The Use of Streptomyces Hyaluronidase in Connective Tissue Specimens for Electron Microscopic Histochemistry of Acid Mucosaccharides

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Summary

Streptomyces hyaluronidase was used as a means of selectively digesting connective tissue specimens for the electron microscopic identification of hyaluronic acid. These specimens included human umbilical cord and rabbit aorta. Testicular hyaluronidase was also employed as a means of similar digestion in the same specimens for comparison. The effects of digestions with Streptomyces and testicular hyaluronidas upon the dialyzed iron reaction of acid mucosaccharides can consistently be interpreted in terms of not only the substrate specificities of the enzymes but the biochemically known nature of acid mucosaccharides present in each connective tissue. Streptomyces hyaluronidase is thought to depolymerize selectively and eliminate hyaluronic acid in connective tissue specimens for electron microscopy as in those for light microscopy and to be useful for the electron microscopic identification of hyaluronic acid in mucosaccharide histochemistry.

Introduction

A novel hyaluronidase with an absolute substrate specificity has been purified from cultures of Streptomyces hyalurolyticus nov. sp.14,15, and has proved to be useful for the histochemical identification of hyaluronic acid in the system of light microscopy24,25,27. Biochemical assay studies have demonstrated that digestion with Streptomyces hyaluronidase results in the selective degradation and elimination of hyaluronic acid in histologic sections of different acid mucosaccharide-containing tissues for light microscopy27. Furthermore, the Streptomyces enzyme was found to fail to affect practically no other mucosaccharides than hyaluronic acid such as neutral and sulfated acid mucosaccharides in the system of light microscopy28. The use of Streptomyces hyaluronidase in specimens for electron microscopic histochemistry of connective tissue mucosaccharides should, likewise, be promising for gaining insight into the precise localization of hyaluronic acid at the ultrastructural levels. In electron microscopic histochemistry of mucosaccharides, digestion experiments with mucosaccharidases such as testicular hyaluronidas6,7,8,13,16, siali-
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dases\(^2\),\(^3\),\(^6\) and chondroitinases\(^5\),\(^28\) can be performed upon at least three forms of tissue specimens; tiny blocks, thick sections and ultrathin sections\(^29\). In light microscopy of acid mucosaccharides, however, the usefulness of Streptomyces hyaluronidase for the identification of hyaluronic acid has been confirmed in thick sections of connective tissues\(^27\),\(^30\). Hence, it seems likely that digestion experiments with the Streptomyces enzyme must be done pertinent in thick sections of mucosaccharide-containing connective tissues which are to be processed for electron microscopy.

This communication demonstrates the use of Streptomyces hyaluronidase as a means of selective digestion of hyaluronic acid in thick sections of connective tissues for electron microscopy of acid mucosaccharides, particularly as it gives reasonable results, when the Rinehart-Abul-Haj solution\(^18\) of dialyzed iron is employed as a staining reagent of choice. In order to be sure that the Streptomyces enzyme acts exclusively upon hyaluronic acid, digestion with testicular hyaluronidase was performed in parallel in the same connective tissue specimens as those employed for digestion with the Streptomyces enzyme. The results obtained in the present study are taken to indicate that Streptomyces hyaluronidase is useful for the histochemical identification of hyaluronic acid in connective tissue specimens for electron microscopy as in those for light microscopy.

