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ISOLATION OF AN *N*-ACETYL-DL-PHENYLALANINE β-NAPHTHYL ESTERASE FROM RABBIT PERITONEAL POLYMORPHONUCLEAR LEUKOCYTES

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Summary

An *N*-acetyl-DL-phenylalanine β-naphthyl esterase has been purified 26-fold from rabbit peritoneal polymorphonuclear leukocytes. The purified enzyme was inhibited by 10^{-7} M *p*-nitrophenylethyl-5-chloropentylphosphonate. The apparent K_m for hydrolysis of *N*-acetyl-DL-phenylalanine β-naphthyl ester is 71 μM. Optimal reaction rates were observed at pH 6—8. No divalent cation requirement for the activation of the enzyme activity was observed. The esterase activity was neither inhibited nor stimulated by bacterial factor, complement component C5a, guanosine 3′,5′-monophosphate (cyclic GMP) and adenosine 3′,5′-monophosphate (cyclic AMP) which are attractants or repellents for polymorphonuclear leukocytes. High chemotactic activity was observed in the partially purified fraction of the enzyme. The chemotactic activity, like the enzyme activity, was completely inhibited by 10^{-7} M phosphonate.

Introduction

The rabbit polymorphonuclear leukocyte (neutrophil, heterophil) contains at least three enzymes, esterases 1, 2 and 3, in the performed, already activated state capable of hydrolyzing *N*-acetyl-DL-phenylalanine β-naphthyl ester (Ac-Phe-ONap) [1]. These enzymes have been defined on the basis of their ability to be irreversably inhibited by *p*-nitrophenylethyl alkylphosphonates. Esterase 1 is inhibited when the enzyme is reacted for 15 min at room temperature with 10^{-7}—10^{-8} M or less of the alklylphosphonates; esterase 2 is not inhibited by 10^{-7} M alkylphosphonate under these circumstances but is

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; MES, 2-(*N*-morpholino)ethane sulfonic acid.

inhibited by 10^{-4}—10^{-5} M alkylphosphonate; whereas, esterase 3 is not inhibited by any obtainable concentration of alkylphosphonate.

Esterase 1 is of interest because a small fraction of this enzyme exists in or on the neutrophil in a phosphonate insusceptible, enzymatically inactive form, termed for convenience "proesterase 1". When the cell interacts with a variety of chemotactic factors, proesterase 1 is transformed into the phosphonate susceptible, enzymatically active, esterase 1. [2,3]. Activation of proesterase 1 is required for the chemotactic response of the rabbit neutrophil [2—4].

Proesterase 1 exists in the cell in a level of activity too low to make isolation possible. We are therefore attempting to isolate and purify the already activated esterase 1 as a necessary first step in learning the role of activated proesterase 1 in the chemotactic response. As part of this endeavor, this paper reports certain of the characteristics of the partially purified esterase 1, and demonstrates that the purified esterase possesses chemotactic activity which is inactivated by 10^{-7} M alkylphosphonate.

Materials and Methods

Polymorphonuclear leukocytes. Rabbit peritoneal neutrophils were obtained 4 h after the injection of 0.1% glycogen into the peritoneal cavity as described previously [5]. Female New Zealand white rabbits of approx. 3 kg weight were the source. The polymorphonuclear leukocytes to be used for the enzyme purification 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 red blood cells. After the treatment of NH_4Cl, the cells were washed twice with Tris/NaCl buffer. The polymorphonuclear leukocytes were stored in a freezer at —70°C before further handling.

Materials. 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. Cyclic AMP and cyclic GMP were purchased from Boehringer Mannheim, (Germany). *p*-Nitrophenylethyl-5-chloropentylphosphonate was the same as previously synthesized in the laboratories of one of us [5]. For purposes of brevity, this will be named, in what follows, as the 5-chloropentylphosphonate, 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 and cytochrome *c* were obtained from Sigma (U.S.A.). Trypsin was obtained from Worthington Biochemical Corp. (U.S.A.). DEAE-cellulose (DE-32) was obtained from Whatman (England). Complement component C5a was prepared by the digestion of highly purified human C5 with twice crystallized trypsin [6]. The bacterial chemotactic factor was a butanol extract of a culture filtrate of *Escherichia coli* prepared as previously described [7].

Assay for the esterase activity. The esterase activity was determined by measuring the release of naphthol after coupling with the fast Scarlet diazonium salt to develop an orange red color as described previously [1]. The standard assay mixture, unless otherwise indicated contained in a final volume of 0.5 ml: 10 mM Tris · HCl buffer, pH 7.4, 0.22 mM Ac-Phe-ONap and 1—70 μg

enzyme protein. The assay mixture was incubated at 37°C for 20 min, and the reaction was terminated by addition of 1 ml acetone. 0.1 ml of freshly prepared Fast Scarlet diazonium salt (2.3 mg/ml) was added to develop orange red color then read at 485 nm. 5-Chloropentylphosphonate inhibition of the esterase activity was assayed 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 naphtol produced per 20 min. Protein concentration was determined by the method of Lowry et al. [8] with bovine serum albumin as a standard.

