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Substrate–Gel Electrophoresis for Composition and Molecular Weight of Proteinases or Proteinaceous Proteinase Inhibitors

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A rapid, sensitive, and generally applicable substrate–sodium dodecyl sulfate–polyacrylamide gel electrophoresis method for detection of proteinases or proteinaceous protease inhibitors in biological preparations is described. Electrophoretic separation of proteinases or proteinaceous proteinase inhibitors in the sample using sodium dodecyl sulfate–polyacrylamide gel slabs is followed by immersion of the gel in (1) a protein substrate solution for detection of proteinases or (2) an appropriate proteinase solution, and then in a protein substrate solution for detection of proteinase inhibitors. Some advantages of the reported method over previously described techniques that incorporate substrate into the gel matrix are: (1) the development of bioactive bands, as well as staining and washing, is accomplished more quickly, i.e., 4 to 6 h; (2) the trailing of proteolysis which often appears in copolymerized substrate systems is eliminated; (3) the method is applicable to assay at pH values other than those used for electrophoresis; (4) molecular weight markers can be visualized on the same gels; (5) proteinaceous proteinase inhibitors can also be examined in the biological samples; and (6) the sensitivity is several times higher than that of former assays.

At the present time, there is increasing interest in new sources of enzymes with specialized properties for food industry and other biotechnologies (1–3). The search for new enzymes includes the evaluation of activity in extracts of various materials and the characterization of the composition of molecules bearing the activity and their molecular weight. These studies are time consuming and, to some extent, expensive.

Electrophoresis in polyacrylamide gels, including sodium dodecyl sulfate (SDS–PAGE),[2] is a powerful biochemical tool which can be used to analyze the heterogeneity and molecular weight (MW) of proteins. This technique has been successfully applied to the assay of proteinases using different approaches. One of them, which includes the hydrolysis of a protein substrate after electrophoresis, is called substrate–electrophoresis (substrate–SDS-PAGE). Several methods for determining the proteinase composition of tissue extracts and biological fluids have been reported (4–9). Lacks and Springhorn (10) pointed out several analytical advantages of these techniques, such as (i) identification of a bioactive protein in complex mixtures; (ii) estimation of enzyme molecular weight; (iii) semiquantitative measurement of specific isozymes; (iv) anodal migrations of proteins regardless of isoelectric point; and (v) applicability to diverse kinds of proteins, including those from membranes. Lacks and Springhorn (10) showed that most proteolytic enzymes remain active after SDS treatment. The activity was recovered even after disruption of their disulfide bonds, when the detergent was removed from the electrophoresis gel. However, in their experience some proteinases such as trypsin could not be renatured. Heussen and Dowdle (4) described a technique for the analysis of plasminogen activators in SDS–PAGE containing copolymerized plasminogen and gelatin. They removed the SDS after

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[2] Abbreviations used: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MWM, molecular weight marker; TCA, trichloroacetic acid; TRT, trout; CHK, Chinook salmon; CRF, crayfish; TRY, trypsin; CHY, chymotrypsin; 4P, langostilla; SBTI, soybean trypsin inhibitors.

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electrophoresis by soaking the gel in a Triton X-100 solution. Brown *et al.* (11) developed a binding-mode method which can be used for the estimation of the amount of enzyme present in the preparation. The method consisted of measuring the length of the track formed by the migration of the enzyme. Kellerher and Juliano (12) prepared conjugates from glutaraldehyde-activated linear polyacrylamide and bovine serum albumin, casein, or gelatin. This technique was used to avoid substrate migration during the electrophoresis. Miskin and Soreq (13) developed a method to detect and quantify proteinases in small volumes of crude biological samples. It included hydrolysis of iodinated substrates and transference onto nitrocellulose filters, autoradiography, and/or counting. Some disadvantages of those methods are that they: (i) are time consuming, i.e., require up to several days; (ii) require several steps in the preparation of the gel and in the development of the zones of activity; (iii) have strong backgrounds, making visualization of protein bands difficult; and (iv) cause tracking of proteinases. Therefore, there are limitations with existing methods for determining the composition and molecular weight of proteinases in crude extracts and biological fluids. The present method overcomes the shortcomings of the already reported methods. Moreover, it allows the observation of activity zones caused by proteinaceous proteinase inhibitors. The method simply involves immersing the gel in a protein substrate solution after electrophoresis. The advantages of this approach over existing methods that copolymerize protein substrate with the polyacrylamide are described.

