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PARTIAL PURIFICATION AND CHARACTERIZATION OF A CHYMOTRYPSIN-LIKE ENZYME FROM HUMAN NEUTROPHIL LEUCOCYTES

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SUMMARY

A chymotrypsin-like enzyme with substrate specificity resembling pancreatic α-chymotrypsin was isolated in small quantities from human neutrophil leucocytes. The enzyme has activity against a variety of chymotrypsin substrates containing a tyrosine residue. It is inhibited by diisopropylphosphorofluoridate and by L-1-tosylamide-2-phenylethylchloromethylketone, suggesting the presence of serine and histidine in the active center. In addition the enzymatic activity is sensitive to the redox reagent 1,4-dithioerythritol. The behaviour of the enzyme in three different separation systems suggested that the molecular weight of the leucocyte enzyme and of its subunits obtained after treatment with 1,4-dithioerythritol is about twice as great as that of pancreatic α-chymotrypsin. The enzyme appears to be localised in similar granules to those containing peroxidase and arylsulfatase A.

INTRODUCTION

Proteolytic activity has been demonstrated in human leucocytes using casein [1], hemoglobin [1] and fibrin [2] as substrates. At least three distinct proteases with different pH optima are known to contribute to this activity. These enzymes, however, have not been characterized in detail. Using synthetic substrates, both trypsin [3] and chymotrypsin-like [4] activities have been described in human neutrophil leucocytes. A chymotrypsin-like enzyme has also been found in the granules of rat mast cells[5, 6].

In this paper, the partial purification and characterization of a chymotrypsin-like enzyme from human neutrophil leucocytes with more specific substrates and inhibitors than those previously reported [4], is described.

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; ATNA, *N*-acetyl-L-tyrosine-4-nitroanilide; BTEE, benzoyl-L-tyrosine ethyl ester; BTChI, *N*-benzoyl-L-tyrosine chloline ester iodide; SPNA, succinyl-L-phenylalanine-*p*-nitroanilide.

MATERIAL AND METHODS

Materials

Benzoyl-L-tyrosine ethyl ester (BTEE), *N*-acetyl-L-tyrosine ethyl ester (ATEE), diisopropylphosphorofluoridate and bovine pancreatic α-chymotrypsin were purchased from Fluka (Switzerland). *N*-Acetyl-L-tyrosine-4-nitroanilide (ATNA), 1,4-dithioerythreitol and sodium dodecylsulfate were purchased from Merck (Germany). *N*-Benzoyl-L-tyrosine choline ester iodide (BTChI) was a gift from Dr Rottenberg (GRD Labor, Wimmis, Switzerland). Succinyl-L-phenylalanine-*p*-nitroanilide (SPNA) was purchased from Boehringer Mannheim (Germany). L-1-Tosylamide-2-phenylethylchloromethylketone and 4-nitrocatechol sulfate were purchased from Sigma (U.S.A.). Dextran T-500 was purchased from Pharmacia (Sweden) and ^{32}P- and ^{3}H-labeled diisopropylphosphorofluoridate were obtained from Amersham (England).

Enzymatic assays

For the activity determinations of the leucocyte enzyme we have modified the BTEE-assay described by Hummel [7] as follows: 1.07 mM BTEE was dissolved in 2% dimethyl sulfoxide. The substrate solution was diluted 2.14-fold with 0.08 M Tris buffer (pH 7.2). $CaCl_2$ was not used. The rate of hydrolysis of BTEE at 25 °C was determined from the change in absorbance at 256 nm. Activity against BTChI was measured under identical conditions by replacing the BTEE dissolved in 2% dimethyl sulfoxide, with a solution of BTChI, 1.0 mM in 0.5 M KCl (Rottenberg, M., personal communication). The activity against ATNA was measured as described by Bundy [8], the activity against ATEE was measured as described by Vandermeers et al. [9] and the activity against SPNA was measured as described by Fritz et al. [10]. All reaction mixtures consisted of a total volume of 1 ml and the spectrophotometric measurements were performed in semi-micro quartz cuvettes using a Zeiss M4QIII recording spectrophotometer. Peroxidase was assayed as described by Bretz and Baggiolini [11]. Arylsulfatase A activity was determined by diluting the leucocyte extract 25-fold and using the method described by Baum et al. [12] for urine.

