Biochimica et Biophysica Acta, 403 (1975) 477—492
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67614

A RAPID METHOD FOR PURIFICATION OF HUMAN GRANULOCYTE CATIONIC NEUTRAL PROTEASES: PURIFICATION AND CHARACTERIZATION OF HUMAN GRANULOCYTE CHYMOTRYPSIN-LIKE ENZYME*

G. FEINSTEIN** and A. JANOFF***

Department of Pathology, State University of New York, Stony Brook, N.Y. 11794 (U.S.A.)

(Received April 14th, 1975)

Summary

A chymotrypsin-like enzyme (EC 3.4.21.-) was purified from granules of human neutrophiles (polymorphonuclear leucocytes). The isolation procedure included differential salt extractions of the granules followed by affinity chromatography on 4-phenylbutylamine-Affi-Gel. This rapid purification method resulted in obtaining pure enzyme in relatively high yield in short time. The purified granulocyte chymotrypsin-like enzyme has a minimum M_r of 22 378, calculated from its amino acid composition. The M_r value obtained by sodium dodecyl sulphate gel electrophoresis was 20 000—23 000. The enzyme did not react with antibodies which are monospecific to granulocyte elastase. The granulocyte chymotrypsin-like enzyme was inactivated by Dip-F and by the chloromethyl ketone derivatives Z-PheCH_2Cl and Z-$(Gly)_2$-PheCH_2Cl but not by Tos-PheCH_2Cl. It therefore appears that the enzyme has serine and histidine side chains in its active site, like pancreatic chymotrypsin. The granulocyte enzyme substrate specificity is similar to that of pancreatic chymotrypsin, it

* Part of this work was previously reported in a preliminary communication (Feinstein, G. and Janoff, A. (1975) Fed. Proc. 34, 234).

** Permanent address: Department of Biochemistry, Tel Aviv University, Ramat-Aviv, Tel Aviv, Israel.

*** Address reprint requests to A. Janoff, Department of Pathology, State University of New York, Stony Brook, New York, U.S.A.

Abbreviations: *N*-*t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester, Boc-Ala-ONp; *N*-benzoyl-L-tyrosine ethyl ester, Bz-Tyr-OEt; *N*-benzoyl-L-leucine ethyl ester, Bz-Leu-OEt; *N*-*p*-tosyl-L-arginine methyl ester, Tos-Arg-OMe; *N*-acetyl-L-tyrosine-*p*-nitroanilide, Ac-Tyr-Nan; *N*-acetyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide, Ac-Ala_3-Nan; *N*-acetyl-DL-alanine-α-naphthyl ester, Ac-DL-Ala-1-ONap; *N*-acetyl-L-phenylalanine-α-naphthyl ester, Ac-Phe-1-ONap; *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone, Z-PheCH_2Cl; *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone, Ac-$(Ala)_2$-Pro-AlaCH_2Cl; *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, Tos-PheCH_2Cl; *N*-carbobenzoxy-glycyl-glycyl-L-phenylalanine chloromethyl ketone, Z-$(Gly)_2$-PheCH_2Cl; Diisopropylfluorophosphate, Dip-F; Human α_1-antitrypsin, α_1-AT; dimethyl sulfoxide, Me_2SO; 4-phenylbutylamine covalently linked to Affi-Gel, PhBuN-Affi-Gel.

being able to hydrolyze Bz-Tyr-OEt, Bz-Leu-OEt, Ac-Tyr-Nan and Ac-Phe-1-ONap. It also has an intrinsic weak hydrolytic activity towards some classical elastase substrates such as Boc-Ala-ONp and Ac-DL-Ala-1-ONap. The granulocyte enzyme is inhibited by human serum and by human α_1-antitrypsin. Its affinity for α_1-antitrypsin is weaker than that of granulocyte elastase for the same inhibitor. The enzyme is stable at neutral pH at 37° C, but unstable at pH 3.5 and at elevated temperature.

Introduction

Several cationic, neutral proteases including elastase-like [1,2] and chymotrypsin-like enzymes [3] have been identified in human neutrophilic granulocytes. Neutral proteases [4], including elastase [5], have been localized to the azurophilic (lysosomal) granules of these cells. Interest in these enzymes has been stimulated by mounting evidence suggesting their participation in a variety of physiologic and pathologic events in man [6,7]. Recently, multiple forms of elastase-like enzymes have been purified [8,9] and several chymotrypsin-like enzymes [10,11] have been partially purified from human neutrophils by a variety of complex techniques. More recently, Rindler-Ludwig and Braunsteiner reported the partial purification of leucocyte chymotrypsin-like enzyme by differential salt extraction followed by affinity chromatography on phenylbutylamine-Sepharose [12]. We now describe a similar, rapid method for purification of both cationic neutral enzymes (elastase and chymotrypsin-like enzyme) from saline extracts of neutrophil granules. The phenylbutylamine ligand was observed to bind both neutrophil elastase and chymotrypsin-like enzyme, so that variation of the eluting buffer conveniently permitted sequential isolation of both kinds of enzymes in a highly purified state. In addition to describing the purification method, data will be presented on physical-chemical, biochemical and immunochemical characterization of the enzymes. The present paper deals with the chymotrypsin-like enzyme, and the following paper [13] concerns the purification and further characterization of granulocyte elastases.

Experimental

Materials

Bovine α-chymotrypsin (lot CDS 1CA), soybean trypsin inhibitor and human α_1-antitrypsin (HAAT, lot 53 E 305) were obtained from Worthington Biochemical Corp., Freehold, New Jersey. Bovine trypsin (3 × cryst., lot 5632) and chicken lysozyme were from Nutritional Biochemicals Corp., Cleveland, Ohio. *N*-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt), *N*-*p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe) and diisopropylfluorophosphate (Dip-F) were obtained from Sigma Chemical Co., St. Louis, Missouri. *N*-*t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp) and *N*-benzoyl-L-leucine ethyl ester (Bz-Leu-OEt) were from Cyclo Chemical Corp., Los Angeles, California. *N*-*p*-Tosyl-L-phenylalanine chloromethyl ketone (Tos-PheCH$_2$Cl) was from Mann Research Laboratories, New York, New York and *N*-acetyl-DL-alanine-α-naphthyl ester

(Ac-DL-Ala-1-ONap) from Fox Chemical Company, Los Angeles, California. *N*-Acetyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (Ac-$(Ala)_3$-Nan) was from Miles Yeda, Rehovoth, Israel and *N*-acetyl-L-tyrosine-*p*-nitroanilide (Ac-Tyr-Nan) from E. Merck, Darmstadt, Germany. 4-Phenylbutylamine was from Aldrich Chemical Company, Milwaukee, Wisconsin and Affi-Gel 10 from Bio-Rad Lab., Richmond, California. The chloromethyl ketone derivatives, Z-PheCH_2Cl, Ac-$(Ala)_2$-Pro-AlaCH_2Cl and Z-$(Gly)_2$-PheCh_2Cl were a generous gift from Dr J. Powers, Georgia Institute of Technology, Atlanta, Georgia. *N*-Acetyl-L-phenylalanine-α-naphthyl ester (Ac-Phe-1-ONap) was a gift from Dr L. Ornstein, Mount Sinai School of Medicine, New York, New York.

