

MOLECULAR SIZE AND CONFORMATION OF PROTEIN RECOVERED FROM CHROME SHAVINGS*

by

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ABSTRACT

Partially hydrolyzed collagen is one product of the treatment for chrome shavings developed in this laboratory. The value of this process to the tanner depends, in part, on the market value of this proteinaceous material. Modification of the process at any of several points may alter the characteristics of the protein product and thus improve its market potential. The molecular size of the collagen fragments and thermal stability of collagen-like conformations are both factors in determining possible uses for this material. Polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the protein fragments. These fragments were extracted from chrome shavings with MgO alone or in combination with NaOH, KOH or the respective carbonates. Results of these analyses showed a wide dispersion of molecular weights in all of the samples with the heaviest concentrations in the 100-200 kDa and 50-100 kDa fractions. About 25% of the protein was degraded into fragments ≤ 50 kDa and a smaller portion was in the form of aggregates ≥ 200 kDa. When aqueous solutions were held at 5° C for at least 15 hrs, these protein fractions developed 30-60% of the triple helical conformation of a comparable concentration of native collagen as measured by circular dichroism. All fractions extracted with MgO/carbonate mixtures developed more than 50% helical structure while those extracted with MgO/hydroxide mixtures developed 30-50% helix. Melting points for the helix to coil transition were 22° C \pm 2° C for all samples studied.

INTRODUCTION

The tanning industry generates substantial quantities of chromium-containing solid waste in the form of shavings and trimmings. Historically much of this waste has been trucked to land disposal sites. Recent increases in the costs of land disposal and decreases in the number of disposal sites have combined to spur research into alternative treatments for this material¹. Ideally the treatment would produce products of sufficient commercial value to at least offset the cost of the process. In previous reports²⁻⁴ we demonstrated that a recyclable chrome cake and a collagen hydrolysate could be recovered from the treatment of chromium-containing leather waste with an alkaline protease. The conditions of this original process resulted in extensive proteolysis of the collagen. Such a process might be economically feasible if land disposal sites were unavailable or if there were a large increase in disposal costs. The limited commercial value of the resulting protein hydrolysate, however, had a negative impact on the overall economics of the process.

Rose⁵ recently described a process for the extraction of gelatin from chromium-containing leather waste. Although the protein fraction obtained was a high quality gelatin, the chromium fraction was heavily contaminated with proteinaceous material. Our own approach was to develop a two-step process that uses a mild alkaline treatment to extract a minimally hydrolyzed, gelable protein fraction in the first step. In the second step, an alkaline protease is used to produce a recyclable chrome cake and an extensively hydrolyzed protein product⁶.

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** Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

In this report, gelable protein fractions obtained from the first step of our two step process are characterized. The effects of various alkaline salts on polypeptide size and the stability of triple helical conformations are evaluated.

EXPERIMENTAL

Extraction of a Gelable

Protein from Chrome Shavings

Chrome shavings, obtained from commercial tanneries, were suspended in water at 200 g/L. A non-ionic surfactant (Pluronic** 25R2 BASF, Parsippany, NJ) was added at 0.1% relative to the weight of shavings. MgO (6%) or a lower concentration (3% to 5%) MgO in combination with up to 2% NaOH, KOH, Na₂CO₃, or K₂CO₃ was used to increase the alkalinity of the mixture. All chemicals were reagent grade. After the mixture was shaken for six hrs at 70-72° C, the soluble protein fraction was isolated by centrifugation. The supernatant was filtered warm through Whatman #1 filter paper (Whatman, Inc., Clifton, NJ) and lyophilized. For greater detail of the isolation procedure see the accompanying paper⁷.

