Acid Mucopolysaccharase Activity in Lysosome of Rat Kidney

Osamu Kawamura

It is well known that connective tissue plays important roles on causing arteriosclerosis, collagen disease as well as various pathological changes in other diseases.

Recently, special attention has paid on metabolism of acid mucopolysaccharides as ground substance of connective tissue.

While, in 1965, McKusick\(^1\)\(^2\) described genetic mucopolysaccharidoses.

But, metabolism of acid mucopolysaccharides, especially enzymes which degrade acid mucopolysaccharides are not yet well known.

Bollet, Bonner and Nance\(^3\) reported low activity of enzyme which degraded hyaluronic acid, similar to testicular hyaluronidase, in several tissue.

In 1955, de Duve et al\(^4\) described acid hydrolases in lysosome some, and showed the presence of hyaluronidase in rat liver lysosome. Recently, similar activities were reported by Hutterer\(^5\).

These facts lead us to the prediction that lysosome plays important roles on metabolism of acid mucopolysaccharides.

This report presents the presence of acid mucopolysaccharase activity in lysosome fraction of rat kidney.

**Materials and Methods**

Acid mucopolysaccharides (AMPS) and testicular hyaluronidase: Hyaluronic acid, chondroitin sulphate A and testicular hyaluronidase (Halldase) were obtained from Nakarai Chemical Co. Limited Japan.

Male Wistar rats weighing 200 to 300 g were used for the preparation of subcellular particles. Fractionation of subcellular particles:

- Lysosomal, mitochondrial and microsomal fractions were prepared from the kidney by the method originated by Shibko and Tappe,\(^6\) and final supernatant fluid was referred to as "supernatant fraction."

**Enzyme assay:**

Acid phosphatase activity was used as a marker for lysosome, succino-oxidase for mitochondria and glucose-6-phosphatase for microsome. The concentration of protein was determined by the method by Lowry et al\(^7\), employing bovine albumin as the standard.

Acid mucopolysaccharase assay: The incubation mixture consists of 0.5 ml subcellular fraction (lysosomal, mitochondrial, microsomal and supernatant fraction) and 0.5 ml of 0.1 M acetate buffer or phosphate buffer (0.15 M NaCl) of various pH containing acid mucopolysaccharide.

Incubation was carried out at 37\(^\circ\)C for various intervals, and then stopped by heating in boiling water for 10 minutes.

Then, acid mucopolysaccharides were precipitated completely in 0.04 M NaCl by forming complex with excessive CPC (cetylpyridinium chloride 3 mg per mg AMPS).

The precipitate was allowed to incubate at 37\(^\circ\)C for 1 hour, then approximately 20 mg of heavy Celite were added per 1 mg of AMPS.

The mixture was stirred thoroughly with a glass rod, centrifuged for 30 minutes at 2,700 rpm. and separated to supernatant and precipitate.

Hexosamine in the supernatant was determined by the method by Elson-Morgan\(^8\) N-acetylhexosamine by Amino, Morgan and Watkins method\(^9\) and uronic acid by Dische carboxylic method\(^10\).

The precipitate of AMPS-CPC complex was heated in the tube with tap in 4 N HCl at 100\(^\circ\)C.

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Scheme I  Method for Determination of Acid Mucopolysaccharase Activity in Subcellular Fraction

Acid mucopolysaccharides + Hyaluronic acid, Chondroitin sulphate A
Subcellular fraction + Lysosomal, Mitochondrial, Microsomal and Supernatant Fraction
Mixed in acetate buffer (0.15M NaCl)
Incubated for 3 hours at 37°C
CPC (Cetylpyridinium chloride) in 0.04M NaCl

ppt. (AMPS-CPC complex) + Supernatant
Hydrolysed in 4N HCl
for 5 hours at 100°C
Hexosamine determination
Hexosamine, N-Acetyl hexosamine and Urionic acid determination

<table>
<thead>
<tr>
<th></th>
<th>Hyaluronic acid, reduced (hexosamine µg/mg protein)</th>
<th>Chondroitin sulphate A, reduced (hexosamine µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosome</td>
<td>43.3</td>
<td>30.7</td>
</tr>
<tr>
<td>Microsome</td>
<td>11.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Supernatant</td>
<td>12.9</td>
<td>16.0</td>
</tr>
<tr>
<td>Testicular hyaluronidase</td>
<td>60.0 µg/10 unit</td>
<td>36.3 µg/10 unit</td>
</tr>
</tbody>
</table>

Acid mucopolysaccharase activities in subcellular fractions were shown using hexosamine (µg) of reduced AMPS as indicator after incubating AMPS for 3 hours at 37°C in acetate buffer at pH 4.0 with each fractions.

for 5 hours, neutralized with 0.1 M NaOH, and filtrated (scheme I).