Preparation of the esterase. All operations unless indicated otherwise were carried out at 0—4°C. About $1 \cdot 10^{10}$—$2 \cdot 10^{10}$ cells of frozen polymorphonuclear leukocytes 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 speed of 2250 rev./min for about 1 min. The completeness of cell breakdown was tested by microscopic observation. The homogenate was centrifuged at 27 000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was added slowly with stirring to the supernatant fraction to 55% saturation. After 30 min of stirring, the precipitate was collected by centrifugation at 16 000 × *g* for 20 min and dissolved in 6% of the crude extract volume of 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 27 000 × *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 (tube Nos 2 and 3, Fig. 1) eluted from the column before the NaCl gradient only contained the esterase activity insensitive to 10^{-7} M 5-chloropentylphosphonate, and will not be discussed. The peak fractions (tube Nos 17—20, Fig. 1) of the esterase activity were pooled and stored at —70°C until used. Except where otherwise indicated, this enzyme preparation was used for all studies reported here.

Chemotaxis assay. The chemotaxis assay was carried out in modified Boyden chamber with filters of 0.65 μm average pore size as described in previous work [9]. The cells in 0.1% bovine serum albumin were placed in the upper compartment of the chemotaxis chamber at a concentration of $2.0 \cdot 10^6$/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. The average standard deviation of the counts of replicate chambers is ±50 cells/five high power fields. In all cases the reported chemotactic activity was corrected for that of the blank (no attractant in the bottom compartment of the chamber).

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 attractant compartment of the chemotaxis chamber. Chemotaxis was then performed in the usual manner.

Results

Isolation of 5-chloropentylphosphonate-sensitive esterase by DEAE-cellulose chromatography. The elution profile of esterase activity from rabbit peritoneal polymorphonuclear leukocytes on DEAE-cellulose column is shown in Fig. 1.

Two peaks of esterase activity were found. The first which was not susceptible to inactivation by 10^{-7} M 5-chloropentylphosphonate eluted before the start of the gradient and was discarded. A second peak, eluted at a NaCl concentration of 0.07 M; about 95—100% of the esterase activity of this peak was inhibited by 10^{-7} M 5-chloropentylphosphonate. Table I summarizes the results obtained in the partial purification of the 5-chloropentylphosphonate-sensitive esterase. In the homogenate, 10% of total esterase activity was insensitive to 10^{-7} M 5-chloropentylphosphonate inhibitor, whereas the purified enzyme from DEAE-cellulose column was almost totally susceptible.

Kinetic results. The pooled fraction from the DEAE eluate was used to determine the kinetics and pH optimum of the enzyme. Under the conditions described above, the enzyme activity is linearly related to the incubation time up to 20 min and the rate of enzyme reaction is directly proportional to the amount of enzyme present in the range employed (1—70 μg of protein). Optimal rates were observed at pH 6—8 (Fig. 2). The apparent K_m for hydrolysis of Ac-Phe-ONap is 71 μM (Fig. 3).

Effect of divalent cations on the activity of the esterase. Addition of the divalent cations, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, a concentration of 10 mM caused no significant change in esterase activity. Fe^{2+}, Cu^{2+} strongly interfered

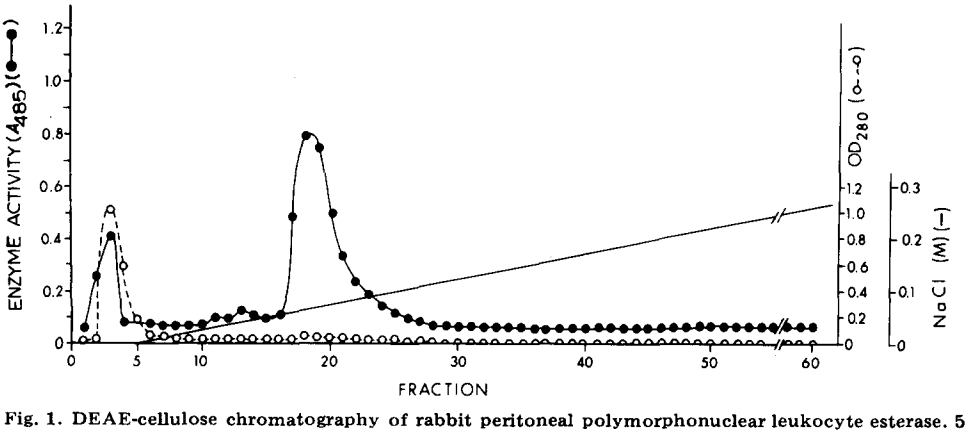

Fig. 1. DEAE-cellulose chromatography of rabbit peritoneal polymorphonuclear leukocyte esterase. 5 ml of the enzyme solution (18 mg of protein) obtained from an $(NH_4)_2SO_4$ precipitate of a 27 000 × *g* supernatant was applied to a 1.5 × 8 cm column equilibrated with 10 mM Tris · HCl buffer, pH 7.4. 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 activity was determined as described under Materials and Methods.

