Specificity of Trypsin and Carboxypeptidase B for Hydroxylysine Residues in Denatured Collagens†

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ABSTRACT: Sequential treatment of denatured collagen with trypsin and carboxypeptidase B caused the release of hydroxylysine in addition to lysine and arginine. Hydroxylysine obtained in this manner was isolated, purified, and identified. This demonstrated that both trypsin and carboxypeptidase B are capable of cleaving collagen at hydroxylysine residues. Using three different collagens containing differing amounts of carbohydrate, it was found that the comparative reactivities with trypsin varied considerably and correlated with the carbohydrate content of the particular collagen. The treatment of the several trypsinized collagens with carboxypeptidase B showed marked differences in the amount of hydroxylysine released as well. Conditions for maximal release of hydroxylysine were determined for each collagen, and maximal values were compared to the results obtained by alkaline and acid hydrolysis. Acid hydrolysis of the material released from denatured collagen following sequential treatment with trypsin and carboxypeptidase B failed to show any increase in hydroxylysine released, indicating that no glycosylated hydroxylysine residues had been released by digestion with the two enzymes. Treatment of denatured bovine nasal cartilage collagen with a combination of α-glucosidase and β-galactosidase resulted in a 41% decrease in the fraction of glycosylated hydroxylysine residues. Pretryptic deglycosylation of that collagen resulted in an increase in the release of hydroxylysine by sequential action of trypsin and carboxypeptidase B nearly identical with the increase in the number of deglycosylated residues formed. Posttryptic deglycosylation did not significantly increase the amount of hydroxylysine released by the proteolytic enzymes. Cleavage of collagen at hydroxylysine residues by trypsin apparently is blocked by carbohydrate-bound residues. With respect to carbohydrate removal, posttryptic deglycosylation was equally effective compared to the deglycosylation of the intact protein, suggesting that the size of the substrate is not of overriding significance in the enzymatic deglycosylation.

The specificity defined for the action of trypsin is that the enzyme splits a peptide bond on the carboxyl side of the basic amino acid residues lysine (Bergmann & Fruton, 1941), arginine (Bergmann & Fruton, 1941), norarginine (Baird et al., 1965), and ornithine (Baird et al., 1965). Susceptibility of modified amino acids such as *S*-(β-aminoethyl)cysteine (Elmore et al., 1967) and monomethyl-ϵ-aminolysine (Benoiton & Denault, 1966) has also been described. However, recent reviews (Neurath & Schwert, 1950; Cunningham, 1965; Bender & Kaiser, 1962; Bender & Kezdy, 1965; Keil, 1971) have not mentioned whether or not the specificity of trypsin includes another natural amino acid, hydroxylysine (Hyl). The interest in this matter has not been great because hydroxylysine is limited to collagenous proteins. Because of the increasing number of collagens and basement-membrane proteins being described and because other proteins such as the C1q component of complement and acetylcholinesterase have been shown to contain collagenous segments, the matter of possible cleavage at hydroxylysine residues is no longer trivial. A small number of studies in the literature relative to the action of trypsin on collagens and to sequencing of collagen chains allows the inference that in certain collagens trypsin is active at hydroxylysine sites (von der Mark et al., 1970; Dixit et al., 1975a,b). On the other hand, Butler & Cunningham (1966) found a tryptic peptide of guinea pig skin collagen which contained an internal residue of hydroxylysine. Several other authors also did not find carboxyl-terminal hydroxylysine in tryptic digests of collagen (Miller et al., 1976). A systematic study of hydroxylysine-specific cleavage by trypsin or of the influence of natural side-chain derivatives of hydroxylysine on tryptic cleavages has not been undertaken. Similar questions arise concerning the action of carboxypeptidase B, which cleaves carboxyl-terminal residues of lysine, arginine (Wolff et al., 1962), and ornithine (Wolff et al., 1962). The specificity of carboxypeptidase B for hydroxylysine has not been studied.

