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Purification of Porcine Leukocyte Lysosomal Hydrolases

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Porcine Leukocyte Lysosomal Hydrolase의 정제에 關한 硏究

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SUMMARY

DEAE-Cellulose, Sephadex column chromatography and polyacrylamide gel electrophoresis were used to purify acid phosphatase, aryl sulfatases, ß-glucuronidase and cathepsin D in *n*-butyl alcohol extracts of porcine leukocyte lysosomes. The degree of purification was quite high for all enzymes studied and some could be identified by histochemical reactions.

INTRODUCTION

Much information has been published concerning the involvement of hydrolases in changing the structure of fibrous proteins during post-mortem aging of meat (reviewed by Parrish, 1971). Most of the past studies of enzymes capable of catalyzing hydrolysis of macromolecules of meat have been made of enzymes removed from muscle tissue by extraction with dilutesalt. A notable exception has been the studies of Parrish and Bailey (1967)who isolated and characterized the hydrolases of bovine muscle lysosomes.

A major difficulty in the above studies was the inability to identify the source of the lysosomes from muscle extracts. Undoubtedly a large quantity of these organelles were derived from blood leukocytes and their constitutent hydrolases possibly participate in macromolecular degradation of muscle tissue during processing.

Cohn *et al.* (1963) have studied properties of lysosomal hydrolases from many types of leukocytes, but most of the cells studied were phagocytes obtained from peritoneal exudates and properties of some of these enzymes have not been studied thoroughly.

The objective of these studies was to purify some of the hydrolytic enzymes in porcine leukocytes using organic solvent extraction, column chromatography and gel electrophoresis.

EXPERIMENTAL SECTION

Lysosome Source. Leukocytes were isolated from porcine blood as described by Venugopal (1970). The granular sediments were separated by centrifugation of the sonicated leukocytes.

Purification Procedures. *n*-Butyl alcohol extracts of aqueous leukocyte suspension, prepared as described by Venugopal (1970), were dialyzed and the dialysate freeze dried. The lyophilized preparations were used to purify and separate the lysosomal hydrolases by chromatography.

DEAE Cellulose Chromatography. N, N-diethylamino ethyl(DEAE) cellulose(Bio-Rad, Cellex D, exchange capacity of 0.8 meg/g; Richmond, California) was used with tris-borate buffer according to the procedure of Peterson and Sober (1962). About 60mg of the lyophilized *n*-butyl alcohol extract was dialyzed against the starting buffer and applied to the column. After eluting the inactive, unabsorbed proteins with the starting buffer (Trisborate buffer 0.01M; pH 6.2), a linear (0-0.34M) NaCl gradient in 0.01M Trisborate buffer, pH 7.2 was used to elute the enzyme proteins and small fractions (10ml, 5ml, 3ml) collected.

Sephadex G-200 Gel Filtration. Sephadex G-200(40-120μ) was used in a jacketed Sephadex column, with tris-borate buffer (0.01M, pH 7.8), employing methods suggested by Flodin (1962). The column was column was calibrated with Blue Dextran 2000. The freezedried *n*-butyl alcohol extract of leukocytes was used after dialysis against tris-borate buffer.

Polyacrylamide Gel Electrophoresis(PAGE). Electrophoresis was performed with an EC 470 vertical gel electrophoresis apparatus as described by Parrish and Bailey (1966). Electrophoretic mobilities were determined by the method of Waldmann-Meyer(1965) at a mean field strength of 16 volts cm^{-1}.

Histochemical Identification of Protein Bands. Enzyme staining reactions were carried out directly on polyacrylamide gel following electrophoresis to identify the enzyme bands.

Acid phosphatase was identified by the post-coupling method of Barka(1961); the PAG electropherogram was incubated in acetate buffer(0.1M, pH 5.0) with sodium 2-naphthyl phosphate (1 mg/ml) as substrate for 2hr at 38°. After washing with acetate buffer, the gel was treated with Fast Garnet GBC Salt (1 mg/ml) in phosphate buffer (0.1M, pH 7.4)for 3 min.

