PARTIAL PURIFICATION OF β-N-ACETYLHEXOSAMINIDASE A BY AFFINITY CHROMATOGRAPHY

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Summary

A glycopeptide derived from bovine nasal septum by sequential treatment with trypsin, chymotrypsin, 0.05N HCl in dry methanol (desulfation), testicular hyaluronidase and β-glucuronidase, was coupled to Sepharose-4B in the presence of cyanogen bromide. β-N-Acetylhexosaminidase A was selectively retarded when crude extracts of human skin fibroblasts or liver were applied to the affinity column and was subsequently eluted with 0.1% Triton X-100 in 0.1M citrate-phosphate buffer pH 4.4, providing a simple method for purification.

Robinson and Stirling (1) reported the existence of two electrophoretically distinct bands of β-N-acetylhexosaminidase activity in mammalian tissue and Okada and O'Brien (2) showed that the more rapidly migrating band ("Hex A") was specifically absent from the tissues of patients with Tay-Sachs disease. This report was confirmed, using iso-electric focussing (3), ion-exchange cellulose (4, 5) and polyacrylamide gels (6) to separate the A and B bands. Reports of the conversion of "Hex A" to the slower migrating band of β-N-acetylhexosaminidase ("Hex B") by viral neuraminidases (1, 7) have not been substantiated. Studies with man-Chinese hamster hybrid cells (8) ruled out the possibility that "HexB" was an intermediate in the formation of "Hex A" but initial studies have shown the two forms to be immunologically identical (9, 10). Both these enzymes

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have been purified by conventional methods and from consideration of the accumulating material in the two types of G_{M2}-gangliosidosis, G_{M2} should be a substrate for "Hex A" and both globoside and asialo-G_{M2} should be substrates for "Hex B" (11, 12). The specific inability of Tay-Sachs tissue to catabolize [^{3}H]-G_{M2} was demonstrated by Kolodny et al (13), but purified "Hex A" does not appear to have any significant level of activity toward G_{M2} (14) and "Hex A" is currently defined on the basis of its electrophoretic mobility (2) and thermal instability (6, 15). The first direct evidence for significant levels of catabolic activity of "Hex A" toward a natural substrate was recently obtained by Thompson et al. (16), who showed that fibroblasts from patients with Tay-Sachs disease did not cleave [^{14}C]-GalNAc from a heptasaccharide having the structure: [^{14}C]GalNAc $\beta(1 \rightarrow 4)$[GlcUA $\beta(1 \rightarrow 3)$GalNAc $\beta(1 \rightarrow 4)$]$_3$. Fibroblasts from controls and patients with other storage diseases, including a variety of mucopolysaccharidoses, showed significant levels of activity. The lack of demonstration of mucopolysaccharide accumulation in Tay-Sachs and Sandhoff-Jatzkewitz diseases is therefore puzzling and suggests that alternative metabolic pathways occur. By selective degradation of bovine nasal septum proteoglycan, we obtained a glycopeptide in which GalNAc was the terminal non-reducing sugar and the terminal sequence of sugars was identical to that of the substrate used by Thompson et al. (16). This material was coupled through the peptide moiety to Sepharose-4B to form an affinity column specific for "Hex A".

Materials and Methods

Chondroitin-4-sulfate proteoglycan was isolated from bovine nasal septum (Wilson Labs. Chicago, Ill) by the method of Telser et al. (17).

Following sequential digestion with trypsin and chymotrypsin, the ethanol precipitate was desulfated by treatment with 0.05N methanolic HCl at room temperature for 24 hours, re-precipitated with ethanol and digested with crude testicular hyaluronidase (Sigma Chem. Co.) (0.5%) at 37° for 48 hours (17). Any remaining terminal glucuronic acid residues were removed by digestion with β-glucuronidase (Sigma Chem. Co., St. Louis, Mo.) as described previously (17).

The proteoglycan glycopeptide was coupled to Sepharose-4B (Pharmacia AB) by the conventional procedure (18) using cyanogen bromide and packed into columns (3 x 0.5 cm.) for affinity chromatography. Human liver (10 g.) in 40 ml of 0.1M citrate-phosphate buffer pH 4.4 was sonicated for 1 minute at 4° (Sonifier, Heat Systems, Inc.) and the extract centrifuged at 600xg for 30 min. to remove nuclei and cell debris. This crude cell extract (40 ml.) was assayed for lysosomal hydrolase activity and applied to the affinity column. Following application of the extract, the column was washed with 0.1M citrate-phosphate buffer pH 4.4 (50 ml) until no protein or enzymic activity was detected in concentrated samples of the eluant. The column was then eluted with 0.1% Triton X-100 in the same buffer and 2 ml. aliquots were collected and assayed for enzymic activity.