Hexosamine in the filtrate was determined by the method by Boas using column of Dowex-50.

β-Glucuronidase activity of subcellular fractions were assayed by the method by Fishman et al.

RESULTS

Subcellular localization of acid mucopolysaccharase activity:

Acid mucopolysaccharase activities of testicular hyaluronidase, and of lysosomal, mitochondrial, microsomal and supernatant fractions of rat kidney were assayed at pH 4.0 to 6.0 in 0.1M acetate buffer for hyaluronic acid and chondroitin sulphate A as substrates.

Acid mucopolysaccharase activity of lysosomal fraction was higher than that of mitochondrial, microsomal and supernatant fractions for each substrate at pH 4.0 (Table 1, Fig. 1).

As it would be mentioned latter, the optimal pH for lysosomal acid mucopolysaccharase for each substrate were very acidic; namely pH 4.1 for chondroitin sulphate A and pH 5.0 for hyaluronic acid. There was no marked difference between acid mucopolysaccharase activity of each fractions at other pH.

As a matter of cause, acid phosphatase activity
Optimal pH for lysosomal acid mucopolysaccharase:

Acid mucopolysaccharase activity of lysosomal fraction was assayed at pH 3.8 to 6.2 in acetate buffer and pH 5.8 to 8.0 in phosphate buffer.

The activity curve showed peaks at pH 4.1 in acetate buffer and 6.8 in phosphate buffer for chondroitin sulphate A as substrate (Fig. 3), and peaks at pH 5.0 in acetate buffer and 6.1 in phosphate buffer for hyaluronic acid (Fig. 4).

While, testicular hyaluronidase activity curve showed broader plateau for each substrates, at the optimal pH of 6.1 for chondroitin sulphate A and of 6.4 hyaluronic acid as substrates.

Acid mucopolysaccharase activities of mitochondrial or mixed solution of microsomal and supernatant fractions were very low and their activity curves showed no peak for each substrates at any pH.

DISCUSSION

Since Meyer described acid mucopolysaccharides as "hexosamine containing polysaccharides of animal origin," many acid mucopolysaccharides have been found in synovial fluid, cartilage, umbilical cord, skin, tendon, cornea, arterial wall, and predominantly in connective tissue.

Acid mucopolysaccharides (AMPS) are one of several components of amorphous ground substance of connective tissue, and Dorfman has suggested that AMPS in connective tissue plays important roles in physiological and pathological processes, such as calcification, wound healing, lubrication, control of electrolytes and water in

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Fig. 1. Acid mucopolysaccharase activities in subcellular fractions.
L: Lysosome, M: Mitochondria, Mc: Microsome, S: Supernatant, T: Testicular hyaluronidase.
Acid phosphatase activities (a marker for lysosome) of each fractions were shown as released Pi from βglycerophosphate.

of lysosomal fraction, the marker for lysosome, was higher than the others.

Release of particle-bound activity:

Similar to β-glucuronidase activity, acid mucopolysaccharase activity of lysosomal fraction showed to be higher after incubating lysosomal fraction for 10 minutes with 0.1% Triton X-100 at pH 4.0 in acetate buffer. The release of enzymes from lysosomal particles by treating with Triton X-100 was accomplished in about 6 hours (Table II, Fig. 2).

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Fig. 2A. Release of lysosomal acid mucopolysaccharase by Triton X-100.
H: Hyaluronic acid, C: Chondroitin sulphate A.

Fig. 2B. Release of lysosomal β-glucuronidase by Triton X-100.
Enzyme activity was shown as phenolphthalein (µg) released from phenolphthalein glucuronic acid.
Table II  Release of Lysosomal Acid Mucopolysaccharase by Triton X-100

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Lysosome</th>
<th>Incubation time</th>
<th>AMPS reduced (hexosamine μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td></td>
<td>3 hrs.</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated with Triton X-100</td>
<td>3</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>73.5</td>
</tr>
<tr>
<td>untreated</td>
<td></td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td>Chondroitin sulphate A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated with Triton X-100</td>
<td>3</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>57</td>
</tr>
</tbody>
</table>

Lysosomal acid mucopolysaccharase released by Triton X-100 was assayed in 0.1M acetate buffer at pH 4.0 as hexosamine μg of reduced AMPS.