Fresh whole human blood was obtained from Inter-County Blood Services, Rockville Centre, New York, a division of the New York Blood Center.

Methods

Preparation of leucocytes. Leucocytes were isolated from fresh whole human blood as described by Janoff and Scherer [1]. The white cells were collected by low speed centrifugation of supernatants obtained by dextran sedimentation of erythrocytes. Contaminating red cells were removed from the leucocyte pellets by hypotonic lysis. The final leucocyte pellets contained over 85% neutrophils. In general, 10—12 units of fresh whole blood were processed to a leucocyte pellet for each purification run.

Preparation of granules. As described before [1], the leucocytes were washed in 0.34 M sucrose and then disrupted by passage through a fine-mesh wire screen under suction. Degree of cell rupture was monitored by phase microscopy. Intact cells and debris were separated by low speed centrifugation and discarded, and the granules were then harvested by centrifugation at 19 000 × *g* for 20 min.

Extraction of granular proteins. The granules were suspended in a small volume (5—10 ml) of pH 7.5, 0.01 M sodium phosphate buffer containing 0.10 M NaCl. The suspension was subjected to freezing in dry ice/ethanol mixture and thawed to room temperature. The freezing and thawing were repeated six times. The suspension was then stirred in the cold for 30 min and was centrifuged for 10 min at 17 000 rev./min in RC2B Sorval ultracentrifuge, rotor SS-34. The supernatant was removed and the precipitate was subjected again to stirring with buffer and centrifugation as before. The last two operations, stirring and centrifugation, were repeated seven more times. Each supernatant was assayed for its enzymatic activities and, later, they were combined. The final pooled extracts constituted the low salt (0.10 M NaCl) granular extract that was used for elastase purification, as described in the following paper [13].

The pellet of washed granular membranes was then subjected to further extraction in the cold in pH 7.5, 0.01 M phosphate buffer containing 1.0 M NaCl. The extractions were repeated as before, except using 1.0 M NaCl containing buffer. After individual enzyme assays, these high salt granular extracts were combined and used for enzyme purification. The pool was first diluted with pH 7.5, 0.01 M phosphate buffer to bring the concentration of NaCl to 0.5 M. It was then concentrated by ultrafiltration through a UM-2 Diaflo mem-

brane (Amicon Corp.) to a volume of about 5 ml. After removing appropriate aliquots for subsequent analyses, the concentrated pool was put on PhBuN-Affi-Gel column for affinity chromatography.

PhBuN-Affi-Gel. One gram (6.7 mmol) of 4-phenylbutylamine was dissolved in 9 ml of ethanol, and 16 ml of pH 7, 0.05 M sodium phosphate buffer were then added to the ethanolic solution. The pH was adjusted to pH 7 with HCl and the solution was cooled to 4° C. The cooled solution was added to 1 g of Affi-Gel 10 (*N*-hydroxysuccinimide derivative of Bio-Gel A) and the suspension was shaken for 2 h at room temperature. The suspension was poured into a column, packed, and washed in the cold with about 500 ml of pH 7.5, 0.10 M phosphate buffer which contained 35% (v/v) ethanol. Washing was continued until the effluent had no more absorbance at 280 nm. The column was then washed with 1000 ml of pH 7.5, 0.01 M phosphate buffer containing 0.5 M NaCl and was ready for use. Regeneration, after the end of affinity chromatography, was accomplished by washing the column with 500 ml of pH 7.5, 0.01 M phosphate buffer containing 1 M NaCl and 20% Me_2SO (v/v) followed by equilibration with pH 7.5, 0.01 M phosphate buffer containing 0.5 M NaCl. The affinity chromatography was performed in the cold using pH 7.5, 0.01 M phosphate buffer containing 0.5 M NaCl.

Purification of chymotrypsin-like enzyme. The high salt (1 M NaCl) UM-2 concentrated granular extract was put on the PhBuN-Affi-Gel column and the column was washed with pH 7.5, 0.01 M phosphate buffer containing 0.5 M NaCl. The flow rate was 24 ml/h and 8-ml fractions were collected. Selected fractions (see Results) showing considerable Bz-Tyr-OEt hydrolytic activity were pooled and concentrated by ultrafiltration on UM-2 membrane. The concentrated enzyme solution was dialyzed overnight, with two changes, against a large volume of pH 7, 0.01 M phosphate buffer containing 0.3 M NaCl. After dialysis, the enzyme solution was lyophilized and stored at −80° C.

Enzymatic assays. All enzymatic assays were carried out at pH 7.5 in 0.10 M phosphate buffer at room temperature (20—22° C). A Gilford recording spectrophotometer (Model 2400-S) was used to determine enzymatic activities against various synthetic substrates. The hydrolysis of Bz-Tyr-OEt, in 5% Me_2SO, was followed at 256 nm according to Hummel [14] using an initial substrate concentration (S_o) of $5 \cdot 10^{-4}$ M. The hydrolysis of Bz-Leu-OEt in 5% Me_2SO, was followed at 245 nm according to Folk and Schirmer [15] using an initial $S_o = 5 \cdot 10^{-4}$ M. The hydrolysis of Boc-Ala-ONp, in 1% acetonitrile, was followed at 400 nm according to Visser and Blout [16] using an initial $S_o = 5 \cdot 10^{-4}$ M. Tos-Arg-OMe hydrolysis (initial $S_o = 1 \cdot 10^{-3}$ M) was determined according to Hummel [14] at 247 nm. Ac-$(Ala)_3$-Nan hydrolysis (10% Me_2SO, $S_o = 5.1 \cdot 10^{-4}$ M) was determined according to Feinstein et al. [17] at 410 nm. Ac-Tyr-Nan hydrolysis (5% Me_2SO, $S_o = 1 \cdot 10^{-3}$ M) was determined according to Bundy [18] at 410 nm.