Lysosomal hydrolases were assayed with synthetic substrates by a modification of the method of Van Hoof (19) as described previously (20), the reaction being stopped by the addition of 3.5% trichloracetic acid as described by Kalke *et al.* (21). Thermal denaturation and starch-gel electrophoresis were carried out according to O'Brien (2, 15). Specific assays for β-*N*-acetylhexosaminidase A ("Hex A") activity were carried out using

TABLE I

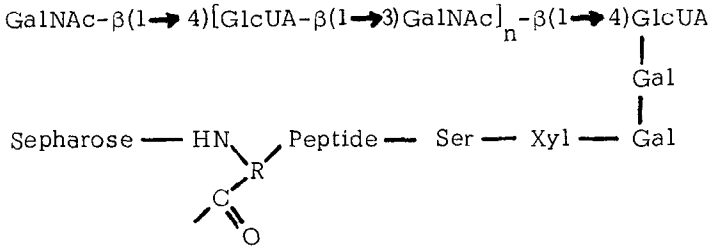
Purification of "Hex A" from normal human skin fibroblasts by affinity chromatography

Lysosomal hydrolase	Crude Extract	Buffer Wash	Triton X-100 eluant
	μmoles substrate cleaved/ mg protein/hr	% of total activity	
β-N-Acetylglucosaminidase	4.45 ± 0.4	100	61
β-Galactosidase	0.85 ± 0.2	90	5
β-Glucuronidase	0.36 ± 0.1	77	< 5
β-Glucosidase	0.43 ± 0.1	113	<10
β-N-Acetylhexosaminidase destroyed by thermal denaturation ("Hex A")	2.15 ± 0.3	3	97

the [^{14}]GalNAc-hexasaccharide as described by Thompson et al. (16).

Results and Discussion

On the basis of previous work on the structure of chondroitin-4-sulfate, we believe the structure of the glycopeptide-Sepharose 4B complex to be:



We attempted to purify the enzyme from normal human skin fibroblasts, since fibroblasts are a convenient source of Tay-Sachs and Sandhoff-Jatzkewitz tissue. This opens up the possibility of future studies on mutant forms

TABLE II

Purification of "Hex A" from normal human liver by affinity chromatography

Lysosomal hydrolase	Crude extract	Buffer Wash	Triton X-100 eluant	
			Fr I	Fr II
	μmoles substrate cleaved/ mg protein/hr	% of total activity		
β-N-Acetylglucosaminidase	2.03 ± 0.3	122	102	33
β-N-Acetylgalactosaminidase	0.75 ± 0.1	106	66	10
β-Galactosidase	0.69 ± 0.1	100	23	1
α-Galactosidase	0.32 ± 0.05	81	< 5	0
β-Glucosidase	0.41 ± 0.05	70	0	0
α-Glucosidase	0.39 ± 0.05	89	< 5	0
β-Glucuronidase	0.38 ± 0.05	103	9	0
β-Fucosidase	0.12 ± 0.02	83	< 5	0
α-Fucosidase	0.22 ± 0.03	64	< 5	0
α-Mannosidase	0.19 ± 0.03	127	< 5	0
Arylsulfatase A	0.21 ± 0.03	58	0	0
Acid Phosphatase	0.31 ± 0.02	57	< 5	0

of "Hex A". Initially, four hydrolases involved in complex carbohydrate catabolism were studied, namely β-N-acetylglucosaminidase, β-D-galactosidase, β-D-glucuronidase and β-D-glucosidase. All four enzymes were detectable in the crude tissue extracts and in the material washed from the column with citrate-phosphate, pH 4.4 buffer, but only β-N-acetylglucosaminidase activity was present in the 0.1% Triton X-100 eluant in more than trace amounts. Approximately 95% of this activity was destroyed by heating at 50° for 2 hours, (Table I) showing it to be virtually all in the "Hex A" form. There was some contamination with β-D-galactosidase but this was less than 5% of the total activity in the wash. Starch -gel

electrophoresis of the non-bound material in the buffer eluant showed "Hex B" to be at least 80% of the total β-N-acetylhexosaminidase activity, whereas analysis of the Triton X-100 eluant showed a single band in the "Hex A" region. To obtain larger amounts of "Hex A" we attempted to purify it from human liver. A wide range of lysosomal enzymes were assayed using extracts of crude human liver, affinity column buffer wash and Triton X-100 eluant. Table II shows that with the possible exception of β-galactosidase, less than 5% of enzymes other than "Hex A" were bound to the column and that very little "Hex B" was bound. The Triton X-100 fraction was assayed for "Hex A" activity using the [^{14}C]-GalNAc-hexasaccharide substrate. A 50 μl aliquot of the first 2 ml Triton X-100 fraction degraded 4% of the substrate when incubated for 16 hrs. at 37°, whereas assay of the wash, which contained large amounts of β-N-acetyl-hexosaminidase activity, showed negligible reactivity toward the natural substrate.

