Characterization of the Chromium(III)-Crosslinked Collagen–Poly(Butyl Acrylate) Graft Copolymer

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Synopsis

The redox, free radical-initiated graft polymerization of butyl acrylate onto chromium(III)-crosslinked collagen has been investigated previously. In the experiments reported here we set out to determine whether true grafting had occurred and, if so, the molecular weight of the synthetic polymer that was grafted to the collagen. The butyl acrylate grafted product was successively extracted with acetone and ethyl acetate to remove homopolymers. The solvent-extracted product was then subjected to enzymatic degradation, followed by chloroform fractionation, and finally gel permeation chromatography of the chloroform-soluble fraction. Viscosity studies of the final fractionated product indicated that the molecular weight was about 1 million. Viscosity studies of the two homopolymers extracted with acetone and ethyl acetate show that the molecular weights of these homopolymers were somewhat less than that of the isolated polymer–peptide fragment. The fractionated polymer–peptide unit contained 2.83% amino acids, indicating that there are about 288 amino acids in the peptide attached to the polymer molecule. This polymer is composed of approximately 8100 monomer units. The IR spectra confirmed that this fraction is principally poly(butyl acrylate) with amide, OH, and NH absorption bands contributed by the peptide. The isolation and characterization of the polymer–peptide fragment provided proof of graft polymerization onto the collagen molecule.

INTRODUCTION

Recently, Korn et al.[1,2] and Harris et al.[3] have shown that it is possible to graft polymerize vinyl monomers onto collagen using a redox initiation system in the absence of air. In these studies, chromium(III)-crosslinked sheepskin collagen was used as the substrate, and the graft polymerization was found to enhance the properties of the leather made from these skins. Since butyl acrylate appeared to be one of the monomers of choice in producing the best overall leather, the collagen substrate in this study was grafted with butyl acrylate. It has been determined that this gives a leather product containing about 18% of the graft copolymer which is irreversibly bound and unextractable with organic solvents. In the present work, we have enzymatically degraded and solvent fractionated the graft copolymer, thereby obtaining a polymeric product which was mainly poly(butyl acrylate) with a relatively small peptide attached. Viscosity determinations, amino acid analyses, and the IR spectra enabled us to prove that true grafting had occurred, and to determine the approximate molecular weight of the grafted synthetic polymer.

EXPERIMENTAL

Materials

Commercially chrominum(III)-tanned Nigerian sheepskins were graft polymerized with butyl acrylate obtained from Rohm and Haas Company. The butyl acrylate contained 5 ppm of the monomethyl ether of hydroquinone (MEHQ) as an inhibitor and was used as received. Other chemicals were obtained from a number of sources and used as received: (1) Poly(butyl acrylate), secondary standard, Aldrich Chemical Co. (2) Triton X-100 (alkylphenoxy polyethoxy ethanol), Rohm & Haas Co. (3) Potassium persulfate, Fisher Scientific Co. (4) Sodium bisulfite, practical, Eastman Kodak Co. (5) Ethyl acetate, Eastman Kodak Co. (6) (Ethylenedinitrilo)tetraacetic acid disodium salt (EDTA), Eastman Kodak Co. (7) Acetone, nanograde, Mallinckrodt Chemical Works. (8) Chloroform, reagent grade, J. T. Baker Chemical Co. (9) Sephadex LH-20, Pharmacia Fine Chemicals. (10) Pepsin, hog stomach mucosa, 3X cryst., Nutritional Biochemicals Co. (11) Pronase, B grade, 45,000 P.U.K./g, Calbiochem.

Graft Polymerization

Approximately 200-g samples (45 g on a dry weight basis) of the substrate were used. These were placed in ½-gallon Mason jars with 400 ml deionized water, 1.8 g of the emulsifer Triton X-100, and, as the redox initiator system, 1.8 g potassium persulfate and 0.9 g sodium bisulfite (to avoid the release of SO_2, do not contact sodium bisulfite with acids). Sufficient Dry Ice was used to displace all the air, and ambient temperature was used throughout. After the Dry Ice had sublimed, the jars were sealed and then tumbled end-over-end for 30 min. At this point, 45 g *n*-butyl acrylate and additional Dry Ice to ensure a continued oxygen-free atmosphere were added, and the jars capped after allowing the dry ice to sublime. Tumbling was continued for 24 hr, at which time all of the monomer had been consumed as determined by gas-chromatographic analysis of a portion of the aqueous phase. The samples were then removed, washed thoroughly in cold running tap water, and allowed to dry at room temperature. Appropriate care must be taken in handling all monomers because of possible flammability and toxic nature of these chemicals.

