ESTERASE AND PROTEASE ACTIVITY OF PURIFIED GUINEA PIG BASOPHIL GRANULES

N.S. Orenstein, M.E. Hammond, H.F. Dvorak, Departments of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Ma. 02114.

and Joseph Feder,
New Enterprise Division, Monsanto Company, St. Louis, Mo. 63166.

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SUMMARY

Highly purified granules of circulating guinea pig basophilic leukocytes were extracted with 0.1% Triton X-100 to yield a mixture of esterases-proteases including caseinolytic activity. By selective inhibition both trypsin- and chymotrypsin-like serine hydrolases have been identified. Sigmoidal pH dependences for hydrolysis of p-tosyl-L-arginine-methyl ester and N-benzoyl-L-tryosine-ethyl ester were observed for both intact granules and Triton granule extracts. Preliminary studies indicate that the enzymes are not solubilized even after Triton X-100 treatment of the granules.

INTRODUCTION

Basophilic leukocytes, the least common of the mammalian granulocytes, have recently been implicated in a variety of biologically significant immunologic reactions of both the immediate (homocytotropic antibody-mediated) and delayed (cell-mediated) types; these include anaphylaxis and skin allograft and tumor rejection (1). Like neutrophils and eosinophils, basophils have prominent cytoplasmic granules whose contents are thought to be largely responsible for the function of this cell. While several low molecular weight mediators (histamine, slow reacting substance of anaphylaxis, an eosinophil chemotactic factor, and platelet activation factor) are thought to reside in basophil granules (2), the macromolecular constituents of these granules have not yet been identified, primarily because of the paucity of circulating basophils

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Abbreviations: PBS, 0.005M phosphate, pH 7.2, 0.075 NaCl; TAME, p-tosyl-L-arginine methyl ester; BTEE, N-benzoyl-L-tyrosine ethyl ester; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl fluorophosphate; PMSF, phenylmethyl sulfonyl fluoride; TCA, trichloroacetic acid.

in most common laboratory animals and man. Recently, a technique has been developed for substantially purifying basophils from guinea pigs with basophilia induced by a series of antigenic injections (3). Using such preparations as a starting material, methods have been developed for isolating and purifying basophil granules by means of differential filtrations and sucrose density gradients (4). Purified basophil granule preparations were found to contain acid mucopolysaccharides (primarily chondroitin sulfate) but lacked lysosomal enzyme activities. We here report studies on the esterolytic and proteolytic properties of the basophil granule.

MATERIALS AND METHODS

Intact basophil granules were prepared as previously described (4). These granules were resuspended in half strength phosphate buffered saline (0.005 M phosphate, pH 7.2, 0.075 M NaCl) (PBS) containing 0.1% Triton X-100 (Fisher Scientific Co.). The Triton X-100 was purified of volatile contaminants in vacuo at 50°C, for four hours. The suspension of granules in PBS/Triton was freeze-thawed twice, incubated overnight at 4°C, freeze-thawed twice more, and centrifuged for 10 min., 10,000 xg, 4°C, in a Beckman minifuge. The resulting supernate was utilized as the granule extract. Intact nonextracted basophil granules were assayed to establish the total enzyme activity. Protein was determined by either a modification (5) of the Lowry (6) method, with centrifugation (2000 xg, 5 min) after addition of the Folin reagent to remove any precipitate caused by the Triton X-100, or by a fluorometric protein assay (7). Proteolytic activity was measured as caseinolytic activity both in a radial diffusion assay on a commercial casein-agarose plate (Worthington Biochemical Co.) and by the method of Anson (8) measuring the TCA soluble 280 nm absorbing products. The enzyme-catalyzed hydrolysis of p-tosyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma) was monitored spectrophotometrically at 247 nm and 256 nm respectively (9). The BTEE assay was modified to use 10 percent methyl alcohol (w/w) and 2.27 x 10^{-4} M substrate. At the 25 percent level of methyl alcohol only pseudo first-order kinetics were observed, indicating that the Km had increased considerably at the higher solvent concentration.

N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), diisopropyl fluorophosphate (DFP) and phenylmethyl sulfonyl fluoride (PMSF) were all obtained from the Sigma Chemical Corporation.

RESULTS AND DISCUSSION

Both the basophil granule and the granule extract catalyzed the hydrolysis of TAME and BTEE (Table 1). Recentrifugation of the Triton

Table 1. Effect of Various Inhibitors on Basophil Granule Extract Catalyzed Hydrolysis of BTEE and TAME.

Inhibitor	Percent Inhibition	
	BTEE[a]	TAME[b]
DFP[c]	100	100
PMSF[d]	100	100
TLCK[e]	12	100
TPCK[f]	100	30

[a](So)=5 x 10^{-4}M; pH 8.0 Tris (0.05M); 10% methyl alcohol (w/w), 25°C
[b](SO)=10^{-3}M; pH 8.0 Tris (0.05M), 25°C
[c]DFP, 10^{-2}M, 10^{-2}M isopropyl alcohol, 2 hours
[d]PMSF, 2 x 10^{-2}M, 16% dioxone, 4.5 hours
[e]TLCK, 5.5 x 10^{-4}M, 4.0 hours, (10)
[f]TPCK, 5.5 x 10^{-4}M, 10% methyl alcohol (w/w), 18 hours, (11).

