PROTEIN-SUGAR INTERACTIONS. PURIFICATION BY AFFINITY CHROMATOGRAPHY OF LIMULIN, AN *N*-ACYL-NEURAMINIDYL-BINDING PROTEIN

Annie-Claude ROCHE, Roland SCHAUER* and Michel MONSIGNY

Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orléans cedex, France, and
**Institut für Physiologische Chemie der Universität Bochum,*
D 4630 Bochum-Querenburg, Postfach 2148, G.F.R.

Received 23 June 1975

1. Introduction

The lectin of *Limulus polyphemus* haemolymph agglutinates erythrocytes [1–4] and other cells [5]. Neuraminic acid has been shown to be the specific ligand of this lectin. The lectin has been isolated by ultracentrifugation and preparative starch gel electrophoresis [6], by DEAE-Sephadex and gel filtration chromatography [7] and by ultracentrifugation, affinity and gel filtration chromatography [8]. Though the protein prepared by the last two methods [7,8] was homogeneous with respect to molecular weight, polyacrylamide gel electrophoresis and immunoelectrophoresis, we got three fractions by affinity chromatography. The first two fractions (Limulin I and II) did not agglutinate erythrocytes, but the third fraction (Limulin III) was very active. We wish to report the affinity chromatography purification of Limulin III and to show that Limulin binds *O*-glycosides of *N*-acyl-neuraminic acids more strongly than free *N*-acryl-neuraminic acids.

2. Materials and methods

N-acetyl-neuraminic acid, *N*-glycolyl-neuraminic and bovine submaxillary mucin (BSM) were obtained from Sigma; 2-*O*-methyl *N*-acetyl-β-neuraminic acid has been obtained by *N*-acetylation [9] of 2-*O*-methyl-β-neuraminic acid which has been prepared according to Schauer and Buscher [10]; 2-*O*-methyl *N*-acetyl-α-neuraminic acid has been prepared according to Meindl and Tuppy [11]. *N*-acyl-α-neuraminidyl acid 2→6 *N*-acetyl-*D*-galactosaminitol has been isolated by treatment of BSM with alkaline sodium borohydride [12] followed by chromatography on Dowex 2X8 [13]. Chromogens derivatives of *N*-acyl-α-neuraminidyl acid-2→6-*N*-acetylgalactosamine (sialyl chromogens I and III) [14] have been isolated by alkaline treatment of BSM, chromatography on Dowex 2X8, chromatography on charcoal-celite mixture (elution with 50 p. 100 aqueous ethanol).

The following submaxillary mucins were used: PSM (porcine) containing 90 p.100 of *N*-glycolyl-neuraminic acid [15], ESM (equine) containing mainly 4-*O*-acetyl *N*-acetyl-neuraminic acid, BSM (bovine) containing predominantly 9-*O*-acetyl *N*-acyl-neuraminic acid [16,17] with 15 p.100 of *N*-glycolyl and 85 p.100 of *N*-acetyl [18], and OSM (ovine) containing *N*-acetyl-neuraminic acid [19].

Sephadex G 50 (fine), DEAE-Sephadex A 50 and Sepharose 4 B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; Ultrogel AcA 22 from L.K.B., Sweden: Dowex 2X8 from Serva, Heidelberg. Frozen haemolymph from *L. polyphemus* was obtained from the Marine Biological Laboratory, Woods Hole, Mass. (U.S.A.).

2.1. *Analysis of sugar*

Purity of sugars and disaccharides or their derivatives was checked by paper electrophoresis on Whatman 3 (10 V/cm, 4 h in pyridine acetic acid water 3/2/400, v/v, pH 5.4) and by paper chromatography on Whatman 1 (*n*-butanol/pyridine/HCl 0.1 N, 5/3/2,

v/v). Sialic acids were detected with the orcinol reagent [20], neutral sugars with the anilineoxalate reagent [21].

Total *N*-acyl-neuraminic acids were determined by the method of Aminoff [22] after hydrolysis of the sample with 0.05 M H_2SO_4 (80°C, 60 min) or by the method of Bial [23]. *N*-acetyl hexosamines were determined by the method of Levvy and McAllan [24]. Chromogen derivatives of *N*-acetyl-osamine were determined by the same method, but without heating after the addition of the alkaline reagent. *N*-acetyl-neuraminic acid and *N*-acetyl-galactosamine were used as standards.

