurophysin and hereafter referred to in this paper as neurophysin II) is a major neurohypophyseal hormone-binding protein which has been purified and studied intensively [1—5]. It is composed of 95 amino acid residues in a single chain which contains 7 disulfide bonds. Determination of its amino acid sequence is now complete [4—8]. The protein has been demonstrated to exhibit microheterogeneity in that the amino acid at position 89 has been found to be either an isoleucine or valine residue. Otherwise, there is no variability in the amino acid sequence of neurophysin II. The sequence of the first twenty amino acids is as follows:

Ala-Met-Ser-Asp-Leu-Glu-Leu-Arg-Gln-Cys-Leu-Pro-Cys-Gly-Pro-Gly-Gly-Lys-Gly-Arg

In this sequence, we and Dr. Elvin Harper observed a domain, from residues 12 through 16, consisting of -Pro-Cys-Gly-Pro-Gly-. That sequence resembles -Pro-X-Gly-Pro-Y-, the repeating peptide arrangement characteristic of various collagens. It should be noted that such a sequence does not occur in any other region of neurophysin II. Of special interest is that a sequence of such composition is precisely the site of cleavage of collagens by the collagenase of *Clostridium histolyticum* [9]. When clostridial collagenase acts on collagen, it cleaves between the -X-Gly- residues giving rise to peptides with amino-terminal glycine. Although many synthetic peptides containing the susceptible sequence are cleaved by highly purified preparations of collagenase, only a few proteins other than the collagens have been shown to be digested by the enzyme. Thus, a protein of the parotid glands, rich in glutamic acid, proline and glycine is cleaved [10]. Also, a soluble form of acetylcholinesterase of the electric eel can be released from a "basement membrane" matrix by collagenase; and indeed, some preparations of acetylcholinesterase retain small numbers of residues of hydroxyproline, an amino acid considered characteristic of collagens [11—13]. Clostridial collagenase also has been shown to act on the collagen-like component of the complement system, Clq [14]. Among non-collagenous proteins, immunoglobulin light chains [15], casein [16], β-casein [17], catalase and cytochrome *c* [16] have been found to be cleaved by collagenase preparations; however, the mode of cleavage has not been established for all of the above.

Our interest was to determine whether purified clostridial collagenase could cleave bovine neurophysin II between residues 13 and 14 (-Cys-Gly-) to yield two peptides with compositions expected from the known sequence of neurophysin II. In addition to establishing the specificity of collagenase in a protein of known sequence, it was felt that the cleavage products would be of interest in studying the mode of action of the neurophysins. In the "native protein", Cys-13 is in disulfide linkage; accordingly, before treatment with collagenase, the protein was reduced with 2-mercaptoethanol and the product alkylated with iodoacetamide. To aid in identification of products, [^{14}C]-iodoacetamide was used. The reduced and *S*-carboxamidomethylated neurophysin II indeed was cleaved as expected.

Finally, if neurophysin II is reduced but not alkylated, the sequence indeed becomes -Pro-Cys-Gly-Pro-. Since cysteine is known to inhibit collagenase [18], presumably by chelation of an intrinsic metal atom such as zinc [18—20], the possibility exists that the reduced non-alkylated form would in fact not be cleaved by collagenase, but instead might inhibit the enzyme. The peptide sequence presumably could allow the enzyme to approach the substrate, but the metal of the enzyme perhaps then would be fixed tightly to the chelating group. In fact, however, reduced neurophysin in the concentrations used did not inhibit the action of collagenase on a collagen substrate.

Experimental section

Materials

Purified bovine neurophysin II used in this study was generously donated by Dr. Esther Breslow. Iodoacetamide was obtained from Sigma Chemical Co. and iodo[1-^{14}C]acetamide (9.35 mCi/mM) from New England Nuclear Corp. Dansyl amino acid standard and dansyl chloride were purchased from Pierce Chemical

Co., collagenase from Boehringer Mannheim GmbH, Sephadex G-200 and G-25 from Pharmacia Fine Chemicals, polyamide layers from Cheng Chin Trading Co. and cellulose layers from Eastman Kodak Co. (Eastman Chromagram Sheets, Cat. No. 13255).

