PURIFICATION OF A HEMAGGLUTININ FROM LIMULUS POLYPHEMUS BY AFFINITY CHROMATOGRAPHY

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SUMMARY: A hemagglutinin has been isolated from the hemolymph of the common horseshoe crab (Limulus polyphemus) using affinity chromatography as the primary purification procedure. The hemagglutinin specifically binds to a bovine submaxillary mucin (BSM) affinity gel and is subsequently released upon the addition of Na citrate to the elution buffer. The purity of the final product was attested to by polyacrylamide gel electrophoresis, electron microscopic observation and by a greater than 1500-fold increase in specific activity as compared to the starting material. The purified hemagglutinin was able to give a precipitin reaction in immunodiffusion gels against purified BSM but not against desialated BSM.

INTRODUCTION: Lectins (agglutinins), have proven to be invaluable tools for studying the many intricacies of cell surfaces. One of the more interesting but least studied of these agglutinins is the hemagglutinin (LPA) isolated from the hemolymph of Limulus polyphemus. It has been reported from agglutination and agglutination-inhibition studies that LPA has a sugar specificity for N-acetyl-neuraminic acid (NANA) and that it will bind to many compounds with terminal neuraminyl groups (1). Marchalonis and Edelman (2) have also demonstrated that Ca^{2+} is required for the expression of biological activity of LPA, that Ca^{2+} chelators such as sodium citrate can inhibit such activity and that such inhibition, itself, can be overcome by the addition of further Ca^{2+}. From the preceding information we have developed a method for the purification of LPA utilizing affinity chromatography. The

end product of this purification is an electrophoretically pure protein, which appears homogeneous in the electron microscope and which exhibits a specific activity considerably higher than that of the same agglutinin purified by other means (2, 3).

METHODS: Adult horseshoe crabs (Limulus polyphemus) of both sexes were obtained from the Marine Biological Labs., Woods Hole, Mass. and were bled immediately upon arrival. The crude hemolymphs obtained from 3-5 animals were pooled and allowed to clot for 1 hr at 20°C and were then centrifuged at 20,000 x g for 30 min at 4°C to remove clots and cellular material. The clarified hemolymph was centrifuged for 6 hr at 95,000 x g in a SW-27 rotor in a Beckman L2-65B ultracentrifuge in order to sediment most of the hemocyanin. The hemocyanin pellets were discarded while the clear supernatants (95,000 x g supernatant fraction) were pooled and dialyzed against 0.05 M Tris HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.01 M $CaCl_2$ (Tris-NaCl-$CaCl_2$ buffer).

The major glycoprotein from bovine submaxillary mucin (BSM), obtained from Worthington Biochemicals, was purified according to procedures described by Gottschalk and Bhargawa (4). 200 mg of the purified BSM was then reacted with 40 ml of hydrated, washed CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the manufacturers' direction. Approximately 90% of the BSM was determined to have been coupled to the gel, as judged by the absorbance at 280 nm of washings obtained during the preparation procedures. After coupling, the remaining CNBr-active sites on the gel were blocked by treatment with buffered ethanolamine. A 1.5 x 20 cm column was then prepared with the BSM-Sepharose and was washed extensively with Tris-NaCl-$CaCl_2$ buffer.

After dialysis, the 95,000 x g supernatant was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant passed through the BSM-Sepharose column. The column was then washed with the Tris-NaCl-$CaCl_2$ buffer until the absorbance of the effluent at 280 nm was at background. The column was then eluted with 0.05 Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.02 M Na-citrate (Tris-NaCl-citrate buffer). All fractions were monitored for absorbance at 280 nm and for agglutinating activity. Fractions showing agglutinating activity were pooled (eluted peak), dialyzed against the Tris-NaCl-$CaCl_2$ buffer and concentrated down to 5 ml in an Amicon Ultrafiltration unit. This sample was then subjected to gel filtration on a 2.5 x 80 cm column of Biogel A1.5 previously equilibrated with the Tris-NaCl-$CaCl_2$ buffer. Elution was carried out with the same buffer at a flow-rate of 15 ml/hr. 10-ml fractions were collected. All fractions were analyzed for absorbance at 280 nm and for agglutinating activity. Those fractions showing agglutination activity were pooled (purified LPA) and concentrated in the Amicon unit.

Hemagglutination assays were performed in test tubes following standard procedure described by Kabat and Mayer (5). Serial dilutions of the hemagglutinin were made in 0.5 ml aliquots of Tris-NaCl buffer containing 0.1 M $CaCl_2$. 0.5 ml of a suspension of 3 x washed horse erythrocytes containing 2 x 10^8 cells in Tris-NaCl buffer, was then added to each tube. After gently mixing, the tubes were incubated at 37°C and macroscopic agglutination was recorded after a 60-min incubation.