ß-Glucuronidase was identified by its action on 8-hydroxyquinoline-glucuronide by a method similar to that of Fishmann and Baker (1956). The PAG containing the ß-glucuronidase band was incubated in a mixture of 8-hydroxyquinoline-glucuronide and ferric sulfate in acetate buffer (0.1M, pH 5.2) for 12 hr at 38°. After incubation, the gel was rinsed in distilled water and treated for one minute in oxalate buffer(0.25M, pH 4.4). After washing, the gel was suspended in a mixture of equal volumes of 1% (w/v) potassium ferrocyanide and 1N hydrochloric acid and gently agitated for a few minutes.

An Aryl Sulfatase band was identified by adapting the method of Baum *et al.* (1959); the PAG electrophoretogram was incubated in acetate buffer (0.1M; pH 4.7) with dipotassium salt of nitrocatechol sulfate for 3 hr at 38°. After washing with 2% tungstic acid and distilled water, the gel was treated for 2min with alkaline quinol reagent (5 ml of 4% quinol in 0.1 HCl mixed with 95 ml of 2.5 N NaOH, containing 5% Na_2SO_3).

RESULTS

DEAE-Cellulose Chromatography. Results of DEAE-cellulose chromatography (of lyophilized *n*-butyl alcohol extract of leukocytes) are shown in Figure 1. The large protein peak was mostly non-enzymic and ß-glucuronidase was not detected. RNA-ase (not shown in the figure) exhibited multiple peaks. Granules (3,500XG and 10,000 XG) separated by differential sedimentation had simiar distribution patterns, but with higher concentrations of non-enzymic protein. There was distinct separation of acid phosphatase peaks in the 3,500XG fraction and a better sparation of aryl sulfatase A from acid phosphatase in the 3,500XG and 10,000XG sediments. Ary sulfatase peaks were identified by their pH optima. Multiple peaks were observed for all three enzymes with small volumes of eluate(3ml), and a slower elution rate on a longer column (Figure 2).

The extent of enzyme purification achieved by DEAE-cellulose chromatography is shown in Table I. Lyophilization of *n*-butyl alcohol extract enhanced the hydrolase activities, acid phosphatase 53.8%, cathepsin D 58.3%, aryl sulfatase 20.9% and RNA-ase 40%. The degree of purification

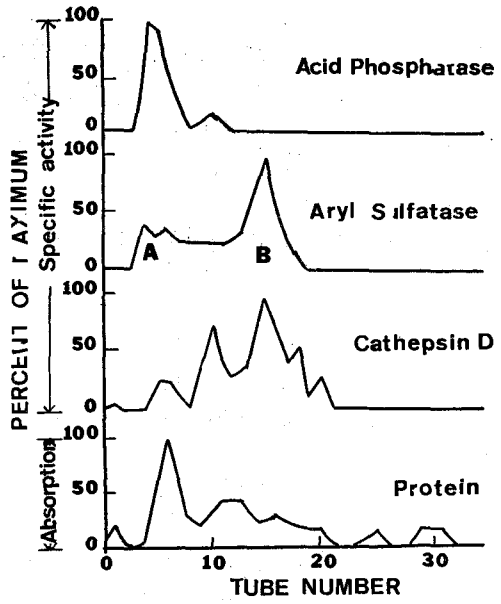

Fig. 1. Elution patterns of porcine leukocyte lysosomal enzymes (*n*-butyl alcohol extract) from DEAE-cellulose(1.5×22cm column). 100 percent absorbance represents 0.46 mg/ml protein nitrogen. Flow rate 45 ml/hr.10 ml fractions collected.

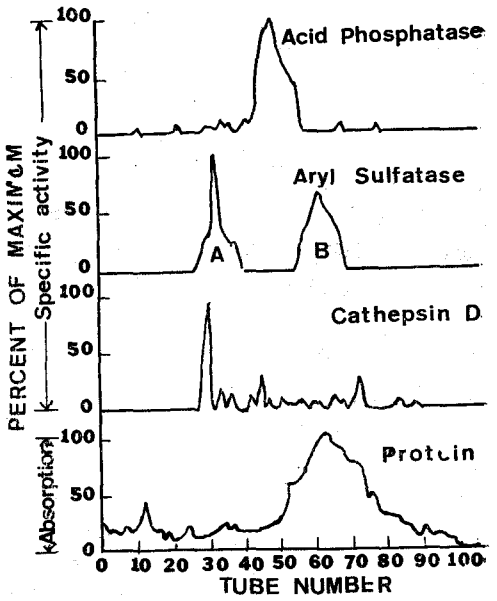

Fig. 2. Elution patterns of porcine leukocyte lysonomal enzymes (*n*-butyl alchol extract) from DEAE-cellulose (2.0×55cm column). 100 percent absorbance represents •4mg/ml protein nitrogen. Flow rate, 30ml/hr. 3ml fractions collected.