Polyacrylamide-gel electrophoresis

Electrophoresis at pH 4.5 was performed as described by Reisfeld et al. [13]. Electrophoresis in the presence of sodium dodecylsulfate was performed essentially as described by Laemmli [14] using a separating gel comprised of 15% acrylamide and 0.1% bisacrylamide. The gels were stained with Coomassie Blue as described by Fairbanks et al. [15]. The gels of the diisopropyl[^{32}P]phosphorofluoridate leucocyte enzyme were autoradiographed as described by Gordon [16] using Kodak PE 4006 X-ray film. In the case of diisopropyl[^{32}P]phosphorofluoridate pancreatic α-chymotrypsin the gel was sliced into 2-mm slices. Each slice was digested with 30% H_2O_2 at 55 °C, diluted with 10 ml Aquasol (New England Nuclear Co.) and measured in a Packard Tri-Carb scintillation counter. The protein concentrations were determined by the method of Lowry et al. [17].

Isolation of neutrophil leucocytes

Neutrophil leucocytes were prepared from fresh blood samples of healthy adult volunteers or from buffy coats of 1-day-old blood conserves (Courtesy of Dr A.

Hässig "Zentrallaboratorium", Swiss Red Cross, Berne). The blood was anticoagulated with 10% of a sodium citrate solution (3.64%). 10 ml of blood were mixed with 2 ml of a solution containing 6% dextran T500 [18, 19] in isotonic NaCl.

After storage for 15–20 min in an oblique position the red cells had aggregated and sedimented to the bottom of the tube. At this moment the leucocytes were still in the supernatant which was removed with a Pasteur pipette and centrifuged at 900 × *g* for 5 min. The pellet was resuspended in 2.5 ml of isotonic NaCl in 8-mm tubes and washed 3 times with isotonic NaCl between centrifugations for 5 min in a refrigerated centrifuge at 6 × *g*. Smears indicated that there were about 90% neutrophil leucocytes and very few eosinophils, basophils and monocytes. Lymphocytes were never more than 1%. The contamination with red cells was 0.5–5%. The smears did not contain platelets.

Enzyme preparation

The leucocyte pellet was resuspended in a small volume of isotonic NaCl (100 000–300 000 cells/mm^3) and homogenized in the cold using a glass–Teflon homogenizer (B. Braun Melsungen) at 1200 rev./min for 5 min. This treatment disrupted more than 80% of the total leucocytes. The intact leucocytes were counted before and after homogenization in a "Neubauer-chamber" after staining with Tuerk's solution.

The homogenate was then centrifuged at 350 × *g* during 10 min in order to remove the intact cells and nuclei. The pellet was resuspended, rehomogenized and centrifuged again. The leucocyte granules which stayed in the supernatant at 350 × *g* during 10 min were sedimented at 50 000 × *g* during 5 min. The granules which we obtained by this centrifugation were disrupted by the use of 1% Triton X-100 and sonication 3 times for 20 s using approx. 50 W in ice water. Membranes and other insoluble material was removed after the solubilization of the enzymes with an ultracentrifugation at 113 000 × *g* during 90 min. As marker enzymes of this granule population we have measured arylsulfatase A and peroxidase. Both enzymes had a similar sedimentation behaviour to the leucocyte chymotrypsin-like enzyme (Table I), thus suggesting a similar localization in granules. The described procedure was used for the preparation of the so-called leucocyte extract. This extract usually contained up to 90% of the total chymotrypsin-like activity, and was used for the characterization and further purification of the enzyme. When the leucocyte extract was not used immediately it was lyophilised and stored at −20 °C.

RESULTS

The leucocyte extract prepared as described contained enzyme activity against the chymotrypsin substrates: BTEE, ATEE and ATNA but no activity could be demonstrated against BTChI or SPNA. This activity occurred immediately in an active form and was not increased by the addition of trypsin. The activity against BTEE had a pH optimum of 7.2, as shown in Fig. 1, and was proportional to the amount of extract added, as shown in Fig. 2.

