FRACTIONATION OF OLIGOSACCHARIDES BY POLYACRYLAMIDE GEL FILTRATION

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A method for separating oligomers of *N*-acetyl-D-glucosamine (NAG) from a partial acid hydrolysate of crude powdered chitin by gel filtration with water on columns of Bio-Gel has been developed. Saccharides are detected in the effluent by measurement of reducing power (Park-Johnson test) and/or by absorbance of 220 nm (or shorter wavelength) length. $(NAG)_1$ through $(NAG)_6$ and larger oligomers may be readily prepared and separated in gram quantities by this method. With a given load size and column dimensions, Bio-Gel P-6 (200-400 m) gave better separation than P-2 or P-4 (both 200-400 m). Essentially complete recovery of the applied reducing power was obtained in the effluent. The columns were used repeatedly at room temperature without loss in resolution.

Fractionation on the basis of size by filtration through crosslinked gels has become a powerful and widely used technique for the purification and study of macromolecules. Although many investigators seek purified oligomers (dimer through hexamer) of *N*-acetyl-D-glucosamine (NAG) for use as inhibitors and substrates for muramidase (hen egg white lysozyme, 3.2.1.17), a well characterized "model" enzyme, these chemicals are not commercially available. The availability of more highly crosslinked polyacrylamide gels for gel chromatography encouraged us to apply the technique of gel filtration for the separation of these smaller oligomers of NAG which differ only in polymerization number. This procedure was found to be preferable, in both ease of execution and extent of purification, to the previously published (1) procedure using charcoal-Celite columns. In attempts to improve the gel filtration system, we found Bio-Gel P-6 (200-400 mesh) to be distinctly preferable to P-2 in efficiency of separation of trimer through octamer and in separation of dimer from a brown contaminant.

MATERIALS AND METHODS

Polyacrylamide gels of various degrees of cross-linking and particle size (mesh ranges) were obtained from Bio-Rad Laboratories, Richmond, Calif. The gels were prepared according to the manufacturer's instructions. Although they were used repeatedly at room temperature without loss of resolution, after several days or weeks without use they sometimes required regeneration by batch washing with alkaline acetate (0.1 *M*, pH 9) followed by 0.1 *N* propionic acid and water. The gels were packed into polycarbonate columns or into glass columns pretreated with organochlorosilanes. Washing and elution were accomplished by upward flow from a peristaltic pump at 3 ml/cm²/hr. An aqueous solution of 0.01% ethanol and 0.01% 1,1,1-trichloro-2-methyl-2-propanol (trichlorobutanol), added as antimicrobial agent, was used as eluant. Chitin was obtained from Mann Research Laboratories, New York, N.Y. (Lot S2344), Calbiochem, Los Angeles, Calif. (Lot 900543), and Miles Laboratories, Inc. (Control No. 4201). It was pulverized to fine powder by ball milling in a cold room for about 20 hr. Hydrolysis was carried out in 6 to 11 *N* HCl at 40 C for 25 min to 4 hr. The acid was removed at 40 C on a rotary evaporator at 2 to 3 mm Hg pressure; the residue was resuspended in gel column eluant and centrifuged at room temperature. The clear supernatant was pumped onto the top of the gel column and washed into the gel before the column was inverted for upward flow. The effluent was analyzed for NAG oligomers by a modification of the Park-Johnson ferricyanide reducing power test (2) or by absorbance of 220 nm light. Peaks thus found were pooled and freeze-dried for storage. The components were identified both by their elution relative to NAG standard (Mann Lot R1155) and by their reducing power per unit weight before and after total hydrolysis (1).

RESULTS AND DISCUSSION

Figure 1 depicts an elution pattern of partially hydrolyzed chitin on Bio-Gel P-6. Although baseline separation between peaks is not achieved with this 75 cm column,

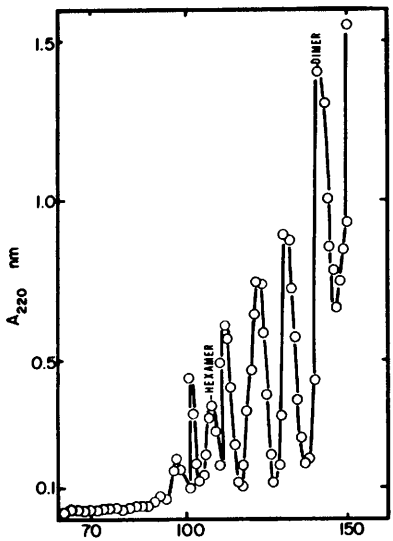

FIGURE 1. Separation on Bio-Gel P-6 (200-400 mesh) of partial hydrolysate of crude chitin in H_2O. Powdered chitin (3 g) was shaken at 40 C in 11 *N* HCl for 1.5 hr and dried at 40 C on a rotary evaporator. The residue which dissolved in 10 ml H_2O was pumped into a 5 x 75 cm column of gel at room temperature and eluted with H_2O containing 0.01% trichlorobutanol and 0.01% ethanol at 60 ml/hr, 10 min/fraction.