Methods

Purification of collagenase. Commercial bacterial collagenase was further purified by gel filtration [21,22]. A 1.5 × 90 cm column of Sephadex G-200 was prepared. A sample (0.5 ml) containing 15 mg of commercial collagenase was layered on top of the column and, after it entered the column, elution was begun. The eluting buffer was 0.05 M Tris · HCl, pH 7.6, containing 0.005 M $CaCl_2$. The temperature was 4°C, the flow rate was 2.0 ml/28 min; and fractions of that volume were collected. Each fraction was assayed for protein by measurement of absorbance at 280 nm and by the method of Lowry et al. [23].

General proteolytic activity was measured using casein as a substrate and determining 280 nm absorbance in a trichloroacetic acid supernate [24]. Assays for collagenolytic activity were carried out with a substrate consisting of gels made from a preparation of guinea pig skin collagen that had been labeled in vivo with [^{14}C]glycine [25,26]. Collagenase activity was expressed as radioactivity (cpm) released into solution in a unit time.

Reduction and alkylation of neurophysin II. The native protein (6.0 mg) was dissolved in 1.0 ml of 0.5 M Tris · HCl buffer (pH 8.2), 8 M urea, containing 10 μl of 2-mercaptoethanol (about 0.1 mmol/ml). The mixture was kept at room temperature for 1 h with occasional shaking. The reduced neurophysin II was then alkylated with labeled iodoacetamide [7]. For this reaction, 0.2 mg (0.01 mCi) of [^{14}C]iodoacetamide were added and, after a few minutes, 10 mg (approx. 50 μmol) of unlabeled iodoacetamide were added to the mixture. The mixture was stirred for 2 h at 4°C and then dialyzed against 4 changes of distilled water at 4°C over a period of 36 h. The preparation was then lyophilized.

Collagenase digestion of S-carboxamidomethylated neurophysin II. Carboxamidomethylated neurophysin II (2.4 mg) was digested with the purified collagenase for 3 h at 37°C in 5 mM $CaCl_2$ dissolved in 50 mM Tris · HCl buffer, pH 7.4; the enzyme:substrate ratio was 1 : 30.

Fractionation of collagenase digest. The digestion mixture was subjected to gel filtration on a 1.5 × 90 cm column of Sephadex G-25 (fine grade). The peptides were eluted with 0.1 M formic acid at a flow rate of 2.0 ml/18 min and collected in 2-ml fractions. The absorbance of fractions was measured at 280 nm and their radioactivity (^{14}C) determined by counting in Bray's solution using a Searle Mark III liquid scintillation spectrometer.

Electrophoresis. Polyacrylamide gels were prepared by a standard method [27] with sample and spacer gels omitted. Electrophoresis was carried out at pH 8.9 using a current of 3 mA per tube for 2 h. Gels were stained for protein with Amido Schwarz, and scanned with a Gilford Model 220 gel scanner.

High voltage paper electrophoresis was conducted on Whatman 3MM paper at 4000 V for 90 min using a Savant high voltage instrument. The solvent was pyridine/acetic acid/water (1 : 10 : 89, v/v), pH 3.5. Papers were then dried and treated with ninhydrin.

Two dimensional chromatography-electrophoresis. In some experiments separation of the peptides from collagenase digests of carboxamidomethylated neurophysin II was performed on a cellulose layer by chromatography in one dimension and electrophoresis in a second. Ascending chromatograms were developed for 5 h at room temperature using a solvent mixture of butanol/acetic acid/water (4 : 1 : 5, v/v). The cellulose layer was then dried, placed on the electrophoresis apparatus and moistened with running buffer (2 M acetic acid/0.6 M formic acid, 1 : 1 v/v, pH 2.0). Electrophoresis then was conducted at 300 V for 1 h.

