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Short Communication

Alanine *p*-Nitrophenyl Esterase Activity of Human Leucocyte Granules

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It has been shown that human leucocyte granules possess elastolytic activity (Janoff & Scherer, 1968). In addition to digesting powdered elastin substrates, granule extracts attack arterial elastic fibres in human kidney *in vitro* and in dog aorta *in vivo*. Thus granulocyte elastase may participate in the pathogenesis of neutrophil-dependent arteritis. The responsible enzyme has been partially purified (Janoff, 1969).

In the past trials of leucocyte elastase with several synthetic substrates have proved unsuccessful (Janoff & Zeligs, 1968). In the previous studies elastolysis was therefore measured by the method of Sachar, Winter, Sicher & Frankel (1955). This technique is based on colorimetry of dyed products solubilized from solid orcein-impregnated elastin, a procedure that involves lengthy incubations and is subject to some error due to non-proteolytic release of dye under certain conditions (Gilfillan, 1969). Recently, however, the NBA* has been described as a synthetic substrate for pancreatic elastase (Visser & Blout, 1969). The use of NBA as substrate greatly improves the sensitivity of pancreatic elastase assays, and the choice is further supported by the fact that alanine peptide bonds constitute a major site of attack of the pancreatic enzyme on native elastin.

The present experiments were undertaken to test NBA as a substrate for human granulocyte (lysosomal) elastase in the hope of improving the sensitivity and specificity of the assay system for the leucocyte enzyme. The results show that the agent in granulocyte lysosomes is even more active against this compound than is pancreatic elastase. By contrast, several other lysosomal enzymes that can weakly solubilize dye from elastin–orcein (Gilfillan, 1969) do not hydrolyse NBA at all. In addition, the previous report on the non-identity of granulocyte and pancreatic elastases (Janoff & Scherer, 1968) is supported by the present observation that DPBB, an inhibitor of NBA hydrolysis by pancreatic elastase, does not affect esterolysis by the leucocyte enzyme.

* Abbreviations: NBA, benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester; DPBB, 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one.

Methods. (*a*) Leucocyte granules. Whole-granule extracts from human peripheral blood leucocytes were obtained as described by Janoff & Scherer (1968). A fraction of the granules, enriched in elastolytic activity, was prepared chromatographically as described by Janoff (1969).

(*b*) Commercial enzyme preparations. Enzymes used were as follows (unless otherwise stated all preparations were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.): carboxypeptidase A (EC 3.4.2.1); chymotrypsin (EC 3.4.4.5); collagenase (EC 3.4.4.19); elastase (EC 3·4.4.7); β-glucuronidase (EC 3.2.1.31); hyaluronidase (EC 3.2.1.–); leucine aminopeptidase (EC 3.4.1.1); lysozyme (EC 3.2.1.17) (from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); papain (EC 3.4.4.10) [crystalline preparation from crude papain extract (Nutritional Biochemicals Corp.), kindly supplied by Dr J. L. Potter, New York University School of Medicine]; trypsin (EC 3.4.4.4).

(*c*) Substrate and assay method. NBA was obtained from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. The assay method, described by Visser & Blout (1969), was briefly as follows: 0·2mM solution of substrate was prepared by dissolving 6mg. of NBA in 1·0ml. of acetonitrile and diluting with 99ml. of 0·05M-sodium phosphate buffer, pH6·5. Cuvettes were filled with 3·0ml. of the substrate solution, and enzyme samples containing 2·5–25μg. of protein were then added to the test cuvette. Liberation of *p*-nitrophenol was followed spectrophotometrically at 347·5nm. for 3min.

(*d*) Inhibition experiments. DPBB was kindly provided by Dr L. Visser (Harvard Medical School, Boston, Mass., U.S.A.). Inhibition studies were carried out as described by him (personal communication). Briefly, this involved preparation of a 10mM stock solution of DPBB in acetonitrile. Samples of the DPBB stock solution were then diluted by addition of pancreatic or granulocyte elastase solutions made up in 0·05M-sodium phosphate buffer, pH7·0, containing $CaCl_2$ (0·1mM). Final concentrations were: granule protein, 0·5mg./ml.; pancreatic elastase, 0·2mg./ml.; DPBB, 0·25mM; acetonitrile, 2·5% (v/v). The mixtures

Table 1. *NBA esterase activity of leucocyte granules and various enzymes*

$\Delta E_{347.5}$/30sec. was calculated from the slope of extinction versus time during the first 2min. of the assay; plots remained linear during this interval. The concentration of DPBB, when added, was 0·25mM.