Inhibition studies. Purified enzyme or crude granular extract were incubated for 10 min, at pH 7.5, with either human α_1-antitrypsin or with human serum. The residual enzymatic activities of non-inhibited enzyme were determined after the addition of substrate.

Inactivation studies. The inactivation studies with Dip-F were done at room temperature, pH 7, 0.10 M phosphate buffer, using $1 \cdot 10^{-3}$ M Dip-F (in

2% isopropanol). The inactivations with the chloromethyl ketone derivatives were done at room temperature, pH 7.5, 0.10 M phosphate buffer. Me_2SO concentration in all experiments and controls with the chloromethyl ketone derivatives was 20%.

Gel electrophoresis studies. Cationic acrylamide disc gel electrophoresis was carried out with 12% acrylamide according to Reisfeld et al. [19]. Proteins were stained in the gels using Buffalo Black (Amido Schwartz B). The staining of the active enzymes in the gels using Ac-Phe-1-ONap and Ac-DL-Ala-1-ONap was done according to Sweetman and Ornstein [20] with slight modifications. The gels, after completion of the electrophoresis, were stored overnight at −80°C and fixing and staining were done on the next day. Apparently freezing and thawing of the gels improved the subsequent penetration of substrates so that color developed quite rapidly (within 5—20 min) at room temperature after the substrate solutions were added to the gels.

Sodium dodecyl sulphate gel electrophoresis was carried out for estimation of molecular weight according to Fairbanks et al. [21]. 12% acrylamide gels containing 0.25% sodium dodecyl sulphate were used and all proteins (chymotrypsin-like enzyme as well as the markers) were first denatured by heating at 56°C for 90 min in 1% sodium dodecyl sulphate containing 0.04 M dithiothreitol and 0.001 M EDTA before layering onto the gel. Chicken lysozyme (M_r, 14 400) and soybean trypsin inhibitor (M_r, 21 500) were used as markers for calculating the molecular weight of the chymotrypsin-like enzyme.

Amino acid analysis. The amino acid composition of the chymotrypsin-like enzyme was determined on a Beckman 120C Amino Analyzer equipped with a computer integrator. Hydrolysis was carried out with 5.7 M HCl at 110°C for 24 and 48 h, after reduction with dithiothreitol.

Protein determination. The Lowry method [22] was used for protein determination using bovine serum albumin for calibration. The protein concentration of bovine α-chymotrypsin and trypsin was calculated from the absorbance at 280 nm. The following absorption coefficients ($A_{280}^{1\%}$) were used: trypsin, 15.4; chymotrypsin, 20.4.

Results and Discussion

Differential salt extraction of granules

A crude separation of elastase and chymotrypsin-like enzyme was first accomplished by differential salt extraction of leucocyte granules, as shown in Fig. 1. Taking advantage of the reported [3,10—12] poor solubility of the chymotrypsin-like enzyme in low ionic strength buffers, granules were first exhaustively extracted with 0.1 M NaCl in 0.01 M phosphate buffer (pH 7.5) as described in Methods, in order to remove the bulk of the elastase activity. As shown in Fig. 1, this could be accomplished while leaving a large fraction of the chymotrypsin-like activity still associated with granule membranes. Subsequent extraction of the latter with 1.0 M NaCl in 0.01 M phosphate buffer, pH 7.5 (see Methods) successfully released this residual chymotrypsin-like activity along with trace amounts of elastase (in comparison to the 0.1 M NaCl extracts). This can also be seen in Fig. 1. In addition, it is apparent from the figure that successive extractions with either 0.1 M or 1.0 M salt gave rise to

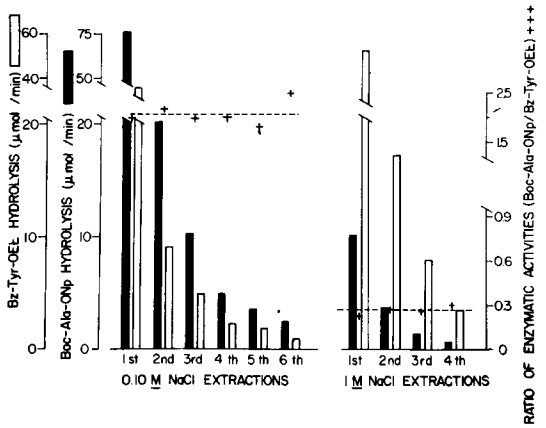

Fig. 1. Enzymatic activities in NaCl extraction of human leucocyte granules. See text for details.

constant ratios of enzyme activities at the two salt concentrations. The 1.0 M saline extracts were adjusted to 0.5 M NaCl final concentration and used as starting material for purification of the enzyme, which is to be described next. It should be noted that the small amount of chymotrypsin activity present in the 0.1 M saline extract was recovered during elastase-purification [13] and pooled with the 1.0 M NaCl extract for isolation of the chymotrypsin-like enzyme.

Affinity chromatography of 1.0 M NaCl granule extract on PhBuN-Affi-Gel

Fig. 2 represents a typical fractionation of 1.0 M NaCl granule extract on the PhBuN-Affi-Gel column. The operation of the column has already been described under Methods. Previous work has shown that PhBuN-Sepharose can be used to purify pancreatic chymotrypsin [23]. The major portion of the

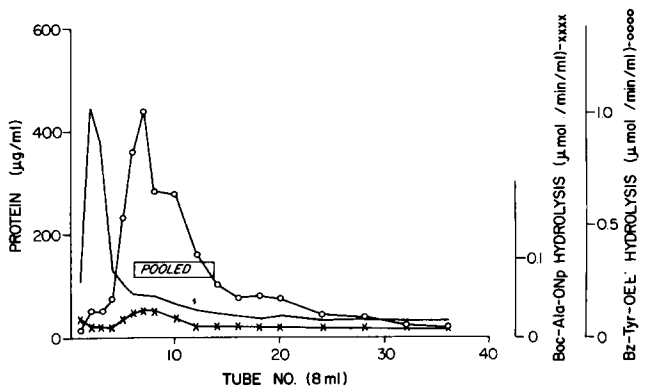

Fig. 2. Affinity chromatography of high salt (1.0 M NaCl) granular extract on PhBuN-Affi-Gel column. The PhBuN-Affi-Gel column (10 cm × 1.2 cm) was equilibrated and run in the cold with pH 7.5, 0.01 M sodium phosphate buffer containing 0.50 M NaCl. The sample was applied to the column in the same buffer. The flow rate was 24 ml/h. Aliquots of the collected fractions were analyzed for their protein concentration (Lowry method) and for hydrolysis of substrates. Bz-Tyr-OEt, o———o; Boc-Ala-ONp, x———x.