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Fig.3. Effect of pH on lysosomal acid mucopolysaccharase and testicular hyaluronidase with chondroitin sulphate A.
Reactions were done in 0.1M acetate buffer at pH 3.8 to 6.2 and in phosphate buffer at pH 5.8 to 8.0. Enzyme activities were shown using hexosamine μg of reduced chondroitin sulphate A as the indicator.

Fig.4. Effect of pH on lysosomal and testicular hyaluronidase.
Reactions were done in 0.1M acetate buffer at pH 3.8 to 6.2, and in phosphate buffer at pH 5.6 to 8.0. Enzyme activities were shown using hexosamine μg of reduced hyaluronic acid as the indicator.

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extracellular fluid and so on.

It can be considered that enzymes which degrade AMPS are widely distributed in the body, because connective tissue containing AMPS are distributed universally in the body.

The first finding of acid mucopolysaccharase was done by Duran-Raynaud\textsuperscript{15} who reported "spreading factor" in testicular extract in 1928, and Meyer\textsuperscript{16} described "pneumococcal hyaluronidase" in 1936.

Afterwards, Chin and Duthe\textsuperscript{17} proved that the spreading factor and pneumococcal hyaluronidase were the same enzyme.

The chemical structure of AMPS consist of hexosamine and glucuronic acid, having repeating structure of each disaccharide involves alternate 1–4 and 1–3 linkage\textsuperscript{18} (Table III).

Testicular hyaluronidase was proved to be endohexosaminidase, acting on β(1–4)hexosaminidic linkage.

Snake and bacterial hyaluronidase are also endohexosaminidase, but leech hyaluronidase is an endoglucuronidase\textsuperscript{19}

Chondroitinase was proved in Proteus vulgaris by Dodgson\textsuperscript{20} and in Flavobacterium heparinum by Hoffman\textsuperscript{21}

In 1963, Bollet, Bonner and Nance\textsuperscript{3} reported low activities of enzyme which was capable of degrading hyaluronate, chondroitin sulphate and protein-chondroitin sulphate complex in various mammalian tissue, including liver, kidney, spleen, plasma, synovial fluid and synovial tissue of rat and guinea pig.

According to their report, the optimal pH of the acid mucopolysaccharase was found to vary with the nature of the substrate (AMPS) and also with buffer used. The optimal pH of hyaluronidase in rat and guinea pig kidney were 4.6 in acetate buffer with chondroitin sulphate and 3.8 with hyaluronic acid.

The properties of the enzyme in tissue were similar to testicular hyaluronidase.

While, in 1955, De Duve et al\textsuperscript{4} reported the release of several acid hydrolase from particulate fraction of rat liver, which they latter designated as "lysosomal fraction," and lysosome particle was confirmed morphologically by Novikoff\textsuperscript{22,23}

Twelve or more discrete enzymes were found in lysosome particle.

Hutterer\textsuperscript{5} reported the specific enzyme for degradation of AMPS, localized in the lysosomal fraction of rat liver.

According to his report, the enzyme was an endohexosaminidase and optimal pH was 3.9 in acetate buffer with hyaluronic acid, and the degradation products were tetra- and oligo-saccharides.

Aronson and Davidson\textsuperscript{24} reported also lysosomal hyaluronidase of rat liver. In their report, hyaluronidase activities were assayed by the rate of liberation of N-acetyl-glucosamine end group after incubating AMPS with the enzyme extract.

There are other method to assay acid mucopolysaccharase activity; viscosity measurement or turbidmetric method.

