Novel Procedures for Isolating Intact Retinal Vascular Beds From Diabetic Humans and Animal Models

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**Purpose.** To improve the 30-year-old “trypsin digestion” procedure for isolation of the complete retinal vasculature, which, in its time, was a revolutionary advance that allowed important discoveries about diabetic retinopathy; to provide a method that will yield more consistent results when applied to retinas representing a wide range of ages, species, and severity of vascular disease, such as that occurring in diabetes.

**Methods.** Because the Difco trypsin preparation (Difco Laboratories, Detroit, MI) is a crude pancreatic extract, containing variable amounts of chymotrypsin, elastase, amylase, lipase, ribonuclease, collagenase, and other contaminants, an attempt was made to determine which of the major enzymes alone (using purified preparations), or what combination of enzymes, might be most effective in providing consistently clean yet intact retinal vasculatures from eyes of different origins.

**Results.** Purified elastase alone (40 U/ml) in 100 mmol/l sodium phosphate buffer with 150 mmol/l sodium chloride and 5 mmol/l EDTA at pH 6.5 and 37°C gave better results than various concentrations of purified trypsin or chymotrypsin alone, or mixtures of trypsin/chymotrypsin, trypsin/elastase, chymotrypsin/elastase, or the crude trypsin preparation.

**Conclusions.** Elastase, which exhibits broad protease activity, and not trypsin, is the most important enzyme of the standard, crude trypsin digestion procedure for removal of the nonvascular tissues of the retina. Invest Ophthalmol Vis Sci 1993;34:2097–2104.

Visualization of the intact vascular pattern and detailed structure of the retinal blood vessels is necessary for critical studies of the early angiopathies associated with diabetic retinopathy. It was the reintroduction of retinal whole mount methods that permitted the histopathologic demonstration (rediscovery) of capillary microaneurysms in diabetic retinopathy. Although retinal whole mounts maintained the vasculature intact and demonstrated microaneurysms, they did not provide a clear view of capillaries. They yielded only gross approximations of the underlying structural detail, even when combined with histochemical and/or contrast enhancement methods such as the benzidine method, the periodic acid-Schiff technique, intra-vascular injections with india ink, injections with latex solutions, and injections with silver nitrate solutions.

The first procedures that removed the nonvascular tissues and permitted adequate isolation and staining of intact retinal vessels involved digestion using “trypsin alone” (actually a crude trypsin preparation) or combinations involving pepsin, crude trypsin, and/or collagenase. The introduction of these procedures had a far-reaching impact on our understanding of the etiology of diabetic retinopathy. Without a so-called “trypsin digestion technique,” it would have been impossible to provide the resolution necessary to discover that pericyte degeneration is the first histopathologic lesion of diabetic retinopathy. Because of its great contribution to our understanding of healthy and diseased human retinal vessels, the trypsin digestion technique has become a standard method in
many pathology laboratories. It generally uses a crude commercial grade of powdered trypsin, 3.0% w/v in 100 mmol/l tris (hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer at pH 7.8 and 37°C.

Although the trypsin digestion procedure has been useful and can work very well, it yields inconsistent results, especially with animal retinas. Even under some of the best conditions it is difficult to obtain good quality mounts of the retinal vasculature from mice, rats, and monkeys with this procedure. We suspected that the frequent overdigestion and variability experienced might be attributable to the fact that the trypsin utilized was truly a crude pancreatic extract, with trypsin being only one of many enzymes present. Also, the enzyme composition might vary greatly among the batches, depending on what vendors the distributors use for their supply. Although it is known that the crude pancreatic extract contains several enzymes in addition to trypsin, including chymotrypsin, elastase, lipase, amylase, ribonuclease, and collagenase, information on the concentrations of the various enzymes from one batch to the next is rarely available to the user, which makes it difficult to assess what enzymes might be most critical.

Therefore, although the standard technique for studying the early stages of diabetic retinopathy has been called trypsin digestion, the primary enzyme involved in the digestion of retinal neural tissue has never actually been determined. The current study on retinal digestion procedures employed purified preparations of several of the various enzymes known to be present in the crude trypsin to test each one or combinations of different ones with minimal contamination from other enzymes.

The findings demonstrate that elastase, an enzyme of broad protease activity, and not trypsin, is the most useful component enzyme of the so-called “trypsin digestion” technique for making digest preparations of the retinal vasculature. Using known concentrations of elastase alone provides a more reliable means of producing good retinal digests than does using purified trypsin alone or relying on the trypsin activity present in the crude trypsin.