Proteins were determined by the method of Lowry et al. (6). Polyacrylamide gel electrophoresis was performed under the standard alkaline conditions using 6% gels, as described by Salton (7) and protein was stained

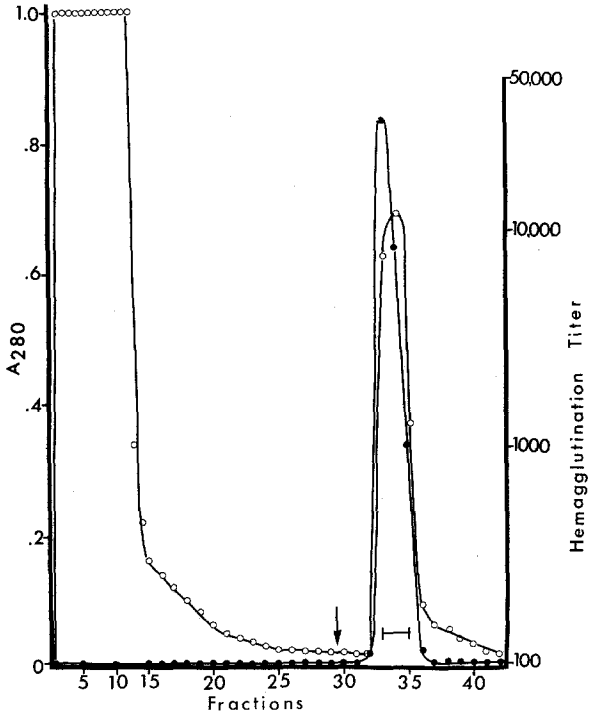

Figure 1. Purification of Limulus polyphemus hemagglutinin (LPA) by affinity chromatography. A 375-ml sample of high speed supernatant of Limulus hemolymph was dialyzed against the Tris-NaCl-$CaCl_2$ buffer and then passed through a column (1.5 x 20 cm) of BSM coupled to Sepharose 4B equilibrated with the same buffer. The column was then washed with the Tris-NaCl-$CaCl_2$ buffer and then with the Tris-NaCl-Na citrate buffer. The change in eluents is indicated by the arrow. Initially 50 ml fractions were collected (1-15) and thereafter 10 ml fractions were collected (16 on). All fractions were monitored for absorbance at 280 nm (—o—) and agglutinating activity (—•—).

with Coomassie blue (8). Immunodiffusion was carried out according to Campbell et al. (9). Desialized BSM was prepared according to the acid hydrolysis method described by Hagopian and Eylar (10).

RESULTS: The initial step in the purification of the hemagglutination from Limulus hemolymph is the removal of most of the respiratory pigment, hemocyanin, which makes up approximately 95% of the hemolymph protein. This is readily achieved by high speed centrifugation. This centrifugation

Table 1

Purification of Limulus polyphemus Hemagglutinin (LPA)

Sample	ml	Protein (mg/ml)	Hemagglutination titer	Specific activity[1]
Whole declotted hemolymph[2]	400	52	1:2048	39
95,000 x g hemolymph supernatant	375	4.6	1:1024	223
Eluted peak from affinity column	30	.55	1:16,364	29,753
Activity peak off Biogel A1.5 column (purified LPA)	50	.14	1:8192	58,500
Concentrated LPA	7	1.0	1:65,456	65,456

[1] Specific activity expressed as hemagglutination titer per mg protein.

[2] Whole declotted hemolymph was obtained by pooling the hemolymph from 3 animals.

step, however, also appears to remove 50% of the hemagglutinin (see Table 1). This is not surprising considering the copious amount of hemocyanin (approximately 40-45 mg/ml) and the relatively large size of the intact hemagglutinin molecules (400,000 molecular weight (2)).

As mentioned in the introduction NANA and compounds with terminal neuraminyl groups specifically inhibit the LPA hemagglutination reaction by apparently binding to the agglutinin's binding site. Utilizing this information, a series of hemagglutination-inhibition test were performed using a number of different glycoproteins which were rich in NANA residues. The major glycoprotein purified from commercial BSM showed the greatest inhibition and was therefore used in the preparation of an affinity column.