Affinity chromatography has been used extensively in the purification of proteins including lysosomal hydrolases such as Arylsulfatase A (22) β-galactosidase (23) α-galactosidase (24) and β-glucuronidase (25). The affinity column technique described in this paper extends the concept to the purification of an individual isoenzyme of a lysosomal hydrolase. Studies on the relationship between "Hex A", "Hex B" and hexosamine-containing compounds would be greatly facilitated by the availability of a technique for rapid purification of these iso-enzymes. Since Mapes and Sweeley (24) found p-aminophenyl α-D-melibiose to be a useful starting compound in the purification of α-galactosidase, the obvious affinity column for the purification of total β-N-acetylhexosaminidase would be to simply reduce the commercially available p-nitrophenyl β-D-galactosaminide

substrate to the amine and couple it directly to succinylated Sepharose (Affinose-202, Bio-Rad Inc.). Such a column could be of great value since the mixture of "Hex A" and "Hex B" expected, could be further separated into "Hex A" and "Hex B" by the affinity column described in this paper.

Using an affinity column in which a GalNAc-glycopeptide derived from bovine nasal septum was coupled to Sepharose, we have demonstrated its ability to selectively bind "Hex A" when crude extracts of liver or human skin fibroblasts were allowed to percolate through the column. One constant finding, is that the yield of β-N-acetylhexosaminidase always greatly exceeded the yield calculated from assays of activity in the original crude tissue extract. Perhaps this reflects the presence of an inhibitor in the total extract. With the exception of β -galactosidase, which remained as a minor contaminant after a single passage through the affinity column, the "Hex A" was essentially free of other lysosomal hydrolases. The binding of β-D-galactosidase could reflect the presence of small amounts of keratan sulfate in our glycopeptide fraction (this might have galactose at the non-reducing end group and therefore bind β-D-galactosidase) but the absence of β-D-glucuronidase is testimony to the efficiency of the commercial β-D-glucuronidase used in the preparation of glycopeptide. This preliminary communication is further evidence of the usefulness of affinity chromatography in the study of glycosphingolipid metabolism in normals and in patients with mutant enzymes.

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REFERENCES

1. D. Robinson and J. L. Stirling, Biochem. J. 107, 321-327 (1968).

2. S. Okada and J. S. O'Brien, Science 165, 698-700 (1969).

3. K. Sandhoff, FEBS Ltrs. 4, 351-354 (1969).

4. B. Hultberg, Lancet ii, 1195 (1969).

5. E. P. Young, R. B. Ellis, B. D. Lake and A. D. Patrick, FEBS Ltrs. 9, 1-4 (1970).

6. J. Friedland, L. Schneck, A. Saifer, M. Pourfar and B. W. Volk, Clin. Chim. Acta, 28, 397-402 (1970).

7. A. Goldstone, P. Konecny and H. Koenig, FEBS Ltrs. 13, 68-73 (1971).

8. H. van Someren and H. B. van Henegouwen, Humangenetik, 18, 171-174 (1973).

9. S. K. Srivastava and E. Beutler, Biochem. Biophys. Res. Commun. 47, 753-759 (1972).

10. M. Carroll and D. Robinson, Biochem. J. 131, 91-96 (1973).

11. K. Sandhoff, K. Hanzer, W. Wässle and H. Jatzkewitz, J. Neurochem. 18, 2469-2489 (1971).

12. P. D. Snyder, W. Krivit and C. C. Sweeley, J. Lipid Res. 13, 128-136 (1972).

13. E. H. Kolodny, R. O. Brady and B. W. Volk, Biochem. Biophys. Res. Commun. 37, 526-531 (1969).

14. Y. T. Li and S. -C. Li, unpublished results.

15. J. S. O'Brien, S. Okada, M.W. Ho, D. L. Fillerup, M.L. Veath and K. Adams, in Lipid Storage Diseases, eds J. Bernsohn and H. J. Grossman, Academic Press, New York, 1971, p. 225.

16. J. N. Thompson, A. C. Stoolmiller, R. Matalon and A. Dorfman, Science in press (1973, August).

17. A. Telser, H. C. Robinson and A. Dorfman, Arch. Biochem. Biophys. 116, 458-46 (1966).

18. P. Cuatrecasas, J. Biol. Chem., 245, 3059-3065 (1970).

19. F. Van Hoof and H. G. Hers, Europ. J. Biochem., 7, 34-44 (1968).

20. G. Dawson, R. Matalon and A. Dorfman, J. Biol. Chem. 247, 5951-5958 (1972).

21. K. Zielke, M. L. Veath and J. S. O'Brien, J. exp. Med., 136, 197-199 (1972).

22. H. R. Sloan, J. L. Breslow and D. S. Fredrickson, in Sphingolipids, Sphingolipidoses and Allied Disorders, eds. B. W. Volk and S. M. Aronson, Plenum Press, New York, 1972, p. 175.

23. M. R. Villarejo and J. Zabin, Nature New Biol., 242, 50-52 (1973).

24. C. A. Mapes and C. C. Sweeley, J. Biol. Chem. 248, 2461-2470 (1973).

25. R. G. Harris, J. J. M. Rowe, P. S. Stewart and D. C. Williams, FEBS Ltrs., 29, 189-191 (1973).