Estimation of Molecular Weight

Ranges in the Gelable Protein Fractions

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was performed using the Phast-Gel System (Pharmacia, Piscataway, NJ). Individual gels are 43 x 50 mm and are precast with a 13 mm stacking gel (4.5% acrylamide) and a 32 mm separating gel containing a continuous 8-25% polyacrylamide gradient on a polyester backing. The 8-25% gradient is recommended for separation of proteins/peptides in the 6000 to 300,000 Da range. For a calibration standard we used the Bio-Rad (Richmond, CA) broad range SDS-Standard (BRM) that contains a mixture of nine proteins ranging in size from aprotinin at 6500 Da to myosin at 200,000 Da. Samples of lyophilized hydrolysate (0.1 mg), containing about 10% protein were dissolved in 50 μ L of sample buffer (10mM Tris-HCl at pH 8.0 containing 1 mM EDTA, 2.5% SDS, 5% β -mercaptoethanol and 0.01% bromphenol blue) and heated at 100° C for five minutes. An eight slot applicator was used to load 1 μ L of each of three samples and the BRM standards in duplicate. Separations required about 30 min, and were achieved at 250 V, 10.0 mA, and 3.0 W for 70 Vh at 15° C in the Phast System apparatus. Gels were stained with Coumassie Blue (Pharmacia) or silver stain (Bio-Rad) following the manufacturer's directions. Gel patterns were

evaluated using a Bio-Rad Model 620 Video Densitometer with 1D Analyst II/PC software.

Helix to Coil Melting Profiles

The method used by Heidemann and Roth⁸ for the study of structural properties of collagen model peptides was adapted for use with these gelable proteins. Melting-profile experiments were performed by measuring the circular dichroism spectra (CD) of the protein fractions in solution. Spectra were recorded by an AVIV62DS spectrometer (AVIV Associates, Lakewood, NJ) equipped with a thermostated cell holder connected to a programmable circulating water bath. Lyophilized protein fractions were dissolved in 0.1 M NaCl buffered with 0.03 M sodium phosphate at pH 7.0. Protein concentrations were estimated from ultraviolet (uv) spectra, recorded by an AVIV-14 spectrophotometer, and adjusted to 1 mg ml⁻¹ using $\epsilon_{218\text{nm}} = 9.43 \text{ mL mg}^{-1}\text{cm}^{-1}$ as reported by Na and coworkers⁹. The CD spectra from 250 nm to 185 nm were recorded for samples in a demountable 0.02 cm pathlength cell. Because the sample tended to evaporate from the demountable cell when the temperature was increased, a fused quartz 1 mm pathlength cell with teflon cap was used for the melting curves. Melting point experiments were conducted as follows: the sample was placed in a 1-mm cell and refrigerated overnight; the cell was transferred to the CD instrument and a spectrum from 250 nm to 208 nm at 5° C was recorded; single wavelength readings at 225 nm were recorded at one degree intervals from 5° C to 65° C; additional spectra (250 nm to 208 nm) were recorded at 65° C and after the temperature was returned to 5° C. For the melting experiments, the temperature was increased one degree in ten minutes with an additional 6 minute equilibration time before recording a CD reading.

RESULTS AND DISCUSSION

A single triple helical molecule of type I bovine collagen has a calculated molecular weight of 277 kDa. Even mild procedures used to extract a soluble protein fraction (gelatin) from skin or bone result in some degree of hydrolysis of the collagen. The number-average molecular weights (M_n) of 50 to 100 kDa and weight-average molecular weights (M_w) of 100 to 1000 kDa reported for commonly available gelatins suggest that although degradation is common, some chains remain crosslinked⁵. Descriptions of the molecular distribution (MWD) for gelatins are often summarized in terms of four size groups: (1) large

crosslinked aggregates $M > 280$ kDa; (2) large fragments (95-190 kDa); (3) intermediate sized fragments (50-95 kDa); and small fragments $M < 50$ kDa⁵.

Figure 1 shows the results of a densitometer scan of an SDS-PAGE experiment. The standard BRM molecular weight mixture is resolved into nine bands, one for each of the component proteins. Also shown are patterns for the gelable protein fractions isolated in three separate treatments of chrome shavings with 6% MgO and no other alkaline agents. The degree of dispersion of bands is similar in these three protein fractions. The overall molecular weight distributions, however, are not identical. In particular, one of these isolates contains significantly less material in the >200 kDa range than the others. A possible explanation for this variability may be found in the range of pH values (8.7 to 8.9) measured in ten extractions of gelable protein with 6% MgO⁷. The MWD of gelable protein fractions isolated from the treatment of chrome shavings with MgO alone or in combination with sodium or potassium hydroxide or carbonate are summarized in Table I. In general appearance the electrophoretogram of each sample of gelable protein was a nonresolvable dispersion spanning the range from <50 kDa to >200 kDa. The categories used in Table I thus represent an arbitrary scale imposed on the data. A high percentage of very large (>200 kDa) aggregates was found in samples from treatments at high pH (>9). Data on these very large aggregates are unreliable because they often do not move into the separating gel and because their size is beyond the range of the BRM.

