Evaluation of Protein Cross-Linking and Biodegradability by Determination of Tryptophan Released by Pronase Using Reversed-Phase HPLC with Photodiode Array Detection

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A bioanalytical method for evaluating the extent of protein cross-linking was developed. The method is based on reversed-phase HPLC determination of tryptophan released from the cross-linked proteins by Pronase, a nonspecific proteolytic enzyme. The amounts of tryptophan released from un-cross-linked bovine serum albumin (BSA), a nanopeptide, and Trp-Phe in control experiments were consistent with the tryptophan content of the protein and peptides. However, the amount of tryptophan released decreased significantly when BSA was cross-linked extensively by heating at 120 °C in the presence of glucose. The decrease in tryptophan showed a good correlation with the amount of cross-linked BSA that remained insoluble after Pronase digestion, suggesting that tryptophan released by Pronase is a useful measure of the cross-linking and biodegradability of a protein-based biomaterial.

INTRODUCTION

When protein molecules are covalently cross-linked, their solubility is decreased and they become hard in extreme cases. Well-known examples include keratin in wool and hair cross-linked by disulfide bonds. Artificial cross-links can also be introduced to proteins (Uy and Wold, 1976). A potentially useful application of protein cross-linking is in the production of protein-based biodegradable materials with desirable physical properties. Increased physical strength of cross-linked proteins, however, implies that extensive cross-linking would also decrease biodegradability of the proteins. It is hoped that, by carefully controlling the extent of cross-linking, it may be possible to produce biodegradable materials with desirable physical properties from a readily available protein such as whey protein, soy protein, or gelatin.

In developing protein-based biodegradable materials it is important to be able to monitor the extent of protein cross-linking either among the proteins or with other components such as starch. As protein cross-linking increases, the proteins become progressively insoluble and unextractable even with such strong extractants as guanidine hydrochloride or sodium dodecyl sulfate (Kim et al., 1984). Therefore, the extent of initial protein cross-linking can be measured by determining the amount of extractable proteins by the dye-binding method (Bradford, 1976) or by observing changes in the gel electrophoretic profile of the extracted proteins. However, once the proteins are highly cross-linked and all proteins become insoluble, further cross-linking cannot be discerned by the above methods.

In highly cross-linked proteins enzymatic hydrolysis wil yield peptide fragments, called "limit-peptide pigments" in the case of protein–carbonyl cross-linking (Clark and Tannenbaum, 1974), that cannot be hydrolyzed further because the peptide bonds around the cross-linking site will be inaccessible to the hydrolytic enzymes. Therefore, the amount of either the limit peptide material or the fully hydrolyzed material could be used as an index of the degree of cross-linking. Obviously, the limit peptides will be inhomogeneous and difficult to determine except by weighing the high molecular weight materials from a size exclusion separation.

Determination of free amino acids released is more straightforward. Among the amino acids tryptophan is the easiest to determine without derivatization because it has a high extinction coefficient at 280 nm and can be easily detected by direct UV absorption. Also, tryptophan can be easily separated from other amino acids by reversed-phase HPLC. Hence, we investigated the possibility of using the amount of tryptophan released by a nonspecific protease as an index of the degree of protein cross-linking and biodegradability. We first established experimental conditions that would yield quantitative determination of tryptophan in native proteins and peptides. Subsequently, the methodology was used to investigate the cross-linking and degradability of highly cross-linked proteins.

EXPERIMENTAL PROCEDURES

Pronase Digestion. A 0.1-mL aliquot of 1 mg/mL Pronase (*Streptomyces griseus* protease, 4.6 units/mg, Sigma) solution in 20 mM sodium phosphate, pH 7.8, buffer was added to 5 mL of 1 mg/mL solution of bovine serum albumin (BSA) (2% w/w) made in the same buffer. The mixture was shaken gently in a 45 °C water bath for 24 h. Aliquots were taken at predetermined time intervals and injected into the HPLC system. A nanopeptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) and a dipeptide (Trp-Phe) (Sigma) were hydrolyzed similarly.

