Carbohydrate Research, 69 (1979) 227–233

ACTION OF 2-ACETAMIDO-2-DEOXY-β-D-HEXOSIDASE FROM *Turbo cornutus* ON PERIODATE-OXIDIZED AND SMITH-DEGRADED, BLOOD-GROUP HLe^b AND Le^a SUBSTANCES FROM HUMAN, OVARIAN-CYST FLUIDS*

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(Received April 28th, 1978; accepted for publication, May 22nd, 1978)

ABSTRACT

Immunochemical and chemical studies were used to monitor and evaluate the structural changes produced by an enzyme from *Turbo cornutus* in periodate-oxidized and Smith-degraded, human blood-group substances from ovarian cysts. After the first step of periodate oxidation and Smith degradation, two blood-group substances, JS (HLe^b) and N-1 (Le^a), were precipitated by mouse-myeloma S117 serum, specific for terminal, nonreducing, β-D-linked 2-acetamido-2-deoxy-D-glucosyl groups, but not by type XIV antipneumococcal horse serum specific for terminal, nonreducing, β-D-linked D-galactosyl groups. An exoglycosidase, 2-acetamido-2-deoxy-β-D-hexosidase (β-*N*-acetylhexosaminidase) from *Turbo cornutus*, split off 2-acetamido-2-deoxy-D-glucose amounting to 22.5 and 20.4% of the total weight of JS and N-1 blood-group substances, respectively. After enzymic digestion, both blood-group substances precipitated with type XIV serum, and did not precipitate with S117 serum. The findings are in agreement with the structure proposed for the water-soluble, blood-group substances [Lloyd and Kabat, *Proc. Natl. Acad. Sci. U.S.A.*, 61 (1968) 1477]. Specific enzymes can be of value in structural studies when used in conjunction with sequential periodate oxidation and Smith degradation.

INTRODUCTION

Our understanding of the relationship between the structures of the water-soluble A, B, H, Le^a, Le^b, and I blood-group determinants and their activities is the result of numerous investigations carried out in different laboratories (for recent reviews, see refs. 1–4). A composite structure for the complex oligosaccharide part

*Immunochemical Studies on Blood Groups, LXVII. Aided by grants BMS-72-02219-AO4 and PCM-76-81029 from the National Science Foundation.

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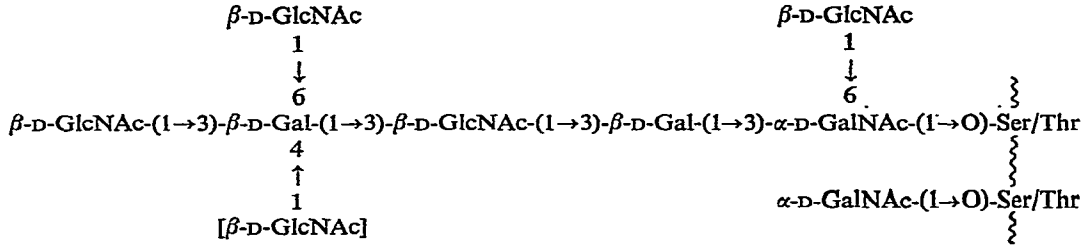

Fig. 1. Proposed composite[6] of the first stage of periodate-oxidized and Smith-degraded blood-group HLe^b, or Le^a substances. The residue in brackets is uncertain.