TABLE I

PURIFICATION OF 5-CHLOROPENTYLPHOSPHONATE-SENSITIVE ESTERASE FROM RABBIT PERITONEAL POLYMORPHONUCLEAR LEUKOCYTES

Esterase assay is described under Materials and Methods. Phosphonate inhibition assay: The enzyme solution was incubated with 10^{-7} M phosphonate at 37°C for 5 min before addition of the substrate. Unit of enzyme activity is expressed as nmol of β-naphthol produced per 20 min.

Fraction	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Inhibition by phosphonate (%)	Purification (-fold)	Yield (%)
Homogenate	262.5	273	71662	90	--	—
27000 × *g* supernatant	107.3	500	53650	86	1.8	75
$(NH_4)_2SO_4$ precipitate	22.2	1189	26396	96	4.4	37
DEAE-cellulose eluate	1.8	7222	13000	98	26.5	18

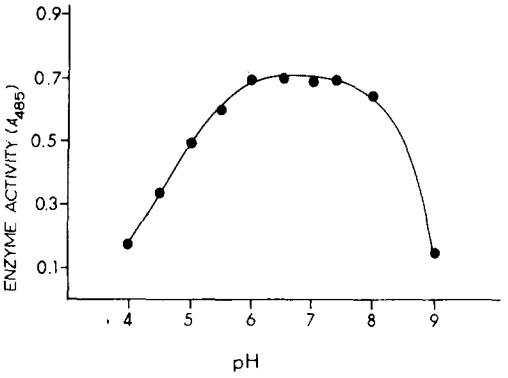

Fig. 2. pH dependence of the esterase of rabbit peritoneal polymorphonuclear leukocytes. Assays were performed as described under Materials and Methods except with respect to the buffer and pH. 10 μg protein of DEAE eluate was used. Buffers: 10 mM acetate buffer was used for pH 4.0—5.5, 10 mM MES buffer was used for pH 6.0—pH 7.0 and 10 mM Tris · HCl buffer was used for pH 7.2—9.

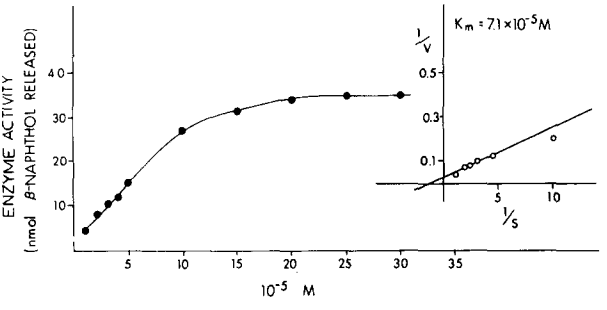

Fig. 3. Effect of substrate concentration on the esterase. Incubation conditions were as described in Materials and Methods except for the variation in concentration of Ac-Phe-ONap.

with the enzyme assay by inhibiting color development of the β-naphthol-diazonium salt.

Efffect of reducing agents. At concentrations higher than 0.1 mM ascorbic acid, cysteine, dithiothreitol and 2-mercaptoethanol strongly interfered with the enzyme assay by suppressing the color development. It was therefore hard to examine the effect of reducing agents on this enzyme.

Effect of bacterial chemotactic factor, C5a, cyclic AMP and cyclic GMP on the activity of the esterase. Cyclic GMP increases and cyclic AMP inhibits neutrophil chemotaxis [10]. Bacterial chemotactic factor and C5a are excellent attractants for neutrophil chemotaxis [3]. To determine whether these agents affect the purified 5-chloropentylphosphonate-sensitive esterase, their effects on esterase activity were examined at every purification step. No significant effect of bacterial chemotactic factor (1 : 10 to 10^4 dilution), C5a (2—200 μg per 0.5 ml, cyclic AMP and cyclic GMP (10^{-3}—10^{-9} M) on the esterase activity was observed.

Chemotactic activity of the esterase. As a first step toward determining whether the esterase is itself chemotactic, varying concentrations of the pooled DEAE-cellulose fraction containing the esterase were assayed for chemotactic activity. Such activity was found; the concentration of the esterase fraction giving optimum chemotactic activity was approx. 30 μg of protein/ml (Fig. 4). Doubling the concentration resulted in a decrease of chemotactic activity of about 50% and this could be decreased even more by increasing the enzyme concentration further (Fig. 4). The inhibition of chemotaxis found at a higher concentration is also seen with other chemotactic factors [11]. In order to get information whether esterase activity is essential for chemotactic activity the esterase was treated with 10^{-7} M 5-chloropentylphosphonate at 37°C for 5 min.