Alkali Hydrolysis. BSA or the peptides were dissolved in 5 N NaOH solution at 1 mg/mL concentration and sealed in a glass ampule under nitrogen atmosphere. The hydrolysis was carried out in an oven at 110 °C for 16 h.

HPLC. A Waters μBondapak C_{18} column (7.8 × 300 mm) was used at room temperature. The eluant was 80% 20 mM sodium phosphate, pH 7.8, buffer and 20% methanol. The flow rate was 1.5 mL/min. A Waters 990 photodiode array detector (PDA)

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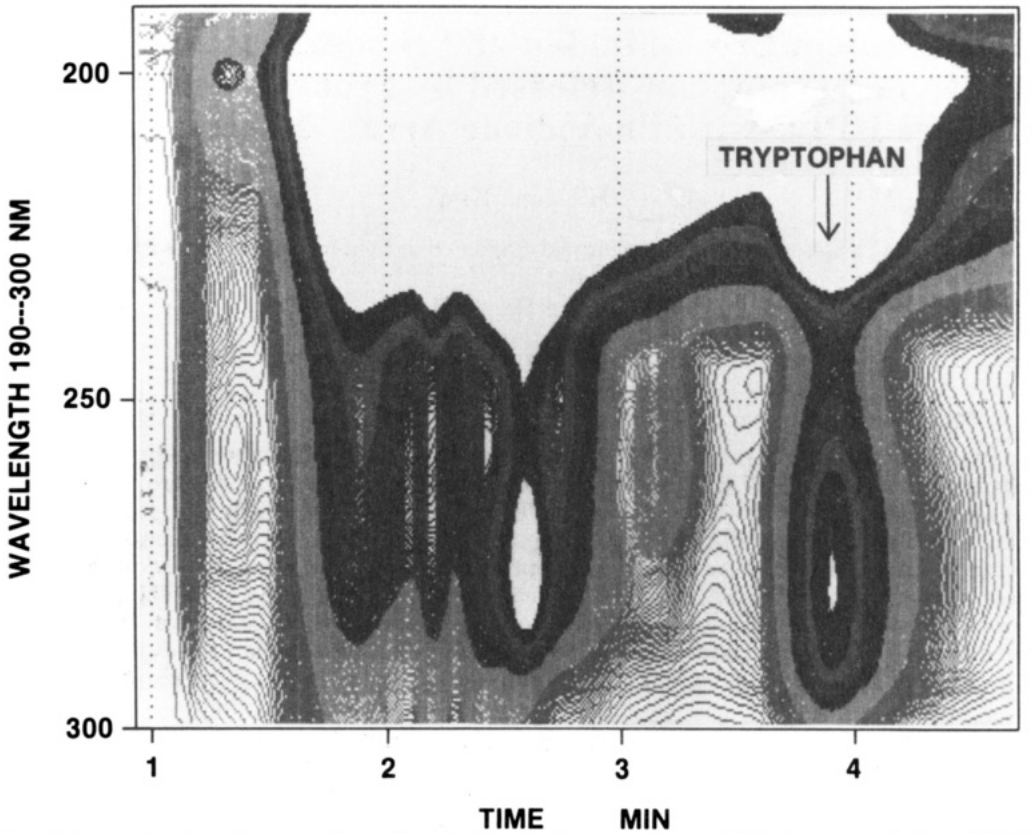

Figure 1. Contour diagram showing the separation of tryptophan in Pronase digest of BSA by reversed-phase HPLC and detection by photodiode array detector.

was used to obtain three-dimensional data (time, wavelength, absorbance). The data were used to obtain a chromatogram at 280 nm or to obtain a UV absorption spectrum for the tryptophan peak. A Schoeffel variable-wavelength UV detector was also used at 280 nm. The peak height of tryptophan observed from the Pronase digest was compared with that from authentic tryptophan at a similar concentration. Column washing between runs was not necessary, and one chromatographic cycle was 10 min.