Isolation of the Synthetic Graft

As outlined in Figure 1, a preliminary Soxhlet extraction with acetone for 6 hr was performed prior to grinding the grafted leather in a Wiley mill. The ground butyl acrylate-grafted product was additionally extracted for 24-hr periods with 150 ml acetone and with two 150-ml portions of ethyl acetate by refluxing using a Soxhlet extractor under atmospheric conditions.

As shown in Figure 2, 5.00 g of the air-dried solvent-extracted sample and 0.5 g EDTA in 400 ml water were autoclaved at 15 psi for 1 hr to degrade the chrome-tanned collagen. The solution was cooled and the pH adjusted to 2.0 with dilute hydrochloric acid. Pepsin, 0.5 g, was added and the sample was agitated intermittently for 72 hr. The pH was adjusted periodically when necessary. The sample was centrifuged, then filtered through sintered glass, and

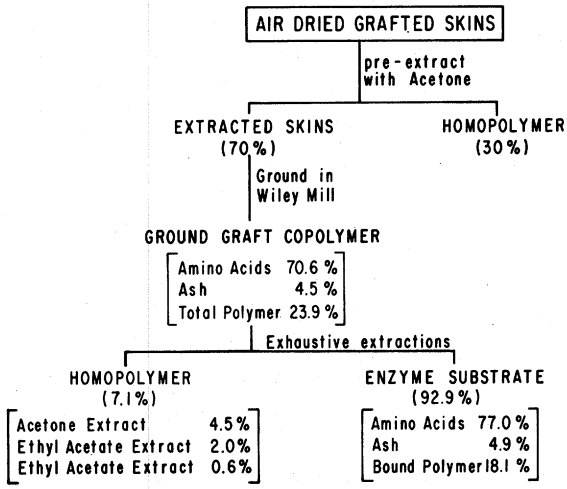

Fig. 1. Properties of initial product.

washed with 0.1*N* HCl, water, 0.1*N* NaOH, and finally water until the filtrate was neutral. The air-dry weight of the residue was 2.3 g. This residue and 0.23 g EDTA in 400 ml water were autoclaved at 15 psi for 1 hr. The solution was cooled to room temperature and the pH adjusted to 8.0 with dilute sodium hydroxide. Pronase, 0.23 g, was added and the enzymatic degradation continued as before for 72 hr. At the end of this period, the residue was filtered and washed with acid, alkali, and water as before. The weight was now 1.02 g.

Gel Permeation Chromatography

As shown in Figure 3, a portion of this residue was solvent fractionated in 50 ml chloroform by stirring intermittently for 72 hr. The sample was centrifuged, and the soluble portion was fractionated on a 2.5 × 75 cm Sephadex LH-20 column using chloroform as the eluant. A reverse flow was used and 8.4-ml fractions were collected in test tubes at the approximate rate of 1 ml/min. The fractions were transferred to 50-ml round-bottom flasks and the chloroform

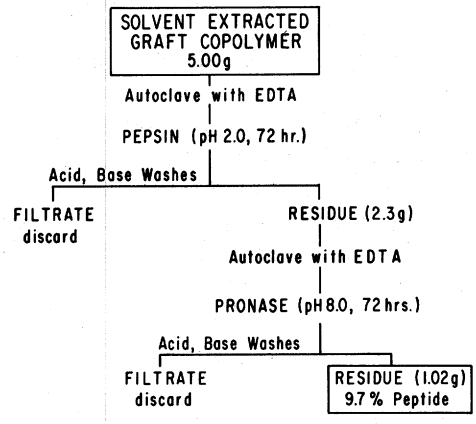

Fig. 2. Enzyme degradation procedure.

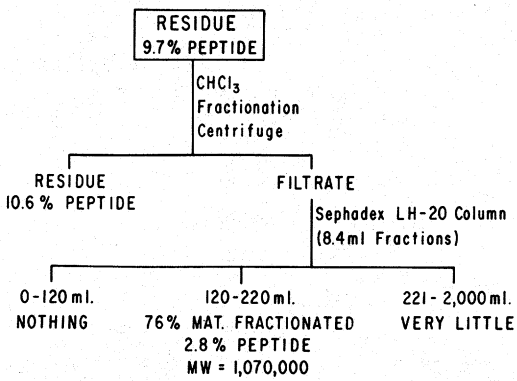

Fig. 3. Fractionation of enzyme-degraded residue.

removed by evaporation under reduced pressure and ambient temperature to obtain any polymer present. Only one component was present, and this material was combined as one fraction.