TABLE I

SUMMARY OF PURIFICATION OF GRANULAR CHYMOTRYPSIN-LIKE ENZYME

Six liters of blood containing approximately $2.7 \cdot 10^{10}$ PMN leucocytes were used to obtain granules for this purification.

Step	Protein (mg)	Bz-Tyr-OEt hydrolysis		Purification**
		Units*	% Yield	
1 M NaCl crude granular extract put on PhBuN-Affi-Gel column (Fig. 2)	10.1	60	100	1
Pooled fractions from column	n.d.	40.4	67	—
Lyophilized material	1.46	30.9	51	3.6

* One unit = one μmol Bz-Tyr-OEt hydrolyzed per min.
** See text for explanation.

starting protein was recovered in the run-through fractions (fractions No. 1—4 in Fig. 2). Thereafter, effluent protein appeared as a small "shoulder" (fractions 5—10) followed by a prolonged "tail" (fractions 12—24). Chymotrypsin-like activity against Bz-Tyr-OEt appeared in fractions 4—20, essentially corresponding to the "shoulder" and "tail" of effluent protein. Activity against Boc-Ala-ONp was also observed in these fractions (see Fig. 2). Subsequent analysis, to be described below, proved that the hydrolysis of Boc-Ala-ONp by these effluent fractions was due to the intrinsic esterolytic activity of leucocyte chymotrypsin-like enzyme against the substrate. As shown in the Fig., fractions 6—14 were pooled, concentrated, dialysed and lyophilized as described under Methods, and this material constituted the purified leucocyte chymotrypsin-like enzyme preparation. A total of four such preparations were obtained in the course of the studies reported here.

Table I summarizes the major steps involved in processing the 1.0 M NaCl extract of the granules to the final lyophilized preparation of purified enzyme. Percent recovery and degree of purification are given for each step. Several different preparative runs gave essentially similar results to those shown in Table I. It should be noted that the degree of purification at each step is based on the specific activity of the starting granule extract, but that the latter value already represents a several-hundred fold purification of enzyme from the original starting material (whole blood).

Electrophoretic monitoring of purification

Electrophoretograms of the starting granule extract (1.0 M salt) and of the purified chymotrypsin-like enzyme preparation obtained by affinity chromatography are shown in Fig. 3. A densitometry scan of these gels revealed that the bands of enzyme shown in Fig. 3 represented 90% of the total protein in the gel pattern. The chymotrypsin-like enzyme migrated rapidly towards the cathode (see Fig.) which is consistent with the M_r and the strongly basic charge previously reported for this leucocyte protease [3,10—12]. Other workers have observed the electrophoretic mobility of leucocyte chymotrypsin-like enzyme to be very similar to that of lysozyme [3,12]. A further discussion of the M_r of this enzyme is given below.

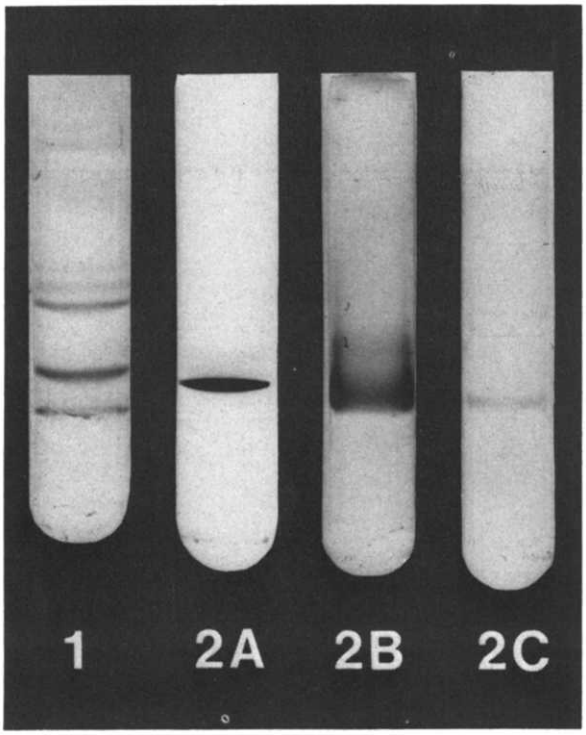

Fig. 3. Acrylamide disc gel electrophoretograms. High salt (1.0 M NaCl) crude granular extract (1) and granular chymotrypsin-like enzyme (2A—2C). Migration from top to bottom. 218 μg protein of crude extract, Buffalo Black stain (1); 39 μg of chymotrypsin-like enzyme Buffalo Black stain (2A); 11.7 μg of chymotrypsin-like enzyme, Ac-Phe-1-ONap stain (2B); 39 μg of chymotrypsin-like enzyme, Ac-DL-Ala-1-ONap stain (2C). See text for details.

Also shown in Fig. 3 are two zymograms of the pure chymotrypsin-like enzyme preparation, one of which (2B) was stained with the chymotrypsin substrate, Ac-Phe-1-ONap, and the other (2C) with the elastase substrate, Ac-DL-Ala-1-ONap, as described under Methods. The presence of strong chymotrypsin activity is evident in the former zymogram. The latter (a control gel stained with Ac-DL-Ala-1-ONap) failed to show any activity at the position usually occupied by elastase [8,13]. However, it is evident that chymotrypsin-like enzyme possessed weak activity against Ac-DL-Ala-1-ONap (Fig. 3, 2C). This result is not surprising in view of the ability of leucocyte chymotrypsin-like enzyme to hydrolyze Boc-Ala-ONp, another elastase substrate (see below). 11.7 μg of the enzyme preparation were present in the gel stained with Ac-Phe-1-ONap, while a considerably larger amount (39 μg) was present in the control gel stained with Ac-DL-Ala-1-ONap. This was done in order to favor the detection of any trace elastase contaminant which might have been present.

