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CHEMOTACTIC ACTIVITY FROM RABBIT PERITONEAL NEUTROPHILS

LACK OF IDENTITY WITH *N*-ACETYL-DL-PHENYLALANINE β-NAPTHYL ESTERASE

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Summary

The chemotactic and *N*-acetyl-DL-phenylalanine β-naphthyl esterase activities of rabbit peritoneal neutrophils are separable from each other by both DEAE-cellulose and Sephadex G-100 column chromatography.

Partially purified esterase obtained from DEAE-cellulose chromatography had molecular weight of 70000. However, the partially purified fraction contained chemotactic activities with major activity in molecular weight of 28000 and minor activities in the molecular weights of 45000, 21900, 14500 and 10500. Esterase activity is inhibited by 10^{-7} M *p*-nitrophenylethyl-5-chloropentylphosphonate but chemotactic activity is not.

Introduction

Previous work has provided evidence that a proesterase capable of hydrolyzing *N*-acetyl-DL-phenylalanine β-naphthyl ester when activated is involved in the chemotactic response of rabbit neutrophils (reviewed in ref. 1). In the process of isolating the activated form of this esterase we found that the partially purified fraction of this esterase also had chemotactic activity [2]. It has been reported that chemotactic material is released by dead or viable neutrophils [3,4]. In order to clarify whether the esterase per se is chemotactic or due to chemotactic factor(s) other than the esterase present in that fraction, the present paper reports the separation of chemotactic activity from esterase activity.

Abbreviations: Ac-Phe-ONap, *N*-acetyl-DL-phenylalanine β-naphthyl ester; esterase, *N*-acetyl-DL-phenylalanine β-naphthyl esterase; 5-chloropentylphosphonate, *p*-nitrophenylethyl-5-chloropentylphosphonate.

Materials and Methods

Polymorphonuclear leukocytes. Rabbit peritoneal neutrophils were obtained 12 h after the injection of 0.1% glycogen into the peritoneal cavity as described previously [2]. The neutrophils used for enzyme purification and isolation of chemotactic factors were washed twice in 10 mM Tris · HCl buffer, pH 7.4, containing 0.15 M NaCl (Tris/NaCl buffer) then treated with 0.15 M NH_4Cl for 10 min at 21° C to lyse any contaminating red blood cells. After the treatment with NH_4Cl, the cells were washed twice with Tris/NaCl buffer. The washed cells were stored at −70° C until used.

Materials. *N*-Acetyl-DL-phenylalanine β-naphthyl ester (Ac-Phe-ONap), was obtained from Schwarz-Mann Research Laboratories (U.S.A.), Fast Scarlet diazonium salt GGN was purchased from GAF Corp., New York, U.S.A. The *p*-nitrophenylethyl-5-chloropentylphosphonate was the same as previously described [2]. For purposes of brevity, this will be named, in what follows, as 5-choropentylphosphonate. the presence of the *p*-nitrophenyl and ethoxy groups being implied. Dextran T-500 and aldolase were purchased from Pharmacia (Sweden). Bovine serum albumin, ovalbumin, α-casein, histone, and cytochrome *c* were obtained from Sigma (U.S.A.). Bovine α-globulin was purchased from Miles (U.S.A.). Hemoglobin was obtained from National Biochem (U.S.A.). α-Chymotrypsin was obtained from Worthington Biochemical Corp. (U.S.A.). DEAE-cellulose (DE-32) was obtained from Whatman (England). The bacterial chemotactic factor was a butanol extract of a culture filtrate of *Escherichia coli* prepared as previously described [5].

Assay for the esterase activity. The esterase activity was determined by measuring the release of naphthol by coupling it with the Fast Scarlet diazonium salt as described previously [2]. The orange red color that developed was measured at 485 nm [2]. The inhibition of the esterase activity by 5-chloropentylphosphonate was determined by incubating the enzyme solution with 10^{-7} M phosphonate for 5 min at 37° C before adding the substrate. One unit of esterase activity is defined as 1 nmol of naphthol produced per 20 min. Protein concentration was determined by the method of Lowry et al. [6] with bovine serum albumin as a standard.

