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Chemical Properties of Porcine Leukocyte Lysosomal Hydrolases

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Porcine Leukocyte Lysosomal Hydrolase의 化學的性質에 關한 硏究

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요 약

돼지 白血球 lysoromal enzyme의 latency를 서로 다른 농도의 sucrose용액(0.0125-0.25M)으로서 조사하고 各 sedimentation fraction에 분포되어 있는 효소들의 specific activity, pH optima 및 activation energy를 측정하였다.

SUMMARY

Lysosomal enzyme latency was demonstrated for hydrolases from porcine leukocyte by suspending sediment sfrom differential centrifugation in 0.125 to 0.250 M sucrose. Specific activities pH optima and activation energies were determined for hydrolases distributed in various sedimentation fractions and for enzymes solubilized by *n*-butyl alcohol extraction. Specific activities of the hydrolases revealed the heterogeneity of the lysosomal fractions relative to enzyme content. pH optima identified the enzyme as acid hydrolases with optima for cathepsin D and aryl sulfatase also at pH 6.8. Activation energies of some hydrolases were low revealing that these enzymes could function efficiently during low temperature aging of meat.

INTRODUCTION

Lysosomal hydrolases such as cathepsin D. acid ribonuclease(RNA-ase), β-glucuronidase, acid phosphatase and aryl sulfatase have been studied in rabbit peritoneal macrophages, beef leukocytes and in meat animal skeletal muscle extracts(Cohn and Hirsch, 1960; Hegner, 1968; Parrish and Bailey, 1967). A report on the morphology of porcine leukocyte lysosomal hydrolases was made recently (Venugopal, 1970).

The objective of this investigation was to study some of the chemical properties of porcine leukocyte lysosomal hydrolases and to compare them with lysosomal hydrolases from other sources.

EXPERIMENTAL SECTION

Enzyme Source. Leukocyte lysosomal sediments

were isolated from porcine blood as described by Venugopal(1970).

Lysis Treatment of Lysosomal Sediments. The lysosomal granular fractions sedimented at $500\times g$, $500\times g$, $10,000\times g$ and $20,000\times g$ were suspended in hypo-osmotic concentrations of sucrose(0.125, 0.063, 0.05, 0.025, and 0.125M) for about 2 hr at 4°(to investigate lysosomal enzyme latency). The control was distilled water instead of sucrose solution. The enzymes released into the medium were separated from unlysed particles by centrifuging the suspension at $20,000\times g$ for 30 min and the supernatant assayed for hydrolase activity. Enzymes studied were acid phosphatase, cathepsin D, β-glucuronidase RNA-ase and aryl sulfatase.

Specific Activities of Hydrolases. Specific activities of leukocyte lysosomal enzymes were measured following lysis of lysosomes by dispersing them in distilled water 1 to 5(V/V), dializing overnight against distilled water at 4° and removing grana membrane by centrifuging at $20,000\times g$ for 30 min.

n-Butanol Extraction of Leukocytes. Leukocytes suspended in water (30ml) were frozen in a porcelin mortar by adding powdered dry ice and triturated with 5ml of *n*-butyl alcohol. Cold (4°) distilled water was added to make the alcohol 4%(V/V). The resulting suspension was shaken (500 ml separating funnel) thoroughly chilled with more dry ice and kept at 4° until the organic and inorgaic phases separated. Frequently, larger quantities of alcohol were required to achieve complete separation of the two phases. The aqueous layer containing the enzyme protein was dialyzed against distilled water at 4° for 24 hr. Residual alcohol was removed from the dialysate by lyophilization.

Assay of Acid Hydrolase Activities. Cathepsin D activity was measured by the method of Anson (1938) as modified by Sliwinsky *et al.*(1959). The incubation was carried out at 38° for 2 hr in pH 4.0 acetate buffer with urea-denatured hemoglobin as the substrate. Specific activity was expressed as the increase in absorbance at 274 nm of trichloroacetic acid (10%) soluble substances during 2 hr reaction per mg enzyme nitrogen times 1000. Another substrate used was urea-denatured actin from porcine muscle.