Determination of amino-terminal residues. Amino-terminal residues of the peptides formed were determined by a modified dansylation procedure [28,29]. Each peptide was dansylated, hydrolyzed and the hydrolyzate spotted on a plate; then a standard mixture of dansyl amino acids was spotted on the obverse of the plate opposite the experimental sample. Development in one dimension was carried out with solvent I (1.5% formic acid in water) and in the second dimension with solvent II (benzene/acetic acid, 9 : 1, v/v), followed by solvent III (ethyl acetate/acetic acid/methanol, 20 : 1 : 1, v/v). The separated dansyl amino acids were visualized by ultraviolet irradiation.

Analysis of amino acids. Samples were hydrolyzed in 6 M HCl at 105°C for 24 h. Amino acid contents were determined with a Technicon Amino Acid Analyzer employing a column (0.6 × 70 cm) with Chromobead Type C-2 resin.

Results

Purification of collagenase. Commercially purified collagenase frequently is contaminated with at least two proteases that in themselves have no significant action on collagen [22]. The commercial preparation used in the present studies separated into at least six components when electrophoresed on acrylamide gel. When that preparation was fractionated on a column of Sephadex G-200, the pattern shown in Fig. 1 was obtained. The first peak, eluted in fractions 31—35, had considerable caseinolytic activity and some collagenolytic activity. The material in that peak had a molecular weight of about 220 000. A second peak containing collagenolytic activity was eluted in fractions 37—44 and corresponded to material with molecular weights ranging from 100 000 to 150 000. This peak contained fractions with little or no caseinolytic activity. The collagenase selected for studies with neurophysin II was fraction 40; that material showed no activity against casein by the method of assay used here.

Peptides obtained from S-carboxamidomethylated neurophysin II after the action of collagenase. The highly purified neurophysin II used in these studies was shown on gels to consist chiefly of a single component with some minor heterogeneity observed as material migrating toward the anode side of the gel. By the methods used, one would not expect to separate the two species referred to earlier that differ only in the amino acid residue found at position 89. This microheterogeneity of the carboxyl end of neurophysin II is assumed in the subsequent discussion of the scission products. Thus, the large peptide (peptide A) is assumed to consist of two peptides differing at position 89. Fig. 2, gel a, shows the electrophoretic pattern of neurophysin II. In the same figure, gel b shows the pattern obtained with carboxamidomethylated neuro-

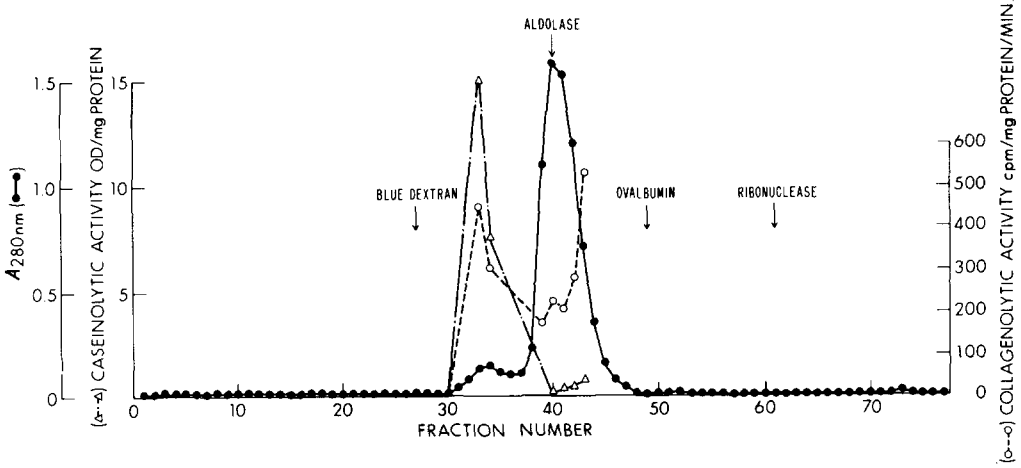

Fig. 1. Purification of clostridial collagenase by gel filtration on a Sephadex G-200 column. The positions of elution of Dextran Blue and several protein markers are indicated. Refer to the text for experimental details.