The Michaelis constant of the BTEE assay of the leucocyte enzyme was compared with that of bovine pancreatic α-chymotrypsin and to that of human pancreatic chymotrypsin which was obtained from duodenal juice. The results indicated a better

TABLE I

SEDIMENTATION BEHAVIOR OF GRANULAR LEUCOCYTE ENZYMES AS COMPARED WITH CHYMOTRYPSIN-LIKE ACTIVITY AFTER CENTRIFUGATION AT 50 000 × *g* FOR 5 min

After removal of the intact cells and nuclei, the leucocyte homogenate was centrifuged at 50 000 g during 5 min. The supernatant and pellet were sonicated 3 times for 20 s in ice water with approx. 50 W on a Sonifier B12 (Branson Sonic Power Co.) and Triton X-100 was added in a concentration of 1% in order to solubilize the enzymes. Insoluble material was removed with 113 000 *g* during 90 min.

Enzyme	Supernatant	Pellet
Chymotrypsin-like enzyme (μM BTEE split/min)	1.141 (10.3%)	9.975 (89.7%)
Arylsulfatase A (μM 4-nitrocatechol/min)	0.002 (0.8%)	0.252 (99.2%)
Peroxidase (μM H_2O_2 split/min)	9.92 (8.6%)	105.74 (91.4%)

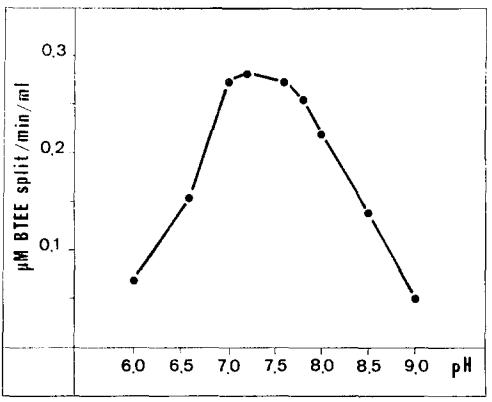

Fig. 1. pH optimum of the leucocyte enzyme. The pH was adjusted by adding different amounts of HCl to a solution of 0.08 M Tris. BTEE activity is expressed per ml of leucocyte enzyme purified by Sephadex G-100 gel filtration.

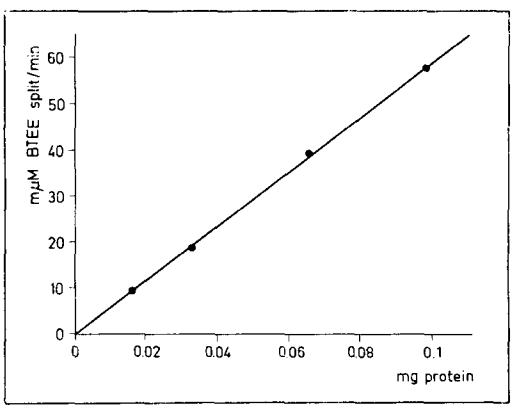

Fig. 2. Correlation of activity and amount of leucocyte extract. Aliquots of leucocyte enzyme were added to the reaction mixture of the BTEE assay. The total volume in the cuvette was 1 ml. ΔA was measured at 256 nm.

TABLE II

EFFECT OF INHIBITORS ON PANCREATIC α-CHYMOTRYPSIN AND LEUCOCYTE ENZYME

Diisopropylphosphorofluoridate $1 \cdot 10^{-4}$ M and 10% propylene glycol, L-1-tosylamide-2-phenylethylchloromethylketone $4 \cdot 10^{-4}$ M and 3% dimethyl sulfoxide, and 0.1 M 1.4-dithioerythreitol were incubated with the enzymes in the presence of Tris buffer (pH 7.2) at 25 °C. The leucocyte enzyme has been purified before by gel filtration on Sephadex G-100. Activity is expressed as nM BTEE split/min/per ml.