of the blood-group substances has been proposed by Lloyd *et al.*[5], based on the mechanism of peeling from the reducing end after elimination from serine and threonine by alkaline borohydride, and on the identification of the reduced oligosaccharides. Further support for the model was provided[6] by Smith degradation of HLe^b blood-group substance JS. After one stage of periodate oxidation and Smith degradation, all of the 2-acetamido-2-deoxy-D-glucose (D-GlcNAc) survived, whereas, during the second step, only D-GlcNAc was decomposed. Moreover, quantitative precipitin and precipitin-inhibition assays with a mouse-myeloma protein (S117) specific for terminal, nonreducing, β-D-linked D-GlcNAc groups showed[7] that, as expected from the composite structure proposed for H substance[5,6], only the product from the first and third stages of periodate oxidation and Smith degradation precipitated, whereas the original JS and the material from the second, fourth, and fifth stages did not. In the composite structure proposed for the first stage of periodate oxidation and Smith degradation of an H substance (see Fig. 1), D-GlcNAc occupies all terminal, nonreducing positions; 2 or 3 residues are at the branch point, and another is linked to 2-acetamido-2-deoxy-D-galactose; from the structures of the oligosaccharide determinants[3-6], the same composite structure would result from one stage of periodate oxidation and Smith degradation of an Le^a substance. In the present study, after a first stage of periodate oxidation and Smith degradation, two blood-group substances, namely, JS(HLe^b) (ref. 6) and N-1 (Le^a), were digested with purified 2-acetamido-2-deoxy-β-D-hexosidase from *Turbo cornutus*[8]. Changes in antigenic determinants were monitored by precipitation with specific sera, the S117 plasmocytoma serum from the BALB/c mice[7], and type XIV antipneumococcal horse serum[9], and correlated with analytical data on the release of D-GlcNAc.

EXPERIMENTAL

Materials and methods. — *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside was obtained from Sigma Chemical Co., St. Louis, Missouri. Ion-retardation resin AG-11A8 was from Bio-Rad Laboratories, Richmond, California. 2-Acetamido-2-deoxy-β-D-hexosidase from *Turbo cornutus* was obtained from Seikagaku Kogyo, Co., Ltd., Tokyo, and from Miles Laboratories, Elkhart, Indiana.

Enzyme activity was determined as previously described[8]. Digestion of blood-group substances presubjected to one stage of periodate oxidation and Smith degradation (1st IO_4)[6] was performed in 33mM citric acid–Na_2HPO_4 buffer, pH 4.0, with 0.33M NaCl and 0.02% of sodium azide. The digestion mixture was incubated at 37°, and aliquots were taken at intervals and analyzed for free 2-acetamido-2-deoxy-hexose[10]. The reaction was stopped by placing the digestion mixture in a boiling-water bath for 4 min. As a control, a small amount of blood-group substance without enzyme was simultaneously treated under the same conditions.

Blood-group substances, JS and N-1 1st IO_4, were prepared as described by Lloyd *et al.*[6]; JS after the 2nd, 3rd, 4th, and 5th stages of periodate oxidation and Smith degradation were also used[6,7]. OG 10% 2x was available[11]. Type XIV horse antipneumococcal sera were the 1938 and 1939 bleedings[9] of horse 635. S117 serum came from the BALB/c mice carrying the nineteenth generation of the S117 plasmacytoma[12], and was specific[7] for β-D-linked, terminal D-GlcNAc. Colorimetric analyses for nitrogen and carbohydrate were conducted as already described[10,13,14]. Sugars released by digestion with 2-acetamido-2-deoxy-β-D-hexosidase were identified as their alditol acetates[15–18] by g.l.c. at 200° on ECNSS-M programmed from 160 to 220° at 4°/min, and isothermally at 200°. 2-Acetamido-2-deoxy-D-glucitol and 2-acetamido-2-deoxy-D-galactitol were used as standards.

