Journal of Chromatography, 240 (1982) 552–555
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,7ɔ7

Note

Rapid procedure for isolation of earthworm bacteriostatic factor isoforms using chromatofocusing

PHILIPPE G. ROCH* and NICOLE DAVANT

Centre de Morphologie Expérimentale du CNRS and Département de Biologie du Développement de l'Université de Bordeaux 1, Avenue des Facultés, 33405 Talence Cédex (France)

(Received January 21st, 1982)

The coelomic fluid of a Lumbricidae *Eisenia fetida andrei* (*E.f. andrei*) c. .tains a molecular product called *E.f. andrei* factor (EFAF), able to hemolyze various vertebrate erythrocytes[1] and to inhibit the growth of some telluric bacteria isolated from manure containing earthworms[2]. As demonstrated by injections, only the EFAF-sensitive bacteria were pathogeneous for the earthworms and rapidly killed the animals[3]. In analytical polyacrylamide gel electrophoresis (PAGE), which separates *E.f. andrei* coelomic fluid into 18 proteic components, EFAF appears as 2 different lipoproteins, with apparent molecular weights of 40.000 and 45.000 (ref. 4). In analytical isoelectric focusing (IEF), EFAF migrates as four different molecules referred to as isoforms characterized by their isoelectric points (p*I*), ranging from 5.9 to 6.3. All the animals possessed either two or three isoforms and among all the populations tested, only six different patterns have been found. Each pattern is genetically defined[5].

According to our present knowledge, although no invertebrates have developed true immunoglobulin, most of them possess humoral defense mechanisms. To understand the invertebrate defense system(s), as well as the phylogenetic evolution of the vertebrate immune system, it is of importance to investigate the biochemical characteristics of invertebrate humoral defense molecules. For that purpose, it is first necessary to isolate pure molecules in quantities compatible with biochemical studies. The present report is devoted to chromatofocusing[6], a preparative technique separating *E.f. andrei* coelomic fluid proteins in a chromatographic column according to their p*I* values.

EXPERIMENTAL

The coelomic fluid of at least 50 earthworms (Annelida, Lumbricidae, *Eisenia fetida andrei*) was harvested by electric stimulation of the worms[5]. After 10 min, 11,000 *g* centrifugation to remove cells, 10 ml of pooled coelomic fluid was filtrated on Ultrogel AcA-44 (LKB)[4]. The two fractions containing EFAF activity as assayed by hemolysis were pooled, then equilibrated with start buffer (0.025 *M* imidazole–HCl, pH 7.4) and concentrated to a volume of 10 ml by ultrafiltration on Amicon PM 10. Chromatofocusing was performed in a K 9-30 column (Pharmacia) filled with 19 ml of PBE 94 gel (Pharmacia) previously equilibrated with starting buffer and degassed. After 1 h packing with starting buffer (60 ml/h), 1 ml of Sephadex G 50 (Pharmacia)

0021-9673/82/0000–0000/$02.75 © 1982 Elsevier Scientific Publishing Company

was layered onto the top of the PBE 94 gel in order to prevent disturbance during sample application. The column was then equilibrated with starting buffer (60 ml/h, 5 h). The sample (10 ml in start buffer) was applied by first running 5 ml (25 ml/h) of eluent PB 74 (Pharmacia) diluted 1:8 with water and adjusted at pH 5.0 with 1 *M* hydrochloric acid, followed by applying the sample and then switching back to the eluent (25 ml/h, 8 h). In this way, the sample proteins were kept close to the physiological pH of the worm coelomic fluid.

RESULTS AND DISCUSSION

The elution profile obtained in chromatofocusing with a sample constituted by the two EFAF fractions of AcA-44 gel filtration of pooled *E.f. andrei* coelomic fluid is indicated in Fig. 1. The pH gradient of the eluate gradually decreased from 7.9 to 5.2. The slight shifting observed when compared to the selected pH gradient (7.4–5.0) was unexplainable but highly reproducible.

The protein diagram was determined by absorption at 280 nm. The first peak obtained corresponded to the elution of sample proteins with p*I* values higher than the gel pH (7.9). Since the eluent pH was not high enough to neutralize the charges of these proteins, they remained positively charged and did not bind to the ion exchanger which was also positively charged. These proteins were carried along in the eluent buffer and simply filtrated, leaving the column in the first 1–3 bed volumes of eluate.

The last peak, obtained when the column was regenerated with 2 *M* NaCl, corresponded to strongly bound proteins with p*I* values lower than the final pH of elution (5.2). Under the present experimental conditions, these proteins were negatively charged and bound to the gel matrix. As a pH corresponding to their p*I* was not used, they still remained bound. These proteins were removed instead by increasing