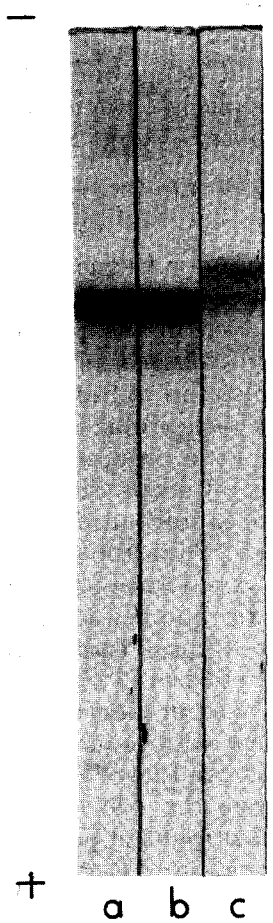

Fig. 2. Polyacrylamide gel electrophoresis patterns of, (a) bovine neurophysin II; (b) *S*-carboxamidomethylated bovine neurophysin II; (c) *S*-carboxamidomethylated bovine neurophysin II after digestion with clostridial collagenase. In each case a 10 μl aliquot of sample which contained 30 μg of the native or modified neurophysin was applied to the gels. For conditions of alkylation and collagenase digestion of neurophysin II, refer to Experimental section of text.

physin II; this is virtually identical with that of unmodified neurophysin II. The pattern given by carboxamidomethylated neurophysin II after digestion with collagenase is shown in Fig. 2, gel c. It indicates that the larger of the two resultant peptides migrated more towards the cathode; it also shows the presence of some undigested carboxamidomethylated neurophysin II. The smaller peptide probably migrated to the buffer front of the gel.

High voltage electrophoresis on paper and two-dimensional electrophoresis and chromatography on cellulose films carried out as described earlier, confirmed that collagenase digestion of carboxamidomethylated neurophysin II yielded two components.

The products of collagenase digestion were also separated by gel filtration. Gel filtration of ^{14}C-labelled carboxamidomethylated neurophysin II on Sephadex G-25 yielded a single symmetrical peak that contained 99% of the total radioactivity recovered from the column. Digestion with collagenase caused the scission of carboxamidomethylated neurophysin II into 2 radioactive components separable by gel filtration. One component (peak A) eluted with the void volume and the second (peak B), was eluted later. Radioactivity was distributed between peaks A and B in a ratio of approx. 9 : 1. The material in Peak A showed absorption at 280 nm but that in Peak B did not. Thus, the positions of elution, the distribution of radioactivity and the ultraviolet absorption due to aromatic amino acids were all consistent with assignment of Peak A to the large fragment expected from the carboxyl-terminal end of carboxamidomethylated neurophysin II and, Peak B to the smaller peptide expected from the amino-terminal end of the protein.

Amino acid contents of S-carboxamidomethylated neurophysin II and of peptide A. The results of the analyses, expressed in terms of residues per molecule, are shown in Table I. The amino acid analysis of carboxamidomethylated

TABLE I

AMINO ACID CONTENTS OF *S*-CARBOXAMIDOMETHYLATED NEUROPHYSIN II (CM-NP-II) AND PEPTIDE A

Amino acid	Residues per mol *	
	CM-NP-II	Peptide A
Lysine	2.6	3.0
Histidine	0	0
Arginine	7.9	5.5
Aspartic acid	5.2	3.8
Threonine	1.9	1.4
Serine	6.5	4.0
Glutamic acid	14.4	11.0
Glycine	16.1	16.3
Alanine	5.9	5.1
Valine	4.4	4.1
Methionine	0.9	0
Isoleucine	2.5	2.2
Leucine	7.5	4.1
Tyrosine	0.8	1.1
Phenylalanine	3.3	3.0

* *S*-Carboxamidomethylated neurophysin II contains 95 total residues per mol; peptide A would contain 82 total residues per mol.

neurophysin II agrees quite well with the published composition of unmodified bovine neurophysin II [4—6]. The half-cystine content is not reported since cysteine was converted to *S*-carboxamidomethyl cysteine which was not quantitated in the analysis. There was also insufficient quantity of proline in the hydrolyzates to permit determining its content accurately.