Cathepsin A, B, and C activities were determined by using carbobenzoxy-*L*-glutamyl-*L*-tyrosine, benzoyl-*L*-arginine amide, and glycyl-*L*-phenylalanine amide as substrates. Rates of hydrolysis were estimated by titration (Davis and Smith, 1955). Acid phosphatase activity was measured as described by Hawk *et al.* (1954) using sodium β-glycerophosphate in acetate buffer(pH 5.0) as substrate. Specific activity was expressed as the increase over the blank per mg enzyme nitrogen times 100.

β-Glucuronidase activity was measured by the procedure of Fishman *et al.* (1948) with 5.0×10^{-2}M phenolphthalein β-*D*-glucuronide, at pH 4.5. Reaction time was 24 hr at 38° and the specific activity was expressed as micrograms of phenophthalein liberated during 24 hr per mg of enzyme nitrogen.

Aryl sulfatase activities were measured by the method of Roy (1953) as modified by Baum *et al.* (1959), with nitrocatechol (2-hydroxy-5-nitrophenyl sulfate, dipotassium salt). For Aryl sulfatase A assay, the substrate contained 1.0×10^{-2}M nitrocatechol sulfate, 5.0×10^{-4}M sodium pyrophosphate, 10% NaCl (W/V) in acetate buffer(0.5 M, pH 4.7). For aryl sulfatase B assay, the substrate contained 1.0×10^{-2}M nitrocatechol sulfate and 1.0×10^{-2}M barium acetate in acetate buffer(0.5M, pH 5.7). The specific activity was expressed as micrograms of nitrocatechol liberated at 38° in one hr per mg enzyme nitrogen.

Ribonuclease activity was measured by a modification of the procedure of Schneider and Hogeboom (1952). The substrate was purified from Nutritional Biochemicals Corporation reagent grade ribose nucleic acid. The reaction mixture of 0.6 ml of enzyme and 0.4ml of substrate (1% RNA and 0.4M KCl in 0.1M citrate buffer, pH 5.0) was incubated at 38° for 30min; 2ml of 2 : 1 *t*-butylalcohol: glacial acetic acid mixture was used to stop the reaction. A reagent blank consisted of

0.6ml of incubated enzyme solution, 2ml of butanol acetic acid mixture and 0.4ml of substrate added in that order. Reaction samples were kept at 0° for at least 2 hr, then centrifuged for 30 min at 7,000×*g*, following protein precipitation. One ml of supernatant was diluted with water and the absorbance measured at 260nm. Specific activity was expessed as change in absorbance during 30 min reaction per mg enzyme nitrogen.

Deoxyribonuclease activity was measured by the method of Schneider and Hogeboom (1952) using highly purified calf thymus deoxyribonugleic acid as substrate. Following incubation at 38° for 30 min, the reaction was stopped with 12% perchloric acid and the absorbance of the supernatant measured at 260 nm. specific activity was expressed as change in absorbance per mg of enzyme nitrogen. Nucleotidase activity was assayed according to the method of Swendseid *et al.* (1952) using adenosine-5-phosphate as the substrate at pH 4.0. The specific activity was expressed as mg of phosphorous liberated at 38° for 30 min per mg enzyme nitrogen time 100.

Protein in the various enzyme fractions was determined by Micro-Kjeldahl (Hawk *et al.*, 1954), whereas protein in the chromatographic eluates was measured by absorbance at 273nm.

Activation Energy Studies. Activation energies were determined for various hydrolases in different sedimentation fractions and in *n*-butyl alcohol exracts from Arrhenius pots of activities at 30, 35, 40 and 45°.

RESULTS

Lysis of Lysosomes by Hypo-osmotic Media. Data obtained for the activity of porcine leukocyte lysosomal cathepsin D from differential sedimentation fractions in hypotonic media are graphically represented by Figure 1. Decreasing the concentration of sucrose from 0.250M to 0.063M increased activity of this enzyme in supernatant following centrifugation at 20,000×*g* very little. At sucrose concentration lesss than 0.063M, the increase in activity was dramatic and reached maximum at 0.0125M sucrose compared to lysosomes dialized against distilled water prior to analysis.