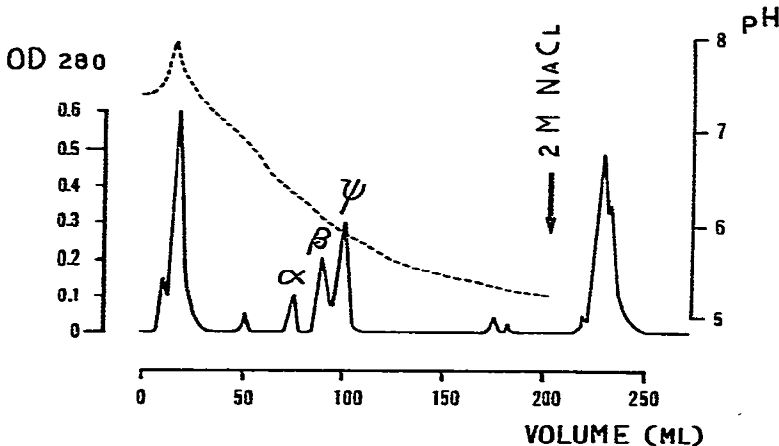

Fig. 1. Separation of the EFAF isoforms from the coelomic fluid by chromatofocusing. Column: K 9-30. Gel: 19 ml of PBE 94. Sample: 10 ml of hemolytic fractions obtained by gel filtration of 10 ml crude coelomic fluid. Elution conditions: Start buffer: 0.025 *M* imidazole–HCl, pH 7.4; Elution buffer: PB 74 adjusted to pH 5.0; Flow-rate: 25 ml/h. Elution profile measured by absorption at 280 nm (solid line) and pH gradient measured in fractions leaving the column (dotted line).

the ionic strength of the eluent with NaCl. Immediately after regeneration, the column was re-equilibrated with start buffer (60 ml/h, 5 h).

The peaks α, β and ψ were eluted respectively by pH values of 5.90, 6.15 and 6.30. The fractions corresponding to each peak were pooled, giving a volume of 12–15 ml that corresponded to 1.2–1.5 sample dilution. For each peak, the polybuffers contained in the eluate were eliminated by ultrafiltration on Amicon PM 10 and the peak volumes adjusted to the start sample volume. The purity of each peak was assayed by analytical IEF in flat-bed polyacrylamide gel containing 6 *M* urea according to the method previously described[5]. In a pH gradient of 5–8, the protein content of peak α focused as a single band of pH 6.30 (Fig. 2). This band corresponded to the isoform of p*I* 6.30 previously described in the hemolytic patterns A, C and E[5]. The 2 preparative steps considered in this paper, gel filtration and chromatofocusing, were sufficient to isolate this particular isoform in a pure form.

Peak β was constituted by two molecules of p*I* 6.00 and 6.30. As they were eluted as a single peak of p*I* 6.15, these two different isoforms must be associated in the pooled coelomic fluid used as sample. The presence of 6 *M* urea in the analytical IEF gel split the molecule into two fractions: one with a p*I* identical to the protein of peak α (6.30), the other with a p*I* of 6.00 (Fig. 2) which represented the EFAF isoform common to all the *E.f. andrei*[5].

Finally, peak ψ was also constituted by two different molecules corresponding to the EFAF isoforms of p*I* 5.90 and 5.95. With the protocol described above, these two isoforms have p*I* values too close to be separated. But according to the hemolytic patterns, some animals did not possess the p*I* 5.95 EFAF band and others did not possess the 5.90 band; thus, their coelomic fluid can be used as a sample to isolate the other band. Although they were characterized by close p*I*, the proteins of peaks β and

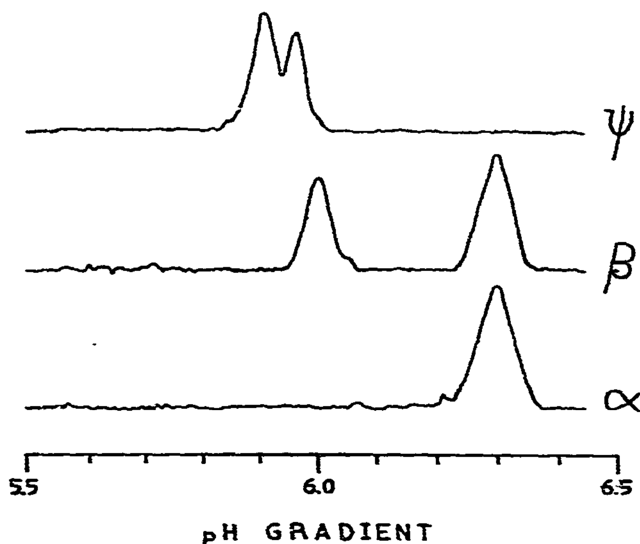

Fig. 2. Analytical flat-bed IEF of peaks α, β and ψ isolated in chromatofocusing. Densitometric scan of Coomassie Blue stained gel. Experimental conditions: pH gradient 5–8; Samples: 20 μl, salt free; Focusing: 3 h, 4°C, 10 W constant power.

ψ were different as evidenced by running a mixture of β and ψ fractions in analytical IEF.

Chromatofocusing performed with a sample containing all the hemolytic patterns led to the isolation of one isoform. Repeating the same protocol with a sample containing only one hemolytic pattern will (1) elucidate the *in vivo* relationships between the isoforms and (2) isolate the three other isoforms in a preparative way for further biochemical studies of one component of the invertebrate humoral defense system.

REFERENCES

1 L. Du Pasquier and P. Duprat, *C. R. Acad. Sci.*, 266 (1968) 538–541.
2 P. Valembois, Ph. Roch and M. Lassègues, *Invert. Pathol.*, in press.
3 M. Lassègues, Ph. Roch, P. Valembois and N. Davant, *C.R. Acad. Sci., Série III*, 292 (1981) 731–734.
4 Ph. Roch, P. Valembois, N. Davant and M. Lassègues, *Comp. Biochem. Physiol.*, 69 B (1981) 829–836.
5 Ph. Roch, *Dev. Comp. Immunol.*, 3 (1979) 599–608.
6 L. A. Æ. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17–30.