Protein Cross-Linking. A sealed glass ampule containing 1 mL of aqueous solution of 10% BSA and 4% glucose was heated for 140 min in a laboratory oven at 100 °C (sample 1). Sample 2 was prepared by heating sample 1 for an additional 70 min at 100 °C. Then the temperature of sample 2 was increased to 120 °C over a period of 30 min (sample 3). These heated samples as well as the unheated control were suspended in an additional 8 mL of the pH 7.8 buffer and hydrolyzed by Pronase as described above for 48 h. The suspension was filtered, and the insoluble material, collected on the filter paper, was weighed after drying. The difference between the initial amount of BSA (100 mg) and the insoluble material was taken as the soluble material. The amount of tryptophan in the filtrate was determined by HPLC as described above.

RESULTS AND DISCUSSION

Reversed-Phase HPLC and Photodiode Array Detection. To use tryptophan released by Pronase as an index of the degree of protein cross-linking and biodegradability, we needed to ensure that the method was capable of determining all of the tryptophan in native proteins. Tryptophan is susceptible to oxidation during hydrolysis by 6 N HCl commonly employed in amino acid analysis of proteins and peptides. Therefore, either alkaline hydrolysis (Hugli and Moore, 1972) or acid hydrolysis with added compounds, such as methanesulfonic acid (Simpson et al., 1976) or β-mercaptoethanol (Ng et al., 1987), is used to release tryptophan from the proteins for subsequent chromatographic separation and detection. A possible alternative is to use a combination of proteolytic enzymes, such as pepsin, trypsin, chymotrypsin, carboxypeptidases, and aminopeptidases, to simulate the complete digestion of proteins in the body.

Fortunately, there is a bacterial protease, called Pronase, which is nonspecific and hydrolyzes all peptide bonds in proteins (Nomoto et al., 1960). Pronase has been used for determination of tryptophan in proteins (Spies, 1967; DeVries et al., 1980; Delhaye and Landry, 1992; Garcia and Baxter, 1992).

Tryptophan can be determined easily without derivatization by reversed-phase HPLC and UV detection at 280 nm because only tyrosine and tryptophan have appreciable absorption around 280 nm and tryptophan is eluted later than tyrosine (Delhaye and Landry, 1986). Figure 1 shows a contour diagram obtained by PDA detection from Pronase digest of BSA. An absorption peak with a maximum at 280 nm was observed at a retention time of 3.9 min when the flow rate was 2.0 mL/min. Tryptophan standard was also eluted at 3.9 min. The absorption spectra for tryptophan standard and tryptophan in the Pronase digest of BSA, both obtained by spectral analysis of the 3.9-min peak, were identical and showed a unique structure in the shoulder around 284 nm (Figure 2). The advantage of such spectral analysis of the three-dimensional spectrochromatogram obtained by PDA detection has been demonstrated for tryptophan and tryptophan-containing peptides (Fell et al., 1984; Nyberg et al., 1986). The spectral purity of the tryptophan peak was also confirmed in the separation of partially hydrolyzed proteins such as BSA. Even though peptide fragments containing tryptophan should be present in such incomplete digest, none seemed to interfere with tryptophan. Of course, if the tryptophan-containing peptide is eluted at exactly the same time as tryptophan, the absorption spectrum will be identical unless the peptide contains

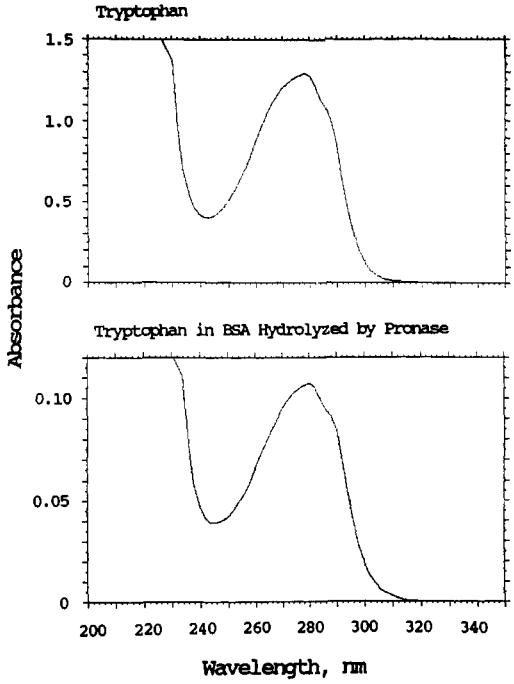

Figure 2. Absorption spectrum of authentic tryptophan and tryptophan in the Pronase digest of BSA obtained by spectral analysis of the three-dimensional spectrochromatogram.