For biopolymers in solution, CD gives a particularly sensitive measurement of conformation and has become a favored technique for monitoring changes in conformation as a function of temperature, pH, or a variety of salts. The CD spectrum of native calf skin collagen exhibits a positive peak ($\theta = 6000$ deg-cm² dmole⁻¹) at 221 nm and a negative peak (-45000 deg-cm² dmole⁻¹) at 198 nm⁹. The apparent melting temperature, T_m , defined as the midpoint of the helix-coil transition, is 38° C for native collagen in a buffered aqueous solution¹⁰. As collagen is denatured, the CD spectrum becomes flatter, both positive and negative peaks are diminished in magnitude, and the positions of the maxima are red-shifted. CD spectra recorded at 5° C and at 55° C for a typical gelable protein fraction are shown in Figure 2. Spectra recorded at 5° C after the completion of the melting experiment (not shown) indicate substantial reversibility of the conformational changes, with a sufficiently long re-equilibration period at least 90% recovery was observed.

Figure 3a is a composite picture of the melting curves obtained in this study. Although the maximum helical content of these samples as measured by CD at 5° C varied by 25%, the optical activity of the heat denatured samples (65° C) was much less variable. The midpoint of the helix to coil transition, T_m , was even less variable at $22^\circ \text{C} \pm 2^\circ \text{C}$. Figure 3b shows melting curves for several samples treated with MgO alone. The small offsets for initial and final optical activity may be a function of the various batches of chrome shavings used. In Figure 3c, melting curves for shavings treated with MgO/carbonate mixtures (4% or 5% MgO with 2% or 1% Na or K carbonate), show the greatest amount of helical structure at 5° C and little variation. Treatment with MgO/hydroxide mixtures (Figure 3d) gave a wider dispersion of helical character at 5° C. The maximum helical structure decreased significantly with increases in hydroxide concentration used for the isolation. Protein isolated from a treatment of chrome shavings with MgO/(KOH 1% or 2%) had maximum helical contents of 49% and 33%, respectively. The wider range of NaOH concentrations used for treatment of shavings⁷ (MgO/(NaOH 0.25-2%)) resulted in a range of helical contents from 51% for protein isolated from the 0.25% NaOH treatment to 34% for protein isolated from the 2% NaOH treatment.

CONCLUSIONS

Partially hydrolyzed collagen fractions extracted from chrome shavings treated with MgO ($\leq 6\%$) alone or in combination with Na or K hydroxide (0.25-2%) or Na or K carbonate (1% or 2%) were evaluated in terms of MWD and thermal stability. All of the samples were from the gelable protein category⁶. SDS-PAGE showed that in most cases about 60% of the protein fragments were equally divided between intermediate (50-100 kDa) and large sized fragments (100-200 kDa), about 25% were in the small fragments or peptides (≤ 50 kDa) fraction and the remainder in the ≥ 200 kDa fraction. When kept in solution at 5° C for at least 15 hrs, these protein fractions developed 30-60% of the triple helical conformation of a comparable concentration of native collagen. Melting points, T_m , for the helix to coil transitions were $22^\circ \text{C} \pm 2^\circ \text{C}$ for all samples studied. Those fractions extracted with MgO/carbonate mixtures developed the most helical structure and those extracted with MgO/hydroxide mixtures the least. Although this study covers a relatively narrow pH range (8.4 to 9.4) and was not designed to explore specifically the effect of pH on quality of protein isolates, there appears to be an increase in the formation of large aggregates and a decrease in maximum attainable helical structure with increasing pH.