To answer more definitively the questions of specificity of trypsin and carboxypeptidase B with respect to residues of hydroxylysine, and then of the effect of glycosylation of hydroxylysine on such actions, we devised the following experimental strategy. (1) Three kinds of collagen, each containing a different number of hydroxylysine residues and having a different extent of glycosylation, would be studied. (2) All samples would be heat denatured, and following treatment with trypsin, further digestion with carboxypeptidase B would be performed to prove or disprove cleavage of hydroxylysine by trypsin by analysis of free hydroxylysine released. (3) By those means, we would establish the number of hydroxylysine residues released for each kind of collagen, and comparison would be made with values for glycosylated and nonglycosylated hydroxylysine as determined by classical hydrolytic methods. (4) Finally, an α-glucosidase and β-galactosidase would be used for removal of the carbohydrate moieties of the several collagens, and the products would be subjected to sequential treatment with trypsin and carboxypeptidase B. In each case, the amounts of hydroxylysine released would be determined and the values thus obtained compared with those obtained by chemical methods.

Experimental Procedures

Materials

Calf skin collagen was purchased from Calbiochem Co. Bovine nasal septum cartilage collagen was a generous gift

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from Dr. Mercedes Paz, Children's Hospital, Boston, MA. Ichthyocol (carp swim bladder collagen) was prepared as described previously (Gallop & Seifter, 1963). All collagen samples, 2.5 mg/mL, were dissolved in distilled water and denatured by heating to 60 °C for 5 min. The samples were filtered through a Millipore filter (0.45-μm pore size). All samples were checked by polyacrylamide disc gel electrophoresis and found to be without significant contamination. The digestion buffer for proteolysis consisted of 0.04 M NaCl and 0.10 M Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer adjusted to pH 8.0 by addition of NaOH.

Crystalline trypsin and ovomucoid trypsin inhibitor were obtained from Boehringer Co. Trypsin was dissolved in the digestion buffer at a concentration of 0.25 mg/mL. The inhibitor was dissolved to a concentration of 0.78 mg/mL with distilled water. Carboxypeptidase B from porcine pancreas (0.515 mg/mL, 333 units/mL) was obtained from Boehringer Co.; polypropylene plastic tubes with caps for alkaline hydrolysis were obtained from Falcon Plastics Co. Glass ampules were purchased from Virtis Co. [^{14}C]Hydroxylysine (used to calculate recovery) was obtained from New England Nuclear Co. 2,2'-Dipyridyl, grade A, was obtained from Calbiochem Co. and diluted to a concentration of 5.0 mg/mL. α-Glucosidase, analytical grade, was obtained from Boehringer Mannheim Co. β-Galactosidase, chromatographically purified (300 units/mg) from *Escherichia coli*, was purchased from Aldrich Chemical Co. The digestion buffer for the deglycosylation consisted of 0.06 M Hepes adjusted to pH 6.0 by addition of dilute HCl. Ninhydrin values at each time point were obtained according to the method of Rosen (1957). Absorption at 570 nm was measured with a Cary 14 spectrophotometer. Separation of amino acids from the digestion mixture was accomplished by pressure dialysis using an Amicon Model 12 dialysis chamber and UM-05 membranes (exclusion limit 500 daltons). High-voltage paper electrophoresis was performed with a Savant vertical tank model at pH 3.5, using a buffer consisting of pyridine–acetic acid–water at a concentration of 1:10:89. Electrophoresis was performed at 800 V for 1 h. Nuclear magnetic resonance spectra were obtained with a Varian Model T60 spectrometer. Mass spectral analysis was performed by Schrader Analytical Labs. All amino acids were quantitated by amino acid analysis using a JEOL Model JLC-6AH amino acid analyzer.

