ウナギ体表面粘質物中のsialic acid含有糖蛋白質に関する研究3

誌名	日本水産學會誌
ISSN	00215392
著者	浅川, 牧夫
巻/号	43巻6号
掲載ページ	p. 727-731
発行年月	1977年6月

Bulletin of the Japanese Society of Scientific Fisheries 43 (6) 727–731 (1977)

Sialic Acid-containing Glycoprotein in the External Mucus of Eel, *Anguilla japonica* TEMMINCK et SCHLEGEL—III Alkaline-reductive Cleavage of Sialic Acid-containing Glycoprotein

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(Received December 11, 1976)

To elucidate the effect of alkaline sodium borohydride on sialic acid-containing glycoprotein (SA-glycoprotein), it was treated with 0.3 M $NaBH_4$ in 0.1 N NaOH.

The carbohydrate moiety in SA-glycoprotein was released from the protein moiety by a β-elimination mechanism. Especially, on treatment at 100°C, about 84% of the carbohydrate moiety was rapidly released in the first 10 min. All of the amino sugar in the carbohydrate moiety obtained from the alkaline-reduced SA-glycoprotein was converted into N-acetylgalactosaminitol.

From the results obtained, it was elucidated that SA-glycoprotein contained the carbohydrate moiety which was attached to the protein moiety by alkali-labile linkage, presumably as *O*-glycosides of threonine and serine residues.

It has become clear that the prosthetic group, NANA (α, 2→6) GalNAc, of ovine submaxillary glycoprotein (OSM) is *O*-glycosidically linked to the hydroxy group of serine and threonine residues in the protein moiety[1] and easily released from OSM by a β-elimination reaction under alkaline or alkaline-reductive condition.[2–6]

In the preceding paper[7] of this series, it has been elucidated that sialic acid-containing glycoprotein (SA-glycoprotein) secreted from the epidermis of eel is very similar in chemical properties to OSM. Thus, the carbohydrate moiety of SA-glycoprotein consists mainly of the equimolar amounts of NANA and GalNAc and the protein moiety is also characterized by the high content of the hydroxy amino acids such as threonine and serine, which are demonstrated to be involved in *O*-glycosidic linkage between the carbohydrate chain and protein moiety in some glycoproteins.[8]

On the basis of these facts, it would be expected that the linkage between the carbohydrate and protein moieties of SA-glycoprotein is alkali-labile and the carbohydrate moiety is released from SA-glycoprotein by treatment with sodium hydroxide or alkaline sodium borohydride as in the case of OSM. In fact, it was observed that in a preliminary experiment, about 30% of the carbohydrate moiety in SA-glycoprotein was released with 1.0 N NaOH in 2 hr at room temperature.

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The abbreviations used are: NANA, *N*-acetylneuraminic acid; GalNAc, N-acetylgalactosamine; $GalNH_2$, galactosamine; GalNAc-ol, *N*-acetylgalactosaminitol: $GalNH_2$-ol, galactosaminitol.

The present paper deals with the effect of alkaline sodium borohydride on SA-glycoprotein.

Experimental

Material SA-glycoprotein used was purified by the procedure described previously.[9]

Isolation of the carbohydrate moiety from SA-glycoprotein SA-glycoprotein (40 mg) was treated with 5 m*l* of 0.3 M $NaBH_4$ in 0.1 N NaOH for 29 hr at 100°C. After this alkaline-reductive treatment, the reaction mixture was adjusted to pH 5 by addition of 0.1 N acetic acid in order to destroy excess $NaBH_4$, applied to a column (1.9×78 cm) of Sepharose 4B, previously equilibrated with 0.05 M Tris-HCl, pH 8.5, and separated into two fractions (Fr. I and Fr. II) as shown in Fig. 1. As described later, Fr. I was a glycopeptide fraction and Fr. II contained carbohydrate moiety contaminated with significant amounts of peptides and amino acids. Therefore, the pooled Fr. II was further chromatographed on Sephadex G–50 (Fig. 2) and Dowex 50W–X8 (H^+) columns to remove the contaminants. Then the effluent containing the carbohydrate moiety was freeze-dried.

The fractions eluted from the columns were analyzed for NANA by the thiobarbituric acid method,[10] for protein by the method of LOWRY *et al.*,[11] for amino acid by the method of YEMM and COCKING,[12] and for $GalNH_2$ by the ELSON-MORGAN reaction[13] after hydrolysis in 2.0 N HCl for 16 hr at 100°C.