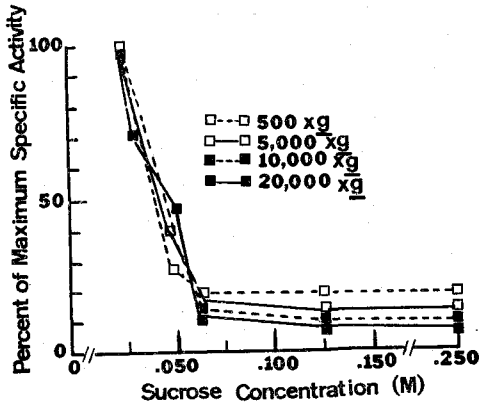

Fig. 1. Activity of porcine leukocyte lysosomal cathepsin D from differential sedimentation fractions in hypotonic media.

Activities of aryl phosphatase, β-glucuronidase and RNA-ase followig suspension of differential sedimented fractions in hypotonic media were similar to that of cathepsin D. For these enzymes, activities increased rapidly in sucrose solutions less than 0.063M and reached a maximum in 0.0125M sucrose.

Lysosomal aryl sulfatase activity was different in that the enzyme was freed to the supernatant beginning at 0.125M sucros and reached a maximum in 0.0125M sucrose.

Specific Activities of Leukocyte Lysosomal Hydrolases. The specific activities of various leukocyte lysosomal enzymes in supernatant solutions following lysis and dialysis of lysosomes are in Table I. Lysosomal heterogeneity was demonstrated by distribution of their hydrolases in different fractions. Acid phosphatase activity was high in 3,500×*g* fraction, cathepsin D activity was high in 10,000×*g* fraction, while RNA-ase and β-glucuronidase activities were high in both 3,500×*g* and 20,000×*g* fractions. High activities in the 500×*g*, and 3,500×*g* fractions were due to grana in unruptured cells or free grana attached to cellular debris.

Low activities for the various hydrolases in the supernatant indicate that these enzymes were

Table I. Specific activities of porcine leukocyte lysosomal enzymes[a,b]

Fractions	Acid phosphatase	Cathepsin D	RNA-ase	Aryl sulfatase	β-Glucuronidase	DNA-ase	Nucleotidase
Homogenate	2.60	67.0	0.16	13.4	7.0	0.14	1.30
500×*g*	28.50	920.0	1.90	107.0	28.0	0.48	3.70
3,500×*g*	47.10	1355.0	7.20	110.0	130.0	3.24	21.34
10,000×*g*	21.72	2945.0	3.20	120.0	67.0	0.74	7.40
15,000×*g*	19.01	2052.0	2.10	29.0	43.0	0.68	5.00
20,000×*g*	10.52	1160.0	7.10	37.0	112.0	2.84	28.20
Supernatant	11.00	614.0	1.90	14.0	nil	0.43	1.20
l-Butanol extract	83.30	3870.0	9.40	268.0	138.0	4.10	32.00

[a] Specific activity expressed per mg of protein nitrogen.
[b] Average values of 18 experiments.

originally present in organelles sedimenting at 20,000×*g* or lower and not in microsomes or in cytoplasm. High hydrolase activities of *n*-butyl alcohol extracts of leukocytes demonstrated the efficiency of this procedure for lysing both leukocyte and lysosomal membranes and in releasing lysosomal hydrolases.

pH Optima. Results obtained by assaying the enzymes obtained from 10,000×*g* sediment and *n*-butyl alcohol extract of leukocytes are illustrated in Figure 2, 3 and 4. Figure 2 shows the pH optima curve of cathepsin D. Sodium acetate-HCl buffer was used for pH 2.0-5.5, phosphate buffer for pH 6.0-8.0 and glycine-NaOH buffer pH 8.5-10.0. Activity maxima occurred at pH 4.0, 8.0 and 10.0, although activity at pH 4.0 was more than twice that at pH 8.0 and 10.0.

