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Abstract

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STRUCTURE OF BLOOD GROUP SUBSTANCES

PROTEOLYSIS OF GROUP SUBSTANCE A

Among the numerous carbohydrate-protein compounds, or glycopeptides, that perform essential functions in the organism, the so-called blood group substances are of particular importance. These complex biopolymers, consisting of monosaccharide and amino-acid residues and having molecular weights reaching several million, determine the group affiliation of the organism and play a highly responsible role in the manifestation of immunity.

Although the number of works devoted to the study of blood group substances is large ([1]), there are still no more or less definite ideas about the structure of these most important mixed biopolymers. The available data, especially those obtained very recently ([2]), make it possible to express certain limited judgments only about the structure of the terminal carbohydrate residues of the biopolymer, but leave completely open even such a central question as the nature of the bond between the carbohydrate and peptide parts of the biopolymer. The suggestions that blood group substances contain an N-glycosidic ([3]) and an O-glycosidic bond ([4]) have remained purely hypothetical; moreover, the fact, published back in 1955 in a brief communication, of the isolation from a group substance of a fragment with a simple ether bond between a sugar and an amino acid ([5]) appears in itself improbable.

We have begun a systematic investigation aimed at studying the structure of blood group substances and, first of all, the type of bond between the carbohydrate and peptide components and the general architectonics of these biopolymers. One of the primary tasks in solving the problem posed is to find a route for destruction of the biopolymer that would make it possible to cleave the carbohydrate and protein parts from one another, so that the fragments obtained and the linkages between them could then be studied.

The investigation was carried out on blood group substance A, isolated from the mucous membrane of pig stomachs essentially according to the method of Kabat ([6])*.

In order to liberate the carbohydrate part of the polymer, which constitutes its main portion, from the peptide chains, we undertook a search for an enzyme

capable of cleaving off the peptide part in the form of amino acids or small fragments. Such a proteolytic enzyme was successfully isolated from the Asian influenza virus, strain A-2 "Krasnodar" 101-59.

For the isolation of the enzyme, allantoic fluid was used, prepared by infecting 10-day-old chick embryos. This fluid was freed from erythrocytes and other insoluble particles by centrifugation (2000 rpm; 30 min). The resulting supernatant, containing virus, was decanted, and the viral particles were collected by centrifugation (45,000 rpm; 2 hours). This sediment was resuspended twice in 0.005 M phosphate buffer, pH 7.0, and centrifuged again. The resuspended sediment, in order to remove aggregated particles (if present), was subjected to

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* Experimental data concerning the isolation, individualization, and study of the monomeric composition of the biopolymer under investigation are being published by us separately in the journal *Izvestiya AN SSSR, Seriya Khimicheskaya.*

centrifugation at 700-800 rpm for 30 min. To the supernatant was added twice-recrystallized trypsin in the amount of 1 mg per 20-30 ml of allantoic fluid. After incubation at 37° for 16-18 hr, the preparation was dialyzed for 72-96 hr against 200 volumes of distilled water at 4°. After dialysis, the aqueous solution was centrifuged (45,000 rpm; 2 hr) to remove destroyed virus particles and aggregated materials.

The enzyme was isolated from the solution by precipitation with acetone. The fraction precipitating in the interval of saturation of the aqueous solution with acetone 0.35-0.5 was separated by centrifugation and dissolved in the minimum amount of water. The undissolved portion was removed by centrifugation, and the solution was lyophilized.

The enzyme obtained was homogeneous electrophoretically and chromatographically (electrophoresis in veronal buffer 0.025 M; pH 8.6, 800 V; in acetate buffer pH 5.7, 800 V; in pyridine buffer, pH 4.2, 900 V; chromatography in buffer solutions pH 6.5-7 and chromatography in conventional systems).

It possesses neither glycosidase activity (tests with maltose, cellobiose, lactose, sucrose) nor esterase activity (tests with O-aminoacyl derivatives of glucose and with phenylalanine esters).

The enzyme has a sufficiently high proteolytic activity (proteolytic coefficient $C' = 123$) and broad substrate specificity. We did not carry out special enzymological studies of the enzyme; it was established only that the optimum of its activity at 36-38° lies within the range pH 6.35-7.1.

Proteolysis of group substance A was carried out by incubating it in phosphate buffer at pH 7.0 and 37° for 48 hr, with addition of 1-5% enzyme relative to the weight of the biopolymer. To evaluate the overall picture of proteolysis, the

mixture obtained after incubation was dialyzed against distilled water. Chromatographic analysis of the diffusate showed the presence of a broad set of amino acids formed as a result of proteolysis of the peptide chains of the biopolymer and autolysis of the enzyme. Free monosaccharides were absent from the diffusate. However, after acid hydrolysis of the diffusate (0.5 *N* HCl; 8 hr), fucose and galactose were detected chromatographically (system: pyridine–butanol–benzene–water (3 : 5 : 1 : 3)). Amino sugars were practically absent. Evidently, the indicated monosaccharides were present in the diffusate in the form of small oligosaccharides or were bound to amino-acid residues and were released only after acid hydrolysis.

