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ISOLATION OF AGGLUTININS FROM LYSINS IN EARTHWORM COELOMIC FLUID BY GEL FILTRATION FOLLOWED BY CHROMATOFOCUSING

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SUMMARY

Agglutinin, hemolysin and bacteriostatin activities have been detected in several fractions of earthworm coelomic fluid separated by gel filtration. Chromatofocusings performed on fractions A (pH gradient 7.4–4.5), B (pH gradient 7.6–5.0) and C (pH gradient 6.2–4.0) have yielded several single proteins characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and analytical isoelectric focusing. Four different molecules are capable of agglutination: MW 11,500 (p*I* 7.5); MW 20,000 (p*I* 4.8); MW 32,000 (p*I* 4.8) and MW 40,000 (p*I* 6.3). Three molecules have an hemolytic activity: MW 40,000 (p*I* 6.3); MW 40,000 (p*I* 5.9) and MW 45,000 (p*I* 6.0). The molecule having MW 40,000 (p*I* 6.3) appears to be a multivalent lytic protein also capable of agglutination.

INTRODUCTION

The coelomic fluid of the Lumbricidae *Eisenia fetida andrei* (*E.f. andrei*, Oligochaete) is known to possess a strong hemolytic activity[1,2]. Using erythrocytes stabilized by glutaraldehyde, an hemagglutinin activity has also been found in coelomic fluid[3]. This activity is different from the hemolytic one as revealed by its sugar dependence, low activity and heat stability. Moreover, a bacteriostatic activity, only directed against worm pathogenic bacteria such as *Bacillus megaterium* or *Aeromonas hydrophila*, has been reported[4]. An approach to the elucidation of the molecular systems involved in these various activities was attempted using chromatofocusing.

The present report focuses on the use of chromatofocusing to study the relationships between hemolysin and agglutinin protein systems and to isolate the active molecules for further biochemical analysis.

EXPERIMENTAL AND RESULTS

The coelomic fluid was harvested by electric stimulation[5] of more than 1000 *E.f. andrei* earthworms collected in farm manures. After centrifugation, pooled coelomic fluid was divided into several portions kept at −80°C. The various activities

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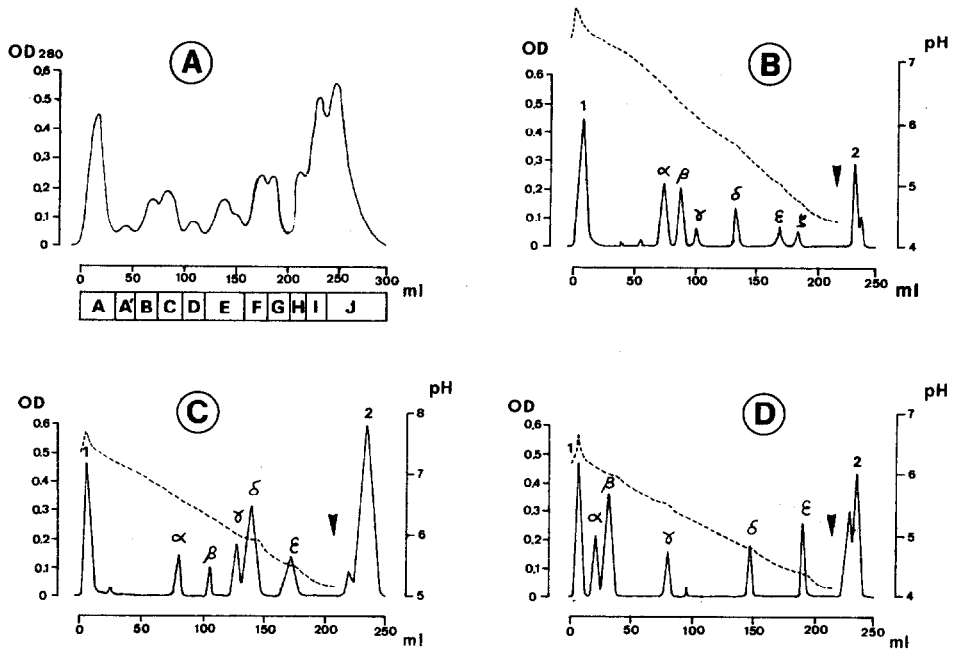