Materials and Methods

Surgical specimens of human umbilical cords and autopsy specimens of rabbit aortas were used. Immediately after being removed from donors, these tissues were placed in either of the two fixatives; (a) chilled (4°C) cacodylate buffered (pH 7.2) 2.5% glutaraldehyde-2.0% paraformaldehyde\(^9\) and (b) chilled (4°C) cacodylate buffered (pH 7.2) 2.5% glutaraldehyde\(^8\). In the fixatives, the tissues were sliced by a razor blade into small strips with a thickness of approximately 1-2 mm and fixed at 4°C for 1-2 hr. After fixation, the materials in the form of small strips were rinsed in 7.5% sucrose and then sectioned at 40-60 µ with a tissue sectioner of Smith-Farquhar type or in a cryostat. The thick sections thus obtained were rinsed in 7.5% sucrose and kept intact or subjected to the procedures of either the enzyme digestion or control experiments described below. Subsequently, the tissue sections were reacted 12-24 hr for the Rinehart-Abul-Haj solution\(^18\) of dialyzed iron at room temperature. The dialyzed iron stock solution was prepared according to the Hardin-Spicer modification\(^4\) of the original method\(^18\). Before use, 1 volume of glacial acetic acid and 4 volumes of the dialyzed iron stock solution\(^6\) were mixed, yielding a staining solution of pH 1.8-2.0. After staining with the dialyzed iron reagent, the tissue sections were rinsed in cacodylate buffer (pH 7.2) and refixed 1-1.5 hr at room temperature in cacodylate buffered (pH 7.2) 2.0% osmium tetroxide. The tissue specimens were then rinsed in distilled water, dehydrated with graded ethanol series of ascending concentrations, embedded in Epon 812\(^11\) and sectioned with a LKB or Porter-Blum ultramicrotome. The ultrathin sections doubly stained with uranyl acetate\(^21\) and lead\(^19\) were examined in a Hitachi HU 11-C or HS-4 electron microscope.

I. Digestion experiments with hyaluronidases
(a) Digestion with Streptomyces hyaluronidase:
It was found appropriate to use 40 μ thick sections of glutaraldehyde-paraformaldehyde fixed tissues to digest hyaluronic acid and eliminate it in tissues consistently. Satisfactory results were obtained by digestion procedures similar in incubation conditions to those employed for tissue sections for light microscopy\(^27,30\). Tissue sections were incubated at 39-41°C for 4-6 hr in 0.1 M phosphate buffer (pH 5.0) solution which contains Streptomyces hyaluronidas (Amano Pharmaceutical Company, Nishiharu, Aichi-ken, Japan) at an activity concentration of 100-150 TRU (Turbidity Reducing Unit)/ml.

(b) Digestion with testicular hyaluronidase:

As in the case with digestion with Streptomyces hyaluronidase, 40 μ thick sections of glutaraldehyde-paraformaldehyde fixed tissues were used for digestion experiments with testicular hyaluronidase. Tissue sections were incubated at 37°C for 4-6 hr in 0.1 M phosphate buffer (pH 5.5) containing testicular hyaluronidase (Sigma Chemical Company, St. Louis, U.S.A.) at an activity concentration of 1.0-1.5 mg/ml.

II. Control experiments for enzyme digestions

Two types of control experiments were conducted for the enzyme digestion experiments.

(a) Controls with buffer solutions without enzymes:

Some control tissue sections were incubated at 39-41°C or 37°C for 4-6 hr in 0.1 M phosphate buffers (pH 5.0 or 5.5) without enzymes.

(b) Controls with buffer solutions with inactivated enzymes:

Other control tissue sections were immersed at 39-41°C or 37°C for 4-6 hr in 0.1 M phosphate buffers (pH 5.0 or 5.5) with heat-inactivated Streptomyces or testicular enzymes.

The results of these control experiments were compared with those obtained with intact tissue sections which underwent none of the experimental procedures.

**Results**

The two types of the present connective tissues, human umbilical cord and rabbit aorta consist of a number of different histologic structures; umbilical cord (stroma, arterial and venous stroma, lakes etc.) and aorta (interfibrillar spaces in intima, media and adventitia etc.). In the present study, electron microscopic observations were focussed upon such histologic structures as constitute the major proportions of each connective tissue; stroma and arterial stroma in human umbilical cord and interelastic spaces of the media in rabbit aorta.

I. Human umbilical cord

In the intact controls of human-umbilical cord tissues, the stroma reacts positively for dialyzed iron and dialyzed iron reactive structures are demonstrated not only in the spaces between fibrils but in close association with fibrils (Figs. 1 and 2). As high power views of the stroma disclose, these dialyzed iron reactive structures are found to consist of fine granules (Fig. 3). In the spaces between fibrils, dialyzed iron reactive fine granules are aligned in filamentous figures of different thicknesses (50-200 Å) and these figures form ramifying meshworks which are here and there continuous with fibril-associated structures consisting of similarly reactive fine granules (Fig. 3). Under the
histochemical conditions employed in the present study, the dialyzed iron reaction of undifferentiated mesenchymal cells appears to be capricious, and no systematic observations were made on the cytochemical features of such cells. In the arterial stroma of the umbilical cord tissues, dialyzed iron reactive fine granules exhibit dual (fibril-associated and unassociated) distribution patterns which are essentially identical with those recorded for the stroma of the umbilical cord tissues.