MATERIALS AND METHODS

Source of Enzymes

Samples containing proteinase activities were obtained from fish viscera and decapods extracts. Fish pyloric caeca were obtained from rainbow trout (*Salmo gardneri;* Mt. Lassen strain; TRT) maintained in tanks at the Aquaculture and Fisheries Center (University of California, Davis) and from Chinook salmon (*Onchorrynchus tschawytscha;* CHK) provided by the University of Washington.

Langostilla extract (*Pleuroncodes planipes;* 4P) was prepared according to the method described by García-Carreño (9). Crayfish (*Pacifastacus astacus*) were purchased from California Crayfish Marketing Association (Sacramento, CA) and transported live in a cold ice chest to the laboratory. The hepatopancreas was removed from decapitated animals, frozen, and homogenized in a Waring blender. The high proportion of water in the organ permits homogenization without addition of any buffer solution. The preparation was centrifuged at 2500*g* for 15 min and the aqueous supernatant (CRF) was stored at −70°C.

Reagents

Commercial enzymes, inhibitors, and general reagents such as trypsin (TRY; porcine pancreas Type IX), chymotrypsin (CHY; bovine pancreas Type II), tris(hydroxymethyl)aminomethane base (Tris), and azocasein were obtained from Sigma Chemical Co. (St. Louis, MO). SDS–PAGE reagents were from Bio-Rad (Richmond, CA). A molecular weight marker (MWM) kit for electrophoresis was acquired from Pharmacia (Uppsala, Sweden). Hammersten casein was purchased from ICN Pharmaceuticals Inc. (Bucks, England).

Enzyme Assay

Proteolytic activity of the samples was assayed using 2% azocasein as substrate according to a modification of García-Carreño (9). Briefly, samples (20 μl) of enzyme preparation were mixed with 0.5 ml of the substrate in 50 mM Tris, pH 7.5, at 25°C. The reaction was stopped 10 min later by the addition of 0.5 ml of 20% trichloroacetic acid (TCA). The reaction mixture was centrifuged in Eppendorf tubes for 5 min at 6500*g*. The supernatant was separated from the undigested substrate and the absorbance at 440 nm for the released dye was recorded. The assay included appropriate blanks and commercial enzymes (1 mg/ml) as internal controls. The activity unit was the change in absorbance (440 nm) per minute per milligram of enzyme protein. The influence of 0.1% SDS on the hydrolysis of azocasein by biological extracts and commercial enzymes was also determined.

Electrophoresis

The electrophoresis of samples containing proteinases or inhibitors was done according to Laemmli (14) using 12% acrylamide. Vertical electrophoresis units for 20 × 20 × 0.08-cm (Bio-Rad) and 8 × 10 × 0.07-cm (Hoffer; San Francisco, CA) gels with temperature control was used. The enzyme extracts were diluted (1:4) in sample buffer containing SDS but no reducing agents. The diluted samples were not boiled before loading onto the gels. Volumes of 5 to 10 μl were applied to the gels. The amount of sample protein applied is indicated in the legends to the figures. Five microliters of MWM was loaded on each gel for easy comparison of the MW of the activity zones. Electrophoresis was performed at constant current of 15 mA per gel for 90 min at 10°C.

Development of Activity Zones

Protein substrate hydrolysis. After electrophoresis, gels were immersed in 50 ml of 2% casein in 50 mM Tris/HCl buffer, pH 7.5, or universal buffer (15), pH 5.0, for 30 min at 5°C, in order to allow the substrate to diffuse into the gel, at reduced enzyme activity. Then, the temperature was raised to 25°C and the gels were

incubated for 90 min for the digestion of the protein substrate by the active fractions.

Inhibition of protein substrate hydrolysis. After electrophoresis of the proteinaceous proteinase inhibitor, i.e., SBTI, (i) the gels were immersed in 0.1 mg/ml of porcine trypsin in 50 mM Tris/HCl, pH 7.5, and the enzyme was allowed to diffuse into the gel at 5°C for 30 min; and (ii) the gels were washed with distilled water and assayed for protein–substrate hydrolysis as described above.