MATERIALS AND METHODS

Tissues Used

New enzyme digestion procedures were developed using elastase on retinas from normal Sprague-Dawley rats ranging in age from 2 to 27 months. However, the procedures were applied successfully to retinas from monkeys (diabetic and normal), rats (diabetic, normal, galactose-fed, and hypertensive), mice (diabetic and normal), and human donors (diabetic and nondiabetic) by using slight variations in incubation and wash times. The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (published May 1992) and the NIH Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 85-23 (revised 1985) were adhered to in the care and handling of all animals. The human donor eyes were obtained through the National Disease Research Interchange (Philadelphia, PA), the Rochester Eye and Tissue Bank (Rochester, NY), and the Old Dominion Eye Bank (Richmond, VA). No human subjects were involved in the study.

Tissue Fixation and Handling

Eyes were removed from fully anesthetized or recently killed animals. During enucleation the eye was held by the nictitans with toothed forceps and pulled gently forward so at least 3 mm of optic nerve was left connected to the eye. This prevented displacement of myelin from the optic nerve into the vessels and/or posterior regions of the retina. The retinas were fixed at room temperature for at least 4 days by immersing the whole eye (slit at limbus) or the posterior segment in 4.0% w/v paraformaldehyde in 50 mmol/l sodium/potassium phosphate buffer with 6.0% sucrose at pH 7.2. All microdissection procedures were performed with great care to keep the retina intact. Avoiding retinal tears and precise cutting in the area surrounding the optic nerve were found to be especially critical to maintain the original pattern of the retinal vasculature during digestion. The retinas were handled and transferred throughout all procedures by using a large-bore pipette rather than forceps.

The fixed retinas were rinsed in deionized water for 4.5–6 hr and then were incubated for 10–35 min in an agitating water bath preheated to 37°, in 40 units/ml elastase in 100 mmol/l sodium phosphate buffer with 150 mmol/l sodium chloride and 5.0 mmol/l ethylenediamine tetraacetic acid (EDTA) at pH 6.5. The shortest enzyme incubation times were used for the youngest rats and the longest times were used for the oldest rats, because of increases in neural tissue resistance and vascular tissue stability in older animals. The tissues were washed overnight in 100 mmol/l Tris-HCl (pH 8.5) at room temperature and then transferred to deionized water for removal of the now-loosened vitreous and digested neural elements by gentle agitation using the sides of forceps and the sides and ends of very fine sable brushes as needed. After all loose tissue was removed, as determined using a Zeiss (Zeiss Inc., Thornwood, NY) operating microscope and darkfield illumination stage, the retinas were incubated in fresh enzyme for 3–5 min and then subjected to a second overnight wash at room temperature in fresh Tris-HCl buffer. On the third day, the retinas were again transferred to deionized water for additional removal of digested neural elements. If the
vessel network was completely free of nonvascular elements then it was mounted as described later. If not, another 3- to 5-min incubation in fresh enzyme was followed by a third overnight wash in fresh Tris-HCl buffer at room temperature. On the third or fourth day, removal of the final remnants of adhering nonvascular tissue was performed in deionized water. In preparation for a flat mount, each retinal vascular bed (still cup-shaped) was cut once from its periphery to the void where the optic nerve had been and then notched two or three times in the periphery as needed to permit even flattening. All remnants of optic nerve were cut off. Then the retinas were mounted by flotation in calcium-magnesium-free Dulbecco's phosphate-buffered saline over slides coated with 0.25% gelatin (Knox, unflavored; Knox Gelatine, Inc., Englewood Cliffs, NJ). Once air-dried in a dust-free environment, the mounts of the retinal vasculature were stained using the periodic-acid-Schiff reaction and hematoxylin counterstaining, described by Luna. Similar tissue handling procedures were used to test various enzyme solutions at different concentrations. These are summarized in Table 1. The sources of the reagents are included in the following section.

### Reagent Sources

Crude trypsin (1:250) was obtained from Difco (Detroit, MI). All the other enzymes used were of the highest purity available. The purified trypsin was received as a lyophilized and crystallized powder (code TRTPCK) from Worthington Biochemical Corporation (Freehold, NJ) and contained an inhibitor of chymotrypsin activity. Alpha chymotrypsin (code CDS) was obtained as a lyophilized powder, also from Worthington. Lyophilized pancreatic elastase (catalog #324689), lyophilized bovine testicular hyaluronidase (catalog #385931, with <0.001% trypsin and chymotrypsin activity), lyophilized neuraminidase (catalog #480714), and crystalline papain were all obtained from Calbiochem Corporation (La Jolla, CA). Crystallized and lyophilized Nagarse (protease type XXVII, catalog #P4789) was obtained from Sigma Chemical Company (St. Louis, MO). Cell culture grade L-cysteine (catalog #8152), which was used (4.0 mg/ml) to protect the sulfhydryl group at the active site of papain (Table 1), was also obtained from Sigma Chemical Company.