Methods

Digestion of Denatured Collagen by Trypsin. Each collagen sample was diluted 5-fold with digestion buffer, and to this was added 0.25 mg of trypsin (substrate to enzyme ratio of 20:1). The solutions were shaken vigorously and incubated at 25 °C. At various time intervals, aliquots were removed, and the digestion was halted with 50 μL of trypsin inhibitor. A blank consisting of identical concentrations of trypsin plus inhibitor was also included. Triplicate samples were used for each time point. The digestion was also carried out with a substrate to enzyme ratio of 10:1 without any significant change in the results.

Digestion of Trypsinized Collagen with Carboxypeptidase B. A large sample of calf skin collagen, 200 mg, was incubated with 10 mg of trypsin for 3 h. Ovomucoid trypsin inhibitor, 15.6 mg, was then added and the pH of the mixture adjusted to 7.6. The mixture was then incubated with carboxypeptidase B, 10 mg, for an additional 3 h; the entire sample was then pressure dialyzed. The dialysate was concentrated and examined by high-voltage paper electrophoresis. After identification of ninhydrin-reactive bands, the material was eluted from the paper, concentrated, and submitted for nuclear magnetic resonance and mass spectral analysis.

Time Course of Hydroxylysine Release from Trypsinized Collagen by Carboxypeptidase B. A sample of each of the three kinds of collagen was treated by the sequential trypsin–carboxypeptidase B method as described above except that aliquots were removed at varying time intervals and carboxypeptidase action was stopped by addition of 2,2'-dipyridyl in a 20-fold excess of inhibitor over enzyme. Each aliquot was pressure dialyzed and analyzed for amino acids. Alkaline and acid hydrolysis for the determination of glycosylation of amino acid residues was carried out as previously described (Spiro, 1967).

Enzyme Specificity and Glycosylated Hydroxylysine. For determination of whether or not trypsin and carboxypeptidase B acting in sequence had been successful in removing glycosylated residues of hydroxylysine, the dialysate was obtained as described above and hydrolyzed in 6 N HCl for 24 h. Control studies consisted of digestion of collagen by trypsin alone and carboxypeptidase B alone. All samples were analyzed for amino acids.

Removal of Protein-Bound Carbohydrate. To focus on the effect of removal of protein-bound carbohydrates in promoting the digestion of collagen by the two proteolytic enzymes, we employed deglycosylation of collagen prior or subsequent to exposure to proteolysis. In order to avoid artifactual cleavages that might occur during chemical hydrolysis, an enzymatic approach was chosen.

Bovine nasal cartilage collagen, 2.50 mg, was added in a 1:2 v/v ratio to the digestion buffer. To this mixutre was added 0.4 mg each of α-glucosidase and β-galactosidase. The solution was allowed to incubate at 25 °C for 24 h. An aliquot was removed for alkaline and acid hydrolysis. The remainder of the sample was adjusted to pH 8.0 and subjected to sequential trypsin and carboxypeptidase B digestion. Samples underwent acid or alkaline hydrolysis and then were analyzed for amino acids.

An identical collagen sample was trypsinized prior to incubation with α-glucosidase and β-galactosidase. A control sample was hydrolyzed in 6 N HCl after treatment with the glycosidases alone.

Results

Kinetics of Digestion of Collagen by Trypsin. The digestion of the three kinds of collagen was complete in all cases within 1 h, and was very rapid during the first 15 min. The net increase in absorption at 570 nm following ninhydrin reaction was markedly less for bovine nasal cartilage collagen compared to the other two collagens, whose maximal values were very similar to each other (Figure 1). Since the sample weight and the molecular weight of each kind of collagen were nearly the same, the difference in the ninhydrin reactivity indicates that the number of new amino groups per molecule, and, hence, the number of cleavages, is less for the bovine nasal collagen than for the other two collagens. The differences in susceptibility to trypsin, however, are not due to differences in basic amino acid composition. The sums of the number of lysine, arginine, and hydroxylysine residues are quite similar: ichthyocol, 84 residues/1000 residues (Piez et al., 1963); calf skin, 79 residues/1000 residues (Eastoe, 1967); bovine nasal collagen, 88 residues/1000 residues (Miller & Lunde, 1973; Miller, 1971).

