CLEAVAGE OF THE PEPTIDE CHAIN OF BLOOD-GROUP SUBSTANCES AT THE HYDROXYAMINO ACID LINKS

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The development of convenient methods for the selective cleavage of the polymer at definite links or bonds is a necessary condition for the study of the structure of a biopolymer. For the selective cleavage of proteins and glycoproteins it is highly desirable to introduce reactive enamine groupings into the peptide chains. The methods of cleaving these groupings are well developed at the present time with model compounds [1]. A possibility of introducing such groupings into the peptide chain is the chemical modification of the residues of cysteine and the hydroxyamino acids serine and threonine directed to the introduction of substituents into the thiol and hydroxy groups. The latter facilitate β-elimination taking place under the action of alkali and leading to the formation of an unsaturated amino acid (α-aminoacrylic or α-aminocrotonic acid) in the peptide chain.

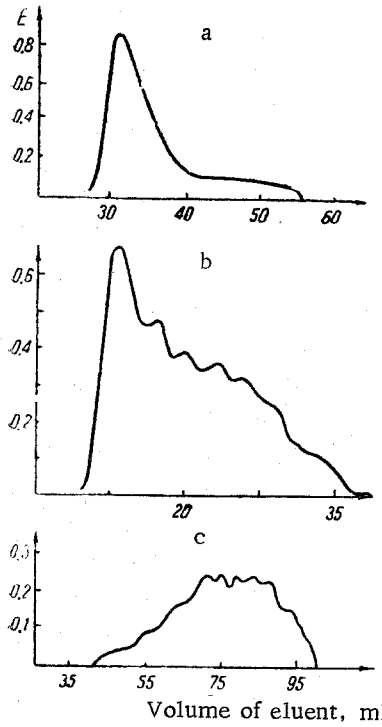


Fig. 1. Gel filtration of the products obtained after the treatment of fragment B with bromine and alkali at pH 11 (30 min, 20° C). a) Sephadex G-25 (1.2 × 75 cm); b) Sephadex G-50 (1.2 × 25 cm); c) Sepahdex G-100 (1.2 × 70 cm).

In some cases, the modification of cysteine gives good results [2]. However, in the case of the hydroxyamino acids the performance of the necessary modification and the elimination itself still encounter considerable difficulties in the investigation of proteins. In the glycoproteins, in which the hydroxyamino acids act as nodes for joining the carbohydrate and peptide components [3–6] the hydroxy groups of serine and threonine are involved in an O-glycosidic bond with oligosaccharides. These oligosaccharides are themselves substituents facilitating β-elimination in the hydroxyamino acid radicals.

We have used the selected cleavage of the peptide chain at residues of hydroxyamino acids in the study of the structure of the blood-group substance A + H from the mucous membranes of porcine stomachs (BGS). The presence of O-glycosidic bonds with serine and threonine has been established in this substance [5, 6].

Experimental

The isolation and characteristics of BGS and the methods of the gel filtration and analysis of the fractions have been given previously [5, 7].

For the cleavage of the peptide chain of BGS we took the high-molecular-weight fragments obtained after the treatment of BGS with 0.1 N caustic soda (fragment A), with 0.1 N caustic soda containing 1% of $NaBH_4$ at 20° C (fragment B) or with 0.05 M Na_2CO_3 at 100° C (fragment C) under the conditions described previously [5], and freed from low-molecular-weight substances by dialysis or gel filtration on a column of Sephadex G-50.

Determination of histidine in the chromatographic fractions. The content of histidine (free and bound in the peptide chain) was determined by the reaction with diazotized 5-amino-1-H-tetrazole [8]. With cooling (0° C), a solution of 70 mg of 5-amino-1-H-tetrazole in 2 ml of 1.6 N hydrochloric acid was diazotized with a solution of 60 mg of $NaNO_2$ in 1 ml of water (to give solution 1). To an aliquot part of a chromatographic fraction (0.5–1 ml) were added 1 ml of 4% aqueous $NaHCO_3$ solution and 0.1 ml of solution 1 brought to approximately pH 5 by the addition of 5 N caustic soda. Then the mixture was left for 20 min at room temperature, water was added to bring the volume to 3 ml, and the optical density was measured at 480 mμ.

Alkaline degradation of fragments A, B, and C after treatment with bromine. Bromine water was added in drops to a solution of 90 mg of the polymer (fragment B) in 3 ml of 0.1 N CH_3COOH until a permanent coloration appeared, and then the excess of bromine was immediately destroyed by the dropwise addition of 0.02 M $Na_2S_2O_3$. The resulting

solution was brought to pH 11 by the dropwise addition of 2 N caustic soda (during this process, the viscosity of the solution fell immediately). After 30 min, the solution was acidified with CH_3COOH and divided into three equal parts which were subjected to gel filtration on columns of Sephadex G-25, G-50, and G-100, respectively (Fig. 1). The cleavage of 35 mg of fragment A and 28 mg of fragment C was carried out similarly (Figs. 2 and 3).