In this report, acid mucopolysaccharase activi-

<table>
<thead>
<tr>
<th>Mucopolysaccharide</th>
<th>Monosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>D-glucuronic acid, 2-acetamido-2-deoxy-D-galactose</td>
</tr>
<tr>
<td>Chondroitin sulphate A</td>
<td>D-glucuronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose</td>
</tr>
<tr>
<td>Chondroitin sulphate B</td>
<td>L-iduronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose</td>
</tr>
<tr>
<td>Chondroitin sulphate C</td>
<td>D-glucuronic acid, 2-acetamido-2-deoxy-6-O-sulpho-D-galactose</td>
</tr>
<tr>
<td>Heparin</td>
<td>D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose</td>
</tr>
<tr>
<td></td>
<td>(both residues containing O-sulphate groups)</td>
</tr>
<tr>
<td>Keratosulphate</td>
<td>D-galactose, 2-acetamido-2-deoxy-6-O-sulpho-D-glucose</td>
</tr>
<tr>
<td>Heparitin sulphate</td>
<td>D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose</td>
</tr>
<tr>
<td></td>
<td>(also containing O-sulphate groups),</td>
</tr>
<tr>
<td></td>
<td>2-acetamido-2-deoxy-D-glucose</td>
</tr>
</tbody>
</table>

List derived from reference (74).
ity of rat kidney was assayed by the rate of total residual AMPS after incubating AMPS with subcellular fractions.

Acid mucopolysaccharase activity in rat kidney was also localized in lysosomal fraction, and optimal pH was 4.1 in acetate buffer with chondroitin sulphate A and 5.0 with hyaluronic acid, but in phosphate buffer, 6.8 with chondroitin sulphate A and 6.1 with hyaluronic acid. While, the optimal pH of testicular hyaluronidase showed much broader plateau with both substrate.

The difference of pH optimum in acetate and phosphate buffers may be dependent on the difference of buffer used.

Lysosome contains both N-acetyl-hexosaminidase and β-glucuronidase, and their combined action may degrade AMPS, but exohexosaminidase is inhibited with acetate.

It is physiologically important that lysosome contains acid mucopolysaccharase, whereas the membrane of lysosome particles are easily broken down by several condition and drugs; hypotonic condition, low pH, X-ray irradiation, UV-irradiation, streptolysin O and S, Triton X-100 or anoxia as labilizer, while cortisol, antihistaminics, colchicine or salicylate as stabilizer.

The degradation of AMPS in vivo may be initiated by the lysosomal acid mucopolysaccharase and brought to completion by exohexosaminidase and β-glucuronidase.

Clinical interest in AMPS and acid mucopolysaccharase have been evoked since a substance, presumably chondroitin sulphate B, was isolated from the liver of patient with Hurler syndrome by Brante in 1952.

Dorfman and Loring reported abnormal excretion of AMPS in the urine of patient with Hurler syndrome, and Brown isolated chondroitin sulphate B and heparitin sulphate, although chondroitin sulphate A and C are the principal AMPS in normal urine.

Many researchers have reported urinary excretion of AMPS in Hurler syndrome.

Meyer considered that abnormal storage and excretion of AMPS in Hurler syndrome was caused by abnormality of fibroblast which produced AMPS, and Dorfman suggested there might be defect of enzyme which combined AMPS with protein, but tissue culture of fibroblast of the skin of patient with Hurler syndrome produced AMPS-protein complex as normal fibroblast.

On the other hand, chondroitin sulphate B in the urine of patient with Hurler syndrome is deficient in amino acid.

Van Hoof and Hers suggested that defect in AMPS was due to the deficiency in one or another of lysosomal enzymes.

Abnormal excretions of AMPS were proved in the urine of patients with Hunter syndrome, Sanfilippo syndrome, Morquio syndrome and Scheie syndrome as well as Hurler syndrome, and McKusick has described these syndromes as "genetic mucopolysaccharidoses".

Abnormal excretion of AMPS was also reported in the urine of patient with Marfan syndrome by Berenson et al., but the AMPS in the urine of patient with Marfan syndrome has normal structure.

Meyer and Hoffman reported abnormal deposition of keratosulphate in cartilage of patient with Marfan syndrome.

It is not yet clear if lysosome is concerned with abnormal metabolism of connective tissue and AMPS in Marfan syndrome, but Szabo reported increase in chondroitin sulphonate B and heparitin sulphate in the urine of patient with complex of Hurler and Marfan syndrome, although chondroitin sulphonate B and heparitin sulphate cannot be degraded with lysosomal enzyme.

Changes of AMPS composition in connective tissue in arterial wall may greatly influence upon the physiology of arteries or aorta; viz. permeability, elasticity, calcification and so on.

