Histochemistry 38, 331—338 (1974)
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Protease Substrate Film Test

H. W. Denker

Arbeitsgruppe Prof. Gottschewski am Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany

Received November 28, 1973

Summary. An improved variant of the protease substrate film test is presented which combines high sensitivity and more accurate localization while allowing control of the chemical purity of the substrate. Directions for the preparation of gelatin films are given in detail, critical steps being the fixation and swelling in alkaline solution. Post-incubation staining rather than the use of prestained films is preferred to avoid inhibition of enzymes. Post-staining also makes histologic details easily discernible. The substrate films are suitable for routine use. They have been applied specifically for the investigation of sperm acrosomal proteases and blastocyst proteases; the result of these tests are also briefly described.

Introduction

Recently there has been an increase of interest in protease substrate film tests, especially for the study of sperm and embryonic proteases (Benítez-Bribiesca and Velázquez-Meza, 1972; Bergström, 1970; Denker, 1971; Gaddum and Blandau, 1970; Kirchner, 1972; Owers and Blandau, 1971; Penn *et al.*, 1972). In most cases some kind of "stain" is incorporated into these substrate films before incubation: viz silver grains in the photograph emulsions (methods of Adams and Tuqan, 1961; Fratello, 1968; Kirchner, 1972; Roitt *et al.*, 1965; Shear, 1969), Indian ink in the method of Owers and Blandau (1971), and a Fast blue B—azo stain in Cunningham's method (1967) (similarly used by Owers, 1971). To avoid inhibition of enzymes, we were interested in the use of "unstained" gelatin substrates. Such unstained membranes are indeed used in a protase substrate film test which was described earlier (Denker, 1969), but whose sensitivity and accuracy of localization seem to be unsatisfactory. The purpose of this study therefore is to present a modified technique by which the sensitivity and sharpness of enzyme localization is improved and in which the principle of using pure gelatin and staining *after* incubation is kept. It will also be demonstrated that by this method the same range of sensitivity can be obtained as provided by recent variants of the photographic emulsion techniques.

Material and Methods

Animal Procedures. Rabbit ejaculate was obtained using an artificial vagina. It was then diluted about 5-fold (v/v) with TC-199-medium (pH 6.7) at 37° C. Segments of rabbit uteri enclosing blastocysts were obtained at 5–$7^1/_3$ days post coitum (d p.c.). They were immediately frozen with liquid nitrogen, stored in a deep freezer at −25 to −30° C and then cut on a cryostat at −20° C at a thickness of 14 μm.

Chemicals. Formaldehyde solution: Fisher Scientific Corp. No. F-79 or Merck No. 3999; Gelatin: Eastman No. 5247 (purified, pigskin) or Merck No. 4070; Hydroxide of hyamine 10-x: Packard No. 1248 (1 m solution in methanol); TC Medium 199: Difco No. 5477-72.

Histochemical Techniques. In order for us to achieve the optimal combination of physical and chemical requirements by which we could devise and recommend a suitable procedure for the protease test, an investigation of the influences of varying the following conditions was first attempted, basing the experiments on the general schedule of the previously described test (Denker, 1969):

1. The effects of changes in the conditions of fixation of the substrate films using varying concentrations of formol and ethanol in the fixing fluid.
2. The effects of differences in temperatures, and in the duration of fixation.
3. Post-treatment with varying concentrations of NaOH, KOH and NH_4OH solutions.
4. Investigations of the appropriate conditions for the storage of the films and for incubation during the performance of the test.

Results

All of the above mentioned conditions of fixation and posttreatment were found to influence sensitivity and/or sharpness of localization of the test. For optimal results, therefore, the following procedure was devised:

Recommended Method

A. Preparation of Substrate Films

A 1. Spread 0.1 or 0.15 ml of gelatin solution (5% w/v, dissolved in distilled water, ~50° C) on a carefully defatted slide over an area of 2.5 × 5 cm.

A 2. Let solidify in a horizontal position on a cool surface. Let dry completely at room temperature (22–23° C).

A 3. Store overnight in a wet chamber (covered staining jar with distilled water on bottom) at + 4° C.

A 4. Transfer directly from the refrigerator to fixative: 10 ml commercial formaldehyde solution (~37%) + 90 ml 50% ethanol, room temperature (22–23° C), for 1 hour.

A 5. Wash 20 min in running tap water (≤ 20° C), rinse in distilled water.

A 6. Air drying at room temperature (22–23° C).

A 7. Let swell in 7% NH_4OH solution at room temperature (22–23° C) until the gelatin film detaches from the slide (~3 min). It is helpful to use an upright jar (*e.g.* Coplin type) with only a low level of NH_4OH solution so that part of the gelatin film stays outside; this part then will keep sticking to the slide and by this way prevent the gelatin film from getting lost. Gently remove the slide from the solution in such a way that the gelatin will readhere to the slide.