Enzyme	Inhibitor	Concn (mM)	Control value without inhibitor	5-min incubation	10-min incubation	15-min incubation	20-min incubation
Leucocyte enzyme	Diisopropylphosphoro fluoridate	0.1	116.90 (100%)	6.30 (5.4%)	3.50 (3%)	1.40 (1.2%)	0.70 (0.6%)
Bovine pancreatic α-chymotrypsin			71.40 (100%)	3.50 (4.9%)	3.50 (4.9%)	2.10 (2.9%)	0.70 (1%)
				1-h incubation	2-h incubation	3-h incubation	4-h incubation
Leucocyte enzyme	L-1-Tosylamide-2-phenyl-ethylchloromethylketone	0.4	78.05 (100%)	31.85 (40.8%)	18.90 (24.2%)	13.12 (16.8%)	5.78 (7.4%)
Bovine pancreatic α-chymotrypsin			77.0 (100%)	23.80 (30.9%)	11.20 (14.5%)	9.80 (12.7%)	6.30 (8.2%)
Leucocyte enzyme	1.4-Dithioerythreitol	100	96.05 (100%)	20.52 (21.4%)	14.74 (15.3%)	13.68 (14.2%)	7.37 (7.7%)
Bovine pancreatic α-chymotrypsin			147.06 (100%)	25.20 (17.1%)	16.80 (11.4%)	12.60 (8.6%)	6.72 (4.5%)

correspondence of the leucocyte enzyme to human chymotrypsin than to bovine. Leucocyte chymotrypsin-like enzyme K_m: $2.0 \cdot 10^{-3}$; human pancreatic chymotrypsin K_m: $1.25 \cdot 10^{-3}$; bovine pancreatic α-chymotrypsin K_m: $7.14 \cdot 10^{-4}$.

The effect of various inhibitors on pancreatic α-chymotrypsin and on the leucocyte enzyme are summarized in Table II. The two chymotrypsin-specific inhibitors [20–22], diisopropylphosphorofluoridate and L-1-tosylamide-2-phenylethylchloromethylketone inhibited the leucocyte enzyme, suggesting the presence of serine and histidine in the active center. The thiol reagent, 1,4-dithioerythreitol, also inhibited the leucocyte enzyme. With all three inhibitors studied, the inhibition occurred at a comparable rate for both leucocyte enzymes and bovine pancreatic α-chymotrypsin.