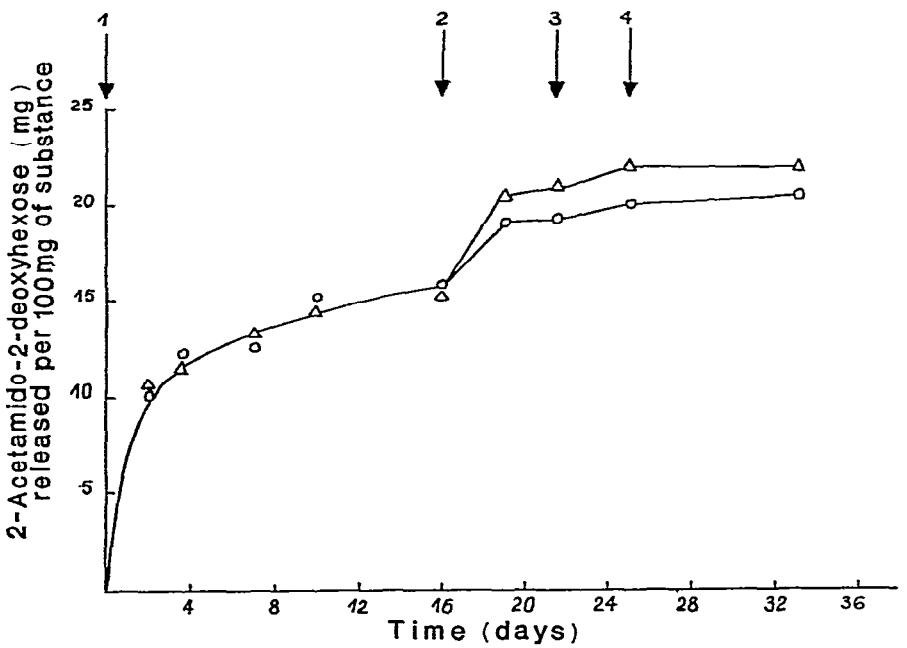

Fig. 2. Time course of digestion of blood-group substances with 2-acetamido-2-deoxy-β-D-hexosidase from *Turbo cornutus*. [△, JS 1st IO_4; ○, N-1 1st IO_4. Arrows mark enzyme addition; to N-1 1st IO_4: 1, 0.3 unit; 2, 2.9 units; 3, 0.8 unit; and 4, 0.7 unit of enzyme; to JS 1st IO_4: 1, 0.3 unit; 2, 1.9 units; 3, 0.8 unit; and 4, 0.7 unit of enzyme.]

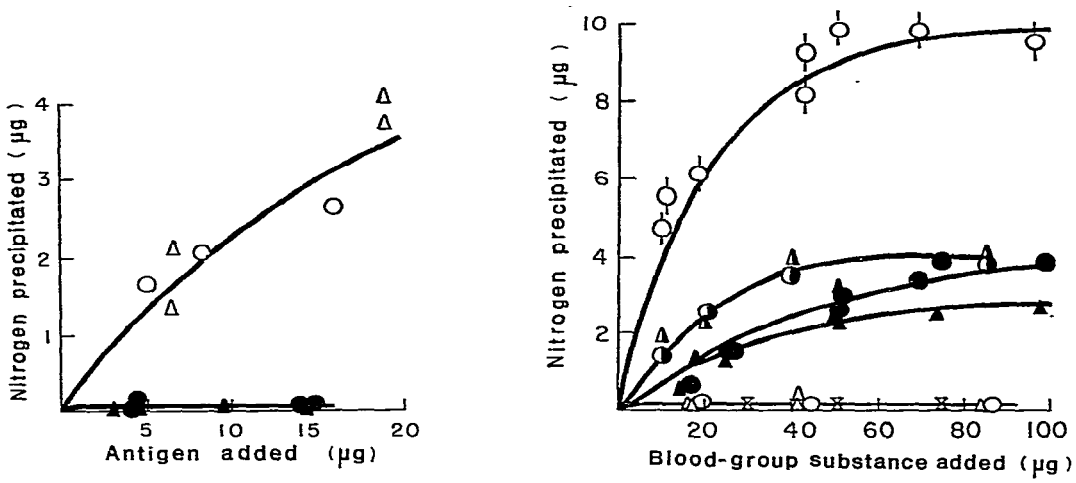

Fig. 3. Quantitative precipitin curves of S117 mouse plasmacytoma serum with blood-group substances, before and after digestion with 2-acetamido-2-deoxy-β-D-hexosidase. [JS 1st IO_4: △, not digested; ▲, digested. N-1 1st IO_4: ○, not digested; ●, digested. The results of both experiments are included.]

Fig. 4. Quantitative precipitin curves of type XIV horse antipneumococcal serum with blood-group substances before and after digestion with enzyme. ϕ, reference blood-group substance was OG 10% 2X. JS 1st IO_4: △, not digested; ▲, digested, △, redigested. N-1 1st IO_4: ○, not digested; ●, digested; ◑, redigested; X JS 2nd IO_4. Results of both experiments are included.]