Table II shows that the esterase preparation had chemotactic activity which was comparable to that given by the known chemotactic factors, bacterial chemotactic factor and C5a. However, the esterase treated with 10^{-7} M

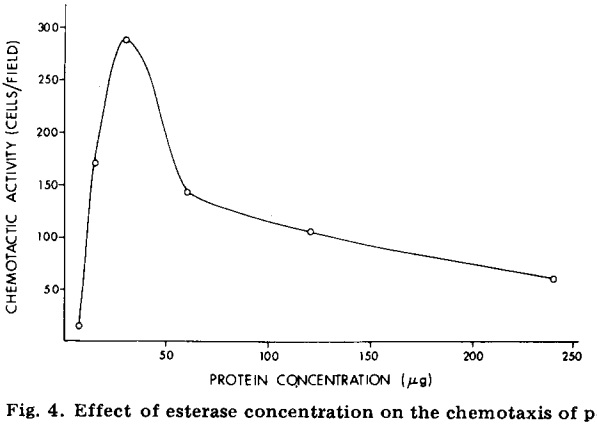

Fig. 4. Effect of esterase concentration on the chemotaxis of polymorphonuclear leukocytes. Assays were performed as described under Materials and Methods. The activity of the control (no esterase) has been subtracted from each value for chemotactic activity. The average activity of the control was 50 cells per five high power fields.

TABLE II

CHEMOTACTIC RESPONSE OF POLYMORPHONUCLEAR LEUKOCYTES TO THE ESTERASE

Chemotaxis assay is described under Materials and Methods. 30 μg of esterase, 100 μg of C5a and 1 : 4000 dilution of bacterial chemotactic factor were used. The esterase and 10^{-7} M 5-chloropentylphosphonate mixture was incubated at 37 °C for 5 min before being placed in an attractant chamber. Mean of three experiments. The deviation was ±50 cells per five high power fields.

Attractant	Chemotactic activity
Hank's medium	0
Esterase	238
Esterase + 10^{-7} M phosphonate	18
Bacterial chemotactic factor	332
Bacterial chemotactic factor + 10^{-7} M 5-chloropentylphosphonate	310
Bacterial chemotactic factor + esterase	556
C5a	176

5-chloropentylphosphonate lost essentially all of its chemotactic activity. As a control, it is seen that the same concentration of 5-chloropentylphosphonate had no effect on the bacterial factor. A purely additive effect was observed when bacterial factor and esterase were combined and tested for their chemotactic activity.

Discussion

This paper describes the partial purification and characterization of the esterase 1, an enzyme from rabbit polymorphonuclear leukocytes implicated in the chemotactic response of these cells. After isolation and partial purification the enzyme hydrolyzes acetyl-DL-phenylalanine β-naphthyl ester and is essentially totally inhibited by 10^{-7} M *p*-nitrophenylethyl-5-chloropentylphosphonate. The enzyme had a low K_m (71 μM) for Ac-Phe-ONap which is in accord with the suggestion from wholly indirect evidence that the esterase 1 is particularly active against aromatic amino acid derivatives [12]. In this respect, it is of interest that Ac-Phe-ONap was originally introduced as a chymotrypsin substrate [13].

The esterase activity is neither stimulated nor suppressed by cyclic AMP or by cyclic GMP suggesting that the role of these cyclic nucleotides in polymorphonuclear leukocyte movement does not involve their action on esterase 1. The lack of an affect of divalent cations on the esterase activity indicates that the requirements for external Ca^{2+} and Mg^{2+} for the chemotactic response [7] is not due to their effects on esterase 1. The inability of chemotactic factors to affect the esterase activity can be attributed either to the fact that the esterase is in the already activated state or to the possibility that the effect of chemotactic factors on the esterase in the cell is an indirect one.

The partially purified fractions are chemotactically active and this activity is essentially completely inhibitable by concentrations of 5-chloropentylphosphonate which completely inactivate the esterase activity. The evidence is presently insufficient to decide whether the chemotactic activity is due to the esterase 1 content of the partially purified fraction or to an impurity which is

also highly phosphonate susceptible. However, at the very least, it is probable from the phosphonate inhibitability that the chemotactic activity of the fraction is due to the enzymatic activity of some kind of esterase.

Borel [14] has reported that a cytoplasmic constituent released by dead neutrophils is chemotactic. In contrast, Zigmond and Hirsch [15] observed a chemotactic material released from viable neutrophils. There is insufficient information characterizing the activities reported by either group to attempt to relate them either to each other or to the chemotactic activity we have found.

Acknowledgement

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