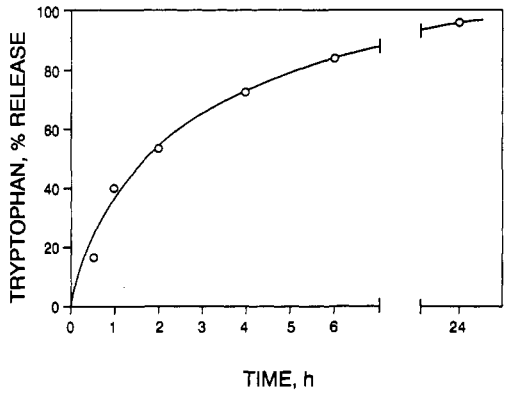

Figure 3. Time course for release of tryptophan from BSA by Pronase at 45 °C.

tyrosine or phenylalanine. However, in the analysis of partial hydrolysate of pure proteins or meats, tryptophan-containing peptides were eluted earlier and no other peak was observed at 280 nm near the tryptophan peak. Therefore, fixed-wavelength detection at 280 nm was routinely used under the above reversed-phase conditions for tryptophan determination.

Time Course of Pronase Digestion. When tryptophan in the BSA–Pronase mixture was determined after several time intervals, results shown in Figure 3 were obtained. The amount of tryptophan released increased gradually at 45 °C and reached 98% of the theoretical value after 24 h. Hydrolysis of the nanopeptide was complete within 1 h at 45 °C. Therefore, we believe that the release of tryptophan from most proteins and peptides should be complete after 24 h at 45 °C unless the proteins are heavily cross-linked. The optimum temperature for Pronase was reported as 40–60 °C (Nomoto and Narahashi, 1959). Nomoto et al. (1960) also showed that casein is almost completely hydrolyzed after 24 h at the optimum temperature.

Determination of Tryptophan. Using the optimized hydrolysis and HPLC conditions, the numbers of tryptophan residues in BSA and the peptides were compared against the theoretical value. As shown in Table 1, the data from alkali hydrolysis and Pronase digestion agreed very well. The values for BSA and for both the nanopeptide and the dipeptide are in agreement with the expected values. The measured values for Trp-Phe were approximately 10% lower than 1, which seems to be indicative of the purity of the material. These data suggest that the Pronase–HPLC method can be used as an alternative technique for determining the tryptophan content in biotechnologically produced proteins and peptides as well as in foods and feeds.

Table 1. Number of Tryptophan Residues in a Protein and Peptides Determined by Reversed-Phase HPLC after Alkali Hydrolysis or Pronase Digestion

protein, peptide	alkali hydrolysis	Pronase digestion	expected value
BSA	1.96	1.95	2
nanopeptide	1.11	0.95	1
Trp-Phe	0.88	0.94	1

Cross-Linked Protein. When BSA was heated in solution extensively with added glucose, the protein turned brown and coagulated. After hydrolyzing for 48 h with Pronase, approximately 1, 9, and 27 mg of insoluble material were obtained in samples 1, 2, and 3, respectively. Some of the material in sample 3 may be due to condensed glucose forming the cross-link. Since it is difficult to estimate the contribution of glucose, we assumed that 27% of the initial 100 mg of BSA became insoluble. This may be an overestimate. Clark and Tannenbaum (1974) investigated glucose-induced cross-linking of insulin using Pronase and termed the high molecular weight brown peptides "limit-peptide pigments". They noted that the brown color of a 7800 molecular weight limit-peptide is due to condensation of up to 31 sugar residues. They also noted that lysine, the N-terminal amino acids, arginine, and probably histidine are the loci for attachment of the condensed sugars to the peptide.