Fig. 1. Elution profiles of *Eisenia fetida andrei* coelomic fluid separated in gel filtration on Sephacryl S 200 (A) and in chromatofocusing of fractions A (B), B (C) and C (D) previously obtained in gel filtration. The solid line represents the protein absorption monitored at 280 nm. The dotted line corresponds to the pH gradient measured in fractions leaving the column. Arrows indicate addition of 2 *M* NaCl to the elution buffer to regenerate the column.

were tested as previously described for hemolysis[2], hemagglutination[3] and bacteriostatin[4].

The various fractions obtained by chromatography were pooled, concentrated and desalted by ultrafiltration then tested for biological activities and submitted to molecular analysis bo polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the fractions was also controlled by analytical flat bed isoelectric focusing (IEF).

Preparative gel filtration

As the first step of analysis, crude coelomic fluid was fractionated using gel filtration. After several attempts combining various gel matrices in various column sizes, the best results were obtained on a Sephacryl S 200 (Pharmacia) matrix in an optimized column of 1.6 × 116 cm. Equilibration and filtration were done with 0.05 *M* Tris–HCl and 0.5 *M* NaCl, pH 8.0. A volume of 9 ml undiluted coelomic fluid, previously centrifuged at 11,000 *g*, was applied to the column and subjected to filtration at 15 ml/h during 24 h. The elution profile monitored at 280 nm revealed the presence of eleven major fractions (Fig. 1A). Hemolytic activity has been detected among the B and C fractions (Table I). Hemagglutination principally occurred with fractions A and B, but was also found in A′ and C. Bacteriostatin activity against *Bacillus megaterium* is maximal in fractions A and B but was also found in H, I and J (Table I). Unfortunately, electrophoretic separation revealed that each gel filtration fraction contained many different proteins. The most intriguing question concerned

TABLE I

BIOLOGICAL ACTIVITIES DETECTED AMONG THE GEL FILTRATION FRACTIONS OF POOLED COELOMIC FLUID

A minus sign indicates no activity, a plus sign represents activity.

Fraction	*Hemolysis*	*Agglutination*	*Bacteriostatin*
A	–	++	++
A'	–	+	–
B	++	++	++
C	++	+	–
D	–	–	–
E	–	–	–
F	–	–	–
G	–	–	–
H	–	–	+
I	–	–	+
J	–	–	+

fraction B where all the three activities were present. One-step gel filtration, even under the best conditions according to sample composition, appeared to have insufficient resolution to reveal the molecular basis of each activity and to isolate them.

Chromatofocusing

The originality of this technique consists in its ability to separate proteins according to their isoelectric points[6]. The separation took place in a small chromatographic column (0.9 × 27 cm) filled with 17 ml of PBE 94 gel (Pharmacia) topped with 1 ml of Sephadex G-50 (Pharmacia). Packing and equilibration were carried out as previously described[7]. The sample, one fraction from gel filtration, was concentrated to 10 ml, equilibrated with starting buffer (see below) and eluted at 25 ml/h during 8 h with PB 74 (Pharmacia) adjusted to the appropriate pH with 1 *M* HCl. The pH and protein (monitored at 280 nm) profiles were recorded simultaneously.

Fraction A. The best separation was obtained with a pH gradient of 7.4–4.5: starting buffer 0.025 *M* imidazole–HCl, pH 7.4; elution with PB 74–HCl, pH 4.5. Eight different peaks were separated, evidencing the heterogeneity of the sample (Fig. 1B). Peak 1 corresponds to the elution of sample proteins with p*I* values heigher than the initial pH of the gel. The last peak or peak 2 represents the proteins with p*I* values lower than the final pH of elution. These proteins were recovered when the column was regenerated by increasing the ionic strength of the elution buffer with 2 *M* NaCl. After concentration and elimination of polybuffers by ultrafiltration, agglutinin activity was found in peak ζ. Electrophoretic analysis revealed that this peak contained one protein chain of apparent MW 32,000 and p*I* 4.8 (Table II). Based on UV absorption and Coomassie blue staining, this protein seems to be normally present at very low concentration in coelomic fluid.