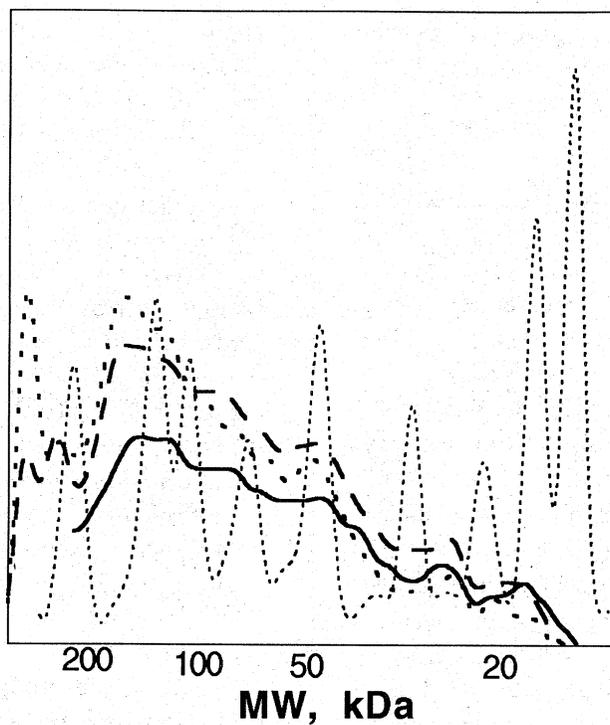


FIGURE 1. — Densitometric analysis of an 8-25% acrylamide Phastgel. The protein standards (-----) range from myosin (200 kDa) to aprotinin (6.5 kDa), the molecular weight scale is logarithmic. Molecular weight distributions from three separate treatments of chrome shavings with 6% MgO are also shown (-----), (-----) and (-----).

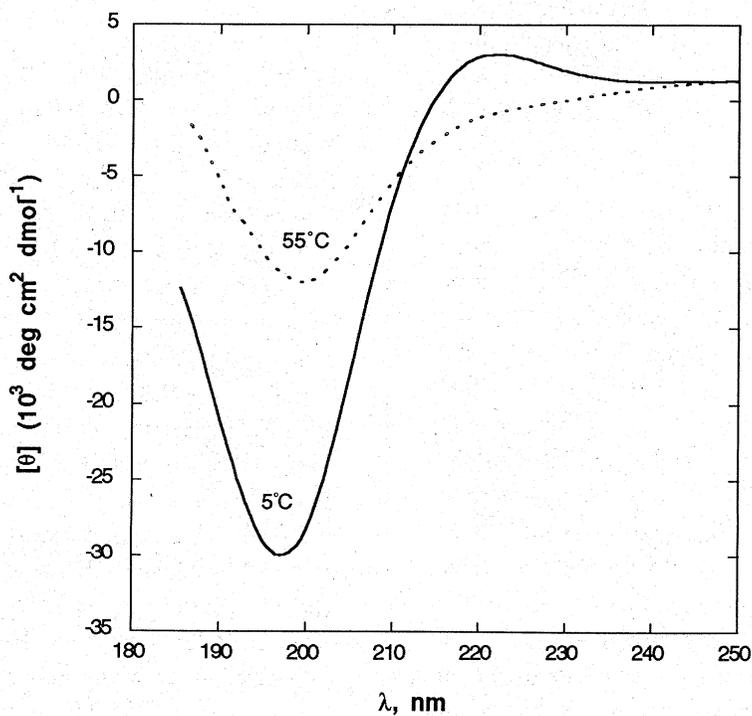


FIGURE 2. — CD spectra of a gelable protein fraction in 0.03 M Na phosphate, 0.1 M NaCl, pH 7.0 at 5°C (-----) and at 55°C (-----). This fraction was extracted from chrome shavings by treatment with 5% MgO, 1% Na₂CO₃ at pH 8.7.