Acid degradation of fragment A. A 30-mg sample of the polymer was hydrolyzed for 3 hr at 100° C in 1.5 ml of dilute hydrochloric acid (pH 1.5) or 0.4 N hydrochloric acid, and then the mixture was cooled, neutralized, and subjected to gel filtration on a column of Sephadex G-25 and G-100 (Figs. 4, 5).

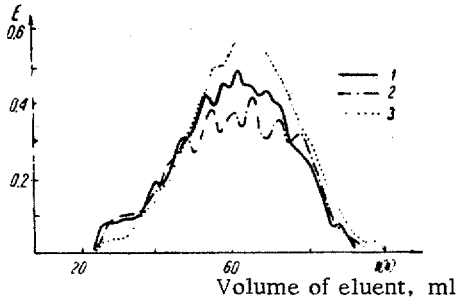


Fig. 2. Gel filtration of the products obtained after the treatment of fragment A with bromine and alkali (conditions as for Fig. 1), Sephadex G-100, (1.2 × 70 cm). 1) Optical density in the reaction with anthrone in 65% sulfuric acid; 2) optical density in the Elson-Morgan reaction; 3) optical density in the Dime reaction.

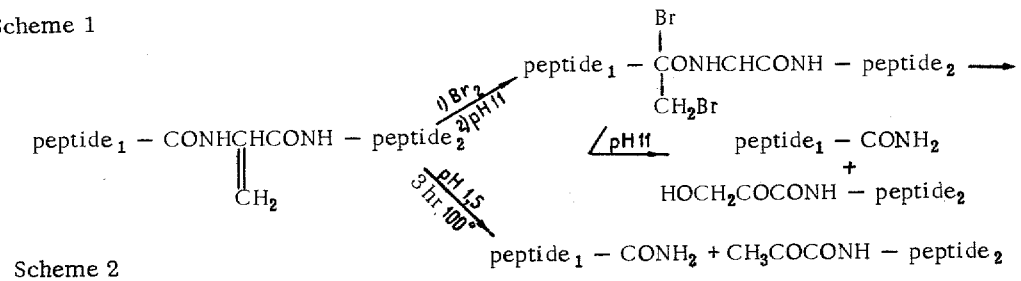
The methods of biopolymer degradation used up to the present time in the study of the structure of BGS have permitted a fairly selective cleavage of only the carbohydrate component or the connecting link between the carbohydrate and peptide components – the glycosidic bond of serine and threonine. These methods include, in the first place, mild acid hydrolysis, cleavage under the action of alkali and glycosidase [5, 9–12]. The methods mentioned have made it possible to establish the presence of an O-glycosidic carbon-peptide bond in the polymer and to obtain valuable information on the structure of the oligosaccharide fractions as well as some information on the over-all structure of the biopolymer. A fundamental gap in this information is due to the absence of a selective method for the degradation of the peptide chain alone, since BGSs are stable to the action of protease, which is generally used for this purpose. At the same time, the cleavage of the peptide chain at the bonds of any amino acids may assist in the determination of the position of the peptide fraction in the molecule of BGS and its dimensions, i.e., it would solve the main question of the structural design of the biopolymer.

In the present paper we describe a cleavage of the peptide part of BGS based on the appearance in the peptide chain under the action of alkaline reagents of the biopolymer of residues of α-aminoacrylic and α-aminocrotonic acids, which arise when the oligosaccharide residues are removed from serine and threonine by a β-elimination mechanism [5].

This reaction can be carried out under the mildest conditions after the addition of bromine in a weakly acid medium to such a peptide. When the solution is subsequently made alkaline to pH 10.8, the decomposition of model peptides takes place completely in 10 min at room temperature. This decomposition also takes place fairly completely without bromine treatment under conditions of mild acid hydrolysis (pH 2.2, 100° C, 1 hr).

We have made use of these results to study the degradation of the high-molecular-weight fragment formed after the alkaline treatment of BGS under various conditions. A change in the conditions of alkali treatment enabled fragments to be obtained with different contents of unsaturated α-amino acids and pictures to be drawn up of their cleavage at the enamine groupings. This decomposition was carried out in two ways:

Scheme 1



Scheme 2

The results of the degradations carried out are given in the form of the results of the analytical gel filtration of the products obtained, which makes it possible to compare the molecular weights and compositions of the fragments.