Gas-liquid chromatography To identify the amino sugar, the carbohydrate moiety (3 mg) obtained from the alkaline-reduced SA-glycoprotein was hydrolyzed with 2.0 N HCl for 16 hr at 100°C. The amino sugar in the hydrolyzate was purified with a small column of Dowex 50W–X8 (H^+) according to BOAS' method.[14] Then, the amino sugar obtained and authentic compounds were converted into trifluoroacetyl[15] and trimethylsilyl[16] derivatives without further reduction with $NaBH_4$.

The GLC instrument used was Shimazu Model GC–1C equipped with a hydrogen flame ionization detector. The trifluoroacetyl derivative was analyzed on a glass column (0.4×200 cm) packed with 2% XF–1105 on Chamelite CS (80–100 mesh, Kishida Chemical Co , Ltd) at column temperature of 165°C. The analysis of trimethylsilyl derivative was carried out at 205°C on a glass column (0.4×260 cm) packed with 5% SE–30 on Shimalite W (60–80 mesh, Wako Pure Chemical Industries, Ltd.). As a carrier gas, nitrogen was used at 50 m*l*/min.

Estimation of the carbohydrate moiety released during the alkaline-reductive treatment SA-glycoprotein (8 mg) was treated with 5 m*l* of 0.3 M $NaBH_4$ in 0.1 N NaOH at 5, 19, 45 and 100°C for 1 hr. An aliquot (0.5 m*l*) of the reaction mixture was taken at various intervals and to it was added 0.2 ml of conc. HCl. Subsequently, the hydrolysis was carried out at 100°C for 16 hr in a sealed tube. Then, $GalNH_2$ in the hydrolyzate was determined by the ELSON-MORGAN reaction.

Results

SA-glycoprotein was treated with alkaline sodium borohydride and chromatographed on a Sepharose 4B column (Fig. 1). The alkaline-reduced SA-glycoprotein was eluted far behind the native SA-glycoprotein which was eluted at the void volume of the same column,[9] and was separated into two fractions, *i.* e., Fr I (tube number 51–63) and Fr. II (tube number 64–77). The carbohydrate moiety in Fr. I composed of about 16% each of NANA and GalNAc present in native SA-glycoprotein and it was recognized to be a glycopeptide fraction. Fr. II contained about 84% of NANA as a bound form. This fraction also contained significant amounts of peptides and amino acids, which were found to be degradation products of SA-glycoprotein. In this fraction, $GalNH_2$ was hardly detected by the ELSON-MORGAN reaction after acid hydrolysis. If GalNAc is located at

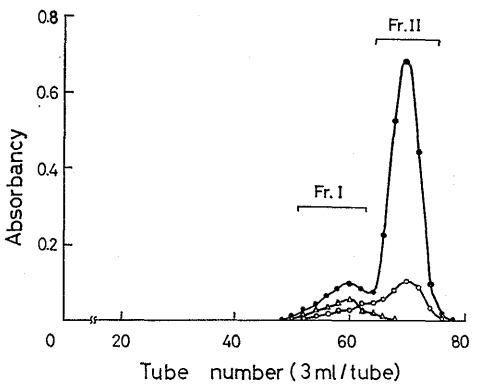

Fig. 1

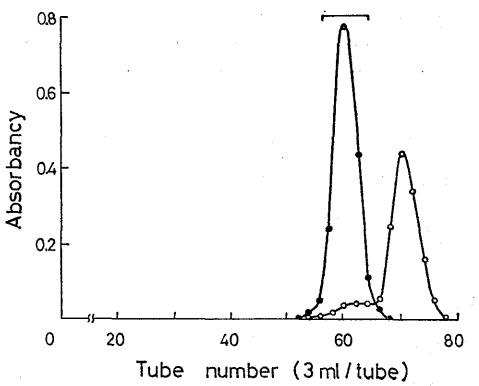

Fig. 2

Fig. 1. Gel filtration of SA-glycoprotein treated with alkaline sodium brohydride on Sepharose 4B. ●—●, NANA; ○—○, protein; △—△, $GalNH_2$.

Fig. 2. Gel filtration of the pooled Fr. II on Sephdex G–50. Fr. II obtained from the alkaline-reduced SA-glycoprotein was applied to a column (1.7×87 cm) and then eluted with deionized water. ●—●, bound NANA; ○—○, peptides and amino acids.