Stability of leucocyte chymotrypsin-like enzyme

Fig. 4 shows the effect of incubation at two different temperatures upon the stability of the leucocyte enzyme. Aliquots were removed at specified time intervals and tested for esterolytic activity using Bz-Tyr-OEt. Enzyme activity

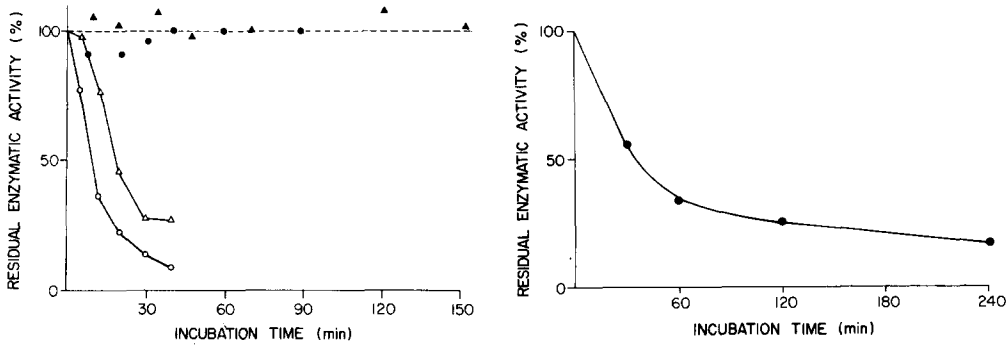

Fig. 4. The effect of temperature on the stability of purified granular chymotrypsin-like enzyme and on the same enzyme in crude extract. The incubations were carried out at pH 7.5 and the rate of Bz-Tyr-OEt hydrolysis was determined using aliquots. A sample was kept at 4°C and was used as a control. Purified enzyme, 37°C (▲———▲) and 58°C (△———△). Crude extract, 37°C (●———●) and 58°C (○———○).

Fig. 5. The effect of pH 3.5 on the stability of granular chymotrypsin-like enzyme in crude granular extract. Granular extract was diluted with pH 3.5, 0.05 M acetic acid containing 0.5 M NaCl and left at room temperature. The enzymatic activity was determined using Bz-Tyr-OEt as a substrate.

was unaffected during incubation at 37°C for 2½ h at pH 7.5, but was significantly depressed after incubation at 58°C for 30 min (pH 7.5). These results were obtained with both purified chymotrypsin-like enzyme and crude granule extract. The purified enzyme showed greater stability at 58°C compared to the stability of the enzyme in the crude mixture. The latter might be attributed to degradation of the enzyme by other proteases present in the crude extract. Control samples, incubated at pH 7.5 and 0°C, did not change in activity over the course of the experiment.

Fig. 5 shows the effect of acid pH on the stability of the leucocyte chymotrypsin-like enzyme. Crude granule extract (1.0 M NaCl) was incubated at pH 3.5 and 25°C for the times shown and activity of aliquots against Bz-Tyr-OEt was tested at specified intervals. Nearly half the starting activity was lost after 30 min of incubation, and a 75% loss occurred after two hours. Control samples, incubated at pH 7.5 and 25°C, did not change in activity over the course of the experiment. Thus, stability of the leucocyte chymotrypsin-like enzyme is favored at neutral pH and cold temperatures.

Inhibition of leucocyte chymotrypsin-like protease by human serum and human alpha 1-antitrypsin

Inhibition of leucocyte chymotrypsin-like enzyme and of bovine trypsin by α_1-AT is shown in Fig. 6. In this experiment, the molar concentration of leucocyte chymotrypsin-like enzyme used was about twice that of pancreatic trypsin. This calculation is based on an estimated M_r of 22 000 for the leucocyte enzyme. As Fig. 6 shows, 50% inhibition of trypsin required 7.5 μg of the purified α_1-AT whereas 50% inhibition of leucocyte enzyme required 40 μg of this same preparation. Thus, on an equimolar enzyme basis, nearly three times the amount of α_1-AT was needed to inhibit leucocyte enzyme as was needed to inhibit pancreatic trypsin.

Fig. 7 shows that whole human serum and purified α_1-AT both inhibit

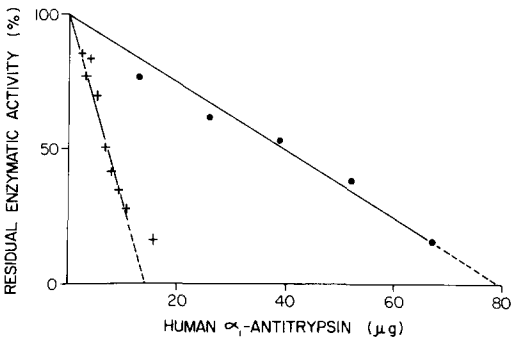

Fig. 6. The inhibition of granular chymotrypsin-like enzyme and of bovine pancreatic trypsin by human α_1-antitrypsin. The residual enzymatic activities of trypsin (1 μg, substrate = Tos-Arg-OMe, +——+) and of granular chymotrypsin-like enzyme (1.6 μg, substrate = Bz-Tyr-OEt, •——•) were determined after 10 min incubation with α_1-AT at room temperature and at pH 7.5.

elastase and chymotrypsin esterase activities of crude granule extracts (0.1 M NaCl), as has been reported previously by ourselves and other workers [1,2,9—11,24]. It is interesting to note that inhibition of leucocyte chymotrypsin-like activity by serum did not begin until 50% inhibition of the leucocyte elastase activity had occurred. Even more striking, inhibition of leucocyte chymotrypsin activity by purified α_1-AT was negligible until 100% inhibition of elastase had been achieved. Since the molar concentration of chymotrypsin-like enzyme is certainly not greater than that of elastase in the 0.1 M NaCl granule extracts (and very likely is much less), the results obtained with purified α_1-AT in both inhibition experiments (Figs 6 and 7) suggests that the latter inhibitor has a lower affinity for leucocyte chymotrypsin-like enzyme than it does for either leucocyte elastase or bovine trypsin. Qualitatively similar results were obtained with whole serum. These observations raise the possibility that leucocyte