Demonstration of the Release of Hydroxylysine by Digestion of Trypsinized Collagen by Carboxypeptidase B. An amino acid from the dialysate after proteolysis was found to elute in the position of hydroxylysine in the amino acid analysis. Its identity was further suggested when coinjection

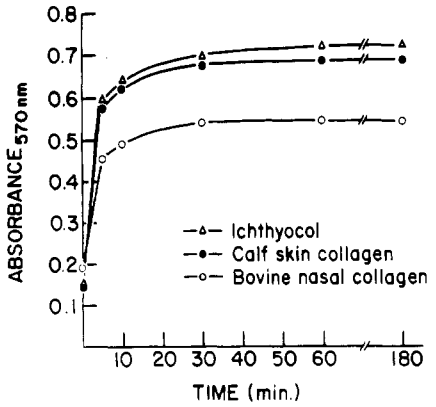

FIGURE 1: Time course of tryptic digestion of denatured collagens as followed by ninhydrin reaction. Three kinds of collagen were denatured and digested with trypsin. Aliquots of the digest were removed, treated with inhibitor, and reacted with ninhydrin.

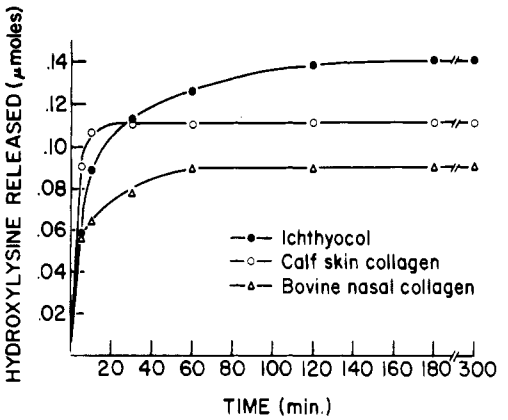

FIGURE 2: Time course of hydroxylysine release from trypsinized collagen by carboxypeptidase B. Trypsinized denatured collagens were digested by carboxypeptidase B. Aliquots were treated with inhibitor, pressure dialyzed, and analyzed for hydroxylysine.

Table I: Determination of Glycosylation by Chemical Methods[a]

denatured collagen	procedure[b] A	procedure[b] B	Hyl glycosylated (% of total Hyl)
ichthyocol	7.0 ± 0.6	5.9 ± 0.3	16
calf skin collagen	8.8 ± 0.5	6.1 ± 0.3	31
bovine nasal cartilage collagen	17 ± 0.8	8.2 ± 0.4	52

[a] Hydroxylysine residues/1000 total residues. [b] Procedure A, hydrolysis in 6 N HCl; B, hydrolysis in 2 N NaOH.

Table II: Release of Hydroxylysine by Enzymatic Digestion of Collagen[a]

denatured collagen	procedure[b] A	procedure[b] B	efficiency of release: % of total Hyl	efficiency of release: % of non-glycosylated Hyl
ichthyocol	3.7 ± 0.2	3.8 ± 0.2	53	63
calf skin collagen	2.6 ± 0.1	2.6 ± 0.1	30	43
bovine nasal cartilage collagen	2.4 ± 0.1	2.4 ± 0.1	16	29

[a] Hydroxylysine residues/1000 total residues. [b] Procedure A, digestion by trypsin followed by addition of trypsin inhibitor, and then treatment with carboxypeptidase B, pressure dialysis, and amino acid analysis. Procedure B, same as above except that the dialysate was hydrolyzed in 6 N HCl prior to amino acid analysis.

of the dialysate together with standard DL-hydroxylysine resulted in a perfect cochromatogram in the amino acid analyzer. As further confirmation, the amino acid was isolated and purified by high-voltage paper electrophoresis. A ^1H nuclear magnetic resonance spectrum showed a multiplet centered at δ 3.9. This is a superimposition of the α-proton absorptions. A doublet at δ 3.2 is due to the ϵ protons, and a multiplet occupying the region from δ 1.4 to δ 2.4 is a superimposition of the β and γ protons. This spectrum was found to be identical with a spectrum of commercially obtained hydroxylysine. A mass spectrum confirmed the amino acid to be hydroxylysine.