X-100 granule extracts at 109,000 xg for 90 minutes resulted in recovery of virtually all of the esterase activity in the pellet, indicating that the extracted enzymes were not solubilized. Selective inhibition was used to further characterize the esterase enzymes. Both the TAME and BTEE hydrolyzing activities were inactivated by DFP and PMSF (Table 1). Treatment of the extracts with TLCK completely inactivated the trypsin-like activity against TAME but reduced hydrolysis of BTEE by only 12 percent. Conversely, TPCK completely inactivated the chymotrypsin-like activity toward BTEE but reduced TAME hydrolysis by less than one-third. These results strongly suggest that two separate "serine hydrolases" have been extracted from basophil granules by Triton X-100. Lagunoff and Benditt have reported the presence of similar enzymes in dog and human mast cells (12).

The pH dependence of the basophil granule- and granule extract-catalyzed hydrolysis of TAME is shown in Table 2. Lineweaver-Burk

Table 2. Comparison of the pH Dependence of Basophil Granule Extract and Unextracted Basophil Granules Catalyzed Hydrolysis of TAME[a]

pH	Buffer (0.1M)	$K_m \times 10^4$ M		$V_{max} \times 10^5$ M/min	
		Granule[b]	Extract[c]	Granule	Extract
5.0	Acetate	4.04	5.76	0.46	2.23
5.5	Acetate	-	10.46	-	7.30
6.0	Phosphate	7.05	3.30	4.10	7.40
6.5	Phosphate	6.25	3.08	5.13	12.27
7.0	Phosphate	7.08	1.48	7.33	13.16
7.5	Tris	6.22	0.53	8.22	11.22
8.0	Tris	6.0	0.57	10.77	15.29
8.5	Tris	-	0.50	-	17.05

[a](So)=4.76 x 10^{-4}M, 25°C

[b]protein concentration, 4.8 µg/ml

[c]protein concentration, 2-4.0 µg/ml

plots were obtained from single reactions run to completion and least squares values were obtained for the calculation of V_{max} and K_m. No product inhibition was observed for TAME. This was demonstrated by a second addition of TAME to a concentration of 4.65 x 10^{-5}M at the completion of the first reaction. Both reactions fitted the same Lineweaver-Burk plot, indicating the absence of measureable inhibition by product from the first reaction. Sigmoidal pH dependencies for V_{max} were observed for both the basophil granule and the extract. It is interesting to note that the K_m for the basophil granule was considerably larger than the K_m for the extract-catalyzed reaction. Figure 1 illustrates the pH dependence of V_{max}/K_m for both the basophil granule- and granule extract-catalyzed hydrolysis of BTEE. The extract exhibited good

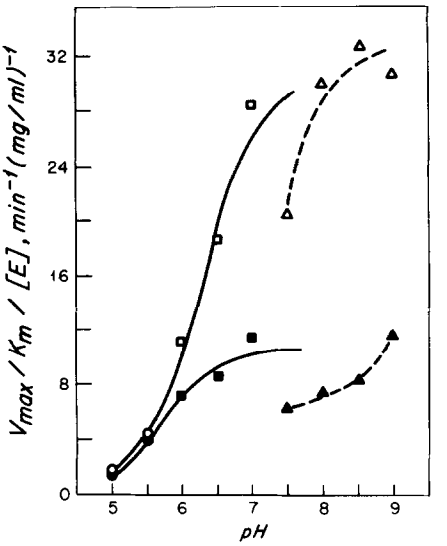

Figure 1. Comparison of pH dependence of basophil granule and granule extract catalyzed hydrolysis of BTEE. ○□△, granule extract; ●■▲, granule; ○●, acetate (0.1M); □■, phosphate (0.1M); △▲, Tris (0.1M). (So)=2.27 x 10^{-4}M, granule and extract protein concentration=1.71-3.27 x 10^{-2} mg/ml, 25°C.

Michaelis-Menten kinetics yielding K_m values of 2-4 x 10^{-4}M in the pH 6-9.0 range. The basophil granule, however, exhibited pseudo first-order kinetics over most of the pH range, indicating that the K_m for the granule was considerably larger than 2.3 x 10^{-4}M, the substrate concentration. Treatment of these reactions as first order processes yielded rate constants, $k=V_{max}/K_m$. For both substrates the sigmoidal pH profiles reflected a dependence on an ionizable group with a pK in the region of 6-7, reminiscent of the role of imidazole in the catalysis of many serine hydrolases including trypsin and chymotrypsin (13).

The specific activities of the two enzyme activities as measured in the basophil granule and the granule extract from a single preparation were compared. The $V_{max}/(E)$ values, using the protein concentration (mg/ml) for (E), obtained from single complete reactions for TAME in Tris buffer (0.1M) pH 8.1 were 1.33 x 10^{-2}M/min/(mg/ml) and 1.87 x

10^{-2}M/min/(mg/ml) for the granule and the extract respectively. The $V_{max}/(E)$ values for BTEE in Tris buffer (0.1M) pH 7.8 were 4.02 x 10^{-3}M/min/(mg/ml) and 6.22 x 10^{-3}M/min/(mg/ml) for granule and extract respectively. Thus, although the K_m values were 3 to 10 fold greater for the granule than the extract, the specific activities agreed within 70 percent.

Substantial caseinolytic activity also was found in these preparations by both assays employed. Similar to the esterase activity, treatment with 5 x 10^{-3}M DFP for 4 hours completely inactivated the proteolysis.

In conclusion, guinea pig basophil granules have been shown to contain trypsin- and chymotrypsin-like esterase as well as DFP sensitive caseinolytic activity.

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