### Microscopic Evaluation

The preparations were examined and photographed using a Leitz Orthoplan microscope (Ernst Leitz Ltd., Midland, Ontario, Canada) equipped with an Orthomat camera and a Nikon micro/macrophotography unit (Nikon Inc., Melville, NY). If the entire retinal vasculature was not intact or the vessel patterns were greatly distorted, the preparation was scored "0."

### Table 1. Summary of Main Retinal Digestion Procedures Tested

<table>
<thead>
<tr>
<th>Enzyme Mixture</th>
<th>Units/ml</th>
<th>Quality*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude trypsin (3.0% DIFCO 1:250)</td>
<td>100–1200</td>
<td>4</td>
</tr>
<tr>
<td>Purified papain</td>
<td>30–100</td>
<td>0</td>
</tr>
<tr>
<td>Purified papain with cysteine (4.0 mg/ml)</td>
<td>30 + 8</td>
<td>0</td>
</tr>
<tr>
<td>Papain with cysteine (4.0 mg/ml) + chymotrypsin†</td>
<td>0.5–13000</td>
<td>0</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>2635 + 27</td>
<td>5§</td>
</tr>
<tr>
<td>Hyaluronidase followed by elastase‡</td>
<td>27 + 1580</td>
<td>2</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>1–2</td>
<td>0</td>
</tr>
<tr>
<td>Neuraminidase followed by elastase‡</td>
<td>2 + 27</td>
<td>5§</td>
</tr>
<tr>
<td>Nagarse</td>
<td>0.83–4.0</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin + chymotrypsin + Nagarse</td>
<td>9 + 23 + 1–3</td>
<td>1</td>
</tr>
<tr>
<td>Purified trypsin</td>
<td>1–33</td>
<td>0</td>
</tr>
<tr>
<td>Purified chymotrypsin</td>
<td>1–16</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin + elastase‡</td>
<td>15 + 10</td>
<td>1</td>
</tr>
<tr>
<td>Chymotrypsin + elastase</td>
<td>9 + 10</td>
<td>1</td>
</tr>
<tr>
<td>Trypsin + chymotrypsin</td>
<td>27 + 9</td>
<td>1</td>
</tr>
<tr>
<td>Trypsin + chymotrypsin + elastase</td>
<td>27 + 9 + 5</td>
<td>3</td>
</tr>
<tr>
<td>Purified elastase in Tris-HCl buffer, pH 8.5</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>Purified elastase in phosphate buffer + NaCl</td>
<td>+ EDTA, pH 6.5</td>
<td>40</td>
</tr>
</tbody>
</table>

* Relative quality was assessed subjectively on a scale of 1 to 10 with 10 being the best and 0 indicating virtually impossible to obtain an analyzable mount of the entire retinal vasculature.
† Purified enzymes were used in all the tests involving more than one enzyme.
‡ The enzymes were used in sequence rather than together so the appropriate buffers could be used.
§ The tissue was only slightly disintegrated after incubation in the hyaluronidase.
||
Otherwise, the quality of the preparations was assessed subjectively on a relative scale of 1–10 based on the stainability, freedom from nonvascular tissue, the level of microscopic detail, and the degree to which the overall vascular patterns were preserved in the isolated retinal vessels. To receive the rank of ten, the particular method had to consistently provide preparations of high quality in all categories. All other ranks were based on relative subjective comparisons among the best preparations rendered by the different methods.

Results

Crude trypsin preparations (1:250, Difco, Detroit, MI) were used in the initial attempts to obtain whole and intact retinal vasculatures that were completely free of neural tissue. There was a great deal of variability and it was very difficult to obtain preparations that did not exhibit both underdigested neural elements and over-digested retinal vessels (Fig. 1A). Therefore, most of this study used various purified enzymes of the types known to be present in the crude Difco trypsin on paraformaldehyde-fixed retinas from normal Sprague-Dawley rats. All these enzymes, including trypsin itself, were received as lyophilized and/or crystallized powders of the highest purities available. Many tests were run using various combinations of these enzymes and also other enzymes and additives that are not present in pancreatic extracts, but which might provide potential benefit for the selective dissociation of neural retinal tissue. The main findings are summarized in Table 1. In successful cases (those rated 1–10) the only enzyme concentrations shown are those that gave the best results. In unsuccessful cases, in which it was essentially impossible to obtain an undistorted mount of the entire retinal vasculature (those rated 0), the full ranges of the enzyme concentrations tested for the specific incubation mixtures are indicated. Some of the other mixtures and the concentrations tested are included in the descriptions of the general findings that follow.