A 8. Air drying at room temperature (22–23° C).

A 9. Store dry films in covered staining jars or in plastic bags at −25° C.

B. Performance of Protease Test

B 1. Remove substrate films from freezer (keep jars covered and bags closed until slides reach room temperature).

B 2. (Optional, for sperm proteases) Immerse substrate films in $3 \cdot 10^{-3}$ m hyamine hydroxide in 0.05 m ethylene diamine-acetic acid buffer (Fasella *et al.*, 1957) for 2 min. A pH of 7.0 was chosen for the present investigations. Air drying is carried at room temperature.

B 3. Spread 1 drop of sperm suspension on the substrate film (using the blood smear technique) or attach fresh frozen sections of organs (*e.g.* uterus containing blastocysts). Let dry 1 min at room temperature.

B 4. Incubate in a wet chamber (Petri dish, sealed with vaseline) containing either distilled water or volatile buffer of chosen pH (ethylene diamine-acetic acid buffer, Fasella *et al.*, 1957) at 38° C. Incubation time for sperm enzymes 15–45 min, for rabbit blastocyst proteases $1^1/_2$–4 hours.

B 5. Stop reaction by fixing in formaldehyde solution (commercial formaldehyde solution, ~37%, 30 ml + distilled water 70 ml) at 4° C, $\geq$15 min.

B 6. Wash ~3 min in running tap water and distilled water.

B 7. Stain in 0.2% toluidine blue in borate buffer pH 10.0 at 4° C for 30 min (If desired, any specific protein stain may be used instead).

B 8. Wash briefly in running tap water and distilled water (If too much stain gets lost by this treatment, rinse only in a diluted NH_4OH solution instead of water). Air drying.

B 9. (Optional) For mounting, dehydrate air dryed films in graded alcohols (starting from 80%), clear in xylene, mount with a synthetic resin. These preparations are stable for many years.

The whole process can be interrupted (and the slides be stored) at either step B 5 or after B 8.

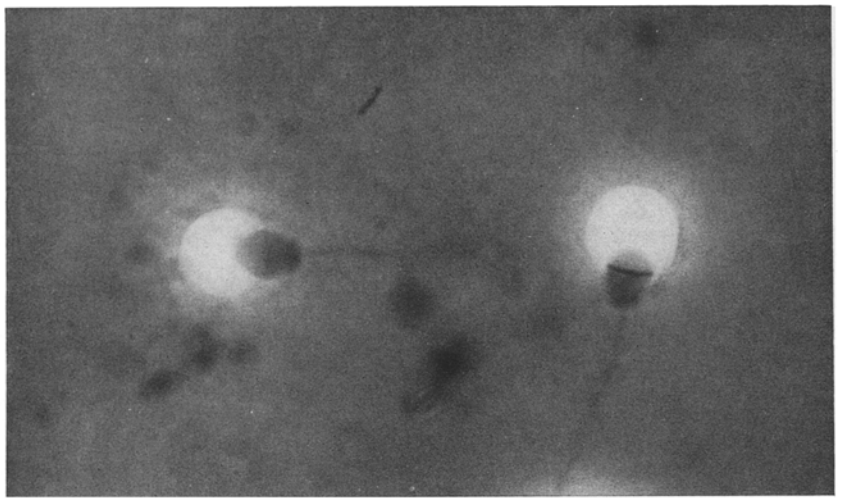
C. Principal Result of the Test

Sites of enzyme activity are indicated by unstained or only slightly stained halos in the gelatin film.

Individual Observations on Rabbit Sperm and Blastocysts

In the case of rabbit sperm, sharply circumscribed round-shaped halos appear in the gelatin surrounding the anterior part of the sperm head (Fig. 1). When hyamine is applied (B 2), in all parts of the slide most sperm show this reaction. The diameter of the halos nevertheless is found a little different in different regions of the slide. On contrast, when hyamine is omitted, the reaction is usually positive only in the thick end parts of the smear, only a low percentage of the sperm react, and the diameter of the halos varies considerably.

In rabbit blastocysts, the revised procedure allows to localize the main protease activity in the blastocyst coverings, whereas the trophoblast does not show much activity. This is especially evident in the stages $6^2/_3$ and 7 d p.c. (Fig. 2). With prolonged incubation (*e.g.* over night), a positive reaction is also obtained in the wall of 5 d and 6 d p.c. blastocysts. At $7^1/_3$ d p.c. there is a marked difference in reactivity between the embryonic and the abembryonic part of the blastocyst: At the embryonic pole (embryonic disc), still well-preserved remnants of the blastocyst coverings show only a very week reaction; on contrast, in the abembryonic part where the coverings are, in this stage, nearly completely dissolved and often hardly recognizable anymore, the activity is very high.—In all stages, the lysis zones are usually surrounded by an area in which the gelatin shows increased dye uptake.