Preparation of the esterase fraction. The esterase fraction was prepared as described previously [2]. About $1 \cdot 10^{10}$—$2 \cdot 10^{10}$ cells of frozen neutrophils obtained from 8 to 10 rabbits were thawed and homogenized with 20 ml of 10 mM Tris · HCl buffer, pH 7.4, at 0° C with teflon/glass homogenizer at a speed of 2250 rev./min for about 1-min. The completeness of cell breakdown was tested by microscopic observation. The homogenate was centrifuged at 27000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ to 55% saturation was added slowly with stirring to the supernatant fraction. After 30 min of stirring, the precipitate was collected by centrifugation at 16000 × *g* for 20 min and dissolved in 6% of the volume of the crude extract with 10 mM Tris · HCl buffer, pH 7.4. The resulting solution was dialyzed against 1 l of the same buffer with two changes of buffer during approx. 14 h. After dialysis, the solution was centrifuged at 27000 × *g* for 30 min, and the resulting precipitate was discarded. The enzyme solution was applied to a column of DEAE-cellulose (1.5 × 8 cm) which had been equilibrated with the same buffer. The flow rate was 1.25 ml

per min. The enzyme was eluted with 300 ml 10 mM Tris · HCl buffer, pH 7.4, containing a linear gradient of NaCl from 0 to 0.3 M. The eluates were collected in 5-ml fractions. The esterase activity was determined as described in the previous section. The peak fractions of esterase activity (tube Nos. 23—28, Fig. 1) were pooled.

Gel filtration on Sephadex G-100. The pooled fractions of peak esterase activity obtained by DEAE-cellulose chromatography were concentrated at 4°C to one-tenth volume in a Diaflow pressure filtration apparatus using PM-10 filter that retained substances of molecular weight greater than 10000. The concentrate was then fractionated on Sephadex G-100 column (1.5 × 92 cm) equilibrated with 10 mM Tris · HCl buffer, pH 7.4, containing 0.15 M NaCl. The flow rate was 0.4 ml/min. The eluates of the column were collected in 1-ml fractions. The column was calibrated with globular proteins of known molecular weights; cytochrome *c* (mol. wt. 12 500), α-chymotrypsin (22 500), ovalbumin (45000), bovine serum albumin (70000). The void volume was taken as the elution volume of blue dextran. Cytochrome *c* was detected by its absorbance at 420 nm. Other standards were detected by their absorbance at 280 nm. Molecular weights were calculated as described by Whitaker [7].

Chemotaxis assay. The chemotaxis assay was carried out in modified Boyden chamber with filters of 0.65 μm average pore size as described previously [2]. The washed peritoneal neutrophils in 0.1% bovine serum albumin were placed in the upper compartment of the chemotaxis chamber at a concentration of $2.5 \cdot 10^6$/ml. All the cells which migrated below the top monolayer were counted which were contained in the projection in the microscopic field of a square grid placed in the ×10 objective of the microscope. The total magnification was 400 times. The counts are reported as the sum of the counts of five such fields. Chemotactic activity is inhibited if the attractant is tested at too high a concentration [2,8]. It was therefore, necessary to assay several dilutions of each sample for the chemotactic activity.

The effect of 5-chloropentylphosphonate treatment on the chemotactic activity of the esterase preparation was tested by incubating the esterase with 10^{-7} M 5-chloropentylphosphonate at 37°C for 5 min and then placing the mixture in the bottom compartment of the chemotaxis chamber. Chemotaxis was then performed in the usual manner.

Results

Separation of chemotactic activity from 5-chloropentylphosphonate-sensitive esterase activity by DEAE-cellulose chromatography. In order to test whether the esterase is chemotactic or not, fractions from the DEAE-cellulose chromatography of the $(NH_4)_2SO_4$ precipitate of the extract of the neutrophils were assayed for esterase and chemotactic activity. Chemotactic activity was assayed at a 1 : 20 dilution in each fraction. Fig. 1 shows the elution profiles of esterase activity and chemotactic activity. It is evident that even though the chemotactic and esterase activities overlapped they were separable from each other.

