

## AMINO ACID SEQUENCE OF THE GLYCOPEPTIDE DERIVED FROM A MAJOR GLYCOPROTEIN IN BOVINE PERIPHERAL NERVE MYELIN

Kunio KITAMURA, Akemi SUZUKI, Masaru SUZUKI and Keiichi UYEMURA  
*Department of Physiology, Saitama Medical School, Iruma-gun, Saitama 350-04, Japan*

Received 30 December 1978  
Revised version received 2 February 1979

### 1. Introduction

The major protein in peripheral nerve myelin, which is not found in central nerve myelin, is a glycoprotein [1,2]. This glycoprotein, which accounts for  $\geq 50\%$  of total peripheral myelin protein [3], increases rapidly in parallel to myelination of peripheral nerve at developmental stages [4] and is suggested to localize in interperiod line of myelin lamellae [5]. The glycoprotein may play important roles in myelin membrane organization or adhesion.

One of the difficulties in studying the membrane glycoprotein was its solubilization and subsequent purification. Recently, it became possible to obtain the myelin glycoprotein, referred to as BR protein (mol. wt 28 000), in highly purified form from bovine peripheral myelin by a gel filtration method with SDS solution [6].

The glycoprotein as PO protein was also purified from rabbit sciatic nerve myelin by a similar method [7]. The amino acid composition, carbohydrate composition and N-terminal amino acid of the glycoprotein have been reported [6,7].

This study describes the amino acid sequence of the BR-PO protein in the neighbourhood of a carbohydrate moiety.

### 2. Materials and methods

#### 2.1. Purification of a major glycoprotein from bovine peripheral nerve myelin

Peripheral nerve myelin was prepared from bovine

intradural spinal roots by sucrose density gradient centrifugation as in [8].

The major glycoprotein, referred to as BR-PO protein here, was purified from the myelin as in [6]. The protein was homogeneous judging from SDS-polyacrylamide gel electrophoresis and N-terminal amino acid analysis.

#### 2.2. Protease digestion of the BR-PO protein

BR-PO protein (40 mg) was suspended in 4.8 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 2.5 mM  $\text{CaCl}_2$ , then digested with *Streptomyces griseus* protease (Sigma, type VI) as in [9] with minor modifications. The mixture was incubated at 37°C for 5 days in the presence of a saturating amount of toluene. A 10 mg/ml protease solution (40  $\mu\text{l}$ ) was added initially and 20  $\mu\text{l}$  additions were made on days 2 and 4.

#### 2.3. Purification of the glycopeptide

After digestion, the solution was lyophilized and the residue dissolved in 2 ml 0.1 M NaCl solution. The insoluble materials were removed by centrifugation at  $26\,000 \times g$  for 30 min, then washed with 1 ml 0.1 M NaCl. The combined supernatant was chromatographed on a Sephadex G-25 column with 0.1 M NaCl as eluent. The eluate was collected in 2.4 ml fractions, portions of which were analyzed for  $A_{230}$  and with anthrone reaction for hexoses.

The void volume fraction of Sephadex G-25 column was lyophilized and the residue dissolved in 1.5 ml 0.05 M pyridine-acetic acid. The supernatant after centrifugation was applied on a Sephadex G-50

column with 0.05 M pyridine–acetic acid as eluent. Each 2.0 ml fraction was collected. For chromatographic profiles, portions of the eluate were subjected to anthrone reaction and to ninhydrin reaction.

#### 2.4. Determination of the amino acid sequence of the glycopeptide

The amino acid sequence of the glycopeptide eluted from Sephadex G-50 was determined by Edman degradation as in [10]. The yield of the phenylthiohydantoin (PTH)-amino acid of each cycle was calculated from  $A_{269}$  in 99% ethanol [11]. The PTH-amino acid was identified by high-performance liquid chromatography using Partigil 5 (Reeve Angel) as in [12].

#### 2.5. Analytical methods

Anthrone reaction was carried out after the appropriate portion of the eluate from the Sephadex columns had been dried as in [13]. Ninhydrin reaction was carried out after alkaline hydrolysis of dried sample as in [14]. Mannose and leucine were used as standard for anthrone reaction and for ninhydrin reaction, respectively. Amino acid and hexosamine analyses were done by automated amino acid analyzer as in [6]. Individual hexoses were determined as trimethylsilyl derivatives by gas–liquid chromatography equipped with a flame ionization detector. A column (3 mm  $\times$  2 m) of 5% silicone OV-17 on chromosorb WAW DMCS (80–100 mesh) was used. The glycopeptide was hydrolyzed in 1 N  $H_2SO_4$  at 100°C for 6.5 h and the hexose fraction was obtained from the hydrolysate as in [6]. A thiobarbituric acid assay was used to determine the sialic acids in the glycopeptide after the sample had been hydrolyzed in 0.1 N  $H_2SO_4$  at 80°C for 1 h.