2.2. *Affinity chromatography column*

Sepharose 4 B (40 ml) activated with cyanogen bromide [25] was coupled with bovine submaxillary mucin (200 mg) and then with 2-amino 2-hydroxymethyl-propane 1,3 diol (100 mg) in order to block any unreacted activated groups. The substituted Sepharose was washed with a 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris-HCl buffer (pH 8.5) and poured into a column (2.5 × 13 cm). After acidic hydrolysis [22] (80°C, H_2SO_4 0.05 M, 1 h) it was found that 500 μg of sialic acid per ml of substituted Sepharose was released. The amount of bound mucin can then be evaluated at 2.5 mg per ml of substituted Sepharose.

3. Results and discussion

3.1. *Purification of haemagglutinating limulin*

The first purification steps were done as previously described [7]. At the last step an affinity chromatography was used instead of gel filtration chromatography. The active material obtained after the chromatography on DEAE Sephadex column was applied directly (without concentration or dialysis) onto the column of BSM-substituted Sepharose equilibrated with 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris-HCl buffer, pH 8.5. A large part of material passed through the column and was devoid of agglutinating activtiy (Limulin I). In preliminary experiments, the column was then washed stepwise, increasing the ionic strength (NaCl, 0.2 M to 1 M). Various amounts of proteins were eluted at each step, but no agglutinating activity could be detected. In routine experiments, the column was washed with the higher ionic strength buffer (1.0 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris-HCl, pH 8.5). Under these conditions, the eluted protein (Limulin II) was devoid of agglutinating activity. However, when agglutination tests were done with Limulin I or Limulin II in presence of $MnCl_2$ 0.1 M instead of $CaCl_2$ 0.1 M, a slight agglutinating activity was detected; the lower protein concentration required to agglutinate horse red blood cells was about 100 μg/ml.

The active protein (Limulin III) was eluted with a calcium-free buffer 1.0 M NaCl, 0.05 M Tris-HCl, pH 8.5. In order to test the effectiveness of this purification procedure, the protein (11 mg) purified according to Oppenheim et al. [8] was applied onto the column: A minor fraction (1 mg) was not retarded, a large fraction (9 mg) was eluted with 1.0 M NaCl, calcium-containing buffer, and the active protein (1 mg) was eluted with the calcium-free buffer. Fractions containing Limulin III were pooled and dialyzed against 0.1 M NaCl, 0.01 M $CaCl_2$ 0.05 M Tris-HCl, pH 8.5.

The purification procedure is summarized in Table 1 and the affinity chromatography step is shown in fig.1. By this procedure, the purification factor (33 000) is much higher than that obtained previously 1700 [8] 60 [7] and 20 [6]. The yield of active material is almost quantitative, and the amount of Limulin III obtained from 1000 ml of crude haemolymph is about 4 mg. The lower concentration required to agglutinate horse red blood cells is about 2 ng/ml. Limulin III gave one band when passed through an ultrogel AcA 22 column (1.5 × 100 cm), and the agglutinating activity followed strictly the protein concentration. The sedimentation constant of Limulin III is $S^{\circ}_{20,w}$ 13.9, as previously described for Limulin [7]. Polyacrylamide gel electrophoresis in presence of sodium dodecylsulfate 0.1 p.100 and mercaptoethanol 0.1 p. 100 leads to a molecular weight identical to that found previously [7].

3.2. *Specific inhibition of agglutinating activity*

The inhibition experiments were done as we have already described [7]. The results are summarized in Table 2. Free *N*-acetyl-neuraminic acid and free *N*-glycolyl neuraminic acid, which are known to be mainly β-anomers [26], inhibit the agglutination at the same high concentration (200 mM), therefore

Table 1
Purification of *Limulus polyphemus* lectin

Procedure	Volume ml	A_{1cm}^{280nm}	Total protein content (mg) *	Aggluatination titre **	Total activity (agglutination units)	Specific activity (units/mg)	Purification factor
Whole haemolymph	1000	113	100 000	10^3	10^6	10	1
Sephadex G 50	2500	38	85 000	2×10^2	5×10^5	6	0.6
DEAE Sephadex	1400	1.8	2250	10^3	1.4×10^6	6×10^2	60
Affinity: Limulin I	1400	1.7	2100	0	0	0	–
Limulin II	36	1.15	36	0	0	0	–
Limulin III	17.5	0.24	3.8	7×10^4	1.25×10^6	3.3×10^5	33 000

* The protein content was estimated assuming an $A_{280\,nm}^{1\%,\,1cm}$ of 11.3
** Horse erythrocytes 3% in 0.1 M NaCl, 0.1 M $CaCl_2$.

