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DETECTION OF TRYPSIN- AND CHYMOTRYPSIN-LIKE PROTEASES USING *p*-NITROANILIDE SUBSTRATES AFTER SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

Specific chromogenic *p*-nitroanilide substrates have proved useful for localizing proteolytic enzymes, such as trypsin, chymotrypsin and elastase after separation by agarose gel electrophoresis and when immobilized on nitrocellulose. This procedure was further developed for use with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated for 10–60 min with Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide as a substrate for detection of trypsin-like proteases and with MeO-Suc-Arg-Pro-Tyr-*p*-nitroanilide for detection of chymotrypsin. The yellow *p*-nitroanilide released at the site of proteolytic activity was converted into a visible and stable red azo dye. By this method was identified and determined the molecular weight of a trypsin-like protease that occurs at high concentrations in mucinous ovarian tumour cyst fluid together with its specific inhibitor peptide, tumour-associated trypsin inhibitor (TATI). The method was also used to visualize trypsin and chymotrypsin in human pancreatic juice. Using the trypsin substrate, three proteolytic bands, corresponding to M_r of 22 000, 24 000 and 26 000 daltons, were visualized in pancreatic juice, while the proteolytic zones in cyst fluid had M_r of 25 000 and 28 000 daltons. With the chymotrypsin substrate, a band of 29 000 daltons was visualized in pancreatic juice, whereas no activity was detected in cyst fluid. By incubation of the blotted cyst fluid proteins with ^{125}I-labelled TATI, a pattern of bands at 25 000 and 28 000 daltons was detected identical to that obtained with the chromogenic substrate.

INTRODUCTION

Several methods have been developed for the qualitative detection of proteases after electrophoresis. Protein substrates have been impregnated in polyacrylamide gel prior to electrophoresis[1,2], or included in an agarose or agar film, placed in contact with an electrophorogram[3–6]. Alternatively, proteases have been localized by staining with specific chromogenic peptide substrates. In these methods, the liberated chromophore, usually *p*-nitroanilide or β-naphthylamide, must be visualized in the

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gel by coupling to an azo reagent[7,8]. However, with *p*-nitroanilide substrates a practical problem has been the poor resolution and preservation of the dye that does not precipitate in the gel. These disadvantages were recently overcome by enzymoblotting[9]. The substrate reaction was carried out on a nitrocellulose membrane, onto which proteases were transferred by capillary diffusion after agarose gel electrophoresis. This immobilizing matrix restricts diffusion of the enzymatic products and preserves a red azo dye generated from *p*-nitroanilide.

Here it is reported that the chromogenic substrate reaction can be utilized for the detection of proteinases after their separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and after electroblotting. This method was used to visualize a trypsin-like protease in cyst fluid of mucinous ovarian tumours. Previous studies have suggested that this protease is similar to or closely homologous with pancreatic trypsin[10]. It is strongly and specifically inhibited by the Kazal-type inhibitor peptide, tumour-associated trypsin inhibitor (TATI). For comparison, the procedure was also used to study the molecular weight of trypsin and chymotrypsin in human pancreatic juice. In addition, it was shown that [^{125}I]TATI provides an alternative method for localizing trypsin-like proteases immobilized on nitrocellulose.

EXPERIMENTAL

Materials

Bovine trypsin (Type 1, twice crystallized) was obtained from Sigma (St. Louis, MO, U.S.A.). TATI was purified from urine of cancer patients, as described[11]. It was iodinated by the lactoperoxidase method[12].

Cyst fluid of mucinous ovarian tumours was obtained from patients undergoing surgery for removal of the tumour. Pancreatic juice was collected by catheterization of the pancreatic duct in patients with biliary or pancreatic diseases. All samples were stored at –20°C until used.

The chromogenic substrates were dissolved in 50 m*M* Tris–HCl + 20 m*M* $CaCl_2$ + 0.1% Triton X-100 (pH 8.0) at a concentration of 1 mg/ml. The solution was stored at –20°C. N-Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S-2222) was used as a substrate for the measurement of trypsin-like activity and 3-methoxycarbonyl propionyl-L-arginyl-L-propyl-L-tyrosine *p*-nitroanilide (S-2586) (Kabi, Stockholm, Sweden) for chymotrypsin-like enzymes.

SDS-PAGE

Sample preparations and electrophoresis were performed according to Laemmli[13] under non-reducing conditions. Prior to electrophoresis, trypsin standards, cyst fluid and pancreatic juice were mixed with an equal volume of Laemmli sample buffer (double concentration), and heated for 2 min in boiling water or incubated for 2 h at 25°C. A sample of 50 μl was applied per lane in the vertical slab gels (16 cm × 18 cm). The acrylamide concentration in the resolving gel was 10 (Fig. 3) or 12.5% (Figs. 1 and 2).