### 3. Results and discussion

#### 3.1. Purification of the glycopeptide

Approximately 80% of the BR–PO protein hexose was recovered from the supernatant after centrifugation of the digested BR–PO protein with *Streptomyces griseus* protease. Most of hexose-containing materials were eluted at the void volume of the Sephadex G-25 column (fig.1) and were fractionated further on a column of Sephadex G-50

(fig.2). Two hexose-containing peaks were observed on the Sephadex G-50. The first peak at the void volume of the column did not react with ninhydrin after alkaline hydrolysis, while the second peak did.

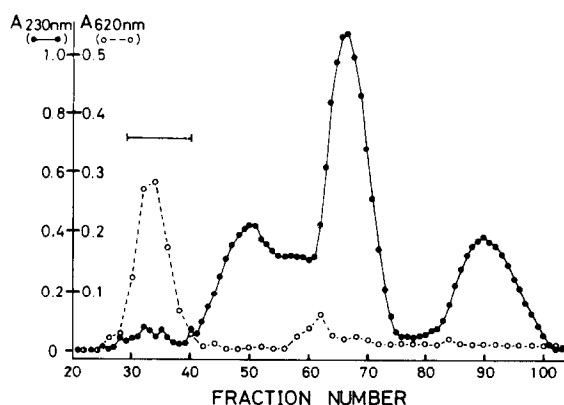


Fig.1. Sephadex G-25 gel chromatography of protease digests of the BR–PO protein. The protease digests of the BR–PO protein (20 mg) were applied on a Sephadex G-25 (fine) column (1.5  $\times$  97 cm) and were eluted with 0.1 M NaCl at 10 ml/h flow rate. Fractions (2.4 ml) were collected and their  $A_{230}$  measured (●—●). Portions (0.2 ml) of the fractions were subjected to anthrone reaction for hexoses (○—○). The void volume fraction was pooled as indicated by the bar.

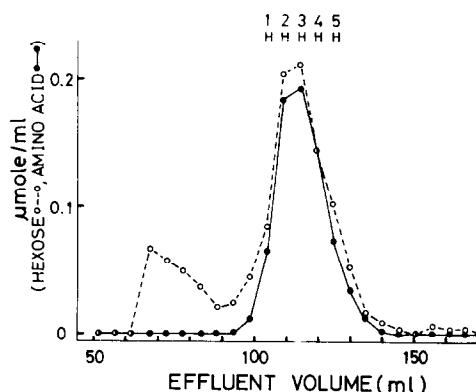


Fig.2. Sephadex G-50 gel chromatography of the void volume fraction eluted from Sephadex G-25. The void volume fraction of Sephadex G-25 was applied on a Sephadex G-50 (fine) column (1.5  $\times$  97 cm) and was eluted with 0.05 M pyridine–acetic acid at 10 ml/h flow rate. 0.2 ml and 0.3 ml of each fraction were subjected to anthrone reaction using mannose as standard (○—○) and to ninhydrin reaction after alkaline hydrolysis using leucine as standard (●—●), respectively.

The first peak contained glucose only judging from hexose analysis, therefore, was probably due to contamination by polysaccharide during the handling procedure. The second peak was considered to contain the glycopeptide, but was broader than would be expected for a single glycopeptide. Therefore, the peak was divided into 5 fractions as indicated in fig.2, and analyses were made of each fraction. As shown in table 1, these fractions contained virtually the same amino acids; Asx(2), Gly(2) and Thr (1 mol). However, the carbohydrate composition varied significantly among these fractions. These results indicate that the peak contains homogeneous pentapeptide but with heterogeneity of its carbohydrate moiety. Heterogeneity of the carbohydrate moiety was also suggested when this peak was fractionated further on DEAE-Sephadex A-25 (data not shown). Therefore, the carbohydrate moiety of the glycopeptide obtained was considered to have charge and size heterogeneities which were of the BR-PO protein per se.