Table 1. Gas-liquid chromatographic identification of the amino sugar in the carbohydrate moiety isolated from the alkaline-reduced SA-glycoprotein

Authentic compounds and amino sugar	Retention time, min
Column A:	
Galactosamine	5.93 (1.00)
Galactosaminitol	8.35 (1.41)
Amino sugar	8.35 (1.41)
Column B:	
Glucosaminitol	6.65 (1.00)
Galactosaminitol	7.61 (1.14)
Mannosaminitol	8.60 (1.29)
Amino sugar	7.62 (1.15)

Column A, 5% of SE-30 on Shimalite W.
Column B, 2% of XF-1105 on Chamelite CS.

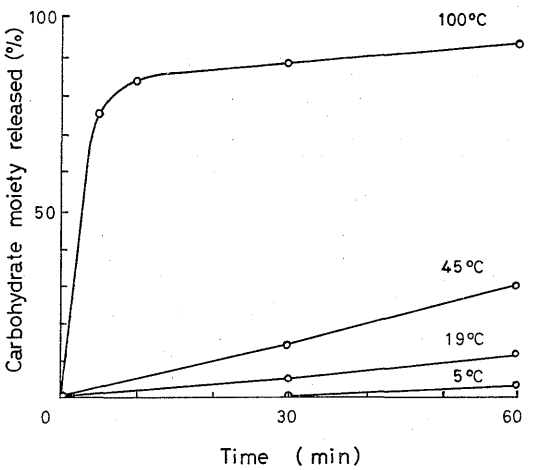

Fig. 3. The liberation of the carbohydrate moiety from SA-glycoprotein during treatment with alkaline sodium borohydride at various temperatures.

the reducing end of the carbohydrate moiety and converted with $NaBH_4$ in alkaline medium into GalNAc-ol, it does not form the characteristic purple chromogen by the ELSON-MORGAN reaction.

Therefore, to identify the amino sugar, the carbohydrate moiety in the pooled Fr. II was purified on Sephadex G–50 (Fig. 2) and Dowex 50W–X8 (H^+) columns and its hydrolyzate was subjected to GLC. As shown in Table 1, all of the amino sugar was identified as $GalNH_2$-ol, indicating that GalNAc was located at the reducing end of the carbohydrate moiety and involved in the carbohydrate-protein linkage in SA-glycoprotein.

In addition, SA-glycoprotein was treated with 0.3 M $NaBH_4$ in 0.1 N NaOH for 1 hr at various temperatures to estimate the amounts of the carbohydrate moiety liberated during the alkaline-reductive treatment. As shown in Fig. 3, the carbohydrate moiety liberated from SA-glycoprotein increased significantly with the rising of temperature. In the experiment at 100°C, about 84% and 92% of the carbohydrate moiety were liberated in 10 and 60 min, respectively.

Discussion

GRAHAM *et al.*[2] reported that about 89% of the prosthetic group in OSM was released during the first 10 min by treatment with 0.1 N NaOH at 100°C in accordance with the first-order kinetics and that the remaining carbohydrate chain was liberated much more slowly. It was also pointed out that on treatment of OSM with 0.3 M $NaBH_4$ in 0.1 N NaOH, the carbohydrate moiety was liberated from the protein moiety and converted into NANA (α, 2→6) GalNAc-ol.[5,6] On the other hand, it was demonstrated that in some glycoproteins, hydroxylysine was involved in *O*-glycosidic linkage between the carbohydrate chain and protein moiety, and such linkage was resistant to hydrolysis even in the strong alkaline medium. That is, the hydroxylysine-linked disaccharide, glucosylgalactosyl-L-hydroxylysine, was isolated in high yield by treatment with 2.0 N NaOH at 105°C for 20–24 hr of glycoproteins obtained from glomerular basement membrane,[17] skin,[18] tendon,[18] swim bladder[18] and lens capsule.[19]

On the basis of the facts that SA-glycoprotein was similar to OSM in respect to the

carbohydrate and amino acid compositions[7] and of the results obtained from the experiment with alkaline sodium borohydride, it was elucidated that the carbohydrate moiety was easily released from SA-glycoprotein by a β-elimination reaction and GalNAc located at the reducing end of the carbohydrate moiety was attached to the protein moiety by alkali-labile linkage, presumably as *O*-glycosides of threonine and serine residues.

Acknowledgement

The author wishes to thank Professors Masamichi TOYOMIZU, Faculty of Agriculture, Kyushu University and Hiroki NAKAGAWA, Faculty of Agriculture, Saga University for helpful suggestions and a critical reading of the manuscript.

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