The leucocyte extract was fractionated by gel filtration on Sephadex G-100

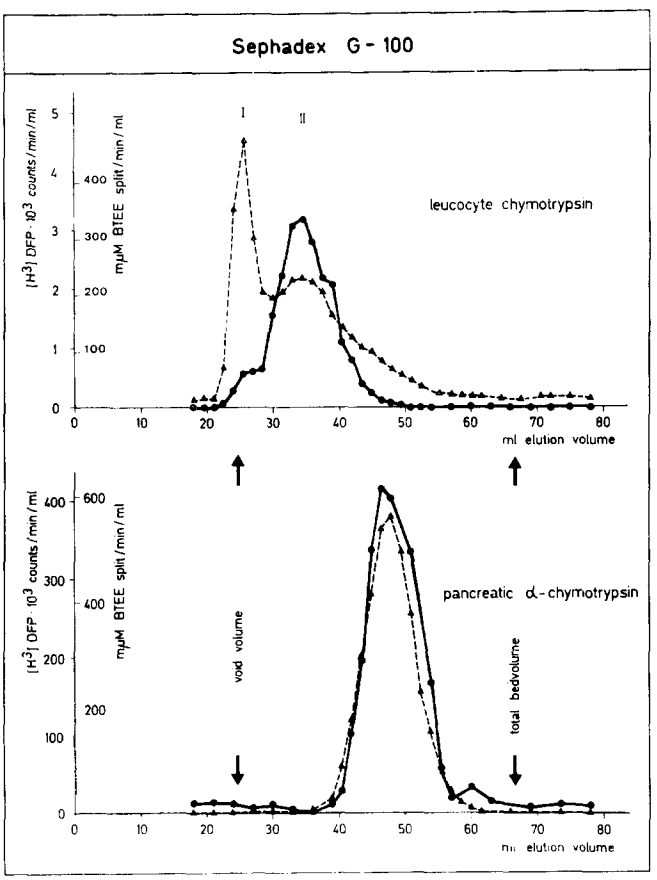

Fig. 3. Gel filtration on Sephadex G-100 of leucocyte extract and pancreatic α-chymotrypsin. The elution buffer was 0.05 M sodium citrate (pH 6.3), the flow rate was 20 ml/h and 1.5-ml fractions were collected. Aliquots were tested for activity in the BTEE assay (●—●). For the radioactivity experiments the leucocyte extract and pancreatic α-chymotrypsin were incubated with $1 \cdot 10^{-6}$ M (^{32}P- or ^{3}H-labeled) diisopropylphosphorofluoridate (DFP) for 1 h at pH 7.2, then exhaustively dialyzed against the elution buffer, before application onto the column. Aliquots of the fractions were mixed with 5 ml Aquasol and counted (▲---▲). The counts are expressed as dpm.

(Fig. 3). The enzymatic activity against BTEE eluted in one peak with an elution volume of 35 ml, whereas bovine pancreatic α-chymotrypsin had an elution volume of 47 ml. This difference in the elution volume suggested a molecular weight of 50 000–70 000.

The small part of activity eluted before the main peak probably represents an aggregation product of the enzyme, since a similar phenomenon was observed when human duodenal juice chymotrypsin was fractionated on the same column. 0.05 M sodium citrate elution buffer (pH 6.3) was chosen as the optimum for the stability of the enzyme and for the activity determinations.

When the leucocyte extract was incubated with radioactive diisopropylphosphofluoridate (^{3}H- or ^{32}P-labeled) to label the enzyme prior to fractionation, part of the protein-bound radioactivity eluted together with the chymotryptic activity. Peak I represents the radioactivity bound to excluded protein. This was again present when the top fractions of BTEE activity were combined, incubated with radioactive diisopropylphosphorofluoridate and fractionated in the same way. While the radioactivity Peak II shape is not identical with BTEE activity, there is a correspondence between the two maxima. The material in Peak II was concentrated by lyophilisation and analysed by polyacrylamide-gel electrophoresis at pH 4.5, and also in the presence of sodium dodecylsulfate at pH 8.8. In both systems the diisopropyl[^{32}P]phosphorofluoridate radioactivity coincided with the Coomassie Blue-stained material for pancreatic α-chymotrypsin.

From the electrophoresis at pH 4.5 (Fig. 4) it appears that the Peak II material contains one protein labeled with diisopropyl[^{32}P]phosphorofluoridate and that this protein migrates more slowly, in this system, than pancreatic α-chymotrypsin. Peak II

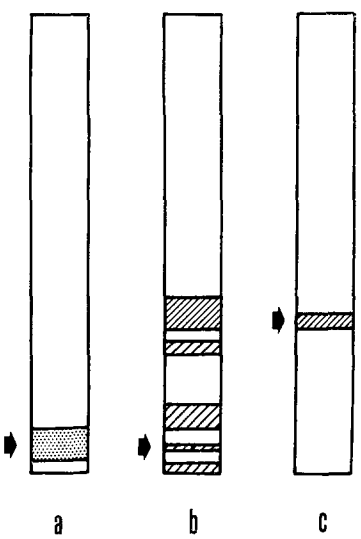

Fig. 4. pH 4.5 polyacrylamide electrophoresis of leucocyte enzyme and pancreatic α-chymotrypsin. The diisopropyl [^{32}P]phosphorofluoridate material of Peak II (fractions 33.0, 34.5, 36.0 ml), was concentrated and dialyzed against 0.05 M sodium citrate buffer (pH 6.3). The concentrated material was separated on 15% polyacrylamide at pH 4.5. Pancreatic α-chymotrypsin was treated identically. (a) Autoradiograph of (b) exposure time: 10 days Kodak PE 4006 X-ray film. (b) Peak II material stained for protein. (c) Pancreatic α-chymotrypsin stained for protein.

material was heated in the presence of sodium dodecylsulfate and 1,4-dithioerythritol in order to dissociate it into subunits and was subsequently subjected to electrophoresis in the presence of sodium dodecylsulfate. This allowed the separation of subunits on the basis of their molecular weights [22]. Two major and one minor protein bands were found to carry diisopropyl[^{32}P]phosphorofluoridate (Fig. 5). The