The percentage of tryptophan released by Pronase from the cross-linked BSA was consistently lower than the percentage of BSA rendered soluble by Pronase for the following reason. The un-cross-linked BSA is completely hydrolyzed by Pronase to individual amino acids. There is no insoluble material remaining, and tryptophan is quantitatively determined. When the proteins are cross-linked, the peptide bonds become less accessible to hydrolytic enzymes and some portion of the cross-linked proteins will remain insoluble. Obviously, the amount of proteins insoluble after Pronase digestion will be a useful index of biodegradability. The soluble material from the cross-linked protein will contain, in addition to free amino acids, some peptide fragments that contain tryptophan residue. This will be especially true if the tryptophan residue is near the cross-linking site and, therefore, inaccessible to the enzyme. Such tryptophan residues in the limit-peptide will not be determined as tryptophan. Therefore, the percentage of tryptophan determined relative to the control will be less than the percentage of the soluble material. This situation is depicted in Figure 4. The limit peptide fragments may contain one or more cross-links between peptide backbones from the same or different protein molecules. Also, they may contain no tryptophan or only one or more tryptophan residues. As the extent of cross-linking increases, the discrepancy between the soluble material and determined tryptophan becomes greater as seen in Table 2.

Obviously, either the decrease in the cross-linked material that is solubilized by Pronase or the decrease in tryptophan released by Pronase and determined as

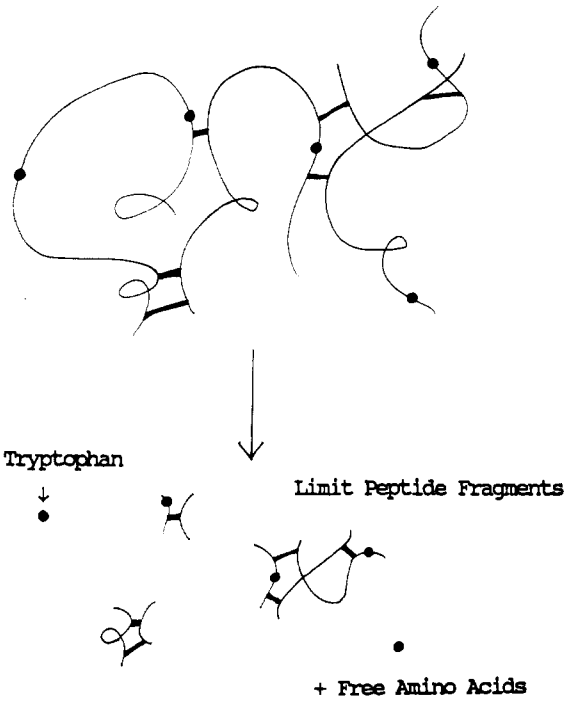

Figure 4. Hypothetical diagram depicting hydrolysis of cross-linked proteins by Pronase releasing free amino acids and limit-peptide fragments, some of which contain tryptophan residues.

Table 2. Relationship between the Amount of Soluble Material and the Amount of Tryptophan Released by Pronase Digestion from Cross-Linked Proteins

heated sample	soluble material, %	released tryptophan, %
control	100	100
sample 1	99	95
sample 2	91	77
sample 3	73	44

tryptophan can be used as an index of the degree of protein cross-linking. However, as results in Table 2 show, released tryptophan is a much more sensitive indicator of the degree of cross-linking because only completely hydrolyzed tryptophan is measured.

When proteins with no tryptophan, such as gelatin, are cross-linked, a different amino acid can be selected that can be easily determined from the Pronase digest. The approach needs to be extended to proteins cross-linked by other mechanisms with or without other components such as starch. Since a bacterial enzyme is used for hydrolysis, the released tryptophan may approximate the true biodegradability of protein-based materials in the natural environment.

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