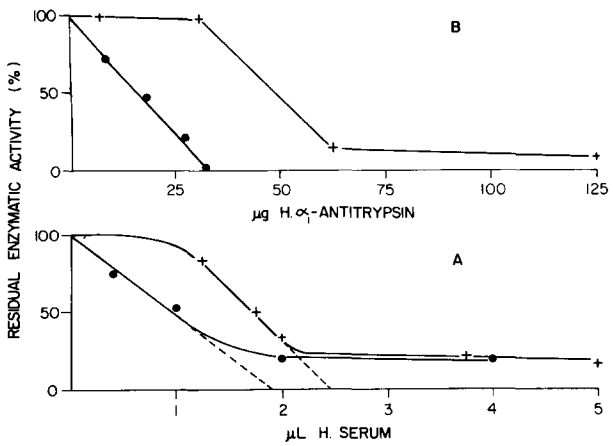

Fig. 7. The inhibition of granular enzymes by human serum (A) and by human α_1-antitrypsin (B). 0.1 M NaCl granular extract (1 μl) was incubated with either serum (A) or α_1-AT (B) at room temperature and at pH 7.5 for 10 min. The residual activity of elastase was assayed with Boc-Ala-ONp (•——•) and of chymotrypsin-like enzyme with Bz-Tyr-OEt (+——+).

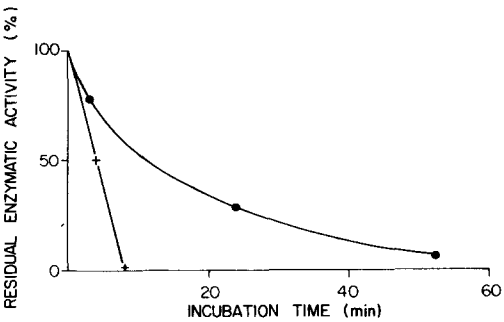

Fig. 8. Inactivation by Dip-F of granular chymotrypsin-like enzyme and of bovine α-chymotrypsin. The incubation of the enzymes with Dip-F ($1 \cdot 10^{-3}$ M) was carried out at pH 7.5 at room temperature. The residual activities were determined with Bz-Tyr-OEt as substrate. Granular chymotrypsin-like enzyme in crude granular extract, ●———●; bovine α-chymotrypsin, +———+.

chymotrypsin-like enzyme may be an important mediator of tissue injury, because of its relative resistance to serum proteinase inhibitors when the latter are operating in the vicinity of other leucocyte proteases.

Inactivation of leucocyte chymotrypsin-like enzyme by Dip-F and by various chloromethyl ketones

Fig. 8 shows that the chymotrypsin-like activity of crude granule extract upon Bz-Tyr-OEt was essentially completely inhibited after 1 h incubation of the extract with 10^{-3} M Dip-F at room temperature and neutral pH. The more rapid inactivation of pancreatic chymotrypsin, under similar conditions, is also shown in the figure.

Fig. 9 reveals that some, but not all, phenylalanine chloromethyl ketone derivatives inhibited the hydrolysis of Bz-Tyr-OEt by purified leucocyte enzyme. At equivalent molar concentrations, Z-PheCH$_2$Cl and Z-(Gly)$_2$-PheCH$_2$Cl inactivated about 50% of the chymotrypsin-like enzyme after 30—45 min of incubation. By contrast, the same molar concentration of Tos-PheCH$_2$Cl failed

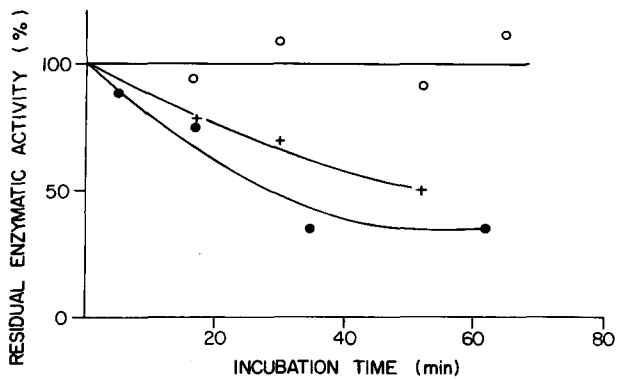

Fig. 9. The effect of chloromethyl ketone derivatives on Bz-Tyr-OEt hydrolysis by purified granular chymotrypsin-like enzyme. The concentrations of the chloromethyl ketone derivatives in the incubation mixture were as follows: Z-PheCH$_2$Cl, $2.2 \cdot 10^{-4}$ M (●———●); Z-(Gly)$_2$-PheCH$_2$Cl, $2.2 \cdot 10^{-4}$ M (+———+); Tos-PheCH$_2$Cl, $2.1 \cdot 10^{-4}$ M (○———○). For details see Methods.

to give any detectable inactivation after 1 h of incubation with the pure enzyme, whereas, in our studies, bovine α-chymotrypsin was 93% inactivated by this concentration ($1 \cdot 10^{-4}$ M) of Tos-PheCH$_2$Cl, which is a well established inactivator of bovine α-chymotrypsin [25].

Barrett (personal communication) has observed a lack of inactivation by Tos-PheCH$_2$Cl of the chymotrypsin-like enzyme he has purified from human spleen (cathepsin G). However, Gerber and co-workers [10] found inactivation of leucocyte chymotrypsin by Tos-PheCH$_2$Cl. We have no explanation for this discrepancy in results, although higher concentrations of Tos-PheCH$_2$Cl were used by the latter workers. Similarly, Rindler-Ludwig and Braunsteiner [12], who partly purified the leucocyte chymotrypsin-like enzyme using methods essentially identical to those described here, reported inhibition by Tos-PheCH$_2$Cl. However, the concentration of inactivator was 10-fold greater and the incubation with enzyme was considerably longer (20 h) in their experiments. Moreover, the enzyme studied by them was obtained from leukemic rather than normal PMN leucocytes, whereas the latter were the source of enzyme studied by us. It may be that the relatively large sulphonamidotosyl moiety in Tos-PheCH$_2$Cl sterically interferes with its interaction with leucocyte enzyme. The carbobenzoxy chloromethyl ketone derivatives of phenylalanine, which proved to be more active in our studies, would presumably not be similarly constrained.

