ROLE OF LYSOZYME AND PEROXIDASE IN LYMPHOCYTE MITOGENIC ACTIVITY REGULATION

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The presence of receptors for many antigens and mitogens on lymphocytes suggests that the range of natural regulators of their mitogenic activity *in vivo* is not confined to the complement factors, lymphokines, and individual mediators of inflammation described in the literature [2, 3]. The basis for the present investigation was provided by indirect evidence of the adjuvant activity of lysozyme [7] and the ability of the blood phagocytes, containing peroxidase, to stimulate blast transformation of lymphocytes [5], which call for an explanation of the precise mechanism of these phenomena.

EXPERIMENTAL METHOD

Lymphocytes were isolated from the venous blood of 50 blood donors by differential centrifugation on a Ficoll—Verografin layer with a density of 1.077 g/ml. The final concentrations of phytohemagglutinin (PHA, from Reakhim, USSR), purified and commercial preparations (From Reakhim, USSR, and Reanal, Hungary) of hens' egg and salivary lysozymes, and horseradish and salivary peroxidase were 25 mg/liter, $(140\text{-}2) \times 10^4$, and $(550\text{-}5) \times 10^4$ units/liter respectively. Peroxidase and lysozyme were isolated from mixed human saliva by gel-filtration on Sephadex G-200 (from Pharmacia, Sweden) and by ion-exchange chromatography, using carboxymethylcellulose CM-52 (from Whatman, England) as the adsorbent. Columns measuring 73 × 2.4 and 49 × 1.3 cm were used. Fractions 3 ml in volume were collected. The purity of the isolated enzymes was verified by disc electrophoresis in polyacrylamide gel (from Reanal). During purification of the egg white lysozyme under these same conditions the enzyme was eluted in two peaks on Sephadex G-200 and one peak on CM-cellulose before the beginning of gradient elution with 0.1 M NaCl solution. The specific activity of the purified egg lysozyme was 15 units/mg protein, of the salivary lysozyme 1 unit/mg protein, and of salivary peroxidase 25 units/mg protein. The protein content and activity of peroxidase [4] and lysozyme [6] were determined spectrophotometrically and mitogenic activity of the lymphocytes by a radioisotope method. Solutions of the enzymes in a volume of 0.1 ml were added to a culture of lymphocytes with and without PHA. After incubation for 68 h at 37°C the cell culture was treated with [^{3}H]thymidine, [^{3}H]uridine, or [^{14}C]glycine with a radioactivity of 3.7×10^4 Bq. Incorporation of the labeled amino acids was analyzed 4 h later by counting radioactivity on an SBS-2 scintillation radiometer. The results were subjected to statistical analysis by Wilcoxon's paired test [1].

EXPERIMENTAL RESULTS

Analysis of the results of investigation of the mitogenic effect of commercial preparations of egg white lysozyme and horseradish peroxidase in a dose equal to the mean physiological concentration of these enzymes in mixed human saliva showed that lysozyme increased incorporation of [^{3}H]thymidine fivefold into DNA, while inhibiting incorporation of [^{14}C]-glycine by several times, whereas horseradish peroxidase did not possess this property (Table 1).

The study of mitogenic activity of purified salivary enzymes, however, gave the opposite results. For instance, salivary lysozyme in a concentration of 140 units/liter did not affect DNA synthesis, whereas salivary peroxidase in a concentration of 550 units/liter

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TABLE 1. Effect of Egg White Lysozyme in Concentration of 2×10^4 units/liter and Horseradish Peroxidase in Concentration of 5×10^4 units/liter on Lymphocyte Blast Transformation Reactions (intensity of incorporation of labeled precursors, in Bq; M ± m)

Enzyme	Intact lymphocytes			PHA-stimulated lymphocytes		
	[^{3}H]-thymidine	[^{3}H]-uridine	[^{14}C]-glycine	[^{3}H]-thymidine	[^{3}H]-uridine	[^{14}C]-glycine
Control	184,6±44	348,6±111	3828,3±823	575,6±84	319,3±83	1775,6±327
			P=0,01			
Lysozyme	342±41	280,3±107	787,3±304	273±55	427±147	2096,4±1216
	P=0,05		P=0,01	P<0,05		
				P_1=0,05		
Peroxidase	227±61	357±127	4316±2067	347±143	283±83	1830±812
				P_1=0,01		

Legend. Here and in Tables 2-4: P and P_1 indicate levels of significance calculated by Wilcoxon's test by comparison with intact and stimulated lymphocytes respectively.