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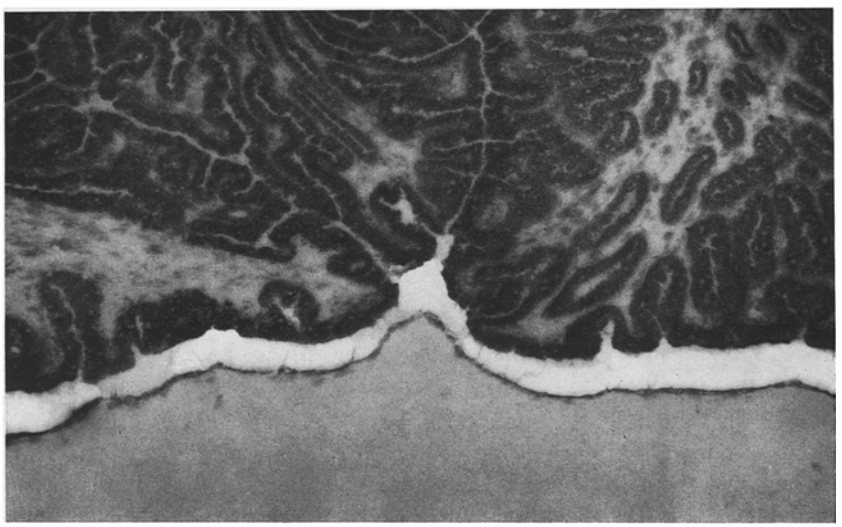

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Fig. 1. Smear of rabbit sperm. Substrate film pretreated with hyamin. Incubation 45 min. × 1400. The round-shaped and sharply circumscribed halos surround the anterior part of the sperm head (acrosomal region)

Fig. 2. Section through rabbit uterus with blastocyst, 7 d p.c., mesometrial. Incubation $2^1/_2$ h. × 90. The gelatin is digested in the area between trophoblast and endometrium, *i.e.* the location of the disintegrating blastocyst coverings (which, themselves, cannot be seen anymore in this section). Uterine secretion (in the uterine crypts), blastocyst fluid and trophoblast do not react

Discussion

The major modifications we introduced now into the protease substrate film test bring about an important increase in *sensitivity:* For example, with the variant

of the method we used previously, overnight incubation (16 h) was needed for the demonstration of rabbit blastocyst proteases (Denker, 1972). With another variant, using a higher alcohol concentration in the fixing fluid, 3–5 hours were found sufficient (Denker, 1971), but in this case the localization was not very sharp. Using the method described in the present paper, the incubation time for rabbit blastocysts can be shortened to $1^1/_2$–4 hours, for sperm acrosomal proteases even to 15–45 min. The *localization* seems to be accurate, the lysis zones being restricted, after short incubation periods, to structures known to be sites of protease activity (acrosomal region of sperm heads). In rabbit blastocysts, we previously could not decide whether the activity would be localized in the trophoblast or in the blastocyst coverings, although the former was thought to be the true site (Denker, 1971, 1972). The modified procedure enables us to localize the main protease activity in the coverings. This is in agreement with results presented by Kirchner (1972), whereas our observations about the correlation between the state of coverings dissolution and pattern of protease activity are not (cf. results). It is evident that the post-staining of gelatin *and* section as applied in our method is a valuable means to study the correlation between enzyme activity and morphological features.

As compared to previously used methods (Denker, 1969, 1972), the major *modifications of the test* are: reduced thickness of the film; changed time and temperature of fixation; adding of a further step: swelling of the film in alkaline solution followed by detachment and re-mounting of the gelatin films. Additional modifications are: storage of the films at −25° C and, for the demonstration of sperm acrosomal enzymes, application of hyamine. The latter is done in order to break the acrosomal membranes (cf. the biochemical work of Hartree and Srivastava, 1965; Stambaugh and Buckley, 1969; Zaneveld *et al.*, 1969). Hyamine application increases considerably the sensitivity of the sperm protease test as well as the percentage of sperm reacting and the uniformity of the reaction over the whole slide. If hyamine is omitted, only certain areas of the slide (where the sperm did not dry completely prior to incubation) will show the reaction. This lack of uniformity was also previously seen with other methods not including detergents (Penn *et al.*, 1972). Triton X-100 has about the same improving effect as hyamine except that the localization obtained by the test seemed to us to be a little less sharp.