Table I. Specific activities and purification of porcine leukocyte lysosomal enzymes separated by DEAE-cellulose chromatography[a,b]

Enzyme	Specific activity				Increased purity relative to		Recovery of enzyme activity (%)
	Leukocyte homogenate	*n*-Butyl after dialysis	Alcohol extract after lyophilization	Most active fraction of eluate	Homogenate	Dialyzed alcohol extract	
Acid phosphatase	2.6	52	80	7,500	2,920	146	82
Cathepsin D	67.4	1,348	2,238	18,563	250	12.5	84
Aryl sulfatase A	13.4	268	828	25,569	1,926	96	79
Aryl sulfatase B	—	—	—	18,310	1,366[c]	68[c]	
RNA-ase	0.16	3.2	16	1,994	13,268	663	87

[a]Recovery of total protein from the column=85%.
[b]Specific activity expressed per mg of protein nitrogen, protein measured by absorbance at 280 nm.
[c]Purity based on the homogenate activity of aryl sulfatase A.

compared to the original sonicated leukocytes was more than 1,000-fold for all enzymes except cathepsin D which was 250-fold. Purification relative to the dialyzed *n*-butyl alcohol extract of sedimented granules was 100-fold or more except for cathepsin D.

Sephadex G-200 Gel Filtration. Figure 3 contains of Sephadex G-200 gel filtration of lyophilized *n*-butyl alcohol extract of porcine white blood cells. The quick elution of most of non-enzymic protein improved the enzyme purification. ß-glucuronidase was well separated from acid phosphatase, aryl sufatases and cathepsin D. The latter enzymes however, were not completely

Table II. Specific activities and purification of porcine leukocyte lysosomal enzymes separated by Sephadex G-200 Gel fiiltrations[a,b]

Enzyme	Specific activity				Increased purity relative to		Recovery of enzyme activity (%)
	Leukocyte homogenate	*n*-Butyl after dialysis	Alcohol extract after lyophil ization	Most active fraction of eluate	Homogenate	Dialyzed alcohol extract	
Acid phosphatase	2.6	52	80	11,960	4,600	230	89
Cathepsin D	67.4	1,348	2,238	50,012	644	37	91
Aryl sulfatase A	13.4	268	828	34,031	2,540	127	90
Aryl sulfatase B	—	—	—	12,983	978[c]	50[a]	—
RNA-ase	0.15	3	17	2,438	15,340	767	92
β-Glucuronidase	6.30	130	160	750	119	6	48

[a]Recovery of total protein=93%.
[b]Specific activity expressed per mg of protein nitrogen, protein measured by absorbance at 280 nm.
[c]Purity based on the homogenate activity of aryl sulfatase A.

separated. Cathepsin D was separated from acid phosphatase but not from aryl sulfatase B. Acid phosphatase was partially separated from aryl sulfatase A. Elution of cathepsin D in two peaks suggested two enzymes with different molecular weights but with the same pH optimum.

The extent of enzyme purification achieved was higher than that obtained by DEAE-cellulose chromatography (Table II).

Polyacrylamide Gel Electrophoresis. PAGE was carried out at pH 8.8 on lyophilized *n*-butyl alcohol extract of porcine leukocytes. The prote

Table III. Electrophoretic mobilities of lysosomal proteins lyophilized *n*-butyl alcohol extract and their identification

PAGE[a] bands	Electrophoretic mobilities[b] (cm^2 $volt^{-1}$ sec^{-1}) $\times 10^{-5}$	Identification[c,d]
1	0.59	
2	1.56	Acid phosphatase
3	1.72	β-Glucuronidase
4	2.72	Aryl sulfatase
5	3.63	
6	6.50	
7	7.80	

[a]Polyacrylamide gel electrophoresis.
[b]Average of eight experiments.
[c]Average of six experiments.
[d]Identified by enzyme staining reactions.