Blotting onto nitrocellulose

Porteins were transferred electrophoretically from SDS gels onto nitrocellulose

(Transblot, Bio-Rad), as described by Towbin *et al.*[14]. Transfer was performed at room temperature for 90 min using a constant current of 250 mA or overnight at 50 mA. Proteins blotted onto the nitrocellulose membrane were first stained reversibly with 0.2% Ponceau S (Chroma, Stuttgart, F.R.G.) in 3% trichloroacetic acid for 2 min in order to facilitate cutting of the lanes into strips. The strips were destained and simultaneously saturated with 2% bovine serum albumin (BSA) in 50 m*M* Tris buffer (pH 7.4), containing 0.9% Triton X-100. Protein standards (Pharmacia, Uppsala, Sweden) were stained with Amido black (Merck, Darmstadt, F.R.G.) Apparent molecular weights of proteases were estimated by comparison with the protein standards.

Detection of proteolytic activity

Incubation with [^{125}I]TATI was performed for 16 h at 25°C, using 500 000 cpm/ml in 50 m*M* Tris buffer (pH 9.0), containing 20 m*M* $CaCl_2$ and 0.1% Triton X-100. After extensive washing of the nitrocellulose sheets with 2% BSA in 50 m*M* Tris buffer (pH 7.4), containing 0.9% Triton X-100 and 0.1 *M* NaCl (BSA buffer), an autoradiogram was generated by placing the dried nitrocellulose paper against X-ray film for 3 d at –70°C. To inhibit immobilized proteases after blotting, nitrocellulose strips were preincubated with TATI at a concentration of 1 μg/ml in BSA buffer for 60 min at 37°C, and then with the chromogenic substrate. The substrate was allowed to react for 10–60 min, and to prevent background staining the incubation was stopped when faintly yellow *p*-nitroanilide bands appeared. The released *p*-nitroanilide was then diazotized by immersing the nitrocellulose membrane for 5 min in a solution of 0.1% sodium nitrite in 1.0 *M* HCl and then for another 5 min in 0.5% ammonium sulphamate in 1.0 *M* HCl. Dark red bands were developed with a fresh solution of 0.05% N-(1-naphthyl)ethylenediamine (Sigma) in 47.5% ethanol[9,15]. After the bands had reached their maximum intensity (1–5 min), the nitrocellulose membrane was washed with water and stored at –20°C. The red bands were best visualized by wetting the nitrocellulose with water.

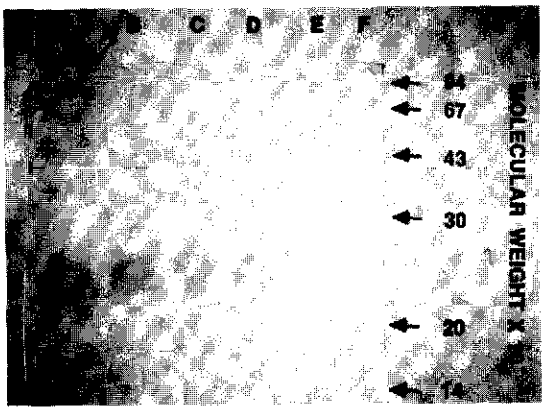


Fig. 1. Estimation of the detection limit for bovine trypsin with substrate S-2222. Lanes A–F contained 500, 300, 100, 50, 20 and 1 ng of trypsin, respectively, separated by SDS-PAGE and electroblotted onto nitrocellulose. The molecular weight marker proteins, treated in parallel, are shown to the right: phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soy bean trypsin inhibitor (20 000) and lactalbumin (14 000 daltons).

RESULTS

Sensitivity of the method

The sensitivity of this method was tested by analysing decreasing amounts of commercial bovine trypsin. The trypsin activity on the nitrocellulose membrane was developed by incubating for 40 min with S-2222 as a substrate. Fig. 1 shows that bovine trypsin migrates with an apparent M_r of 23 000 daltons, the minimum detectable amount of trypsin is 20 ng. When samples were reduced with 2-mercaptoethanol before electrophoresis, no proteolytic activity was detected.