The *detachment and re-mounting steps* of preparation of the substrate film (A 7) merit special discussion for being evidently most important for the increase in sensitivity of the substrate films. It seems interesting that the method recently proposed by Penn *et al.* (1972) includes steps which are comparable to these: the autoradiographic stripping film used by them is developed in a highly alkaline solution, afterwards it is floated on water where it swells again, and finally mounted and dried on a slide. But these authors did not discuss a special importance of this treatment for the high sensitivity of the membranes which they observed.

We have done a small series of experiments to prove whether swelling of gelatin films and subsequent attachment to a slide might influence the sensitivity of the method (Denker, unpublished data). We used the method of Penn *et al.* (1972). Kodak AR-10 autoradiographic plates were prepared and processed as described by these authors, except that for fixation, reagent grade sodium thio-

sulfate (20% w/v) was used. Rabbit sperm suspended in Hanks' solution pH 7.6 were tested. Each developed AR-10 plate was divided into two parts. From one part strips were prepared and (a) floated in distilled water at 22°C (or 0°C) and mounted, or (b) floated in 60% (b 1) or 80% (b 2) ethanol and mounted. The other part (c) was used unstripped and unfloated.

We made the following observations: Unstripped (and unfloated) films (c) and strips floated in 80% ethanol (b 2), which did not swell during floating, showed far less sensitivity than the water-floated (and swollen) controls (a). Differences were considerable when sperm smears were dried for about one minute and then incubated for long periods (4–5 hours). Furthermore, in these unsensitive films (b 2 and c) the halos formed were irregularly shaped and not sharply circumscribed so that any correlation with distinct parts of the sperm head was difficult to achieve, whereas on water-floated films the halos were round-shaped and distinct. Changing the temperature of the floating water from 22°C to 0°C had little effect. Films floated in 60% ethanol (b 1) apparently had reduced sensitivity as compared to water-floated ones. In general, there was a *correlation between sensitivity and the degree of swelling* of the films during floating: the most sensitive films were those which were allowed to swell maximally (a). Strips floated in 60% ethanol (b 1) swelled moderately and exhibited medium sensitivity, but films floated in 80% ethanol (b 2) (where they did not swell at all) were as insensitive as unstripped (and unfloated) films (c) were.

In discussion of these observations, we suggest that swelling and mounting of the gelatin substrate films are critical steps. In earlier investigations, we already observed augmented sensitivity of films which were swollen during floating and thereafter mounted (Denker, 1971). These observations were made in the course of experiments with glycogen-gelatin films, which of course we did not use further for lack of specificity. We suppose that the procedure of swelling, floating, remounting and drying of the film changes the physico-chemical properties of the gelatin in some way. The film thereby would gain an increased tendency to retract itself when incubated in a humid atmosphere. If then the film becomes disintegrated in a certain area (in our test due to the action of proteolytic enzymes), the retracting forces would cause the formation of a hole. Such a hole could be expected to be relatively sharply circumscribed and the surrounding area might gain increased protein density (as indeed suggested by our observation of increased dye uptake in this margin area). The formation of a hole might start already after a short incubation time, *i.e.* after the breakdown of only few protein molecules (which would not yet change the staining properties of the gelatin enough to become apparent). This means that the ability to retract would provide the gelatin films with especially high sensitivity, and the physical "enlarging" process would make the holes formed easily visible. There might also be some relationship to biochemical work showing that tension accelerates the hydrolysis of reconstituded and denatured collagen fibres by proteolytic enzymes (Helfman and Bibby, 1967; Sizer, 1949).

Up to now, this explanation for the increased sensitivity of our modified substrate films remains hypothetical. Further work is needed to prove its validity. It has also to prove the possible involvement of other mechanisms, *e.g.* an effect of the alkali treatment on the carbohydrate sidechains found in gelatin glyco-

proteins (Schwarzmann and Micheel, 1970). Such sidechains inhibit the action of proteases (for discussion see Denker, 1970).

Acknowledgements. I am grateful to Prof. Dr. E. S. E. Hafez for providing me with the possibility to finish the experiments in his laboratory at Wayne State University, Detroit, Michigan, USA. Appreciation is extended to Dr. Daoust, Montréal, for valuable discussion. The excellent technical assistance of Mrs. Sophia El-Deeb is gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (De 181/3).

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Dr. Dr. H. W. Denker
Arbeitsgruppe Prof. Dr. G. H. M. Gottschewski
Max-Planck-Institut für Immunbiologie
D-7800 Freiburg i. Br.
Stefan-Meier-Straße 8
Federal Republic of Germany