Viscosity Determinations for Molecular Weight Calculations

The samples were dissolved in acetone and several dilutions made. The specific viscosities were determined at 25°C using a Cannon-Manning semimicro viscometer. The data for the specific viscosities at various concentrations were extrapolated to zero concentration to obtain the intrinsic viscosity. The intrinsic viscosities thus obtained were used in the equation $[\eta] = KM^a$ to determine the molecular weights using literature values[4] for K and a.

Amino Acid Analyses

The samples were prepared for amino acid analysis by hydrolyzing in 6N hydrochloric acid solution for 24 hr under an atmosphere of nitrogen. The polymer residue was filtered off, and the excess hydrogen chloride was removed by repeated evaporations under reduced pressure with the intermittent addition of deionized water. The final residues were then diluted to a known volume with 0.1N hydrochloric acid solution. The analyses were run on a Piez–Morris[5]-type ion exchange column with a continuous-gradient elution buffer. The results were calculated and tabulated on an IBM 1130 computer using programs developed at our laboratory.

Due to the small amount of sample remaining after the molecular weight studies, another method was used for hydrolyzing this sample. An aliquot was transferred to a test tube; and after evaporating the acetone, 2 ml 6N hydrochloric acid was added and the tube sealed under vacuum. It was hydrolyzed in an oven at 100°C for 24 hr and then prepared for the amino acid analyzer as previously described.

IR SPECTRA

The secondary standard, poly(butyl acrylate), and the unknown polymer fractions in acetone were applied dropwise to separate sodium chloride crystals.

Several applications were made to form a film, and the crystals were dried with an infrared lamp between applications. A Perkin–Elmer 137 spectrophotometer was used for the studies.

Nitrogen Analyses

The percent nitrogen was determined by the semimicro Kjeldahl method.

RESULTS AND DISCUSSION

Although some research has been done recently on the graft polymerization of vinyl monomers onto hides and skins, little is known of the actual grafting site on the collagen molecule or the size of the polymer formed. Rao et al.[6,7] have enzymatically hydrolyzed collagen–vinyl graft copolymers and isolated the grafted vinyl polymer side chains. They determined proof of grafting through the detection of amino acid endgroups by using a ninhydrin solution on the isolated grafts. It appeared to us that this product might have contained entrapped proteinaceous material as a result of the isolation methods used, although in all probability it was a true graft copolymer. Subjecting the residue from enzymatic digestions to acid and base washes, dissolution in organic solvents and chromatographic fractionation procedures should eliminate this possibility.

As shown in Figure 1, the collagen–poly(butyl acrylate) graft copolymer was acetone preextracted to remove most of the homopolymer prior to grinding in the Wiley mill, since the presence of homopolymer made the grinding difficult or even impossible and made the Wiley mill overheat. This extraction removed 30% of the original material as homopolymer. Analysis of the ground graft copolymer showed that amino acids accounted for 70.6% of the material (calculated on a molecular weight basis), and the ash content was 4.5%. In addition to the homopolymer extracted prior to grinding the sample, three 24-hr periods of Soxhlet extraction removed only 7.1% homopolymer. The first 24-hr extraction using acetone yielded 4.5% homopolymer with a molecular weight of about 500,000. The second 24-hr extraction with ethyl acetate gave 2.0% homopolymer with a molecular weight of about 800,000. The final extraction with ethyl acetate gave only 0.6% homopolymer, which was insufficient material for a molecular weight determination. These exhaustive extractions were done to avoid contaminating the isolated graft fraction with homopolymer fractions. The extracted graft copolymer containing 18.1% bound polymer was enzyme degraded and then solvent fractionated to obtain the isolated graft fraction. The percent bound polymer was calculated from Kjeldahl nitrogen determinations before and after treatment with butyl acrylate. If we add the amino acids, the ash content, and the total polymer, 99.0% of the starting material is accounted for in the ground graft copolymer. This sample was used for all of the analytical work; however, analysis of the enzyme substrate was obtained by calculation and is shown in Figure 1.