Digestion with Streptomyces hyaluronidase results in the disappearance of ramifying meshworks of filamentous figures consisting of dialyzed iron reactive fine granules in the spaces between fibrils throughout the stroma tissues (Figs. 4 and 5). In the stroma of the umbilical cord tissues undergone digestion with Streptomyces hyaluronidase, fibril-associated dialyzed iron reactive fine granules are relatively smaller in amount and weaker in stainability, as compared with those observed in the stroma of intact control tissues (Figs. 4 and 5). Digestion with Streptomyces hyaluronidase yields a nearly identical effect upon the dialyzed iron reactive fine granules exhibiting dual distribution patterns in the arterial stroma of the umbilical cord tissues.

In the human umbilical cord tissues digested with testicular hyaluronidase, ramifying meshworks of filamentous figures consisting of dialyzed iron reactive fine granules are never observed in the spaces between fibrils throughout the stroma tissues (Figs. 6 and 7), as in the case with the tissues digested with Streptomyces hyaluronidase. Digestion with testicular hyaluronidase gives, likewise, rise to a suppressive effect upon fibril-associated dialyzed iron reactive fine granules which is significantly more marked than the effect induced by digestion with Streptomyces hyaluronidase (Figs. 6 and 7). Thus, dialyzed iron reactive fine granules associated with fibrils are far smaller in amount and apparently less intense in stainability, in comparison with those in the tissues digested with Streptomyces hyaluronidase. Similarly, digestion with testicular hyaluronidase results in a markedly suppressive effect upon dialyzed iron reactive fine granules exhibiting dual distribution patterns in the arterial stroma of the umbilical cord tissues.

II. Rabbit aorta

In the intact controls of rabbit aorta tissues, dialyzed iron reactive structures are found to consist of fine granules in the interelastic spaces of the media, and these granules are mostly associated with collagenous fibrils, but some of them are distributed in irregular patterns in the spaces between fibrillar and cellular elements (Fig. 8). In the interelastic spaces of the media, the dialyzed iron reaction of both the connective tissue cells and smooth muscle cells is capricious under the present histochemical conditions, and no attempts were made to observe systematically the cytochemical features of the dialyzed iron stained cellular elements.

Digestion with Streptomyces hyaluronidase diminishes apparently the amount and staining intensity of dialyzed iron reactive fine granules in the interelastic spaces of the media (Fig. 9), but such effect is not uniform throughout the interelastic spaces; in some loci the iron reactive fine granules are more pronounced in amount and stainability than those in other loci (Fig. 9).

If the tissues of rabbit aorta are subjected to digestion with testicular hyaluronidase,
dialyzed iron reactive fine granules in the interelastic spaces of the media are strikingly diminished in amount and stainability and are substantially smaller in amount and more feeble in stainability than those in the tissues undergone digestion with Streptomyces hyaluronidase (Fig. 10).

III. Control experiments.

(a) Controls with buffer solutions without enzymes:
In the two types (umbilical cord and aorta) of control tissues treated with buffer solutions without enzymes, dialyzed iron reactive structures are detected which are largely similar in composition, stainability and distribution patterns to those observed in the two types of intact control tissues. There are, however, exceptions such as dialyzed iron reactive fine granules in some parts of the spaces between fibrils in the stroma of human umbilical cord tissues incubated in buffer solutions (pH 5.5) without enzymes. Dialyzed iron reactive fine granules in such sites are smaller in amount and weaker in stainability, as compared with those in intact control tissues.

(b) Controls with buffer solutions with heat-inactivated enzymes:
In the two types (umbilical cord and aorta) of control tissues subjected to treatment with buffer solutions with heat-inactivated enzymes, dialyzed iron reactive structures are essentially comparable in morphological characteristics to those observed in the two types of intact control tissues.