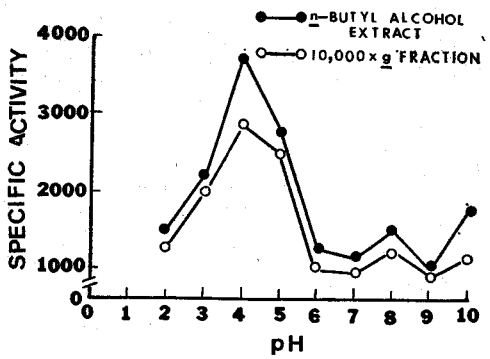

Fig. 2. pH optima of porcine leukocyte lysosomal cathepsin D assayed with hemoglobin as substrate at 38°C.

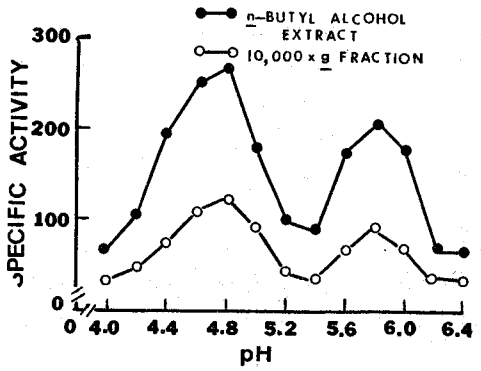

Fig. 3. pH optima of porcine leukocyte lysosomal aryl sulfatase assayed with nitrocatechol sulfate as substrate at 38°C.

Figure 3 is the pH optima curve for aryl sulfatase. Acetate buffer (0.5M) containing 5×10^{-4}M sodium pyrophosphate was used. Activivy maxima were obtained at pH 4.8 and 5.8.

β-Glucuronidase had an optimal pH of 4.5(Fig 4). Identical pH optima for enzymes from 10,000 ×*g*, sediment and *n*-butyl alcohol extract were observed, with alcohol extracted enzmes having greater specific activities.

Activation Energy. The activation energies for acid phosphatase, cathepsin D, aryl sulfatase, RNA-ase and β-glucuronidase in the various sedimentation fractions and in *n*-butyl alcohol extract are given in Table II, Except for β-glucuronidase, activation energies are quite uniform for enzymes in the various fractions,

Table II. Energies of activation of porcine leukocyte lysosomal enzymes[a,b]

Fractions	Acid phosphatase	Cathepsin D	Aryl sulfatase	RNA-ase	β-Glucuronidase
500×*g*	10.94	6.98	12.70	15.90	7.14
3,500×*g*	10.63	7.13	12.72	16.75	6.75
10,000×*g*	10.72	7.23	12.70	16.17	10.63
l-Butanol extract	10.94	7.21	13.20	15.63	8.78

[a] Expressed in kilocalories mole^{-1} from Arrhenius plots.
[b] Each value is an average of two individual plots.

DISCUSSION

Enzyme latency is a characteristic feature of lysosomes. The 10-fold increase in hydrolase activities of sediments from sonicated leukocytes under hypo-osmotic conditions demonstrates this structure linked latency, which is similar to the behavior of mammalian liver lysosomal enzymes (de Duve, 1959; Wattiaux and de Duve, 1956). Evidence from microscopic study of these sediments relate them morphologically to liver lysosomes (Venugopal, 1907). The differences in the stability of membranes of rabbit peritoneal exudate PMN leukocytes and porcine peripheral blood leukocytes are shown by unsuccessful attempts to lyse porcine leukocytes with hyper-omotic sucrose(0.4M) used by Cohn and Hirsch(1960). The sharp increase in aryl sulfatase activity of sediments in 0.125M sucrose indicates either greater fragility of aryl sulfatase containing lysosomes or loose-binding of this enzyme to the membrane or both. Absence of aryl sulfatase activity in muscle tissue extracts (Venugopal, 1970) and studies on factors affecting lysosomal membrane and availability of enzymes(Sawant *etal.*, 1964) substantiates the fragility concept.

n-Butyl alcohol was very effective in rupturing leukocyte and lysosmal membranes and in subsequent release and solubilization of enzymes, as shown by high hydrolase activities(Table I); although *n*-butyl alcohol inhibits enzyme activities in varying degrees it is noted for its ability to destroy lipo-protein complexes(Morton, 1955). Solid carbon dioxide contributed to the physical shock in lysing the membranes and aided in the separation of alcohol and aqueous layers following extraction.