Quantitation of Glycosylated Hydroxylysine Residues by Chemical Methods. The chemical methods used in this section for the determination of glycosylation may be summarized as follows: (1) Hydrolysis in 6 N HCl (Table I, method A) destroys peptide bonds and glycosyl links. This procedure gives the total amount of hydroxylysine present in the sample. (2) Hydrolysis in 2 N NaOH (Table I, method B) destroys peptide links but preserves glycosyl bonds. This procedure allows measurement of nonglycosylated hydroxylysine. By these methods, the fraction of glycosylated residues showed marked differences among the three collagens, with ichthyocol being the least glycosylated (16%) followed by calf skin collagen (31%); bovine nasal cartilage collagen was the most glycosylated, with 52% of its hydroxylysine residues bearing carbohydrate. Published values, already referred to, agree well with these figures.

Kinetics of Hydroxylysine Release from Trypsinized Collagen by Carboxypeptidase B. The time course of the release of hydroxylysine by the sequential action of trypsin and carboxypeptidase B is shown in Figure 2. The time for the completion of digestion varied among the three kinds of collagen. Calf skin was the most susceptible; its digestion was complete in 40 min. Bovine nasal collagen required 70 min for complete reaction, and ichthyocol required 3 h. Identical treatment of collagen by either trypsin or carboxypeptidase B alone did not release any hydroxylysine. A 2-fold increase in concentration of both enzymes did not increase the amount of hydroxylysine released. For each collagen, the amount of hydroxylysine release and the fraction of the total hydroxylysine represented by that amount are shown in Table II. The efficiency of hydroxylysine release (ratio of released to total hydroxylysine) was greater for ichthyocol and for calf skin collagen than for bovine nasal cartilage collagen, and in this respect paralleled the tryptic action on the three collagens. The nasal collagen showed a marked resistance to enzymatic release of hydroxylysine despite the greater quantity of hydroxylysine per molecule in that collagen as compared to the other two. The fact that almost 50% of the hydroxylysine residues in the bovine nasal cartilage collagen bear carbohydrate moieties may be responsible for the relatively poor activity of trypsin and/or carboxypeptidase B on this kind of collagen. Prevention of proteolytic cleavage at hydroxylysine residues by carbohydrate modification of such residues may be tested by searching for the release of glycosylated hydroxylysine.

Enzyme Specificity and Glycosylated Hydroxylysine. Acid hydrolysis splits glycosyl links and should cause an increase in free hydroxylysine if glycosylated residues indeed had been liberated into the dialysate by proteolysis. In fact, following this procedure, no increase was found for any of the samples as shown in Table II, indicating that no glycosylated hydroxylysine had been released by the sequential action of trypsin and carboxypeptidase B.

Effect of Deglycosylation of Trypsinized Collagen on the Release of Hydroxylysine by Carboxypeptidase B. Table III, column A, shows that the glycosidases are able to remove a significant amount of carbohydrate from heat-denatured collagen under the conditions described. The number of

Table III: Effects of Glycosidases on Carbohydrate-Bound Hydroxylysine and the Enzymatic Release of Hydroxylysine[a]

procedure[b]	A	B	C	D	E
Hyl released from bovine nasal cartilage collagen	14 ± 0.8	17 ± 0.9	8.5 ± 0.5	14 ± 0.8	2.8 ± 0.2

