Monitoring Keratin degradation in activated sludge by rapid gel permeation chromotography

Wastewaters from the hide unhairing process comprise a major portion of the effluents discharged from tanneries. These wastes consist predominantly of soluble and insoluble inorganic salts (sulfides and lime for example), keratin protein and its fragments,[1] and melanin. Treatment of such effluents with activated sludge is a possible means of meeting water discharge standards.[2] Research is needed, however, to elucidate the degradation process in order to optimize reactor conditions and design. Parameters such as chemical oxygen demand (COD), biological oxygen demand (BOD), and total organic carbon (TOC), measure only the overall results of the reaction. Chromatography has provided useful information about the intermediate steps in the biological degradation of model amino acid systems.[3]

Recently, new column packing materials, improved solvent delivery systems, and sensitive, low dead-volume detectors have permitted fast analysis of polymer mixtures by gel permeation chromatography (GPC).[4] However, only within the past year have packings become available that are stable over the wide pH range needed to rapidly separate biopolymers. Previously, such separations required many hours. In this report, the potential of rapid GPC for monitoring the biodegradation of keratin-based proteins will be explored.

EXPERIMENTAL METHODS

Chromatographic system. Columns were assembled from 316 stainless steel tubing (0.64 cm OD, 0.41 cm ID) that had been cut to the desired length and thoroughly washed, flushed with acetone, then hexane, and dried. Fritted disks (0.25 μm pore diam) were pressed into holes countersunk into the outlet end of the tubing. Fittings and stainless steel tubing were used throughout the system. Connections between columns in series and to the detector were of capillary tubing (0.16 cm OD) to minimize dead volume. A minipump provided a constant flowing mobile phase. A pressure-limiting switch and gauge placed in the line for safety reasons also provided sufficient pulse dampening. The detector was a high-performance, fixed-wavelength (254 nm) photometer. Sample solutions were injected by syringe into a septum-type port.

Packings. Preliminary experiments with an ethylene glycol dimethacrylate polymer-based support gave useful separations of some protein standards at pH 7. However, at the pH needed to solubilize keratin proteins (pH 7.5), irreproducible chromatograms were obtained, probably because hydrolysis of the support promoted sorption of proteins.

Surface-modified, controlled-porosity glasses were found to have the necessary stability. A column 80 cm long was dry packed by the tap-fill method with Glycophase—G/CPG-550 (*glycerolpropylsibylglass*[5]), 37 to 75 μm particle diam, 550 Å pore diam (Pierce Chemical Company) and coupled with a 1-m column containing Glycophase G/CPG-250 (250 Å pore diam) fitted into the injector.

Mobile phase. The mobile phase (phosphate buffer, pH 7.5) contained 1 percent sodium dodecylsulphate and 0.02 percent sodium azide bacteriostat. Mobile phases were passed through a 0.22 μm pore bacteriological filter before use. The flow rate was 0.5 ml/min (approximately 1 724 kN/m^2 (250 psi) pump pressure) and time for complete elution of components was about 1 hour.

Biodegradation. Simulated tannery effluent consisting of keratin-based proteins, lime and

sodium sulfide were thoroughly mixed with acclimated activated sludge in a batch reactor. Ten-ml aliquots were taken periodically for analysis. After biological filtration, 150 μl of 2 percent sodium azide solution were added to equalize the ultraviolet (UV) absorption of the mobile phase. Two hundred microliters were injected into the chromatograph for each determination.

The keratin-based proteins used in this study were isolated by suspending cattle hair in a lime and sodium sulfide solution, removing the insolubles by filtration, dialyzing the supernatant against phosphate buffer (pH 7.5) for several days to remove nonprotein material, and clarifying the retained material by centrifugation at 20 000 rpm.

RESULTS

As shown in Figure 1, the keratin isolate is a complex mixture with a wide range of molecular weights. Protein standards, chromatographed under the same conditions, are also shown in Figure 1 as a molecular weight reference. Four prominent groups are observed. Peak 1 elutes near the void volume of the column and consists of proteins having apparent molecular weights above 100 000 daltons. Peak 2 contains proteins in the 10 000 to 50 000-dalton range and Peak 3, the smaller proteins or protein fragments of several thousand daltons. Peak 4, elutes near the total volume of the column and consists of low molecular weight materials such as peptides, amino acids, and salts. The molecular weight assignments are approximations because the volume and shape of the protein, which also influence its accessibility to the pores of the support, may be solvent-dependent.[6, 7]

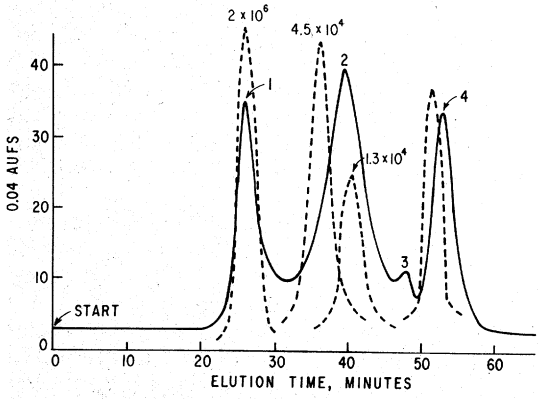

FIGURE 1. Chromatogram of keratin-isolate and of blue-dextran, ovalbumin, ribonuclease, and sodium azide standards. Ordinate is absorption units full-scale.