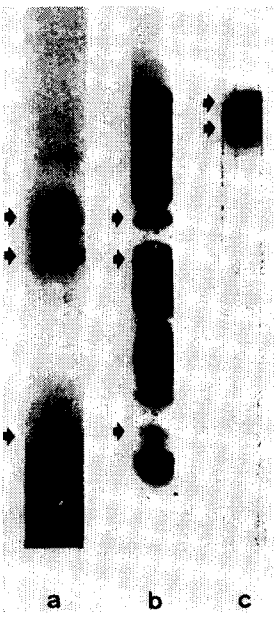

Fig. 5. Sodium dodecylsulfate polyacrylamide-gel electrophoresis of leucocyte enzyme and pancreatic α-chymotrypsin. The diisopropyl[^{32}P]phosphorofluoridate material from Peak II was concentrated and dialyzed as described in the legend to Fig. 4. This material was boiled in the presence of sodium dodecylsulfate (4%) and 1.4-dithioerythritol (0.1 M) and then electrophoresed on a 15% polyacrylamide gel in the presence of 0.1% sodium dodecylsulfate at pH 8.8. Pancreatic α-chymotrypsin was treated identically. The gels were stained for protein with 0.1% Coomassie Blue. (a) Autoradiograph of (b), exposure time: 10 days Kodak PE 4006 X-ray film. (b) Peak II material stained for protein. (c) Pancreatic α-chymotrypsin stained for protein.

two major bands had an apparent molecular weight of approx. 26 000 and 30 000, respectively. This was clearly larger than either of the subunits of pancreatic α-chymotrypsin which appeared with a mobility corresponding to molecular weights of 10 000–12 000. The minor labeled band in the Peak II material had a very large molecular weight (greater than 100 000).

DISCUSSION

This paper describes the partial purification and characterization of an enzyme which appears to be localized in granules of human neutrophil leucocytes. The enzyme has many features in common with pancreatic α-chymotrypsin. It hydrolyzes several tyrosine-containing synthetic substrates, indicating a similar specificity; its Michaelis constant has the same order of magnitude; it is inhibited by L-1-tosylamide-2-phenyl-

ethylchloromethylketone and by diisopropylphosphofluoridate suggesting a resemblance of the active center to that of pancreatic α-chymotrypsin. The enzyme is sensitive to 1,4-dithioerythritol indicating that disulfide bridges are essential for its activity. On the other hand, several properties distinguish the leucocyte enzyme from pancreatic chymotrypsin. It is unable to hydrolyze BTChI or SPNA, both of which are good substrates for the pancreatic enzyme. The pH optimum of 7.2 is clearly lower than the pH optimum of 8.0 of the pancreatic enzyme; the leucocyte enzyme elutes before pancreatic α-chymotrypsin on a Sephadex G-100 column; its electrophoretic migration at pH 4.5 is distinctly slower than the migration of pancreatic α-chymotrypsin; and finally the apparent molecular weight of the subunit carrying the active site of the leucocyte enzyme is at least twice as great as the molecular weight of the two subunits of the pancreatic enzyme. For these reasons we feel that the leucocyte enzyme is obviously different in some respects to the pancreatic enzyme. Experiments are in progress to determine the extent of the structural homology, if any, between the two enzymes. The lack of congruence of the radioactivity with activity in Peak II also deserves comment. It is probable that in addition to chymotrypsin other enzymes which bind diisopropylphosphorofluoridate are eluted in the neighbourhood of this peak. This contention would also explain the number of diisopropyl-[^{32}P]phosphorofluoridate containing subunits in the sodium dodecylsulfate gels.

The physiological function of the enzyme in leucocytes is not clear. However, the fact that it is apparently localized in granules makes it likely that this enzyme is involved in the digestion of phagocytosed material. Activity-enhancing experiments with trypsin gave no positive results, so that leucocyte chymotrypsin appears to be present in the cell in an active form, whereas pancreatic chymotrypsin is stored in the form of zymogen.

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