Although papain, hyaluronidase, neuraminidase, and Nagarse are very useful for the dissociation of fresh tissues, including some neural tissues, they were essentially ineffective in selectively removing the neural tissue from formalin-fixed retinas, whether used alone or in combination with other enzymes. This occurred despite being used in a wide range of concentrations. The range was from 30 U/ml to 1200 U/ml for papain, from 0.5 U/ml to 15000 U/ml for hyaluronidase, from 1 U/ml to 2 U/ml for neuraminidase, and from 0.83 U/ml to 4.0 U/ml for Nagarse.

Essentially no success was achieved when either purified trypsin or chymotrypsin were used alone, for a wide range of concentrations. At the lower concentrations and shorter incubation times some of the external layers of the retina were digested, but the vessels were never freed from the tissue of the inner retinal layers. At the higher concentrations and longer incubation times, the inner retinal layers lost structural integrity, and the main vessels as well as the capillaries started to disintegrate because of overdigestion, that is, excess enzymatic attack. In rats, it was virtually impossible to obtain a preparation of the entire vasculature that was completely free of undigested neural remnants yet showed no signs of overdigestion of the vessels. The vessels were always damaged before enough enzyme action occurred in the neural tissue to remove all the debris that was attached to the isolated vessels.

Many combinations of trypsin and chymotrypsin with or without elastase or Nagarse were used, some of which are shown in Table 1. In these tests, the trypsin was used in concentrations varying from 1 U/ml to 27 U/ml, the chymotrypsin from 1 U/ml to 9 U/ml, and the elastase from 0.1 U/ml to 10 U/ml. In all cases, undigested neural tissue remained in the preparations, yet the capillaries were overdigested. The mixture of trypsin (27 U/ml), chymotrypsin (9 U/ml) and elastase (5 U/ml) gave preparations that approached the quality of those produced by the crude trypsin.

Elastase gave the best results of all the enzymes used. When elastase was used after hyaluronidase or neuraminidase, it was only after the elastase was introduced that significant dissociation of the tissues resulted. Purified elastase alone was tested in different buffers at concentrations of 1 U/ml to 46 U/ml. At 27 U/ml in 100 mmol/l Tris-HCl buffer at pH 8.5 and 37°C, elastase gave very good results (Fig. 1B). Elastase alone, 40 U/ml in 100 mmol/l sodium phosphate buffer with 150 mmol/l sodium chloride and 5 mmol/l EDTA at pH 6.5 and 37°C, gave excellent results with rat retinas and also with retinas from human donors, mice, and monkeys (Fig. 2). Longer digestion times were generally required for retinas from the older animals and from diabetic and galactosemic animals, but fortunately the vessels were more stable in these retinas.

Although the sodium phosphate buffer was the ideal vehicle for the enzyme digestion, Tris-HCl (pH 8.5) was clearly the most effective buffer for the overnight washes, because it permitted much easier removal of nonvascular tissue than the phosphate buffer (pH range 5.3–7.8) on the next day. Its effectiveness may be attributable to the higher pH alone, but it is possible that the Tris-HCl buffer actually facilitates the washout of tissue-bound elastase through a mechanism involving the enzyme’s catalytic site.

Mild sonication during enzyme incubation was tested but it did not yield any better results than the agitation provided by the water bath. Many other varia-
FIGURE 1. Representative whole mounts of retinal vessels from a 10-mo-old female Wistar rat (A) and an 18-mo-old male Sprague-Dawley rat (B). Whereas digestion with 27 U/ml of purified elastase (B) usually retains the in vivo structure and arrangement of arteries (a), veins (v), and capillaries (c), digestion with crude trypsin (A) often leaves areas with undigested neural tissue (arrows) even when the enzyme digestion has already caused extreme fragility and breakage of capillaries (arrowheads), suggesting overdigestion of vessels. Calibration bar = 500 μm for both micrographs.
FIGURE 2. Whole mounts of the vasculature beds of retinas from a normal mouse (A) and a normal monkey (B) obtained by incubations in elastase (40 U/ml) in 100 mmol/l sodium phosphate buffer with 150 mmol/l sodium chloride and 5.0 mmol/l EDTA at pH 6.5 and 37°C. Note the maintenance of the in vivo arrangements of the arteries (a), veins (v), and capillaries (c), including the patterns surrounding the macula (m) in the monkey. Calibration bars = 500 μm.
Apparently the "contaminating" enzyme, elastase, and not the trypsin of the classical "trypsin digestion" mixture is the most reliable protease for the digestion of nonvascular components of formalin-fixed retinas. We now report greatly improved methods for the isolation of intact and clean vascular beds from rat retinas based on the use of purified elastase, an enzyme of broad protease activity. Compared to these novel elastase digestion procedures, the trypsin digestion technique, so commonly used in pathology laboratories, is not as consistent or as easily controlled. Our experience using crude trypsin digestion, although reasonably successful (Table 1), often resulted in preparations showing both underdigestion and overdigestion. Our tests with purified trypsin indicate that this enzyme by itself is actually more prone to attack vessels than the neural tissue surrounding the vessels. This agrees with evidence that it is not trypsin alone but a combination of enzymes in the crude trypsin that produces the dissolving effect in several tissues. In the mouse retina, Cuthbertson and Mandel found that purified trypsin was even less effective than distilled water, with some DNase added, for preparing retinal digest preparations of vessels from fresh retinas.