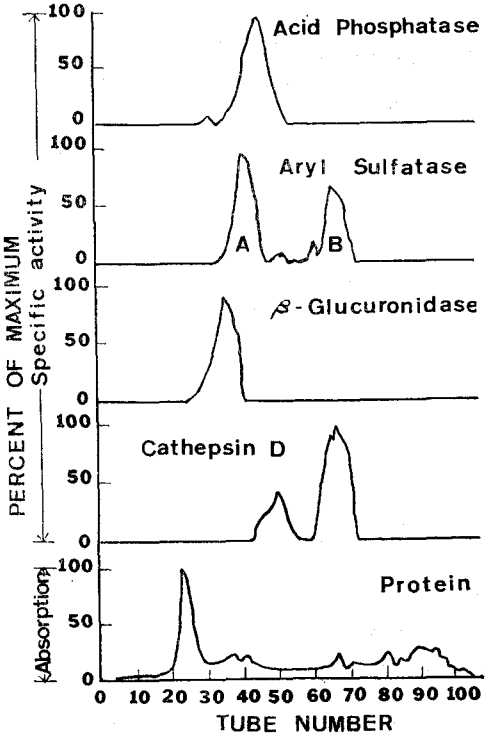

Fig. 3. Elution patterns of porcine leukocyte lysosomal enzymes(*n*-butyl alcohol extract) from Sephadex G-200(2.5×85cm column). 100 percent absorbance representies 0.84mg/ml protein nitrogen. Flow rate 12ml/hr. 3ml fractions collected.

ins in the extract were separated into seven distinct bands identified by electrophoretic mobilits shown in Table III. Three bands were identified

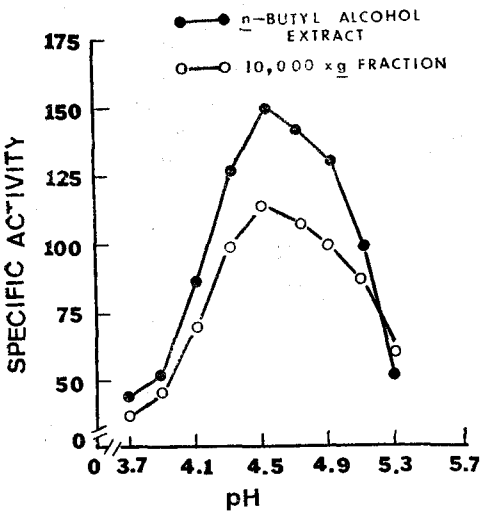

Fig. 4. pH optimum of porcine leukocyte lysosmal β-glucuronidase assayed with phenolphtha lein-β-glucuronide as substrate at 38°.

as acid photphatase, β-glucuronidase and aryl sulfatase by enzyme staining reactions. The acid phosphatase stained as a purple band on a clear background, the β-glucuronidase band was browni sh-purple and the aryl sulfasase band was yellow. All three enzyme-staining reactions were specific for the respective enzymes.

DISCUSSION

Attempts to enrich 10,000XG lysosomal sediment by zonal sucrose density gradient(Ragab *et al.*, 1967) were not satisfactory, due to poor re covery of hydrolases with little increase in their specific activities. Similar attempts to enrich3,500 XG and 10,000XG lysosomal sediments by continuous sucrose density gradient sedimentation (Beaufay *et al.*, 1964) were also unsuccessful due to the distribution of hydrolase activities in different layers of the gradient. *n*-Butyl alcohol-extracted enzymes were far more active than those enriched by the above procedures. The use of this alcohol as an extracting medium for enzymes in sedimented granules and from isolated leukocytes was an effective procedured for removing enzymes from particulate matter. Properties of constituent enzymes appeared to be the same regardless of isola ting procedure. Use of this procedure also assured the absence of bacterial contamination.

Acid phosphatase, cathepsinD, aryl sulfatases A and B and RNA-ase were purified extensively using both DEAE-cellulose and Sephadex G-200 gel filtration. β-glucuronidase was also purified using the latter procedure.

The adaptation of enzyme-staining procedures for identifying enzymes on polyacrylamide gel following electrophoresis was a sensitive qualitat ive procedure and also has potential as a quanti tative tool.

요 약

돼지 白血球 lysosome의 n-butanol 抽出物로 부터 DEAE-cellulose, Sephadex c-200 column chromatography 및 Polyacrylamide gel electrophoresis 로서 acid phosphatase, aryl sulfatase, ß-glucuronidase로서 및 cathepsin D를 分離정제하고 그 성질을 조사하였다.

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