Substrate specificity of leucocyte chymotrypsin-like enzyme

Human leucocyte chymotrypsin-like enzyme was found to have about 1/3 the specific activity of bovine pancreatic α-chymotrypsin when the two enzymes were tested on Bz-Tyr-OEt. Thus, the pancreatic enzyme hydrolyzed $21 \cdot 10^{-3}$ μmol of substrate/min/μg protein while the leucocyte enzyme hydrolyzed $7 \cdot 10^{-3}$ μmol/min/μg protein under identical assay conditions. Neither enzyme hydrolyzed Ac(Ala)$_3$Nan, which is hydrolyzed by porcine pancreatic [17] and by human leucocyte elastase (Feinstein, G. and Janoff, A., unpublished observations). The ability of the leucocyte chymotrypsin-like enzyme to hydrolyze several other synthetic substrates was also tested. It was found that, like bovine pancreatic chymotrypsin, it hydrolyzed Bz-Tyr-OEt faster than Bz-Leu-OEt, but unlike pancreatic chymotrypsin, it hydrolyzed Ac-Tyr-Nan at about the same rate as Bz-Leu-OEt. The latter substrate is hydrolyzed faster than Ac-Tyr-Nan by the pancreatic enzyme.

Hydrolysis of Boc-Ala-ONp by leucocyte chymotrypsin-like enzyme

Previously, one of us [26] found that pancreatic chymotrypsin was capable of hydrolyzing Boc-Ala-ONp at about 10% the rate given by partially purified leucocyte elastase when the enzymes were compared on an equal weight basis. In the course of the studies on leucocyte enzymes reported in this and the succeeding paper [13], it was observed that each enzyme had weak but detectable activity against the other's preferred N-acetyl amino acid alpha naphthyl ester substrate. Thus, the chymotrypsin-like enzyme was very active against Ac-Phe-1-ONap as expected, but was also weakly active against Ac-DL-Ala-1-ONap, while the reciprocal held true for the elastase. Some of these results are shown in the gel zymograms presented in this and the follow-

TABLE II

THE EFFECT OF CHLOROMETHYL KETONE DERIVATIVES ON ENZYMATIC ACTIVITIES OF CRUDE GRANULAR EXTRACT AND PURIFIED GRANULAR ELASTASE

The enzyme preparations (pure elastase and 0.10 M NaCl crude granular extract) were incubated at pH 7.5, room temperature, for 120 min, and then assayed for their activities. The concentrations were $2.7 \cdot 10^{-4}$ M and $3 \cdot 10^{-4}$ M respectively for Z-PheCH_2Cl and Ac-$(Ala)_2$-Pro-AlaCH_2Cl in the incubation mixtures.

Enzyme preparation	Chloromethyl ketone		% Activity	
	Z-PheCH_2Cl	Ac-$(Ala)_2$-Pro-AlaCh_2Cl	Boc-Ala-ONp hydrolysis	Bz-Tyr-OEt hydrolysis
Elastase	–	–	100	—
Elastase	+	–	100	—
Crude extract	–	–	100	100
Crude extract	+	–	83	11
Crude extract	–	+	10	110
Crude extract	+	+	1	11

ing paper [13]. For these reasons, it was suspected that leucocyte chymotrypsin-like enzyme might also have activity against Boc-Ala-ONp which would largely account for the hydrolysis of that substrate by fractions of the PhBuN-Affi-Gel effluent showing Bz-Tyr-OEt esterase activity (see Fig. 2). Such fractions failed to hydrolyze Ac-$(Ala)_3$-Nan, suggesting that their Boc-Ala-ONp esterolytic activity was not due to any elastase contaminant. Experiments were therefore performed using specific chloromethyl ketone inactivators, and the results are summarized in Table II. As can be seen in the Table, the chymotrypsin inactivator, Z-PheCH_2Cl, did not inhibit hydrolysis of Boc-Ala-ONp by purified leucocyte elastase but did partially inhibit the hydrolysis of Boc-Ala-ONp by crude granule extract suggesting that granular chymotrypsin-like enzyme could hydrolyze this substrate. Moreover, the elastase inactivator, Ac-$(Ala)_2$-Pro-AlaCH_2Cl was unable to completely suppress hydrolysis of BOC-Ala-ONp by crude granule extract, but this suppression was rendered complete when Ac-$(Ala)_2$-Pro-AlaCH_2Cl was combined with Z-PheCH_2Cl. This result supported the suggestion that granule chymotrypsin-like enzyme is also capable of hydrolyzing Boc-Ala-ONp. Ac$(Ala)_2$-Pro-AlaCH_2Cl alone had no effect on chymotryptic-like activity, as seen from its failure to inhibit hydrolysis of Bz-Tyr-OEt by crude granule extract, and from its failure to enhance the Z-PheCH_2Cl induced suppression of this hydrolysis (Table II).

From all of the foregoing data, we conclude that leucocyte chymotrypsin-like enzyme has intrinsic esterolytic activity against Boc-Ala-ONp. Some of the apparent elastase activity of the 1.0 M salt extracts of human leucocyte granules may have been due to the relatively high concentration of chymotrypsin-like enzyme in these extracts, although some elastase was doubtless also present (see Fig. 1).

M_r estimation of purified chymotrypsin-like enzyme

Pure leucocyte enzyme was denatured by heating in 1% sodium dodecyl sulphate containing 0.04 M dithiothreitol and 0.001 M EDTA and was then electrophoresed in 12% polyacrylamide gels containing 0.25% sodium dodecyl

sulphate. Egg white lysozyme (M_r = 14 400) and soybean trypsin inhibitor (M_r = 21 500) were used as marker proteins to calibrate the gels. The M_r of the leucocyte enzyme was tentatively estimated by this procedure to be 20 000—23 000. Reduction of disulfide bonds by dithiothreitol did not result in a lower M_r than predicted from routine electrophoretic analysis, suggesting that the enzyme is a single polypeptide chain.

Amino acid analysis of purified chymotrypsin-like enzyme

Table III gives the amino acid composition determined for the purified enzyme. The amino acid histidine gave identical values after 24 and 48 h of hydrolysis. For this reason, and because it was one of the amino acids present in the lowest molar quantity, it was used as a basis for computing the amino acid composition. The choice of five histidine residues in this computation was preferred, since this gave a value most comparable to the results obtained by sodium dodecyl sulphate gel electrophoresis. On the basis of these data, an estimated minimal M_r = 22 378 was calculated. This value is in reasonably close agreement with the estimated M_r derived from sodium dodecyl sulphate electrophoresis above. The actual M_r of this enzyme may be slightly higher, due to the fact that ammonia, tryptophan and carbohydrate were not measured in our

TABLE III

AMINO ACID ANALYSIS AND COMPOSITION OF HUMAN GRANULOCYTE CHYMOTRYPSIN-LIKE ENZYME AND OF PANCREATIC CHYMOTRYPSIN

The amino acid composition of granulocyte chymotrypsin-like enzyme was calculated assuming 5 mol of histidine residues per mol protein (see text for explanation). Data for human pancreatic chymotrypsin are taken from Feinstein et al. [27] and those for bovine chymotrypsin are taken from Hartley and Kaufman [28].