Gel Staining

After substrate hydrolysis and incubation, gels were washed with distilled water and immediately fixed and stained in a one-step process by immersing them in a filtered staining solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. Gels for detection of MWM and proteins other than proteinases were stained also. The staining period was carried out for 2 h. Clear zones on blue background, indicating proteinase activities, or dark zones on clear background, indicating inhibition of trypsin, were observed at this stage. MWM bands and proteins other than proteinases or inhibitors had higher intensity of blue color than the background caused by the staining of the undigested casein. A couple of hours of washing with 40% ethanol–10% acetic acid solution was enough to destain and improve the contrast of the clear zones indicating proteinase activity. This procedure was more important for the inhibition assays. Gels were photographed and dried using a Bio-Rad slab gel dryer (Model 443).

RESULTS

Copolymerization of the protein substrate with acrylamide, as suggested by Lacks and Springhorn (10) and Heussen and Dowdle (4) used in our preliminary studies gave (i) anodic migration of the substrate and (ii) a clear streak in the upper zone of the gel, when highly active proteases were present in the sample (Fig. 1). These results were more obvious when assaying fish pyloric caeca extracts and some commercial preparations. However, some streaking was also observed in other samples such as 4P and CRF extracts. Both protein substrate migration and streaking phenomena decreased to a great extent the contrast in the upper zone of the gel, reducing the possibility of observing activity zones.

Proteinases in CRF, 4P, fish extracts, and commercial preparations retained more than 95% of their activity when assayed for proteolytic activity in test tubes, up to several hours with the inclusion of the SDS in the same concentration as that used in PAGE gels.

The standard procedure described under Materials and Methods was used for the electrophoresis analysis

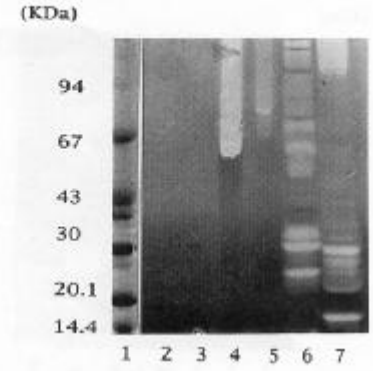

FIG. 1. Substrate-containing gel was prepared by copolymerizing 0.05% casein and acrylamide. Samples were diluted (1:4) in sample buffer. Column (C) 1, MWM comprising phosphatase (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lactalbumin (14,400); C 2, 2.5 μg TRY; C 3, 2.5 μg CHY; C 4, 2.5 μg TRT; C 5, 2.5 μg CHK; C 6, 71 μg CRF; C 7, 14 μg 4P. After electrophoresis the gel was incubated in 50 mM Tris buffer, pH 7.5, for 90 min at 25°C and then immersed in Coomassie stain for 2 h and washed.

of biological preparations containing proteinases. Well-resolved, sharp and clear zones of proteolysis, surrounded by an intense blue background, were observed. Proteinase activity was detected in samples containing from 2.5 to 71 μg of total protein. Zymograms are shown in Fig. 2. A number of activity zones were produced in the biological extracts while one main and some minor (due to impurities) activity zones were produced with commercial enzymes, i.e., trypsin and chymotrypsin. As expected, the main zones produced by trypsin and chymotrypsin corresponded to their reported MW. The MW of proteinases in biological extracts was easily measured using the log MW vs migration plot.

The activity zones in 4P had molecular weights ranging from 16 to 65 kDa. The zymogram for CRF preparation also showed caseinolytic activity in several zones. The molecular weights of the activity zones in CRF ranged from 14 to 81 kDa. The compositions of activity zones in the fish extracts were different from each other. In some samples the zones of activity corresponding to trypsin and chymotrypsin were easily noticed, which confirmed the presence of these types of proteolytic enzymes when assayed with site-directed specific inhibitors (16).