To assure eliminating as much proteinaceous material as possible, several experiments were performed to study the enzyme degradation procedure using conditions other than those reported here. EDTA was found to be helpful in complexing the chromium(III) and preventing its reaction with the enzymes. In one experiment, the solution was discarded after autoclaving with EDTA, and

M.W. OF POLYMER-PEPTIDE UNIT = 1,070,000

1,070,000 X 2.83% AMINO ACIDS = 30,281 M.W. FOR AMINO ACIDS

$$\frac{30,281}{105.67 \text{ (AVG. R.M.W. FOR AMINO ACIDS)}} = 287 \text{ AA PEPTIDE + 1 ON POLYMER}$$

1,070,000 - 30,281 = 1,039,719 M.W. FOR POLYMER

$$\frac{1,039,719}{128.17 \text{ (M.W. OF BUTYL ACRYLATE)}} = 8,112 \text{ MONOMER UNITS}$$

Fig. 4. Composition of graft copolymer.

the same amount of deionized water was added to the residue prior to adding the enzymes. It appeared, however, that more protein was digested in the experiments where the EDTA was present with the enzymes. This is probably due to continued protection of the enzyme from being poisoned by chromium released during protein cleavage. Increasing the temperature to 37°C during the enzyme digestions was not beneficial. However, the dilute acid and dilute alkali washes of the digested residues were necessary for removing some of the entrapped material. The residues were washed exhaustively until a negative ninhydrin test was obtained on the solutions.

After the enzyme digestions, air drying was used to avoid possible problems in subsequent rehydration of a small peptide group attached to a large hydrophobic polymer which might then interfere with the enzyme attack. The air-dried product remaining after the enzyme digestions amounted to 1.02 g, or about 20% of the starting material. Amino acid analysis of the residue after enzyme digestion showed that there was a peptide still bound to the graft, amounting to 9.7% of its weight.

As shown in Figure 3, a portion of the residue was solvent fractionated in chloroform. The insoluble part of the residue (85%) was found to contain 10.6% amino acids on a weight basis as a peptide. This peptide was low in hydroxyproline, proline, glycine, and alanine when compared to the collagen substrate.

In order to carry out fractionation on the Sephadex LH-20 column, it was necessary to apply the sample to the column in a single 50-ml portion of chloroform. Some peak broadening would be expected. The bulk of the sample eluted between 120 and 220 ml; this was combined as one fraction. Although an additional 1780 ml chloroform was run through the column, very little further material eluted. Seventy-six percent of the sample applied to the column was recovered in the one fraction. The molecular weight of this copolymer as determined by viscosity measurements was 1,070,000. This solvent-fractionated product contained 2.83% amino acids on a weight basis as a peptide which was low in hydroxyproline, proline, glycine, alanine, and arginine when compared with collagen.

While we were not able to determine the molecular weight of the chloroform-insoluble enzyme-degraded copolymer fraction, the following considerations lead us to postulate that the poly(butyl acrylate) portion has about the same molecular weight as that of the chloroform-soluble fraction, or about 1,000,000: (1) Poly(butyl acrylate) homopolymers are soluble in chloroform; therefore, the degree of solubility of the copolymers is determined by the size of the polypeptide portion. (2) The enzyme attack should be random; therefore,

the molecular weight distribution of the poly(butyl acrylate) in the soluble and insoluble copolymer fractions should be the same.

If the molecular weight of the polymer–peptide fragment is 1,070,000, then the amino acid portion of this would amount to 30,281 molecular weight units, as shown in Figure 4. On the basis of the amino acids found and the relative amounts of each, the average residue molecular weight was calculated as 105.67. This would indicate that there are 287 amino acids on the peptide chain plus one amino acid that is probably impervious to acid hydrolysis and remains attached to the polymer molecule. Dividing the molecular weight of the polymer by the molecular weight of the monomer indicates that there are about 8112 monomer units in this polymer–peptide fragment.

The IR spectra of the polymer–peptide fragment confirms that it is principally poly(butyl acrylate) when compared with the standard. Amide absorption bands around 1650 cm^{-1} and NH and OH absorption bands around 3200–3500 cm^{-1} are contributed by peptide fragment.

It was also important to determine whether the autoclaving of the grafted collagen alters the homopolymer or graft copolymer in any way that would affect the molecular weight. In another experiment, the preextracted ground graft copolymer was autoclaved and the homopolymer then extracted with acetone. Viscosity determinations showed only a 10% increase in the molecular weight of the homopolymer, indicating that autoclaving has only a negligible effect on the molecular weight. Also, gas-chromatographic analysis of the autoclave liquor indicated there was no hydrolysis of the ester groups of the polymer to butyl alcohol.

In conclusion, these experiments provide evidence for the true graft polymerization of collagen in hides. The sequential enzyme digestions, chloroform solubility, LH-20 chromatography, and IR spectra, taken together, prove that grafting to the collagen molecule has taken place.

It is anticipated that methyl methacrylate graft copolymers will be studied in future experiments, since preliminary experiments show that acid hydrolysis does not appear to attack the ester linkages as it does with the butyl acrylate graft copolymers. These results could then be compared with those obtained using the enzyme degradation procedure. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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