Further separation of chemotactic activity from esterase activity by gel filtration on Sephadex G-100. In order to further confirm that the chemotactic

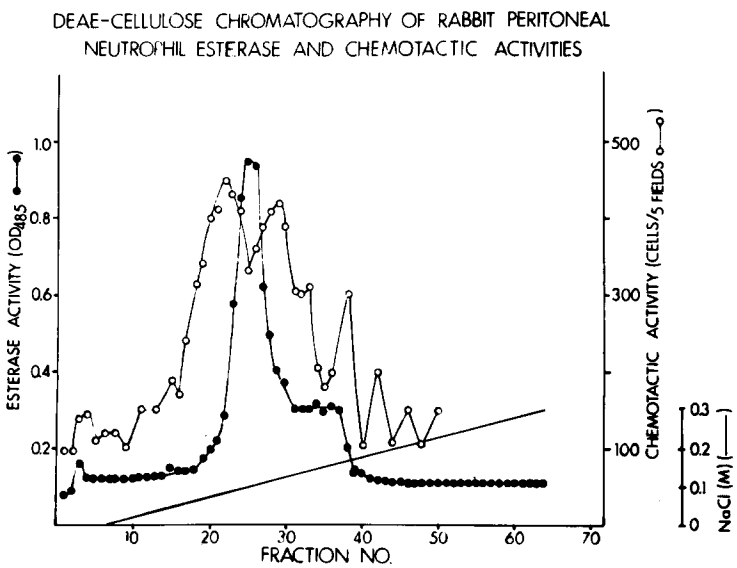

Fig. 1. DEAE-cellulose chromatography of rabbit peritoneal neutrophil esterase and chemotactic activities. 5 ml of the enzyme solution (12 mg of protein) obtained from an $(NH_4)_2SO_4$ precipitate of a 27000 × *g* supernatant was applied to a 1.5 × 8 cm column equilibrated with 10 mM Tris · HCl buffer, pH 7.4. Subsequently, the column was washed with 15 ml of the same buffer before eluting with 300 ml of this buffer containing a linear NaCl gradient from 0 to 0.3 M. Esterase and chemotactic activities were determined as described under Materials and Methods.

and esterase activities are separable, the esterase peak fractions obtained from DEAE-cellulose chromatography (Fig. 1, fraction Nos. 23—28) were pooled and concentrated in a Diaflow pressure filtration apparatus and applied to a Sephadex G-100 column. The gel filtration profiles of esterase and chemotactic activities are shown in Fig. 2. The chemotactic activity was clearly separated from esterase activity. The esterase activity eluted in the region of 70000 daltons the major chemotactic activity eluted in the molecular weight region of 28000 with minor peaks in the regions of 45000, 21900, 14500 and 10500.

Lack of sensitivity of chemotactic activity to 5-chloropentylphosphonate. One of the characteristics defining the specific esterase activity is that it is inhibitable by 10^{-7} M 5-chloropentylphosphonate or even lower concentrations. In order to know whether the isolated chemotactically active peaks were inhibitable by 10^{-7} M 5-chloropentylphosphonate, the major peaks of chemotactic activity eluted from DEAE-cellulose column or Sephadex G-100 were assayed for 5-chloropentylphosphonate inhibition. In order to eliminate acetone effect on chemotactic activity, the acetone concentration in 5-chloropentylphosphonate solution was diluted with Hank's balanced salt solution to 0.01% in the chemotaxis assay. No inhibitory effect of 10^{-7} M 5-chloropentylphosphonate on the chemotactic activity was found. Previously we reported that the chemotactic activity of the pooled esterase peak eluted from DEAE-cellulose column was inhibited by 10^{-7} M 5-chloropentylphosphonate [2]. The reason for the discrepancy between our previous findings and our present ones in this regard can be explained by the following points: (a) The present report was done in the isolated chemotactic activity peak and previous ones contain-