Preparative separation of the complex mixture of products of proteolysis of group substance A proved most convenient and effective with Sephadex. The use of dialysis for the same purposes is less convenient and gives less distinct results. Separation was carried out on medium Sephadex G-25, on a 380 × 18 mm column, collecting fractions of 5 ml each and monitoring them by periodate oxidation and staining with ninhydrin. The results of the separation are presented in Fig. 1. As is evident from the graph, the high-molecular-weight residue obtained upon proteolysis was eluted as a single sharp peak, which indicated its homogeneity. The amount of this high-molecular-weight residue was about 50 wt. % of the original biopolymer. After elution of the residue, with some interruption, lower-molecular-weight degradation products began to elute. For an approximate assessment of changes in the molecular weight of the polymer after proteolysis, the intrinsic viscosity of the high-molecular-weight fragment was determined (0.39% solution in water at 34°). The value obtained, η_{ud}, was 0.145, which, in comparison with η_{ud} of the original biopolymer, equal to 0.286, indicates a sufficiently high molecular weight of the fragment obtained.

This important fragmentation product of group substance A was then subjected to complete hydrolysis (0.5 *N* HCl; 8 hr) with subsequent determi-

composition of its constituent monosaccharides (by the anthrone method ([7])) and amino sugars (by the modified Elson–Morgan method ([8])).

The results obtained showed that the substance contains fucose and galactose, the percentage content of which is close to their content in the original biopolymer, and amino sugars in an amount considerably exceeding their content in the original biopolymer. If one takes into account that the amino sugars were also practically absent from the diffusate and, consequently, were practically not split off during proteolysis, it is clear that a larger amount of amino-sugar residues entering into the biopolymer remains, after proteolysis, in its high-molecular-weight fragment. In contrast, galactose and fucose are partially split off during proteolysis.

Fig. 1. Fractionation of the high-molecular-weight products of proteolysis on Sephadex G-25. Along the ordinate is plotted the amount of periodate consumed for oxidation.

Upon hydrolysis with 6 *N* HCl (100–105°; 16–18 h), practically only one amino

Fig. 1. Fractionation of high-molecular-weight products of proteolysis on Sephadex G-25. Along the ordinate is plotted the amount of periodate consumed for oxidation.

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acid was found in the hydrolysate, identified as aspartic acid and quantitatively liberated during acid hydrolysis.

The hydrolysate was subjected to thin-layer chromatography on silica gel together with authentic samples in the systems phenol—water (75 : 25), *n*-propanol —ammonia (70 : 30), *n*-butanol—acetic acid—water (60 : 20 : 20), ethanol—ammonia (77 : 23); to electrophoresis (900 V; pH 4.2; 1–1.5 h) and to electrophoresis followed by chromatography on paper in the systems butanol—acetic acid—water (4 : 5 : 1) and butanol—pyridine—water (3 : 2 : 1.5). In all cases the amino acid split off from the residue fully corresponds, in R_f value and spot color, to authentic samples of aspartic acid. It is practically the only amino acid found in the hydrolysate; only when very high concentrations of the hydrolysate are applied to the electropherogram can traces of other amino acids be observed, the amount of which is negligible in comparison with aspartic acid and should not be taken into account in assessing the general structure of the biopolymer.

These data are very significant and indicate that aspartic acid is directly bound to the polysaccharide chain of the isolated fragment and, apparently, serves as a linking unit between the peptide and polysaccharide parts of blood group substance A.

To confirm this proposition, the obtained high-molecular-weight fragment was subjected to dinitrophenylation by the Sanger and Le Jette method, followed by complete acid hydrolysis (6 *N* HCl, 100–105°, 16–18 h). Electrophoresis (900 V; pH 4.2; 1 h) and chromatography on paper (isoamyl alcohol saturated with 1% acetic acid) showed the presence in the hydrolysate only of DNP-aspartic acid, which was identified with an authentic sample; a complete absence of any other DNP-amino acids was noted, and only insignificant traces of free aspartic acid, arising probably because of not quite complete dinitrophenylation. The latter was also confirmed by the fact that, upon the action of aminopeptidase on the high-molecular-weight fragment, no splitting off of free amino acids was observed. These data show that indeed all the aspartic acid present in the fragment is directly bound to the polysaccharide part, and is not linked in the form of peptide chains and thus actually serves as a linking unit between the polysaccharide and peptide parts of the blood group substance.

A. Moreover, comparison of the content of aspartic acid in the high-molecular-weight fragment (0.5%) with its total content in the original group substance (0.95%) shows that a considerable amount of it is located in such points of

linkage between the peptide chains and the polysaccharide portion.

Furthermore, if one takes into account that the high-molecular-weight fragment itself has a sufficiently high molecular weight (see above) and, consequently, contains several aspartic acid residues, all of which must be located at the points of linkage between the carbohydrate and peptide portions, then this should indicate a branched structure of the biopolymer.

The material presented in this article shows that proteolysis of blood group substance A opens a path toward a very important fragmentation of this highly complex biopolymer.

Further study of the high-molecular-weight fragment and of the smaller products of proteolysis, currently being carried out, will make it possible to draw important conclusions about the general architectonics of the biopolymer, about the structure of its individual regions, and about the type of linkage between these regions.

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Note: Figure translations are in progress. See original paper for figures.

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