The term "trypsin digestion" is misleading because the Difco 1:250 preparation that is usually used is a crude pancreatic extract containing several other enzymes that vary in amounts from one preparation to another. The unpredictable concentrations of these contaminating enzymes may account for the variability in the incubation times that are required. In their early description of the retinal "trypsin digestion" technique, Kuwabara and Cogan mentioned that the human retinal tissue must be left in the enzyme solution for 1–3 hr, suggesting a wide range of variability in the incubation times. Similarly, the pepsin–trypsin method involves ranges of 20 min–17.5 hr for the enzyme incubation times.

More important than the variation in incubation times required by the crude trypsin method is the inconsistency observed in the quality of the final vascular mounts. When a vascular mount exhibits inadequate removal of neural tissue and/or overdigestion of vessels, it does not lend itself to unambiguous analysis of pathologic changes. This could result in misleading interpretations of experimental results. For instance, the earliest vessel lesions of diabetic retinopathy occur in capillaries of the peripapillary region, often the most difficult area to isolate intact and, at the same time, free of contaminating debris. Loss of this region from retinal digests of some experimental animals and not others, because of inadequate procedures, could bias the data and have profound consequences.

The term "elastase" comes from the very early findings of Baló and Banga who discovered the enzyme in pancreatic extracts, but mistakenly concluded that "elastase" digests only elastin and not other proteins. Although elastase has essentially no trypsic or chymotryptic activity, its specificity is quite broad. Elastase is an endopeptidase; it can hydrolyze a wide variety of peptide bonds on the C-terminal side of neutral amino acids having aliphatic side chains. It is true that elastase is the only pancreatic enzyme that can digest elastin, but elastin is only one of the many substrates for this enzyme. The determination of its complete amino acid sequence and three-dimensional structure has shown that elastase is highly homologous with other serine proteinases. In a crude preparation, trypsin and chymotrypsin are able to hydrolyze certain peptide bonds after initial elastase attack. It found that although purified trypsin was quite ineffective in disintegrating embryonic cells, elastase alone could dissociate cells in high yields from chick embryonic heart, breast muscle, liver, and unkeratinized skin. These findings demonstrate a clear effectiveness of elastase in digesting a broad range of extracellular substrates involved in cell adhesion. Perhaps the relative ineffectiveness of purified trypsin in digesting neural retinal elements is attributable to its specificity for basic amino acid side chains only.

The elastase-based digestion mixtures reported here have consistently provided mounts of the entire rat retinal vasculature that are free of contaminating debris, yet completely intact and arranged in the original, in vivo patterns. Also, these procedures, sometimes with slight modifications, have routinely resulted in equally successful digest preparations from several other types of retinas during the last 3 years, including human and several animals, such as the mouse and monkey. The preparations are of sufficient quality to permit critical analysis of the earliest changes associated with diabetic retinopathy. These early lesions occur in the central retinal region, which were so rarely preserved in previous studies. Unless such a reliable procedure is used, meaningful comparisons cannot be made between control and experimental retinas regarding disease-related changes.

Although based on a crude enzyme preparation, the "trypsin digestion" procedure made possible the early discoveries and descriptions of the microangiopathies associated with diabetic retinopathy, such as degeneration of pericytes and proliferation of endothelial cells. It is hoped that current refinements in retinal digestion procedures will similarly stimulate progress in detecting and developing treatment for early vascular lesions that cause diabetic retinopathy.
and other retinal disorders. Although application of the "trypsin digestion" procedure to nonretinal tissues has shown few successes,8 the use of well-defined enzyme mixtures and a thorough consideration of other potential variables using modern technologies may bring success similar to that obtained in the retina.

**Key Words**

retina, capillaries, diabetes, elastase, trypsin digestion

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**References**