After dialysis against the Tris-NaCl-$CaCl_2$ buffer the high speed

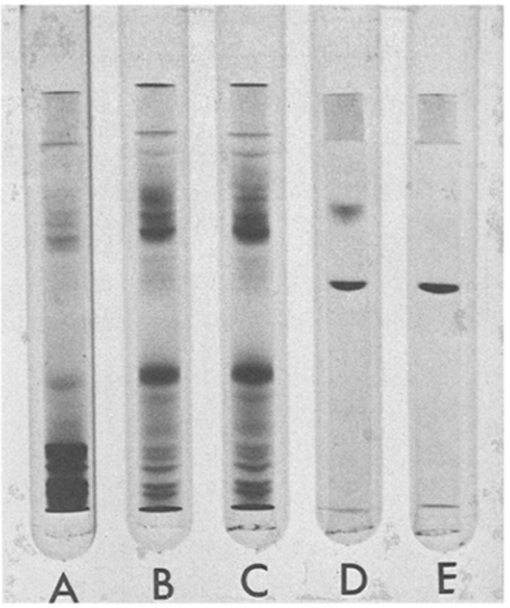

Figure 2. Tris-glycine gel electrophoresis of samples of A whole declotted hemolymph from Limulus polyphemus; B. high-speed supernatant of the hemolymph; C. high-speed supernatant after passage through the BSM affinity column; D. affinity-bound material eluted from the column, and E. activity peak material off the Biogel column (purified LPA) stained with Coomassie blue.

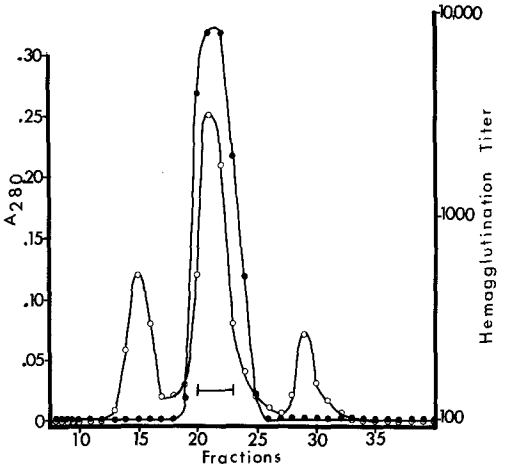

Figure 3. Chromatograph of BSM affinity purified LPA on Biogel A1. 5. The affinity purified LPA (16. 5 mg in 5 ml of Tris -NaCl-$CaCl_2$ buffer) was applied to a column (2. 5 x 80 cm) of Biogel A1. 5. Elution was performed with the Tris-NaCl-$CaCl_2$ buffer. Fractions of 10 ml were collected and analyzed for absorbance at 280 nm (—o—) and agglutinating activity (—•—).

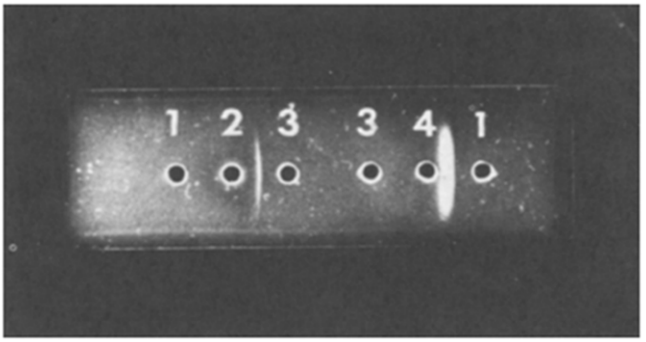

Figure 4. Immunodiffusion gel displaying the precipitin reactions of purified LPA (well 2) and soybean agglutinin (well 4) against BSM (well 3) and desialated BSM (well 1). The gel slide was prepared with agar containing 0.01 M $CaCl_2$.

hemolymph supernatant was passed through the BSM-Sepharose column. The profile of this column is shown in Fig. 1. After thoroughly washing the column with the Tris-NaCl-$CaCl_2$ buffer until the eluent showed no further absorbance at 280 nm, the column was then eluted with a Tris-NaCl-citrate buffer which caused the immediate release of a peak of 280 nm absorbing material. All the agglutinating activity of the high speed supernatant, which was bound to the column on the initial passage, was subsequently released in this eluted material. The pooled material from this peak showed a 140- and 750-fold increase in specific activity as compared to the high speed hemolymph supernatant and the whole hemolymph (see Table 1). The results of polyacrylamide gel electrophoresis of samples of whole hemolymph, high speed hemolymph supernatant, high speed supernatant after passage through the affinity column and the affinity-bound material eluted from the column as seen in Fig. 2 (A, B, C and D) further dramatize the many fold purification achieved. From Fig. 2 D it was obvious, however, that further purification was necessary. After the affinity-bound Tris-NaCl-citrate eluted fraction was dialyzed and concentrated it was chromatographed

on a Biogel A1. 5 column (Fig. 3). The major $A_{280\ nm}$ peak was found to contain all the agglutinating activity. Material from this peak migrated as a single band in gel electrophoresis (Fig. 2 E), showed the highest specific activity (Table 1), appeared as homogeneous molecules in the electron microscope and was able to give a precipitin reaction in immunodiffusion gels against BSM but not against desialated BSM (Fig. 4). The latter result contrasts sharply with the gel reaction of purified soybean agglutinin which shows a precipitin reaction against the desialated BSM but not against native BSM (11).

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