Fraction B. Several pH gradient ranges were tested with fraction B which possessed all three activities. Fig. 1C gives a representative profile obtained with a pH gradient of 7.6–5.0, starting buffer 0.025 *M* imidazole–HCl, pH 7.6. Seven different peaks were eluted. Agglutinin activity was localized in peak 1 (one protein MW 11,500,

TABLE II

CHARACTERISTICS OF AGGLUTININ AND HEMOLYSIN ACTIVITIES AMONG CHROMATOFOCUSING PEAKS OF GEL FILTRATION FRACTIONS A, B AND C FROM *E.F. ANDREI* COELOMIC FLUID

Gel filtration fraction	*Chromatofocusing peak*	*Biological activity*	*Molecular weight*	*Isoelectric point*
A	ζ	Agglutinin	32,000	4.8
B	1	Agglutinin	11,500	7.5
	β	Agglutinin and Lysin	40,000	6.3
	γ	Lysin	45,000	6.0
	δ	Lysin	40,000	5.9
	2	Agglutinin	–	<5.2
C	1	Agglutinin and Lysin	–	>6.2
	α	Lysin	45,000	6.0
	β	Lysin	40,000	5.9
	δ	Agglutinin	20,000	4.8

p*I* 7.5), peak β (MW 40,000, p*I* 6.3) and peak 2 (several proteins, p*I* < 5.2) (Table II). Hemolysis occurred with peaks β, γ (MW 45,000, p*I* 6.0) and δ (MW 40,000, p*I* 5.9). Peaks β, γ and δ constitute the polymorphic *E.f. andrei* factor (EFAF) previously described as highly hemolytic[2]. According to the present results, the molecule having p*I* 6.3 seems to be a multivalent hemolytic protein also capable of agglutination when lysis is prevented, for instance by glutaraldehyde stabilizing the targets.

Fraction C. Fraction C from gel filtration only exhibited hemolysin and agglutinin activities (Table I). Upon chromatofocusing with a pH gradient of 6.2–4.0, starting buffer 0.025 *M* histidine–HCl, pH 6.2, it was resolved into seven peaks (Fig. 1D). The incomplete separation between gel filtration fractions B and C led to some overlaps between the two chromatofocusing elution profiles. Peak 1 exhibited both lytic and agglutinin activities (Table II), due to the presence of unseparated proteins with p*I* values higher than 6.2. Peaks α and β were identical to peaks γ and δ, respectively, of fraction B. Peak δ contained one protein of MW 20,000 (p*I* 4.8), only capable of agglutination. Regeneration of the column (peak 2) did not permit recovery of any activity.

CONCLUSIONS

Several chromatofocusing performed with gel filtration fractions have permitted clear definition of the two humoral systems naturally present in *E.f. andrei* coelomic fluid:

(1) an agglutinating system involving four different multivalent molecules of MW 11,500 (p*I* 7.5), 20,000 (p*I* 4.8) 32,000 (p*I* 4.8) and 40,000 (p*I* 6.3).

(2) a lytic system comprising three molecules of MW 40,000 (p*I* 6.3), 40,000 (p*I* 5.9) and 45,000 (p*I* 6.0). This system in fact comprises two molecules (MW 40,000 and 45,000) with a total of four isoforms (p*I* 5.9, 5.95, 6.0 and 6.3), only two or three of which are expressed in any animal[5]. One of these molecules (MW 40,000, p*I* 6.3) is also capable of agglutination when lysis is prevented.

The molecular composition of the bacteriostatic molecules not reported here is under investigation following the same protocol involving gel filtration fractions, A, B, H, I and J.

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