one additional passage of the individual freeze-dried pools through P-6 (for NAG trimer or larger) or P-2 (for trimer or dimer) columns will effect essentially complete separation. Better separation of monomer and dimer was obtained with P-2, as is illustrated in Figure 2. The large A_{220} peak eluting from P-6 with NAG monomer and slower was quite heterogeneous; it was largely non-reducing material and contained yellow or light brown pigment. Most of this material eluted much more slowly than did monomer from P-2, al-

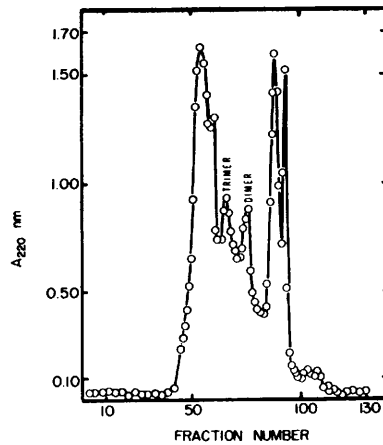

FIGURE 2. Bio-Gel P-2 separation of partial hydrolysate of 1 g crude chitin in 3 ml H_2O. The 3.2 x 75 cm column of P-2 (200-400 mesh) was eluted with H_2O at 30 ml/hr, 10 min/fraction.

though a small amount of color was sometimes eluted with the dimer and monomer from this gel.

Greater than 80% recovery of reducing power (2) applied to the column was obtained routinely. Table 1 contains re-

TABLE 1. *Representative distribution of various oligomer fractions from Bio-Gel P-6 preparative columns (3.2 x 75 cm) chromatography of partially hydrolyzed chitin.*

Fraction[a]	Distribution[b] (%)	Calculated weight (mg)
Monomer	42	43
Dimer	14	28
Trimer	13	39
Tetramer	8.7	35
Pentamer	5.9	30
Hexamer	3.5	21
TOTAL	87	196

[a] Fractions identified by elution position and ratio of reducing power before and after total hydrolysis.

[b] Determined from reducing power of identified oligomer; expressed as percentage of total reducing power applied to column.

covery data from a P-6 run, as measured by reducing power. The recovery of the original weight of chitin in the soluble material

after removing the hydrolyzing acid was much lower if the hydrolysis time was kept short enough to retain significant quantities of hexamer and larger oligomers. We have hydrolyzed (40 C, 2 hr, 11 *N* HCl) the remaining insoluble material as many as three times without appreciably diminishing the quantity or distribution of soluble components in the hydrolysate. Batches of chitin described as "purified" and "highly purified" were not found to yield significantly better hydrolysates than did crude crustacean chitin.

As many as eight A_{220} peaks which elute before NAG monomer have been obtained. The quantity of components eluting before hexamer was sensitive to extent of hydrolysis as controlled by temperature and time of hydrolysis. To obtain desirable yields of soluble material with appreciable quantities of hexamer and larger components, we routinely used 6 *N* HCl at 40 C for 60-80 min in hydrolysis and drying time.

Preliminary investigations have indicated that Bio-Gel P-4 (200-400 m), P-10 (200-400 m), and P-6 (minus 400 m) yield no better, or not as good, separation of the chitin oligosaccharides than does P-2. Although P-6 was better than P-2 in its capacity and its effectiveness of separation of NAG trimer through octamer, even better performance could probably be obtained by eluting the chitin hydrolysate through columns of P-6 *and* P-2 connected in series.

While this investigation was under way, reports on the fractionation of chitin hydrolysates by gel filtration on Sephadex and Bio-Gel P-2 have appeared (3, 4).

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REFERENCES

1. J. A. Rupley, Biochim. Biophys. Acta 83: 245-255 (1964).
2. J. S. Thompson and G. D. Schockman, Anal. Biochem. 22: 260-268 (1968).
3. M. A. Raftery, T. Rand-Meir, F. W. Dahlquist, S. M. Parsons, C. L. Borders, Jr., R. G. Wolcott, W. Berancк, Jr., and L. Jao, Anal. Biochem. 30: 427-435 (1969).
4. C. S. Tsai, Anal. Biochem. 36: 114-122 (1970).