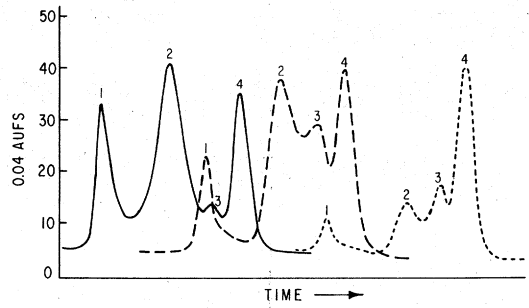

FIGURE 2. Effect of biodegradation on chromatograms of keratin-isolate. Retention times are same as in Figure 1. Aliquots were taken from biomedium at 0 hr (—), 5 hr (- - -), 71 hr (. . . .).

The peak areas do not necessarily reflect the relative amounts of the keratin isolate components. Small amounts of protein containing large numbers of UV-absorbing amino acids could give a large detector response while larger amounts of protein with few chromophores could be almost undetected. However, comparisons of chromatograms obtained on samples taken periodically from the same biomedium are more likely to be valid (Figure 2) comparisons.

Better resolution in the middle range is desirable, but the degree obtained with the system as described is satisfactory, particularly in light of the fast time scale. A computer approach, developed to aid in the interpretation of complex GPC profiles obtained from forage proteins,[8] was applied.

In this approach, peak heights are measured at specified time increments and these data are used to compute the first and second normal moments of the entire chromatogram. The moments measure the mean (central tendency) and polydispersity of the molecular size distribution as determined by gel chromatography and are analogous to, but not identical with, the number and weight average molecular weights.[9] The moments are computed in terms of K_{av}, the column partition coefficient, which is defined in equation 1 and is used because it is independent of column geometry and packing density.[10]

$$K_{av} = \frac{V_i - V_o}{V_t - V_o} \qquad (1)$$

where

V_i is the retention volume of the ith solute molecule,
V_o is the void volume or total exclusion volume,
V_t is the total liquid volume.

Moments quantify a complex distribution and can be used to compare distributions of similar materials. The first moment increased from 0.538 to 0.810 during the biodegradation while the second moments increased from 0.620 to 0.859. Comparison with a calibrating curve of reference proteins (ovalbumin, chymotrypsinogen, ribonuclease) showed that this increase in moments corresponded to a decrease in the apparent molecular weights. The first and second moment apparent molecular weights change from 13 000 and 7 000 at the start of the biodegradation to 2 000 and 1 500 after 75 hours.

The computational method also facilitated comparison of total and individual peak areas. The individual normalized peak areas are shown in Table I. While other mechanisms are possible, the decreases in peaks 1 and 2, the increases in peaks 3 and 4, and the overall decrease in area suggest that in the biodegradation of keratin-based proteins, the larger substrates are decomposed first to lower molecular weight species in addition to the gaseous products. These species are reacting also but at a slower rate.

When total peak area and TOC of the samples periodically withdrawn from the batch reactor are plotted as a function of time (Figure 3), similarly shaped curves are observed, demonstrating that the chromatographic method correlates well with conventional methods for measuring the overall effect of the bio-oxidation. In addition, chromatography is advantageous for monitoring treatment systems because the breakdown or resistance of individual groups of substrates can be observed. Furthermore, fractions can be collected from the chromatograph and the nature of resistant peptides studied in order to improve reactor conditions.

TABLE I. Distribution of peak areas obtained by chromatography of keratin-based proteins.

Biodegradation Time (hr)	Normalized Areas			
	1 (%)	2 (%)	3 (%)	4 (%)
0	22	49	4	25
75	8	23	21	48

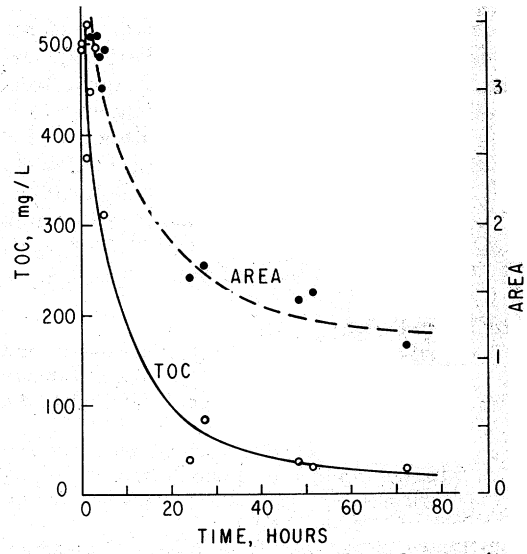

FIGURE 3. Changes of total peak area (arbitrary units) and TOC with time.

Information is obtained more rapidly by chromatography than by standard COD methods which require several hours. In fact, preliminary results in our laboratory, using a new higher pressure pumping system and longer chromatograph columns, indicate that separation time can be reduced to less than 30 minutes. Ambiguities in COD data caused by incomplete oxidation of some compounds using the standard test conditions have been reported,[11] suggesting the need for alternate methods.

Studies are continuing with batch and continuous flow reactors with model and real tannery waste systems to further elucidate the nature of the biodegradation process.

CONCLUSIONS

Gel permeation chromatography can rapidly give important information necessary to evaluate the biodegradation of tannery wastes. Innovations are constantly being made in the field of chromatography and are leading to faster, more efficient analyses. These improved analyses should be even more useful for monitoring wastewater treatment systems.

ACKNOWLEDGMENTS

Credits. The authors thank Joseph E. Cooper, Eastern Regional Research Center (ERRC), for the TOC determinations.

Authors. Robert A. Barford is a Research Chemist at the Eastern Regional Research Center, United States Department of Agriculture, Philadelphia, Pa. Jan Kupec, who was during this work an International Research Exchange Fellow, a Senior Scientist at Faculty of Technology, University, Gottwaldov, Czechoslovakia. M. L. Fishman is a Research Chemist at the Richard B. Russell Center, U. S. Department of Agriculture, Athens, Ga.

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