Discussion

In a series of reagents for the electron microscopic histochemical demonstration of acid mucosaccharides such as colloidal iron\(^{10,23}\), colloidal thorium\(^{11,17}\) and ruthenium red\(^{29}\), the Rinehart-Abul-Haj solution\(^{18}\) of dialyzed iron appears to be one of the best in terms of the staining selectivity for acid mucosaccharides, the property of penetrating into tissues, the size of granulations of products of reaction with acid mucosaccharides and the usability in combination with mucosaccharidase digestion procedures\(^{6,23,29,31}\); staining with the dialyzed iron solution has been shown to demonstrate selectively acid mucosaccharides rich in carboxyl groups and sulfate esters\(^{4,23}\), and the form of iron which binds specifically to the substrates has been reported to be the complex ion FeOH\(^{++10}\). Thus, all the dialyzed iron reactive structures observed in the two types (umbilical cord and aorta) of the present connective tissues are thought to contain acid mucosaccharides rich in carboxyl groups and sulfate esters.

In biochemical system, Streptomyces hyaluronidase is known to exhibit an absolute substrate specificity; hyaluronic acid is the only acid mucosaccharide which can be degraded by this enzyme and various types of isomeric chondroitin sulfates, chondroitin, heparin, keratosulfate and chitin were shown to be unaffected by this enzyme\(^ {14,15}\). In the system routinely employed in light microscopic histochemistry of mucosaccharides, such absolute substrate specificity of Streptomyces hyaluronidase has been certified as a result of previous histochemical\(^ {24,25,28,27}\),\(^ {30}\) and biochemical\(^ {24,27}\) studies on the nature of the enzyme.

It has biochemically been well recognized that testicular hyaluronidase splits endo-\(\beta-\)
N-acetyl-D-glucosaminidic residues specifically. Under the conditions of light microscopic histochemistry, likewise, such substrate specificity of the testicular enzyme has been confirmed and the enzyme has been known to depolymerize and eliminate, in tissues, all the acid mucosaccharides containing endo-\(\beta\)-N-acetyl-D-glucosaminidic residues such as hyaluronic acid, chondroitin and chondroitin sulfates A and C.

In the present study, the following structural components have been found to be extinguished by digestion with Streptomyces hyaluronidase; (a) dialyzed iron reactive fine granules forming ramifying meshworks of filamentous figures in the spaces between fibrils and certain moieties of fibril-associated structures in the stroma and arterial stroma of human umbilical cord tissues and (b) some proportion of fibril-associated and unassociated dialyzed iron reactive fine granules in the interelastic spaces of the media of rabbit aorta tissues. In view of the staining selectivity of the dialyzed iron reagent and the histochemically confirmed substrate specificity of Streptomyces hyaluronidase, all of these dialyzed iron reactive fine granules are conceived to consist of hyaluronic acid.

In the stroma and arterial stroma of human umbilical cord tissues and in the interelastic spaces of the media of rabbit aorta tissues, digestion with testicular hyaluronidase results in further disappearance of structural components consisting of dialyzed iron reactive fine granules, in addition to the components extinguished by digestion with Streptomyces hyaluronidase. In other words, graded effects upon dialyzed iron reactive fine granules forming various structures have been induced by digestions with Streptomyces versus (vs.) testicular hyaluronidases in the human umbilical cord and rabbit aorta tissues. In light of the histochemically known substrate specificity of testicular hyaluronidase, those dialyzed iron reactive fine granules additionally extinguished by digestion with the testicular enzyme can be interpreted to consist of chondroitin, chondroitin sulfates A and/or C in the umbilical cord and aorta tissues.

According to the previous data of biochemical analyses of various mucosaccharides in animal tissues, the major acid mucosaccharides involved in human umbilical cord tissues are hyaluronic acid and chondroitin sulfate C, while as those possibly involved in mammalian aorta tissues chondroitin sulfates A and C, hyaluronic acid, dermatan sulfate and heparan sulfate being enumerated. If these data on the biochemical compositions of acid mucosaccharides in the two types of tissues examined in the present study are taken into consideration, the present effects of digestions with Streptomyces and testicular hyaluronidases upon the dialyzed iron reaction of acid mucosaccharides can consistently be interpreted in terms of the substrate specificities of the two hyaluronidases, which have been confirmed histochemically in connective tissue sections for light microscopy.