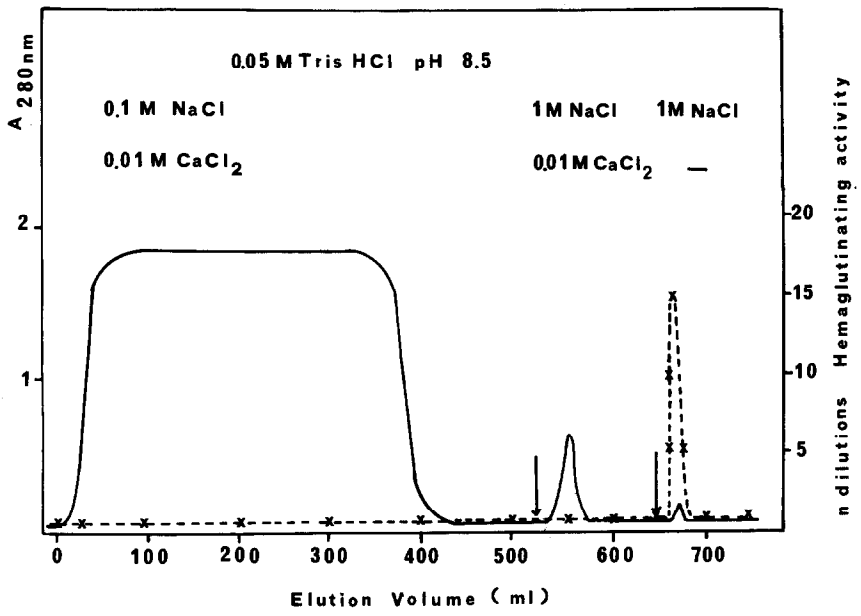

Fig.1. Purification of *Limulus polyphemus* hemagglutinin by affinity chromatography on mucin-sepharose column (2.5 × 13 cm) equilibrated in 0.05 M Tris-HCl, 0.01 M $CaCl_2$, 0.1 M NaCl buffer, pH 8.5. Pooled active fractions from DEAE-Sephadex chromatography column were applied. The proteins were eluted with Tris, $CaCl_2$, 0.1 M NaCl buffer (Limulin I), with Tris, $CaCl_2$, 1 M NaCl buffer (Limulin II) and then with Tris, 1 M NaCl buffer (Limulin III). 10 ml fractions were collected (Limulin I) and thereafter 3 ml were collected (Limulin II and III). ———, absorbance at 280 nm; ——, macroscopic agglutination with 3% horse erythrocytes in 0.1 M NaCl, 0.1 M $CaCl_2$.

Table 2
Inhibition of agglutinating activity

Compounds	Concentration* (10^3 × mol/l)	Specificity** factor
N-Acetyl-neuraminic acid	200	1
N-Glycolyl-neuraminic acid	200	1
2-*O*-Methyl β-neuraminic acid	***	–
2-*O*-Methyl *N*-acetyl β-neuraminic acid	20	10
2-*O*-Methyl *N*-acetyl α-neuraminic acid	10	20
N-Acyl-α-neuraminyl 2→6 chromogens	5	40
N-Acyl-α-neuraminyl 2→6 *N*-acetyl-galactosaminitol	0.04	5000
ESM	0.3	650
OSM	0.1	2000
BSM	0.03	6000
PSM	0.03	6000

* Concentration of inhibitors, expressed as sialic acid, needed to inhibit one unit of agglutination.
** Relative to the activity of free *N*-acetyl-neuraminic acid.
*** No inhibition with a 300 mM concentration.

the N-glycolyl group does not change the capability of binding to the Limulin. 2-*O*-methyl β-neuraminic acid, with a free amino-group, has no effect at all, so the amido-group seems to be important for the binding. The low molecular weight glycosides derivatives of *N*-acyl-neuraminic acid inhibit the agglutination at a lower concentration than free *N*-acyl-neuraminic acid. The β-anomer of 2-*O*-methyl *N*-acetyl-neuraminic acid is slightly less active than the α-anomer.