Enzyme	Amount of protein tested (μg.)	Inhibitor	Time at 37° with DPBB (hr.)	$\Delta E_{347.5}$/30sec.	Inhibition (%)
Part A					
Elastase	10·0	—	—	0·022	—
Granules (whole extract)	25·0	—	—	0·025	—
Granules (enriched fraction)	2·5	—	—	0·010	—
	5·0	—	—	0·019	—
	7·5	—	—	0·030	—
	10·0	—	—	0·036	—
	12·5	—	—	0·042	—
Part B					
Carboxypeptidase A	25·0	—	—	0·000	—
Chymotrypsin	25·0	—	—	0·009	—
Collagenase	25·0	—	—	0·000	—
β-Glucuronidase	25·0	—	—	0·000	—
Hyaluronidase	25·0	—	—	0·000	—
Leucine aminopeptidase	25·0	—	—	0·000	—
Lysozyme	25·0	—	—	0·000	—
Papain	25·0	—	—	0·002	—
Trypsin	25·0	—	—	0·003	—
Part C					
Elastase	10·0	—	2	0·016	
	10·0	—	4	0·014	
	10·0	—	6	0·014	
	10·0	DPBB	2	0·012	25
	10·0	DPBB	4	0·007	50
	10·0	DPBB	6	0·007	50
Granules (whole extract)	25·0	—	2	0·019	
	25·0	—	4	0·016	
	25·0	—	6	0·015	
	25·0	DPBB	2	0·018	5
	25·0	DPBB	4	0·015	6
	25·0	DPBB	6	0·015	0

were incubated at 37° for 6hr., and portions containing 10μg. of elastase or 25μg. of granule protein were removed and assayed with NBA at 2hr. intervals as described above. Enzyme solutions incubated without DPBB were also included as controls.

Results. The results of several experiments are summarized in Table 1. Part A shows that crude extracts of human leucocyte-granule protein have considerable NBA esterolytic activity (about 40% of that of crystalline pancreatic elastase when the two are compared on an equal-weight basis). Further, a chromatographic fraction of this granule extract that had been enriched about fivefold in elastolytic activity (orcein–elastin assay) showed a fourfold increase in specific NBA esterase activity in my experiments. Thus this fraction was nearly twice as active as crystalline pancreatic elastase on an equal-weight basis. As shown in part A of Table 1, the partially purified leucocyte elastase gave linear increases in esterolytic activity with increasing concentration of test protein up to 7·5μg./3·0ml. of substrate. Above that concentration activity increased at a diminished rate.

Part B of Table 1 shows that, with the exception of chymotrypsin, the other enzymes tested did not possess significant NBA esterase activity under the conditions of the assay. Even chymotrypsin, however, was only one-tenth as active as the enriched granule fraction when the two were compared on the basis of equal protein concentration.

Part C of Table 1 shows that preincubation of pancreatic elastase for 4hr. with 0·25mM-DPBB inhibited 50% of the esterolytic activity of this enzyme. Under the same conditions there was essentially no inhibition of granule-mediated esterolysis by DPBB, even after 6hr. of preincubation with the agent.

Discussion. The present results clearly show that NBA is a superior substrate for leucocyte elastase. The use of this synthetic ester should greatly facilitate future studies, especially final purification of the granulocyte enzyme. Moreover, a number of the exo- and endo-saccharases and proteases reportedly active against elastin under certain circumstances (Gilfillan, 1969) were also tested on NBA and, except for relatively slight activity by chymotrypsin, found to be entirely inactive on this substrate. Thus there is good specificity as well as sensitivity of NBA for true elastases. The elastolytic activity of human granulocyte lysosomes therefore appears to be due to a true elastase and not to the action of lysosomal saccharases, papain-like proteases, or collagenase. It is worth noting that esterolysis of NBA undoubtedly involves mechanisms closely related to those involved in elastin-degradation itself, in view of the large increase in exposed alanine residues observed after elastin breakdown by the pancreatic enzyme (Visser & Blout, 1969).

Finally, in keeping with the previous findings of major differences between pancreatic and leucocyte elastolytic enzymes (Janoff & Scherer, 1968; Janoff, 1969), the present experiments also show that these two enzymes behave differently with respect to inhibition by DPBB. Thus it appears reasonable to suggest that the elastase of human granulocyte lysosomes not only is a true elastolytic protease, but, further, represents a second source of this activity in man, independent of that present in pancreas.

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