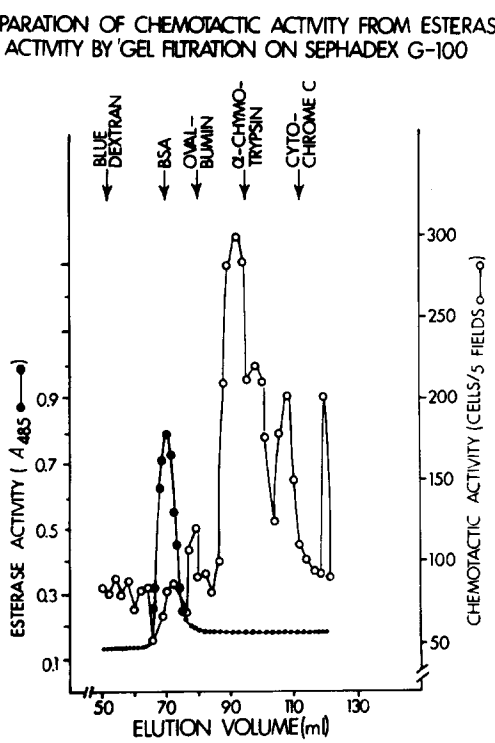

Fig. 2. Separation of chemotactic activity from esterase activity by gel filtration on Sephadex G-100. The esterase peak fractions (Fig. 1, fraction Nos. 23—28) obtained from DEAE-cellulose chromatography were pooled and was concentrated in a Diaflow pressure filtration apparatus as described under Materials and Methods. 1 ml of the concentrate (0.7 mg of protein) was applied on Sephadex G-100 column (1.5 X 92 cm) equilibrated with 10 mM Tris · HCl buffer, pH 7.4, containing 0.15 M NaCl. 1-ml fractions were collected. The flow rate was 0.4 ml per min. Esterase activity and chemotactic activity were determined as described under Materials and Methods except 1 h incubation was used in the esterase assay.

ing chemotactic activity and esterase activity. (b) The previous experiment 0.5% acetone was present with 5-chloropentylphosphonate, whereas, in the present assay acetone concentration was eliminated to 0.01%. (c) 0.5% acetone suppressed the chemotactic activity of isolated chemotactic fraction but not esterase activity or the activity of bacterial chemotactic factor. It appears acetone directly effect on chemotactic molecule(s). Without isolated chemotactic fraction this phenomenon could not be found.

Discussion

After DEAE-cellulose and Sephadex G-100 column chromatography, the esterase and chemotactic activities are clearly separated from each other (Figs. 1 and 2) indicating that the two activities are not associated with the same molecular population. Further separation of the two activities is indicated by the findings that the chemotactic activity was not inhibited by 10^{-7} M 5-chloropentylphosphonate, whereas, the esterase activity was completely abolished by the phosphonate.

The partially purified esterase obtained from DEAE-cellulose chromatogra-

phy had a molecular weight of 70000. However, the chemotactic activities isolated from same fraction had major activities in molecular weight of 28000 and minor activities in molecular weights of 45000, 21900, 14500 and 10500 (Fig. 2).

The basis for this molecular weight heterogeneity of chemotactic activity is unknown. It may be intrinsic to the rabbit neutrophil or a reflection of proteolytic degradation. Compatible with this latter suggestion, is the finding of proteolytic activity in the partially purified esterase obtained from DEAE-cellulose chromatography (Tsung, P.-K., unpublished results).

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References

1 Becker, E.L. and Henson, P.M. (1973) Adv. Immunol. 17, 94—193
2 Tsung, P.-K., Kegeles, S.W., Showell, H.J. and Becker, E.L. (1975) Biochim. Biophys. Acta 403, 98—105
3 Borel, J.F. (1970) Int. Arch. Allergy Appl. Immunol. 39, 247—271
4 Zigmond, S.H. and Hirsch, J.G. (1973) J. Exp. Med. 137, 387—410
5 Becker, E.L., Showell, H.J., Henson, P.M. and Hsu, L.S. (1974) J. Immunol. 112, 2047—2054
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Whitaker, J.R. (1963) Anal. Cem. 35, 1951—1954
8 Becker, E.L. (1972) J. Exp. Med. 135, 376—387