Kaplan and Meyer isolated heparitin sulphate, chondroitin sulphonate A and C and hyaluronic acid in human aorta, and reported decrease in hyaluronic acid and chondroitin sulphonate C, and increase in chondroitin sulphonate B and heparitin sulphate in aorta with aging.

Manley also reported the difference of AMPS component in human aortic wall with age, blood pressure or position in aorta.

Numerous histological observation have suggested that AMPS may play a role in atherosclerosis of aorta. Klynsstra et al. reported that the concentration of hyaluronic acid decreased with increasing stage of atherosclerosis and aging, although the concentration of total AMPS showed little correlation with the stage of atherosclerosis.

Heparin, heparitin sulphate and chondroitin possess anticoagulant activity whereas hyaluronic acid, chondroitin sulphate A and C do not. In addition, heparin is reported to play a role in lipoprotein lipase activity of plasma.

So, if the lysosome in aortic wall is destructed.
by some condition or drugs, released acid mucopolysaccharase alter normal constitution of aortic wall, and causes pathological changes in aorta.

Paterson et al.54 reported that hyaluronic acid increased and chondroitin sulphate and \(^{35}\)S-uptake decreased in arteries of Alloxan diabetic mouse.

Haukes55 observed sulphate AMPS synthesis decreased in diabetic animal, but AMPS metabolism decreased especially in heart, artery, kidney and skin, so deposition of AMPS occurs in these organs.

Ragan and Meyer56 observed a marked increase in total amount of hyaluronic acid in synovial fluid of patient with rheumatoid arthritis. Blax57 proved an excess of incomplete polymerized hyaluronic acid in rheumatoid synovial fluids, resulting impairment of lubrication between joint surface.

Recently, it is proved that abnormal increase in lysosomal hyaluronidase in synovial membrane in rheumatoid arthritis, and increase in lysosomal particles in rheumatoid synovial tissue58.

Other lysosomal enzymes also increase in rheumatoid synovial fluids59.

The AMPS content in human brain arteries were proved to be different from that of other arteries.60 61 Heart valves contain AMPS as well as arterial wall, so lysosomal mucopolysaccharase may play a role on the pathological changes of heart valve62.

There is not so much AMPS in normal liver, but AMPS, especially chondroitin sulphate B, is increased in the liver in case of liver cirrhosis as well as of chronic hepatitis63.

Hutterer64 reported the activity of hepatic lysosomal acid mucopolysaccharase was related to the turnover rate of hepatic connective tissue, and acid mucopolysaccharase was increased in reversible fibrosis, and diminished markedly in irreversible fibrosis. He suggested that the lysosomal acid mucopolysaccharase activity of liver might be a simple expression of the number and activity of lysosome-rich hepatic mesenchymal cells which influenced the turnover of connective tissue in the reversible and irreversible phase of fibroblasts.

Lysosome are also associated with several disease of liver, as Esner and Novikoff65 observed bilirubin intaken in hepatic lysosome in obstructive jaundice, and pigments in Dubin-Johnson syndrome and copper in Wilson disease were proved also in hepatic lysosomes66 67.

Some researchers have suggested that lysosome are related to circulatory diseases; it is reported that myocardial lysosomes increased in cases of atrial septal defect or congestive heart failure68 69. Fischer70 has demonstrated that granules of juxtaglomerular cells was lysosomes, which appeared to release renin, a protease, under some condition.

Renin, acid adenosine triphosphatase and acid angiotensinase were recently proved to be lysosomal enzymes in rat kidney.71-73.

Thus the lysosome and its enzymes have much correlation with physiological and pathological process in the body either in normal or in disease.

Lysosomal acid mucopolysaccharase in kidney may also play a role in hypertensive or renal diseases.

### Summary

1. Acid mucopolysaccharase activity in lysosome of rat kidney was demonstrated using hyaluronic acid and chondroitin sulphate A as substrates.
2. The enzymes were released from lysosomal particles by treating with Triton X-100, as well as lysosomal \( \beta \)-glucuronidase.
3. The optimal pH for acid mucopolysaccharase of lysosome were pH 4.1 for chondroitin sulphate A and pH 5.0 for hyaluronic acid as substrate in acetate buffer, but in phosphate buffer pH 6.8 for chondroitin sulphate A and pH 6.1 for hyaluronic acid.

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