The larger peptide, A, obtained by scission of carboxamidomethylated neurophysin II by collagenase, essentially had the composition one should expect if the first 13 amino acid residues had been removed from the amino-terminal end of carboxamidomethylated neurophysin II. When the amino acid contents of peptide A are compared with those of carboxamidomethylated neurophysin II it can be seen that no differences were observed for those amino acids not expected to be present in the small peptide. For example, the lysine, threonine, glycine, valine, isoleucine, tyrosine and phenylalanine contents are unchanged. On the other hand, those amino acids which are expected to be contained in the small peptide are correspondingly decreased in content in the large peptide. Thus the large peptide contains three residues less of leucine than does carboxamidomethylated neurophysin II, and one less of aspartic acid, alanine and methionine. The arginine, serine and glutamic acid contents of the large peptide are also present in amounts consistent with the deletion of residues 1—13 from neurophysin II.

Determination of dansylated amino acids. Fig. 3a shows the chromatogram obtained with a standard dansylated amino acid mixture using the method of two-dimensional thin layer chromatography on polyamide sheets. In Fig. 3, panels b and c demonstrate the dansylated amino acids of peptides A and B respectively. The larger peptide, A, showed an amino-terminal residue of glycine and a suggestion of amino-terminal alanine; the latter could have arisen from the presence of some undigested carboxamidomethylated neurophysin II. The smaller peptide, B, showed only alanine as the amino-terminal residue.

We did not have a sufficient amount of peptide B to allow direct analysis of amino acid contents; and accordingly, an analysis was performed by the dansylation procedure. A small amount of peptide B was first hydrolyzed in 6 M HCl at 105°C. After removal of HCl by repeated addition of water and evaporation in vacuo, the amino acid mixture was dansylated; at the same time a standard mixture of those amino acids whose presence in peptide B was anticipated was also dansylated. The chromatograms obtained with these mixtures are shown in Fig. 3, panels d and e respectively. One may note that peptide B contained no amino acids other than those anticipated from the composition of residues 1 through 13 in the sequence of neurophysin II. However, one amino acid, methionine, was not quite in the expected position on the chromatogram.

Effect of reduced neurophysin II on collagenase activity. As mentioned in the introduction, one might expect that reduced, but non-alkylated neurophysin II possibly could inhibit the action of collagenase on a collagen substrate. To test that possibility, neurophysin II (1.0 mg) was reduced with 2-mercaptoethanol as described previously, and then dialyzed against distilled water containing 0.1 M 2-mercaptoethanol. The dialysis removed the urea used in the reduction mixture while maintaining the protein in reduced form. Collagenase (5 μg) was pre-incubated with the reduced neurophysin II, and then allowed to