### 3.2. Amino acid sequence of the glycopeptide

The amino acid sequence of the glycopeptide in the fraction 3 on Sephadex G-50 was determined by Edman degradation (fig.3), which proceeded well from the N-terminus to 4th step (Gly). The C-terminal amino acid residue was identified as Thr by amino acid analysis without hydrolysis of the residue after

Table 1  
Amino acid and carbohydrate composition of the glycopeptide fractions eluted from Sephadex G-50

	Fraction				
	1	2	3	4	5
Asp	2.00	2.00	2.00	2.00	2.00
Gly	1.80	1.88	1.80	1.92	2.15
Thr	0.97	1.04	1.06	1.01	0.98
Ser	0.10	0	0	0	0.29
Glu	0	0	0	0	0.27
Glucosamine	3.75	3.12	2.31	2.94	3.01
Galactosamine	0	0	0	0	0
Fucose	1.47	1.37	1.24	1.47	1.65
Mannose	2.95	3.56	3.16	3.86	3.78
Galactose	1.08	1.01	1.23	1.18	1.63
Glucose	0	0	0	0	0
Sialic acids	1.38	1.08	0.98	0.90	0.66

The values are expressed as molar ratios to aspartic acid (2.00). Values <0.1 mol are expressed as 0 or not shown

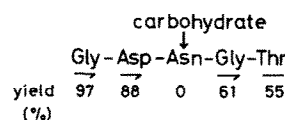


Fig.3. Amino acid sequence of the glycopeptide derived from the BR-PO protein. 21 nmol of the glycopeptide were analyzed. (→) and (—) indicate the sequence proved by Edman degradation and by amino acid analysis without hydrolysis, respectively. The yield of each cycle is shown as the percentage of the amount of glycopeptide used.

the 4th step of Edman degradation. No PTH-amino acid of the 3rd step was detected on high-performance liquid chromatography. Therefore, the carbohydrate-carrying Asx residue was verified to be at the 3rd position. The failure to detect any PTH-amino acid at the 3rd step was because the PTH-Asx linked to carbohydrate was not extracted with the organic solvent used. Because of the presence of glucosamine in the glycopeptide (table 1) and of the established chemical nature of the glycosylamine bond, the Asx is depicted as Asn although this has not been directly established for the sequence shown.

Sufficient data have been accumulated to consider the particular sequence, Asn-X-Thr (or Ser), as a recognition site for the enzyme attaching *N*-acetylglucosamine to the Asn residue in the polypeptide chain [15]. The present results also support the above concept.

In this study, only one glycopeptide was obtained from the BR-PO protein by protease digestion. This result suggests that the BR-PO protein contains a single carbohydrate chain in its molecule with an asparagine *N*-glycosyl linkage.

### Acknowledgements

This study was partially supported by Science Research Grant from the Ministry of Education of Japan and from Igaku Shinko Foundation in Japan.

### References

- [1] Everly, J. L., Brady, R. O. and Quarles, R. H. (1973) *J. Neurochem.* 21, 329-334.
- [2] Wood, J. G. and Dawson, R. M. C. (1973) *J. Neurochem.* 21, 717-719.

- [3] Greenfield, S., Brostoff, S., Eylar, E. H. and Morell, P. (1973) *J. Neurochem.* 20, 1207–1216.
- [4] Uyemura, K., Horie, K., Kitamura, K., Suzuki, M. and Uehara, S. (1978) *Bull. Jap. Neurochem. Soc.* 17, 65–68.
- [5] Wood, J. G. and McLaughlin, B. J. (1975) *J. Neurochem.* 24, 233–235.
- [6] Kitamura, K., Suzuki, M. and Uyemura, K. (1976) *Biochim. Biophys. Acta* 455, 806–816.
- [7] Roomi, M. W., Ishaque, A., Khan, N. R. and Eylar, E. H. (1978) *Biochim. Biophys. Acta* 536, 112–121.
- [8] Uyemura, K., Tobari, C., Hirano, S. and Tsukada, Y. (1972) *J. Neurochem.* 19, 2607–2614.
- [9] Quarles, R. H. and Everly, J. L. (1977) *Biochim. Biophys. Acta* 466, 176–186.
- [10] Van Eerd, J.-P. and Kawasaki, Y. (1973) *Biochemistry* 12, 4972–4980.
- [11] Edman, D. (1970) in: *Molecular Biology, Biochemistry and Biophysics* (Kleinzeller, A. et al. eds) vol. 8, pp. 211–255, Springer-Verlag, Berlin.
- [12] Isobe, T., Black, L. W. and Tsugita, A. (1977) *J. Mol. Biol.* 110, 168–177.
- [13] Spiro, R. G. (1966) *Methods Enzymol.* 8, pp. 3–26.
- [14] Hirs, C. H. W. (1967) *Methods Enzymol.* 11, 325–329.
- [15] Marshall, R. D. (1972) *Ann. Rev. Biochem.* 41, 673–702.