[a] Hydroxylysine residues/1000 total residues. [b] Procedure A, digestion by α-glucosidase and β-galactosidase followed by hydrolysis in 2 N NaOH and amino acid analysis; B, digestion by α-glucosidase and β-galactosidase followed by hydrolysis in 6 N HCl and amino acid analysis; C, digestion by α-glucosidase and β-galactosidase followed by digestion with trypsin; trypsin inhibitor was subsequently added and followed by treatment with carboxypeptidase B, pressure dialysis, and amino acid analysis; D, digestion with trypsin followed by addition of trypsin inhibitor and subsequent treatment with α-glucosidase and β-galactosidase; the sample was then hydrolyzed in 2 N NaOH and analyzed for amino acids; E, digestion with trypsin followed by addition of trypsin inhibitor and subsequent treatment with α-glucosidase and β-galactosidase; the sample was then digested with carboxypeptidase B, pressure dialyzed, and analyzed for amino acids.

nonglycosylated residues of hydroxylysine increased from 8.2 to 14 residues/1000 residues, a 41% increase. Deglycosylation followed by tryptic digestion and subsequent treatment by carboxypeptidase B (Table III, column C) demonstrated a marked increase in hydroxylysine release, i.e., 8.5 residues/1000 residues compared to the value of 2.4 residues/1000 residues obtained prior to deglycosylation (Table II, method A). That incremental number of hydroxylysine residues susceptible to trypsin–carboxypeptidase B treatment subsequent to glycosidase digestion is nearly identical with the number of residues determined to have been enzymatically deglycosylated. That strongly suggests that proteolytic cleavage at hydroxylysine residues does not occur if those residues are glycosylated.

The effect of substrate size on glycosidase activity is shown in Table III, method D. Large protein molecules (column A) appear to be equally effectively deglycosylated when compared to much smaller tryptic peptides (column D).

The effect of reversal in the order of deglycosylation and trypsinization on the release of hydroxylysine by carboxypeptidase B is shown in Table III, column E. Only 2.8 residues/1000 residues were released by that procedure; this is not significantly different from the value of 2.4 residues/1000 residues obtained without prior deglycosylation (Table III, method A). The conclusion is thus confirmed that glycosylated hydroxylysine sites are not cleaved by trypsin since in that case removal of carbohydrates from the new carboxyl-terminal residues should result in an increase in hydroxylysine released by subsequent treatment with carboxypeptidase B.

The value obtained (Table III, column B), 17 residues/1000 residues, is the same as that for acid hydrolysis without prior deglycosylation (Table I, method A) and demonstrates that the above results are not due to artifacts or contaminants accompanying the glycosidases.

Discussion

The experiments described have demonstrated that both trypsin and carboxypeptidase B are capable of cleaving collagen at certain hydroxylysine residues. However, tryptic cleavage does not occur at these sites if the hydroxylysine residues are glycosylated. Since the side-chain length and positive charge requirements are satisfied, it appears that the steric effect of the δ-OH group is, by itself, insufficient to prevent proper approximation of the substrate and enzyme so that peptide bond cleavage does take place. The recognition site for the positive charge, however, apparently cannot accommodate the addition of a sugar moiety to the δ-carbon atom.

Also of interest is the fact that with increasing extent of glycosylation of a collagen the efficiency of proteolytic release of *nonglycosylated* hydroxylysine residues decreases.

It is to be emphasized that all of the above actions of trypsin, as well as those of carboxypeptidase B, were studied on denatured collagen. It remains true that trypsin does not cleave undenatured collagens except to minor extents. Thus, it may remove small segments of terminal sequences ("telopeptides") on either amino-terminal or carboxyl-terminal α chains wound in the triple-stranded collagen structure, but this does not seem to occur at hydroxylysine residues. Type III, of the various collagens that have been studied, is indeed cleaved in the undenatured form by trypsin, but the scissile bond contains an arginine residue as the basic group satisfying the specificity of trypsin; this was shown by removal of that arginine with carboxypeptidase B (Miller et al., 1976).

The use of α-glucosidase and β-galactosidase provides a convenient method for enzymatic removal of carbohydrates for the facilitation of proteolytic cleavage of collagen and may be helpful in sequence studies of basement-membrane collagens and other glycoproteins.

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