Digestion of JS 1st IO_4 and N-1 1st IO_4 with 2-acetamido-2-deoxy-β-D-hexosidase from Turbo cornutus. — Experiments were conducted with 1.045 and 1.067 mg of JS 1st IO_4 and N-1 1st IO_4 and 0.05 unit of enzyme, and with 11.5 mg of JS 1st IO_4 and 16.1 mg of N-1 1st IO_4 and ~0.3 unit of enzyme. Mixtures were incubated at 37° in 0.8 and 10 ml, respectively, of 0.33M citric acid–Na_2HPO_4 buffer, pH 4.1, containing 0.33M NaCl and toluene, or 0.02M sodium azide. Samples (50 μl) were periodically withdrawn during 2 to 16 days, and the 2-acetamido-2-deoxyhexose released was measured. The time course of the digestion is shown in Fig. 2. After 10 days, ~16% of the weight of substance was liberated as D-GlcNAc. After 16 days, and again after 21 days, additional enzyme was added to portions of the materials remaining; 10.1 mg of N-1 1st IO_4 was incubated with 2.9 units and then with 0.8 unit of enzyme. This increased the proportion of liberated 2-acetamido-2-deoxyhexose to 20% by weight. The same result was obtained in a small-scale experiment with 1.5 mg of N-1 1st IO_4 by adding 0.07 and then 0.14 unit of enzyme. Successive addition of 1.9 units and 0.8 unit of enzyme to 6.0 mg of JS 1st IO_4 released up to 22.5% of 2-acetamido-2-deoxyhexose. A fourth addition of 0.7 unit of enzyme to both substances no longer liberated 2-acetamido-2-deoxyhexose.

After the first and fourth digestions, the reaction mixtures were placed in boiling water for 4 min, and dialyzed extensively in preboiled, thoroughly washed, cellophane dialysis-tubing or in Amicon UM10 membrane; the retentates were lyophilized, and analytical solutions prepared, and analyzed for various sugars and

amino sugars, and studied immunochemically. The dialyzable portion was examined for sugars by colorimetric reactions and by g.l.c. as their alditol acetates.

Immunochemical properties of the enzyme-digested retentates. — Figs. 3 and 4 show the effects of enzyme digestion on the reactivity of S117 serum and type XIV horse antipneumococcal serum. It is evident that the reactivity with S117 is completely abolished by enzyme treatment, whereas the precipitating ability for type XIV antiserum is increased. A second digestion with fresh enzyme produced a further increase in cross-reactivity with type XIV antiserum (see Fig. 4).

A sample of JS 2nd IO_4 was found not to precipitate with type XIV antiserum (see Fig. 4); this result was surprising, in view of the finding[6,7] that it should possess terminal, nonreducing D-galactose (D-Gal). It was found, however, that addition of JS 2nd IO_4 to type XIV antiserum inhibited precipitation by OG 10% 2x. Failure to precipitate is ascribable to three factors: (*a*) the smaller number of terminal, β-D-Gal groups per molecule, (*b*) the fact that the linkage in the JS 2nd IO_4 is β-D-(1→3) to D-GlcNAc, as compared with β-D-(1→4) in the original, JS blood-group substance, and (*c*) the higher solubility of specific precipitates in cross-reactions with horse antipneumococcal sera[10].

Analytical properties of the dialyzates. — In the small-scale experiments with JS 1st IO_4 and N-1 1st IO_4, 167 μg and 178 μg, 16.0 and 16.7%, and in the large-scale experiment, 1.72 and 2.52 mg, 14.3 and 15.7%, respectively, of the weights used consisted of dialyzable 2-acetamido-2-deoxyhexose. Removal of these quantities of 2-acetamido-2-deoxyhexose abolished the reactivity with S117 serum (see Fig. 3), and resulted in the appearance of cross-reactivity with type XIV antiserum (see Fig. 4). By incubation with additional enzyme, the proportion of 2-acetamido-2-deoxyhexose liberated increased to ~20%.