Amino acid	nmol in analysis	mol/mol		
		Granulocyte chymotrypsin-like enzyme	Pancreatic chymotrypsin	
			Human	Bovine
Lysine	31.9	5	13	14
Histidine	32.5	5	3	2
Arginine	118.0	18	5	4
Aspartic acid	131.5	20	22	23
Threonine	84.5	13	17	23
Serine	116.4	18	20	28
Glutamic acid	156.2	24	15	15
Proline	54.2	8	13	9
Glycine	135.5	21	21	23
Alanine	85.4	13	20	22
Half-cysteine	22.6	4	8	10
Valine	69.1	11	21	23
Methionine	21.5	3	2	2
Isoleucine	53.2	9	10	10
Leucine	90.0	14	16	19
Tyrosine	28.8	5	2	4
Phenylalanine	36.3	6	6	6
Tryptophan	n.d.	n.d.	6	8
Total		197	220	245

studies. The mole percentage of basic amino acids in granulocyte chymotrypsin-like enzyme is 14.2% compared with 8.2% and 9.5% for pancreatic bovine and human chymotrypsins, respectively. A large amount of ammonia was released during the acidic hydrolysis of the granulocyte protease indicating that most of the acidic amino acids are present in the protein in their amide form, namely asparagine and glutamine. The relatively high proportions of basic amino acids and of amides explains the very high basicity of the enzyme. The strongly basic character of this enzyme could help to account for its relatively poor solubility when granules were first extracted in low ionic strength buffer, and its enhanced recovery in higher ionic strength buffer (1.0 M NaCl). Also, the rapid migration of leucocyte chymotrypsin-like enzyme in cationic electrophoresis (its mobility is close to that of lysozyme) is compatible with this interpretation of the amino acid composition.

Immunochemical analysis of purified chymotrypsin-like enzyme

Purified chymotrypsin-like enzyme failed to cross-react with rabbit antiserum raised against purified leucocyte elastase (see Fig. 6 in the following paper). Antiserum to purified chymotrypsin-like enzyme was not yet available at the time this paper was written.

In conclusion, the foregoing results show that chymotrypsin-like enzyme can be purified from human neutrophil leucocyte granules by differential salt extraction followed by affinity chromatography on PhBuN-Affi-Gel. The entire procedure represents essentially a single step. Isolation and characterization of this enzyme may lead to further knowledge of the action of cationic, neutral proteases in the physiological and pathological functions of human polymorphonuclear leucocytes.

Acknowledgements

We would like to thank the New York Blood Center for their cooperation in supplying human blood for the collection of leucocytes. We also thank James C. Powers and Peter M. Tuhy for generously supplying many of the chloromethyl ketone derivatives used in this study. David Gold and Frederick Miller performed the analysis of the amino acids. We thank Rosemarie Dearing for her technical assistance throughout the experiments. This work was supported by N.I.H. Grant HL-14262 and by a Phebe Cornell Maresi Center Grant of the New York State Arthritis Foundation.

References

1 Janoff, A. and Scherer, J. (1968) J. Exp. Med. 128, 1137—1155
2 Ohlsson, K. (1971) Scand. J. Clin. Lab. Invest. 28, 225—230
3 Rindler, R., Schmalzl, F. and Braunsteiner, H. (1974) Schweiz. Med. Wschr. 104, 132—133
4 Spitznagel, J.K., Dalldorf, F.G., Leffell, M.S., Folds, J.D., Welsh, I.R.H., Cooney, M.H. and Martin, L.E. (1974) Lab. Invest. 30, 774—785
5 Dewald, B., Rindler-Ludwig, R., Bretz, U. and Baggiolini, M. (1975) J. Exp. Med. 141, 709—723
6 Janoff, A. (1972) Am. J. Path. 68, 579—591
7 Janoff, A., Blondin, J., Sandhaus, R.A., Mosser, A. and Malemud, C. (1975) in: Proteases and Biological Control (Reich, E., Rifkin, D. and Shaw, E., eds) Cold Spring Harbor Press, Cold Spring Harbor, New York, in press

8 Janoff, A. (1973) Lab. Invest. 29, 458—464
9 Ohlsson, K. and Olsson, I. (1974) Eur. J. Biochem. 42, 519—527
10 Gerber, A.Ch., Carson, J.H. and Hadorn, B. (1974) Biochim. Biophys. Acta 364, 103—112
11 Schmidt, W. and Havemann, K. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1077—1082
12 Rindler-Ludwig, R. and Braunsteiner, H. (1975) Biochim. Biophys. Acta 379, 606—617
13 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 493—505
14 Hummel, B.C.W. (1959) Can. J. Biochem. Physiol. 37, 1393—1399
15 Folk, J.E. and Schirmer, E.W. (1965) J. Biol. Chem. 240, 181—192
16 Visser, L. and Blout, E.R. (1972) Biochim. Biophys. Acta 268, 257—260
17 Feinstein, G., Kupfer, A. and Sokolovsky, M. (1973) Biochem. Biophys. Res. Commun. 50, 1020-1026
18 Bundy, H.F. (1963) Arch. Biochem. Biophys. 102, 416—422
19 Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281—283
20 Sweetman, F. and Ornstein, L. (1974) J. Histochem. Cytochem. 22, 327—339
21 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
23 Stevenson, K.J. and Landman, A. (1971) Can. J. Biochem. 49, 119—126
24 Janoff, A. (1972) Am. Rev. Resp. Dis. 105, 121—122
25 Schellman, G. and Shaw, E. (1963) Biochemistry 2, 252—255
26 Janoff, A. (1969) Biochem. J. 114, 157—159
27 Feinstein, G., Hofstein, R., Koifman, J. and Sokolovsky, M. (1974) Eur. J. Biochem. 43, 569—581
28 Hartley, B.S. and Kauffman, D.L. (1966) Biochem. J. 101, 229—231