The concentration of BSM and PSM required to inhibit agglutination is low and in the same range for these two mucins (30 μM). ESM is less active (300 μM) and OSM has an intermediary activity (100 μM). Therefore, the *O*-acetyl in position 4 as in ESM seems to lower the affinity; the *O*-acetyl in position 9 as in BSM or the *N*-glycolyl as in PSM seems to increase slightly the affinity, while there is no difference between free *N*-glycolyl- and *N*-acetyl-neuraminic acid. A strong inhibitory effect is obtained with the disaccharide derivatives released from BSM. The dissacharide derivatives obtained by alkaline treatment in the absence of sodium borohydride are forty times more active than free sialic acid and the disaccharide released by alkaline treatment in the presence of sodium borohydride inhibitis agglutination in the same concentration range as BSM. Therefore, the inhibitory effect is not related to the macromolecular structure of mucin but to its *N*-acyl-neuraminic containing disaccharide.

Acknowledgements

This work was supported by INSERM grant n° 74.4.229.03 and D.G.R.S.T. grant n° 74.7.05.79. The authors thank Dr Rossignol (Orsay) for a gift of pure OSM, and R. Veh (Bochum) for a gift of *N*-acyl-neuraminidyl-2→6-*N*-acetyl-D-galactosaminitol.

References

[1] Noguchi, H. (1903) Zent. Bakt. Abt. I. Orig. 34, 286–288.
[2] Cohen, E., Rose, A. W. and Wissler, F. C. (1965) Life Sci. 4, 2009–2016.
[3] Cohen, E. (1968) Trans. N.Y. Acad. Sci. 30, 427–443.
[4] Watne, A. L., Cohen, E., Migaiolo, J. A. and Lyon, V. E. (1966) Acta Cytol. 10, 255–260.
[5] Pardoe, G. I., Uhlenbruck, G. and Bird, G. W. G. (1970) Immunology 18, 73–83.
[6] Marchalonis, J. J. and Edelman, G. M. (1968) J. Mol. Biol. 32, 453–465.
[7] Roche, A. C. and Monsigny, M. (1974) Biochim. Biophys. Acta 371, 242–254.
[8] Oppenheim, J. D., Nachbar, M. S., Salton, M. R. J. and Aull, F. (1974) Biochem. Biophys. Res. Commun. 58, 1127–1134.
[9] White, T. (1940) J. Chem. Soc. 26, 428.
[10] Schauer, R. and Buscher, H. P. (1974) Biochim. Biophys. Acta 338, 369–373.
[11] Meindl, P. and Tuppy, H. (1965) Monatsh. Chem. (1965) 802–815.
[12] Murty, V. L. N. and Horowitz, M. I. (1968) Carbohyd. Res. 6, 266–275.
[13] Gottschalk, A. and Graham, E. R. B. (1959) Biochim. Biophys. Acta 34, 380–391.
[14] Graham, E. R. S. and Gottschalk, A. (1960) Biochim. Biophys. Acta 38, 513–519.
[15] Schauer, R., Schoop, H. J. and Faillard, H. (1968) Hoppe-Seylers Z. Physiol. Chem. 349, 645–652.
[16] Schauer, R. (1970) Hoppe-Seylers Z. Physiol. Chem. 351, 595–602.
[17] Kamerling, J. P., Vliegenthart, J. F. G., Versluis, C. and Schauer, R. (1975) Carbohydr. Res. 41, 7–17.
[18] Schauer, R. and Faillard, H. (1968) Hoppe-Seylers Z. Physiol. Chem. 349, 961–968.
[19] Blix, G., Lindberg, E., Odin, L. and Werner, I. (1955) Nature 175, 340–341.
[20] Klevstrand, R. and Nordal, A. (1950) Acta Chem. Scand. 4, 1320.
[21] Partridge, S. M. (1948) Biochem. J. 42, 238–250.
[22] Aminoff, D. (1961) Biochem. J. 81, 384–392.
[23] Böhm, P., Dauber, St. and Baumeister, L. (1954) Klin. Wochenschrift 32, 289–292.
[24] Levvy, G. A. and McAllan, A. (1959) Biochem. J. 73, 127–132.
[25] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[26] Schauer, R. (1973) Angew. Chem. Internat. Edit. 12, 127–138.