The results given show, in the first place, the nature of the change in the fractions of the concentrations of the carbohydrate components of BGS – fucose, galactose, and hexosamines. It is also very important to estimate the total content of all amino acids in each fraction, but this involves great difficulties since the general methods of determin-

ing amino acids (reaction with ninhydrin and TNBS,* and the formation of copper complexes) are based on reactions of the amino group, so that the considerable amounts of aminosugars contained in BGS interefere with this determination. However, if it is considered to a first approximation that the amino acids in the peptide part of BGS, which has a very high molecular weight, are distributed more or less uniformly, i.e., a large number of individual amino acids can scarcely accumulate at any one part of the molecule, then the values for the content of any single amino acid can be used as a rough guide to the content of amino acids in the chromatographic fractions. For this purpose we have used a sensitive method of determining histidine which enables both free histidine and histidine forming part of a peptide chain to be detected [8]. By means of this method it is possible to follow the change in the content of histidine in the chromatographic fractions even though its amount in BGS is comparatively low (about 0.6%).

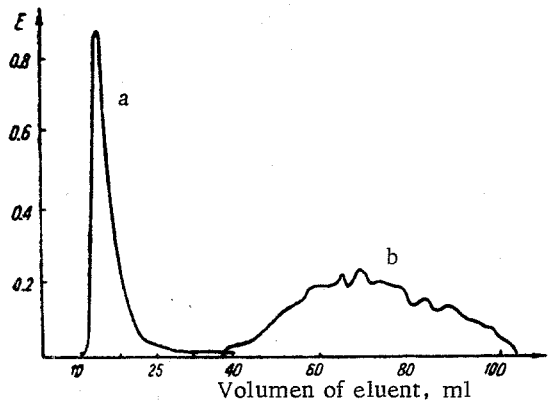


Fig. 3. Gel filtration of the products obtained after the treatment of fragment C with bromine and alkali (conditions the same as for Fig. 1). a) Sephadex G-50 (1.2 × 25 cm); b) Sephadex G-100 (1.2 × 70 cm).

It can be seen from Figs. 1-3 that when the peptide fraction is cleaved by scheme 1, no accumulation of a considerable amount of low-molecular-weight fragments similar to those obtained in alkaline degradation [5] takes place. Consequently, under the same conditions of this selective degradation, the oligosaccharide chains and their links with the peptide fraction do not undergo cleavage. Likewise, the cleavage of the peptide chain takes place specificially at the enamine residues. As control experiments have shown, this is confirmed by the fact that BGS which has not been subjected to treatment with alkali undergoes only very slight degradation under the same conditions.

Sephadex G-25 (Fig. 1a) retains only a small part of the products and the bulk of them issues with the front; the majority of the fragments formed in the degradation have high molecular weights (more than 5000). Some of these fragments are not retained by Sephadex G-50, either (Fig. 1b).

As gel filtration on Sephadex G-100 shows (Figs. 1b and 2), the polymer undergoes cleavage at various parts of the peptide chain, fragments of different molecular weights being obtained; however, no sharp separation into individual fractions occurs.

On comparing the results of the degradation of fragments A, B, and C, it can be seen that the nature of the alkaline treatment of the BGS affects only the amount of reactive residues entering the peptide chain and does not affect the basic course of the decomposition. Thus, fragment C, containing a somewhat smaller amount of unsaturated linkages, decomposes basically in the same way as fragment A (cf. Fig. 3a and 1b), but the fragments formed pass through Sephadex G-50 almost completely and, therefore, possess fairly high molecular weights.

By comparing the results of the gel filtration of the products of decomposition at the enamine groupings under various conditions it is possible to discover a fact which we consider to be very important.

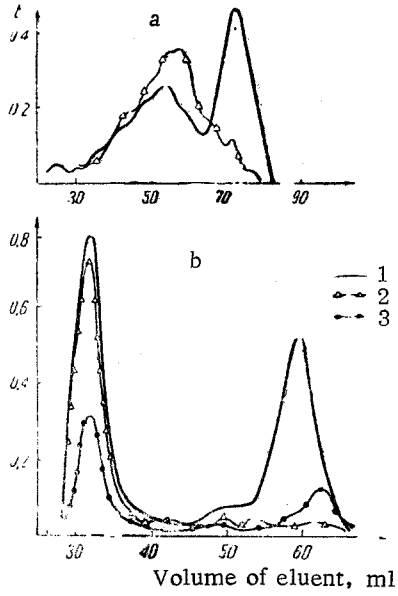


Fig. 4. Gel filtration of the products obtained by the acid treatment of fragment A (dilute HCl, pH 1.5, 3 hr, 100°C). a) Sephadex G-100 (1.2 × 60 cm); b) Sephadex G-25 (1.2 × 75 cm). 1) Optical density in the reaction with anthrone in 65% sulfuric acid; 2) optical density in the reaction with 5-amino-1-H-tetrazole; 3) optical density in the reaction with TNBS.