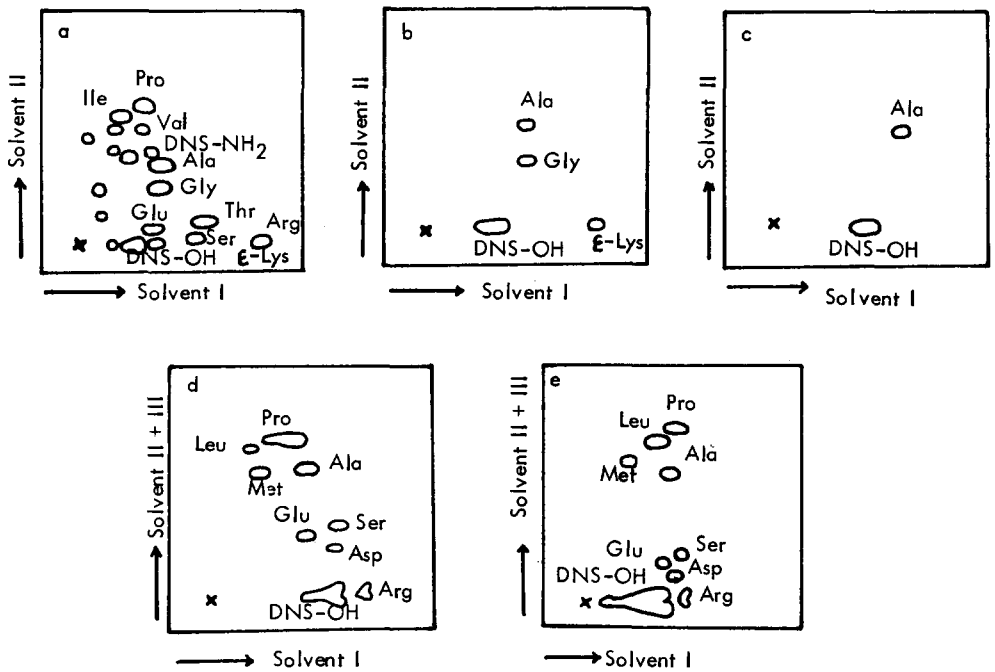

Fig. 3. Panels (a), (b), (c): Two-dimensional thin layer chromatograms of dansylated amino-terminal amino acids of the peptides obtained by collagenolytic cleavage of carboxamidomethylated neurophysin II. (a) Dansyl amino acid standards; (b) Peptide A; (c) Peptide B. Panels (d) and (e): Thin layer chromatograms of amino acids in Peptide B. Peptide B was hydrolyzed in acid prior to dansylation. (d) Standard mixture of amino acids expected to be present in Peptide B. (e) Dansyl amino acids found in Peptide B. Solvent systems used are described in the text.

act on a gel containing 0.5 mg of ^{14}C-labelled guinea pig skin collagen. It should be noted that the concentration of 2-mercaptoethanol used is known not to inhibit collagenase [18]. In fact, however, the amount of reduced neurophysin II used in this experiment caused no inhibition of collagenase activity.

Discussion

Purified clostridial collagenase has been shown to cleave a sequence in reduced and alkylated bovine neurophysin II that meets the postulated requirements of specificity of the enzyme [9]. Thus peptides are produced with the anticipated amino-terminal groups of alanine and glycine respectively, and with amino acid contents corresponding to their respective origins in the neurophysin II molecule (residues 1 through 13 and residues 14 through 95). Because of the limited availability of neurophysin II for these studies, the possible cleavage by collagenase of the unmodified neurophysin was not investigated at this time. Since neurophysin II has disulfide bridges between the cysteines on both sides of the susceptible peptide bond, the products should still be held together even should cleavage occur. In that event the product of cleavage of unmodified neurophysin II by collagenase would have to be reduced and perhaps alkylated in order to yield peptides as separate entities.

In any event, carboxamidomethylated neurophysin II is a protein of known sequence that can be cleaved by clostridial collagenase at a single site between -X-Gly- in the defined sequence of -Pro-X-Gly-Pro-Y-. Most of the other proteins that can be cleaved by collagenase, as mentioned in the introduction, undergo scission in a mode not yet established by detailed study of the products of cleavage. Indeed, in some of the instances cited, the collagenase preparation used was not adequately purified or characterized. Recently, Gilles and Keil [17] reported that a commercially purified preparation of collagenase cleaved β-casein at two sites yielding peptides with amino-terminal leucine residues. However, the results in the present study with neurophysin, in which a more highly purified collagenase was used, confirm the specificity of the enzyme as determined previously with collagens and synthetic oligopeptides. The unique feature of the present study with neurophysin II is that the cleavage occurs at the site of a cysteine residue; this finding may have implications regarding the preparation of inhibitory peptides for collagenase, since cysteine per se is an inhibitor.

In addition, the findings presented here may provide useful means by which to prepare peptide segments from various neurophysins in comparative studies of sequence. If unmodified neurophysin II is also cleaved by collagenase, the effect of that scission on the binding of specific hormones to the neurophysin may afford useful information.

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