All the data obtained in the present study are well comprehended by the concept that digestion of thick tissue sections with Streptomyces hyaluronidase provides a promising means for the electron microscopic histochemical identification of hyaluronic acid. In electron microscopic histochemistry of mucosaccharides, however, digestion experiments with mucosaccharidases can be performed upon at least three forms of tissue specimens; tiny blocks, thick sections and ultrathin sections. In the present study, moreover, only
a limited number of mammalian connective tissues have been employed as materials for testing the utility of Streptomyces hyaluronidase in electron microscopic histochemistry of mucosaccharides. Accordingly, further extensive studies on different forms (tiny blocks, thick sections, ultrathin sections etc.) of various mucosaccharide-containing connective tissues in a number of animal species are needed, in order to determine critically the usefulness of Streptomyces hyaluronidase for the electron microscopic histochemical identification of hyaluronic acid and to find out the most appropriate histochemical conditions for procedures of digestion with the Streptomyces enzyme. These statements of discussion are to be stressed, particularly because in the present study the dialyzed iron reaction of acid mucosaccharides in some structural components examined is found, though exceptionally, to be affected by prior treatment with buffer solutions without enzymes. The true nature of such enigmatic phenomenon remains to be elucidated precisely, even though either possible liberation of acid mucosaccharides into the buffer solutions or perplexing buffer interference of dialyzed iron reaction in staining acid mucosaccharides might be responsible for it.

References

13) Matukas, V.J., Panner, B.J. and Orbison, J.L.: Studies on ultrastructural identification and


33) Zeghe, F. T.: The demonstration of the individual acid mucopolysaccharides in human aortas, coronary arteries and cerebral arteries. 1. The methods. J. Histochem. Cytochem., 10: 441-
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Description of Plate Figures

Figs. 1 and 2. Parts of the stroma of human umbilical cord tissues. Dialyzed iron reactive fine granules are associated with fibrils and are aligned in filamentous figures forming ramifying meshworks (arrows) in the spaces between fibrils. Dialyzed iron stained. ×31,000.

Fig. 3. A high power view of the stroma of human umbilical cord tissues. Ramifying meshworks of filamentous figures (arrows) consisting of dialyzed iron reactive fine granules in the spaces between fibrils and fibril-associated accumulations of similarly reactive fine granules are obvious. Dialyzed iron stained. ×55,000.

Figs. 4 and 5. Parts of the stroma of human umbilical cord tissues. In the spaces between fibrils no dialyzed iron reactive structures can be found at all. Fibrils are associated with dialyzed iron reactive fine granules which are smaller in amount and less intense in stainability than those in Figs. 1 and 2. Streptomyces hyaluronidase digested and dialyzed iron stained. ×31,000.

Figs. 6 and 7. Parts of the stroma of human umbilical cord tissues. In the spaces between fibrils no dialyzed iron reactive structures can be discerned at all. Fibrils are associated with dialyzed iron reactive fine granules which are far smaller in amount and less intense in stainability than those in Figs. 4 and 5. Testicular hyaluronidase digested and dialyzed iron stained. ×31,000.

Fig. 8. Part of the media in rabbit aorta tissues. In the interelastic spaces dialyzed iron reactive fine granules are mostly associated with collagenous fibrils and occasionally distributed in the spaces between fibrillar and cellular elements. E (elastic fiber), C (cellular element). Dialyzed iron stained. ×14,000.

Fig. 9. Part of the media in rabbit aorta tissues. In the interelastic spaces dialyzed iron reactive fine granules are smaller in amount and less intense in stainability than those in Fig. 8. E (elastic fiber), C (cellular element). Streptomyces hyaluronidase digested. ×14,000.

Fig. 10. Part of the media in rabbit aorta tissues. In the interelastic spaces dialyzed iron reactive fine granules are apparently smaller in amount and less intense in stainability than those in Fig. 9. E (elastic fiber), C (cellular element). Testicular hyaluronidase digested and dialyzed iron stained. ×14,000.
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