The products of the degradation of fragment A (cf. Fig. 2) have approximately the same carbohydrate composition, i.e., no fragments differing considerably in their content of any monosaccharide appear. At the same time (see Fig. 4), the content of histidine (and, therefore, to a first approximation, that of the other amino acids) changes in parallel with the change in the content of carbohydrate constituents (neglecting the low-molecular-weight fraction of carbohydrates due to acid

* Presumably 2, 4, 6-trinitrobenzenesulfonyl chloride. (Tr.)

hydrolysis). It follows from this that the cleavage of the peptide chain of BGS also does not form fragments impoverished or enriched in amino acids. It must be particularly stressed that in the experiments on the acid or alkaline degradation of BGS which we have described, in which it was mainly the carbohydrate part of the polymer that was hydrolyzed or the carbohydrate-peptide (O-glycosidic) bond that was ruptured, a considerable enrichment of the high-molecular-weight fragment with amino acids took place [5, 10].

We have studied the acid degradation of fragment A at pH 1.5 (see Fig. 4) and in 0.4 N hydrochloric acid (cf. Fig. 5) at 100° C. The degradation of fragment A in dilute acid (pH 1.5) is accompanied by the partial hydrolysis of the carbohydrate chains. We have previously made a detailed investigation of this hydrolysis of BGS [10]. In this case (decomposition by scheme 2) the polymer (fragment A) undergoes far-reaching degradation as a result of cleavages of the peptide fraction. The results of gel filtration on Sephadex G-100 (cf. Fig. 4a) show the similarity of the degradation to decomposition by scheme 1, with the exception of the fact that there are low-molecular-weight products of the hydrolysis of the carbohydrate chains. The appearance of these products is seen in a particularly striking manner on gel filtration on Sephadex G-25 (cf. Fig. 4b). Under these conditions, the high-molecular-weight fragments issuing with the front are retained, as on degradation after bromination, but because of acid hydrolysis a considerable part of the carbohydrates is split off in the form of monosaccharides and low-molecular-weight oligosaccharides.

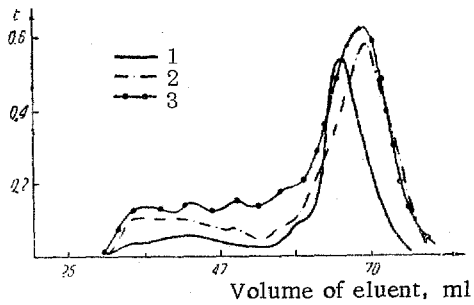


Fig. 5. Gel filtration of the products of the acid hydrolysis of fragement A (0.4 N HCl, 3 hr, 100° C), Sephadex G-25 (1.2 × 75 cm). 1) Optical density in the reaction with anthrone in 65% sulfuric acid; 2) optical density in the Elson-Morgan reaction; 3) optical density in the reaction with TNBS.

The distribution of histidine and amino groups in the fractions (cf. Fig. 4b) shows that under such mild conditions of hydrolysis no accumulation of amino acids or peptides in the low-molecular-weight fractions occurs and therefore degradation is due to specific decomposition and not to the rupture of peptide bonds. Under the conditions of more severe hydrolysis (0.4 N HCl), degradation takes place less selectively – the peptide part is cleaved at "reactive" links and part of the peptide bond and the main bulk of the glycosidic bonds of the carbohydrate component are hydrolyzed. Fragments are obtained (cf. Fig. 5) which consist of glycopeptides of relatively low molecular weight and low-molecular-weight hydrolysis products.

The results given in this paper demonstrate for the first time the selective degradation of the peptide chain of BGS, which is an important step on the road of the study of the structure of this biopolymer. The procedure mentioned is obviously also applicable in principle to the degradation of the peptide chain of other glycoproteins and glycopeptides including the O-glycosidic bonds of serine and threonine.

The results obtained are essential for an understanding of the general structural features of blood-group substances. They show the presence in these biopolymers of a large peptide chain or large peptide chains, which is in agreement with ideas on BGS as a glycoprotein whose main skeleton is based on a long peptide chain.

Summary

1. A method for the cleavage of the peptide chain of glycoproteins containing O-glycosidic bonds of hydroxyamino acids based on the introduction into the peptide chain of reactive enamine groupings through the β-elimination of the carbohydrate moieties has been proposed for the first time.

2. The degradation based on this principle of the peptide chain of blood group substances A+H has been effected. Various conditions for this degradation and also the dependence of the degree of degradation on the conditions of β-elimination have been studied.

3. The basic structural nature of the molecule of blood group substance A + H has been deduced. The skeleton of the molecule is a long polypeptide chain.

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