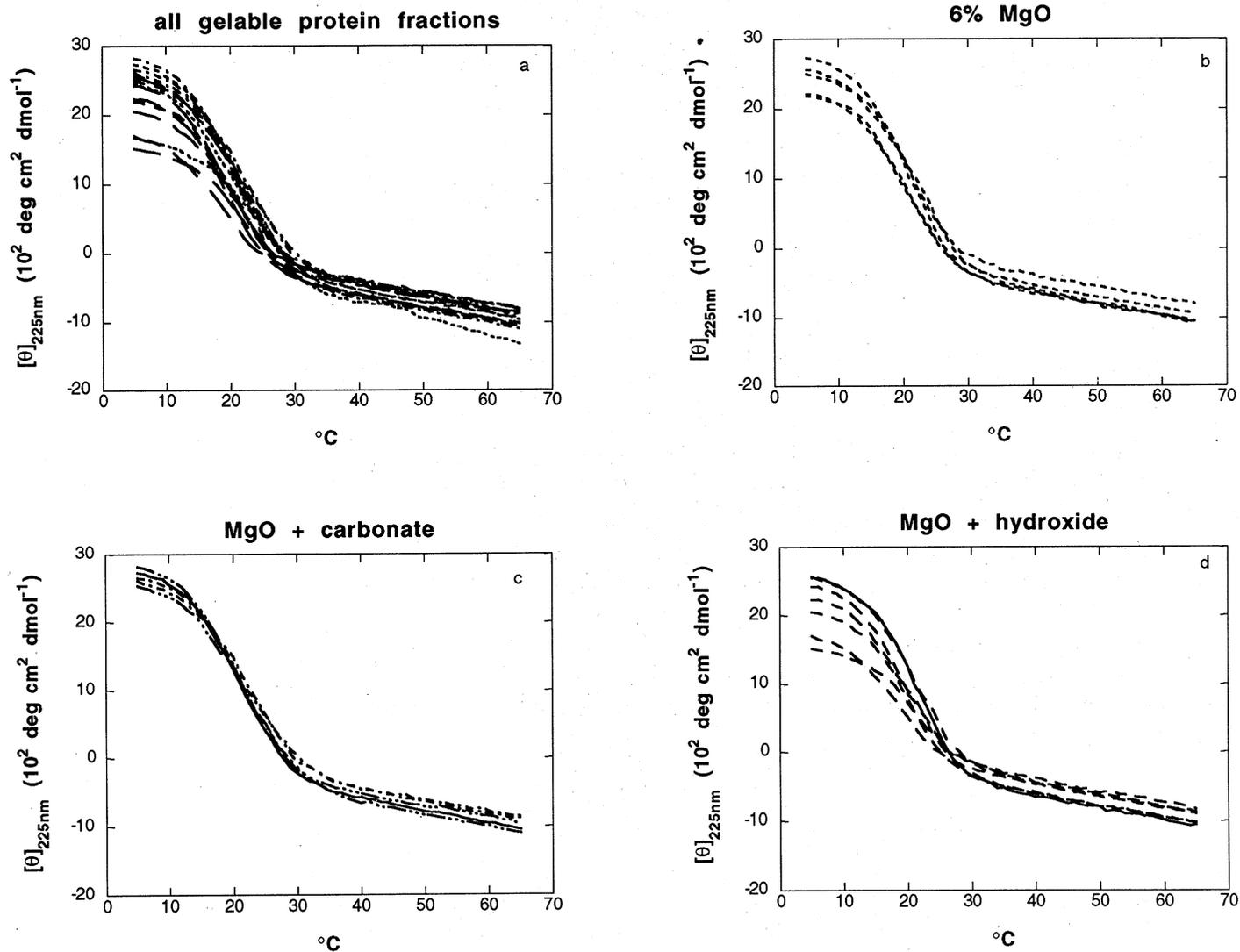


FIGURE 3. — Helix to coil transition curves for gelable protein fractions in 0.03 M Na phosphate, 0.1 M NaCl, pH 7.0.

Panel (a) is a composite of data from all of the extraction treatments used.

Melting curves in Panel (b) represent protein fractions extracted only with 6% MgO.

Melting curves shown in panel (c) were obtained with protein fractions extracted with MgO/carbonate mixtures.

Panel (d) shows melting curves for protein extracted with MgO in combination with NaOH (0.25-2%).

TABLE I
Molecular Weight Distributions of Protein Fragments Extracted From Chrome Shavings

Treatment ¹	# of samples	≥ 200 ²	100-200	50-100	≤ 50
All ³	20	13 ± 15 ⁴	30 ± 8	31 ± 8	26 ± 8
MgO	6	8 ± 8	34 ± 4	31 ± 5	28 ± 6
MgO/carbonate	5	20 ± 24	27 ± 10	32 ± 15	22 ± 12
MgO/NaOH	9	13 ± 13	30 ± 8	30 ± 7	27 ± 5

¹ Treatment refers to the salts used in extraction of protein from chrome shavings; all treatments used either 6% MgO or a lower concentration of MgO in combination with up to 2% Na or K hydroxide or carbonate.

² Protein fragments are assigned to four classes: high molecular weight (≥200 kDa); large (100-200 kDa); intermediate (50-100 kDa) or small (≤50 kDa).

³ Data in this row were obtained by combining the results from six MgO treatments, five MgO/carbonate treatments, and nine MgO/NaOH treatments.

⁴ Values are the % of sample from each treatment in a molecular weight class followed by the standard deviation.

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