TABLE I

ANALYTICAL COMPOSITION OF THE NON-DIALYZABLE PORTION OF JS 1ST IO_4 AND N-1 1ST IO_4 AFTER THE FOURTH ADDITION OF 2-ACETAMIDO-2-DEOXY-β-D-HEXOSIDASE (LARGE-SCALE EXPERIMENT)

Sugar	*N-1 1st IO_4*			*JS 1st IO_4*		
	I[a] (%)	*II*[a] (%)	*Difference (I—II)* (%)	*I*[a] (%)	*II*[a] (%)	*Difference (I—II)* (%)
2-Acetamido-2-deoxyhexose	40.0	22.8	17.2	44.0	18.0	26.0
2-Amino-2-deoxyhexose[b]	41.5 (51.0)[c]	27.1 (33.3)	14.4 (17.7)	47.6 (58.5)	24.9 (30.6)	22.7 (27.9)
2-Amino-2-deoxygalactose[d]	12.3 (15.1)	10.4 (12.8)	1.9 (2.3)	(14.8)[c]	11.7 (14.4)	(0.4)

[a]Key: I = original substance; II = enzyme-treated, nondialyzable substance. [b]Values in parentheses are calculated as 2-acetamido-2-deoxyhexose. [c]Value from ref. 6. [d]Values in parentheses are calculated as 2-acetamido-2-deoxygalactose.

The dialyzates were desalted on an ion-retardation resin, and the yield of 2-acetamido-2-deoxyhexose was ~67%. This product was identified as GlcNAc by g.l.c. of its alditol acetate. With N-1 1st IO_4, the only sugar was 2-acetamido-2-deoxyglucitol. With JS 1st IO_4, it comprised 71% of the total peak-area, but three small, additional peaks preceded that of 2-acetamido-2-deoxygalactitol. These were absent from the blank, and have not yet been identified.

Analytical properties of the enzyme-digested retentates. — The non-dialyzable fractions after the first digestion of JS 1st IO_4 and N-1 1st IO_4 weighed 6.0 and 11.7 mg, respectively (the low yield with JS 1st IO_4 resulting from a leak in the dialysis bag). Analytical data are given in Table I. It is clear that the content of 2-acetamido-2-deoxyhexose decreased, and that of 2-amino-2-deoxygalactose remained unchanged. After the first stage of enzyme digestion, 15.2 and 15.8% of free 2-acetamido-2-deoxyhexose were found in the digests of JS 1st IO_4 and N-1 1st IO_4, respectively (see Fig. 2). Second and third additions of enzyme decreased the content of 2-acetamido-2-deoxyhexose in the retentate of N-1 1st IO_4 and JS 1st IO_4 by ~17 and 26%, respectively, values in agreement with the content of 2-acetamido-2-deoxyhexose in the digest, namely, 20.4 and 22.5%, respectively.

DISCUSSION

The release of ~20% by weight of D-GlcNAc by digestion of the first stage of periodate-oxidized and Smith-degraded JS and N-1 blood-group substances with the 2-acetamido-2-deoxy-β-D-hexosidase of *Turbo cornutus* is in general agreement with the composite structure of the blood-group substances proposed from chemical and immunochemical data[3-7]. The disappearance of reactivity with S117 myeloma protein specific for β-D-linked D-GlcNAc, and the appearance of cross-reactivity with type XIV antipneumococcal horse serum, also support the composite structure, and indicate that D-Gal residues are covered by terminal, nonreducing D-GlcNAc groups (see Fig. 1).

That N-1 1st IO_4 and JS 1st IO_4 have the same reactivity per unit weight with S117 myeloma serum, and, after enzyme treatment, cross-react to the same extent with type XIV antiserum, indicates that they have the same, general structure and approximately the same numbers of determinants.

Fig. 1 shows the average molar composition[6], which favored 4 GlcNAc, 2 Gal, and 2 GalNAc; and the GlcNAc in brackets that is linked β-D-(1→4) to the subterminal D-Gal was hypothesized from the isolation of oligosaccharides linked β-D-(1→6) to 3-hexenetetrols. The proposed mechanism of alkaline borohydride cleavage[5] necessitated substitution on C-3 and C-4 of the original, branched galactose. It is clear, however[5], from the isolation of oligosaccharides linked β-D-(1→6) to 3-deoxyhexitols, and also of oligosaccharides linked β-D-(1→3 or 4) to galactitol, that not all such chains are tri-substituted, and that there is substantial heterogeneity in the oligosaccharide side-chains with respect to the number of substituents on the branched D-Gal. Subsequent studies on blood-group substances[17] showed that (*a*) a

portion of the branched D-Gal chain could be directly linked β-D-(1→3) to D-GalNAc-Ser/Thr, thus bringing the branch closer to the polypeptide backbone, and (*b*) chains are present in which the branched D-Gal is substituted by L-Fuc linked α-L-(1→2). Very short chains, and chains having terminal, nonreducing α-D-GlcNAc groups, were also isolated[19,20]. These findings clearly complicate the calculation of an overall composition. Most of the carbohydrate chains in intact, blood-group substances are larger than the oligosaccharides thus far isolated, and they may be very heterogeneous.