The zymogram in Fig. 3 was performed by incubating the gel in 2% casein solution, in universal buffer, pH 5.0. In this experiment the pH of the substrate solution soaking the gels was monitored during the first minute of incubation. When necessary, some drops of 0.1 M HCl were added to maintain the pH at 5. Most of the zones

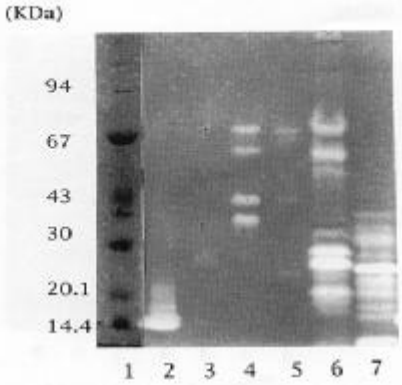

FIG. 2. Substrate–SDS–PAGE. Gels were prepared according to Laemmli (1970). Samples were treated as described in the legend to Fig. 1. Column (C) 1, MWM as in Fig. 1; C 2, 2.5 μg TRY; C 3, 2.5 μg CHY; C 4, 2.5 μg TRT; C 5, 2.5 μg CHK; C 6, 71 μg CRF; C 7, 14 μg 4P. After electrophoresis the gel was incubated in 50 mM Tris buffer, pH 7.5, containing 2% casein for 30 min at 5°C, and then the temperature was raised to 25°C for 90 min. The protein was fixed and stained as described in the legend to Fig. 1.

observed in gels incubated at pH 7.5 were also found. However, the activity zones of low-molecular-weight enzymes were more evident in CRF and 4P. These bands corresponded to molecular weights of 14 to 21 kDa.

The presence and number of active fractions and their MW in a SBTI preparation are shown by a dark zone produced by the undigested protein substrate. This was due to inhibition of enzyme by the trypsin inhibitor present in the sample (Fig. 4). The band had a characteristic shape resembling a hexagon. The position of the MWM was indicated by bands consisting of clear zones enclosing more intense blue bands against a slightly blue background. The lowest concentration of protein (weight basis) assayed was 12 ng of SBTI.

DISCUSSION

The tracking, time-consuming steps and generally unsatisfactory zymograms obtained in fish extracts using the copolymerized substrate methods encouraged the evaluation of an alternative method which overcomes these inconveniences.

In order to determine the effect on activity of the detergent needed in the electrophoresis step, the enzyme activity was assayed in 0.1% SDS. The results suggested that the presence of detergent in the substrate–electrophoresis gels does not alter the activity of digestive proteinases in the studied preparations.

Gels containing 12% acrylamide were chosen because the separation of the fractions in the studied samples was satisfactory. The development of bands for casein hydrolysis was accomplished in 2 h. Longer periods of incubation in the substrate solution resulted in a reduction of sharpness of the activity zones. This happened because of diffusion. Gels were first immersed in a substrate solution at 5°C to allow the diffusion of substrate into the gel containing the separated enzymes. Then, the temperature was raised to 25°C to increase the activity to be measured.

García-Carreño (9), using a similar method with a horizontal electrophoresis chamber and SDS-free gels, found sharp bands with high contrast. However, the porcine trypsin always migrated to the cathode. In the present study the migration of the porcine trypsin was anodal according to its MW. The composition of activity zones of 4P (9) and in the present system, which separated the proteins according to their MW, was, as expected, quite different. The results were confirmed when commercial trypsin, chymotrypsin, and MWM migrated according to their MW.

Electrophoresis using 20 × 20 × 0.08-cm gels can be accomplished in 1 day. However, when using 8 × 10 × 0.07-cm microgels, the period can be reduced to 2 h. About 2 to 4 h more was required to develop the zones of hydrolysis and stain the gels.