TABLE 2. Effect of Purified Human Salivary Peroxidase and Horseradish Peroxidase in Concentration of 550 units/liter on Intensity of Incorporation of [^{3}H]Thymidine into Lymphocytes (in Bq; M ± m)

Lymphocytes	Control	Peroxidase	Salivary peroxidase
Intact	146,6±20,6	291,6±87,2	237,6±42
		P=0,05	P=0,01
PHA-stimulated	276,6±76,5	178±31	202±39
	P=0,05		

TABLE 3. Effect of Purified Human Salivary Lysozyme and Egg White Lyoszyme in Concentration of 140 units/liter on Intensity of Incorporation of [^{3}H]Thymidine into Lymphocytes (in Bq; M ± m)

Lymphocytes	Control	Egg lysozyme	Salivary lysozyme
Intact	146,6±20,6	538±22,4	187±52,5
		P=0,01	
PHA-stimulated	276,6±77	237,3±66,3	148,3±31
		P=0,01	

TABLE 4. Effect of Egg White Lysozyme of Different Degrees of Purity on Intensity of Incorporation of [^{3}H]Thymidine into Lymphocytes (in Bq; M ± m)

Statistical parameter	Control	PHA	Unpurified lysozyme	Purified preparations of lysozyme		
				gel-filtration on Sephadex G-200		ion-exchange chromatography on CM-cellulose
				I protein peak	II protein peak	
$M \pm m$	80,3	143,4	117,3	73	60	62
	10	36	20,3	10	9	6
P	—	0,05	0,05	—	—	—

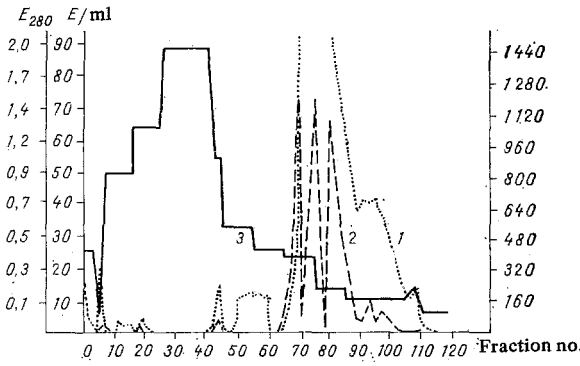

Fig. 1. Fractionation of biologically active substances of egg white on Sephadex G-200. Dotted line — protein concentration in fractions; broken line — lysozyme activity; continuous line — intensity of incorporation of [^{3}H]thymidine into lymphocytes.

significantly stimulated this process. A commercial preparation of peroxidase in the same concentration had the identical action on DNA biosynthesis by lymphocytes (Table 2). Unpurified egg white lysozyme, in this low dose also, preserved marked ability to stimulate DNA biosynthesis, with a strength of action similar to that of PHA (Tables 3 and 4). Consequently, the mitogenic effect of peroxidase is manifested only in low concentrations of the enzyme.

The characteristics of stimulation of lymphocytes by lysozymes of different origin and of different degrees of purity, revealed by these experiments, might be attributable to differences in the biological properties of egg and salivary lysozyme, established in the course of evolution, if one could be confident that the commercial preparation of lysozyme contained no impurities capable of inducing a mitogenic effect. To make sure of this point, a preparation of egg white lysozyme was purified by gel-filtration and by ion-exchange chromatography. The three purified fractions of lysozyme thus obtained were added to a culture of lymphocytes in order to carry out the blast transformation reaction in the same final concentration. These experiments showed that purified egg lysozyme did not affect incorporation of [^{3}H]thymidine into the lymphocytes (Table 4). The results are similar to those of investigation of purified salivary lysozyme (Table 3). To study the nature of this unknown mitogen in lymphocytes, proteins of a commercial preparation of egg lysozyme were fractionated by means of gel-filtration. The protein content and lysozyme activity of the resulting fractions were determined spectrophotometrically, after which their effect of lymphocyte blast transformation was studied. The results (Fig. 1) show that the lysozyme came out with the principal peak (IV) of protein from the 65th to the 95th fraction. Maximal stimulation of blood lymphocytes was reached in response to fractions 25-40 of the eluate, containing minimal concentrations of protein and lysozyme.

On the addition of fresh whole egg white to the samples as the source of lysozyme in concentrations of between 1 and 20%, an increase was observed in the intensity of incorporation of [^{3}H]thymidine into the lymphocytes, which was directly proportional to the increase in its concentration. Comparison of the protein concentrations, lysozyme activity, and blast transformation of the lymphocytes suggests that this mitogen is evidently polysaccharide in nature, and that its isolation and study may prove interesting in both experimental and clinical practice.

These investigations thus revealed a new mitogen in the composition of egg white and demonstrated the hitherto unknown ability of plant and mammalian peroxidases to stimulate the mitogenic activity of lymphocytes.

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