The 20% of D-GlcNAc split off by the enzyme would account for two-thirds of the D-GlcNAc present in JS 1st IO_4. As may be seen from Fig. 1, this would agree quite closely with removal of three D-GlcNAc residues if there were six 2-acetamido-2-2-deoxyhexose units. It is, however, doubtful whether the analyses are sufficiently precise to discriminate between removal of three of six, and four of seven, residues.

Enzyme treatment of periodate-oxidized and Smith-degraded HLe^b or Le^a substances would effect a substantial further diminution in the complexity of the oligosaccharide part, and could be a useful step, prior to alkaline borohydride degradation, for facilitating structural studies.

REFERENCES

1 W. M. WATKINS, in A. GOTTSCHALK (Ed.), *Glycoproteins*, 2nd edn., Elsevier, Amsterdam, 1972, Chapter 7, section 5, pp. 830–891.
2 S. HAKOMORI AND A. KOBATA, in M. SELA (Ed.), *The Antigens*, Vol. 2, Academic Press, New York, 1974, pp. 79–140.
3 E. A. KABAT, *Structural Concepts in Immunology and Immunochemistry*, 2nd edn., Holt, Rinehart, and Winston, New York, 1976.
4 K. O. LLOYD, *MTP Int. Rev. Sci., Org. Chem. Ser. Two*, 7 (1976) 251–281.
5 K. O. LLOYD, E. A. KABAT, AND E. LICERIO, *Biochemistry*, 7 (1968) 2976–2990.
6 K. O. LLOYD AND E. A. KABAT, *Proc. Natl. Acad. Sci. U.S.A.*, 61 (1968) 1470–1477.
7 G. VICARI, A. SHER, M. COHN, AND E. A. KABAT, *Immunochemistry*, 7 (1970) 829–838.
8 T. MURAMATSU, *J. Biochem. (Tokyo)*, 64 (1968) 521–531.
9 E. A. KABAT, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 181–186.
10 E. A. KABAT, *Kabat and Mayer's Experimental Immunochemistry*, 2nd edn., C. C. Thomas, Springfield, Ill., 1961, pp. 504–507.
11 G. VICARI AND E. A. KABAT, *J. Immunol.*, 102 (1969) 821–825.
12 M. COHN, *Cold Spring Harbor Symp. Quant. Biol.*, 32 (1967) 211–221.
13 G. SCHIFFMAN, E. A. KABAT, AND W. THOMPSON, *Biochemistry*, 3 (1964) 113–120.
14 F. LUDOWIEG AND J. D. BENMAMAN, *Anal. Biochem.*, 19 (1967) 80–88.
15 M. E. ETZLER, B. ANDERSON, S. BEYCHOK, F. GRUEZO, K. O. LLOYD, N. G. RICHARDSON, AND E. A. KABAT, *Arch. Biochem. Biophys.*, 141 (1970) 588–601.
16 B. ANDERSON, E. A. KABAT, S. BEYCHOK, AND F. GRUEZO, *Arch. Biochem. Biophys.*, 145 (1971) 490–504.
17 L. ROVIS, B. ANDERSON, E. A. KABAT, F. GRUEZO, AND J. LIAO, *Biochemistry*, 12 (1973) 5340–5354.
18 G. R. SPIRO, *Methods Enzymol.*, 28 (1972) 3–43.
19 W. NEWMAN AND E. A. KABAT, *Arch. Biochem. Biophys.*, 172 (1976) 535–550.
20 F. MAISONROUGE-MCAULIFFE AND E. A. KABAT, *Arch. Biochem. Biophys.*, 175 (1976) 90–113.