The heterogeneity of porcine leukocyte lysosomes is illustrated by the distribution of the hydrolases in various sediments and is similar to those in rabbit PMN leukocytes(Cohn and Hirsch, 1960; Baggiolini *et al.*, 1969); horse and rat eosinophils (Archer and Hirsch, 1963); rabbit peritoneal and alveolar macrophages(Cohn and Weiner, 1963) and beef leukocytes(Hegner, 1968). The acidic pH optimum of porcine leukocyte lysosomal cathepsin D was consistent with the leukocyte lysosoml cathepsin D of other species such as rabbit PMN cells(Cohn and Hirsch, 1960; Stefanovic *et al.*, 1962) and bovine PMN cells(Hegner, 1968). The acid and alkaline pH optima observed in these studies for cathepsin D was similar to those of human leukocytes(Fraenkal-Conrat *et al.*, 1966) and leukocytes from rabbit(Wasi *et al.*, 1966). The optimal pH of muscle cathepsin D of other animals ranges from 3.5 to 4.8. The pH optima of 4.0, 8.0 and 10.0 for porcine muscle cathepsin D (Parrish and Bailey, 1967) are consistent with results from the present studies. The involvement of cathepsin D in proteolytic activity at alkaline pH is uncertain, since neutral proteases present in leukocyte lysosomes are completely inactive at pH 4.0 but active at alkaline PH.

The existence of aryl sulfatases A and B in leukocyte lysosomes was confirmed by their pH optima. These pH optima values are consistent with those of aryl stlfatases isolated from other tissues (Archer and Hirsch, 1963; Tanaka *et al.*, 1962).

The optimal pH of 4.5 for porcine leukocyte

lysosomal β-glucuronidase is similar to those isola ted from rabbit PMN cells (Cohn and Hirsch, 1960), rats and horse eosinophiles (Archer and Hirsch, 1963) and human blood (Fishman *et al.*, 1948), but lysosomal β-glucuronidase from other tissues have higher optimal pH.

The consistent value for acid phosphatase indicated one enzyme involved in splitting phosphate from sodium glycerophosphate. The activation energy of 7.1 Kcal mol^{-1} for cathepsin D agreed well with the 7.3 Kcal mol^{-1} of porcine muscle cathepsin D (Parrish and Bailey, 1967). The two values for aryl sulfatase was not entirely unexpected in view of the occurrence of aryl sulfatase A and B in the lysosomes and aryl sulfatase C in the microsomes but agreed with the value of ox liver aryl sulfatases (Roy, 1953). The activation energy of RNA-ase ranged from 15.6. to 16.7 Cal mol^{-1} suggesting either multiple forms of the same enzyme or different kinds of RNA-ases.

Energy of activation for β-glucuronidase ranging from 5.38 to 10.63 Kcal mol^{-1} was perhaps due to 24 hr assay period and other difficulties of enzyme assay; a similar range of values from 14.0 to 18.9 KCal Mol^{-1} was reported for ox spleen β-glucuronidase (Miles *et al.*, 1953).

Energy of activation, a characteristic of a specific enzyme, is constant over a range of temperatures until the inactivation temperature of the enzyme is reached. Activation energy is not essentially affected by changes in pH, purity, source, change in enzyme or substrate concentrations or environmental changes unless these factors modify the catalytic surface of the enzyme.Enzymes with low activation energy values are active at lower temperatures compares comparable to their activities at optmum temperatures(Sizer, 1943). The low values for acid phosphatase, cathepsin D and β-glucuronidase indicate efficient activity at low temperatures and strengthen the view that cathepsin D is active during low temperature aging of meat.

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