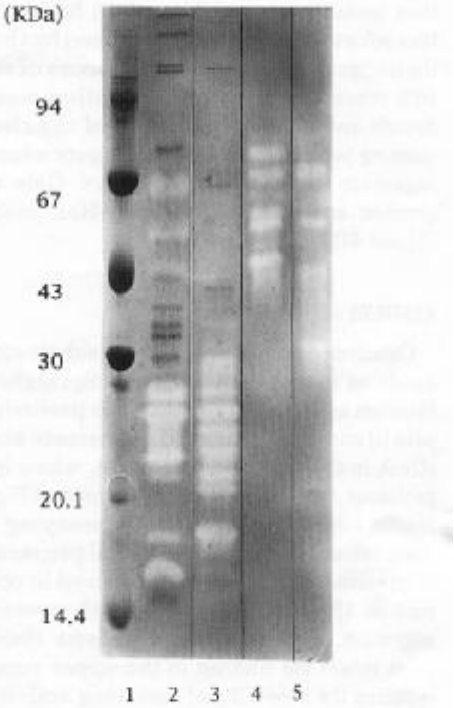

FIG. 3. Substrate–SDS–PAGE. Gels was prepared as described in the legend to Fig. 2. Samples were treated as described in the legend to Fig. 1. Column (C) 1, MWM; C 2, 71 μg CRF; C 3, 14 μg 4P; C 4, 2.5 μg TRT; and C 5, 2.5 μg CHK. After electrophoresis the gel was incubated in universal buffer, pH 5.0, containing 2% casein as described in the legend to Fig. 2. In the first min of incubation some drops of 0.1 M HCl were added to keep the pH constant. Staining was done as described in the legend to Fig. 2.

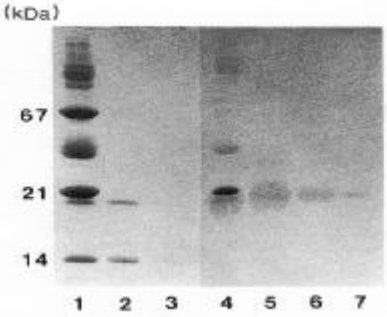

FIG. 4. Substrate–SDS–PAGE for proteinaceous proteinase inhibitor SBTI. Gel was prepared as described in the legend to Fig. 2. Sample (1 mg/ml SBTI) was diluted 1:4, 1:40, and 1:400 in sample buffer. Samples of 10 μl were loaded on the gel. Column (C) 1, MWM as described in the legend to Fig. 1; C 2, 12 μg SBTI; C 3, 120 ng SBTI; C 4, MWM; C 5, 12 μg SBTI; C 6, 120 ng SBTI; C 7, 12 ng SBTI. After electrophoresis samples on columns 1–3 were fixed and stained as described in the legend to Fig. 1. Samples on columns 4–7 were incubated in 50 mM Tris buffer, pH 7.5, containing 0.1 mg/ml porcine trypsin for 30 min at 5°C. Then, the gel was washed with distilled water, assayed for casein hydrolysis, and stained as described in the legend to Fig. 2.

The detection of enzyme activity in 0.1% SDS, both in test tubes and during electrophoresis, is not unusual. Heussen and Dowdle (4) reported what they called trails of proteolysis. They assumed these trails were due to the incubation of the gels at room temperatures. Lacks and Springhorn (10) assumed that trypsin was impaired in its hydrolysis capability when treated with SDS. However, in the present test tube assays, the azocasein hydrolysis by porcine trypsin in 0.1% SDS showed more than 95% of the activity without the detergent. Moreover, porcine trypsin produced an activity zone (Fig. 2) in the substrate–SDS–PAGE. Nevertheless, the assay of enzyme activity in a biological preparation including 0.1% SDS is highly recommended before performing SDS–PAGE. If the enzyme activity is altered by the detergent, the SDS should be removed after electrophoresis by washing the gel for 30 min at 5°C in the soaking buffer without the substrate, or soaking it in a buffer containing 2.5% Triton X-100.

Most of the proteolytic enzymes are composed of only one polypeptide chain. This is an advantage for the procedure reported here. Lacks and Springhorn (10) recognized that refractory enzymes for the treatment with SDS could be those composed of multiple subunits, which could increase the dependency of interchain interactions to regain activity.

Samples containing proteinases in the concentrations of 2.5 to 71 μg of total protein were assayed and several activity zones detected. Concentrations of proteinaceous protease inhibitor as low as 12 ng was assayed and an activity zone detected by the sensitive method. The sensitivity of the method is at least several times higher than a former technique (17). Moreover, the activity zones are sharper and the general procedure is simpler.

The method could be helpful in detecting the fraction with proteolytic activity when conflicting results for the MW of active fractions are obtained from several techniques, as with those reported by Honjo *et al.* (18). Comparison of proteinase composition in enzyme extracts from different especies could be achieved. However, some precautions must be considered before attempting substrate electrophoresis. For example, (i) some enzymes need activators such as Ca^{2+}, cysteine, or EDTA to exhibit activity, and accordingly they must be included in the substrate solution; (ii) other substrates, such as hemoglobin or bovine albumin, could be used; and (iii) some gels could be better stained with another dye, such as amido black.

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