Detection of pancreatic trypsin and chymotrypsin

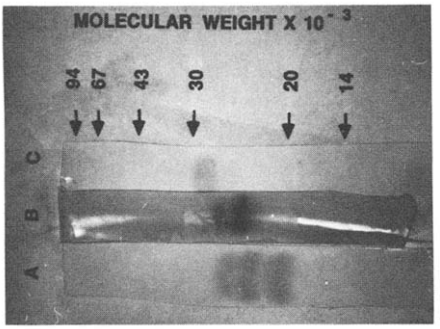
Fig. 2A shows a typical staining pattern with 25 μl of pancreatic juice using S-2222 as a substrate. Three amidolytic zones at 22 000, 24 000 and 26 000 daltons are observed, demonstrating molecular weight heterogeneity. With the chymotrypsin substrate S-2586, only one active band, migrating at 29 000 daltons, was discovered in pancreatic juice (Fig. 2C).

Visualization of the trypsin-like protease in mucinous ovarian cyst fluid

Of twelve cyst fluid samples studied, three contained detectable amidolytic activity migrating at 25 000 daltons, and in one sample a minor active band of 28 000 daltons was also detected, by using S-2222 as a substrate (Fig. 2B). In addition, a faint amidolytic band emerged at M_r 75 000 daltons when the substrate reaction was performed at pH 9.0 (not shown). With the chymotrypsin substrate S-2586, no activity was detected.

Binding of TATI to proteases immobilized onto nitrocellulose

When the nitrocellulose membranes were preincubated with TATI no amido-



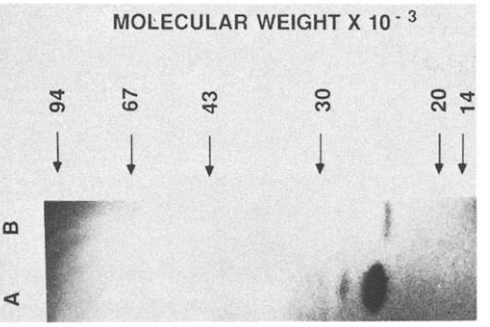


Fig. 2. Visualization of human pancreatic trypsin, ovarian tumour-associated trypsin-like protease and pancreatic chymotrypsin with chromogenic substrates. After SDS-PAGE, proteins were transferred onto nitrocellulose. (A) Pancreatic juice (25 μl), stained for trypsin activity with S-2222 as a substrate (30-min incubation); (B) mucinous ovarian tumour cyst fluid (25 μl), stained for activity against S-2222 (60-min incubation); (C) pancreatic juice (25 μl), stained for chymotrypsin activity with substrate S-2586 (30-min incubation). The positions of the molecular mass standards are indicated on the right (*cf.*, Fig. 1).

Fig. 3. Binding of [^{125}I]TATI to immobilized proteases. Lanes A and B contained 25 μl of mucinous ovarian cyst fluid and of pancreatic juice, respectively, separated by SDS-PAGE and electroblotted onto nitrocellulose. After extensive washing, the complexes labelled with TATI were visualized by autoradiography. The positions of the molecular weight standards are shown to the right.

lytic activity was detected in cyst fluid samples with S-2222 as a substrate (not shown). Incubation of nitrocellulose membranes containing cyst fluid and pancreatic juice proteins with ^{125}I-labelled TATI showed that TATI is bound to the cyst fluid proteins of 25 000 and 28 000 daltons and to pancreatic trypsin of 24 000 daltons (Fig. 3). Weak binding of TATI to the cyst fluid protein of 75 000 daltons was also observed (not shown).

DISCUSSION

This paper describes a simple, sensitive method for visualization of proteases, blotted onto a nitrocellulose membrane after separation by SDS-PAGE. Proteases are detected by incubation with specific *p*-nitroanilide substrates and coupling the hydrolysed *p*-nitroanilide to an azo dye. The dark red bands that form against a white background are stable for years when the nitrocellulose is stored at –20°C.

The chromogenic substrate reaction on the nitrocellulose was originally reported by Ohlsson *et al.*[9] for localizing trypsin, chymotrypsin and elastase, separated by agarose gel electrophoresis. This study shows that proteases, such as trypsin, chymotrypsin and the trypsin-like protease associated with ovarian tumour, are recovered in active form on nitrocellulose, even after the denaturating conditions in SDS-PAGE and electroblotting. An apparent advantage of the use of SDS and heating of samples before electrophoresis is the dissociation of complexes between a protease and its inhibitor, which enables detection of proteolytic activity in crude biologic samples containing an excess of inhibitors.

By the present method the apparent molecular weights of proteases can be determined. Using the substrate S-2222, three trypsin bands (M_r 22 000, 24 000 and 26 000 daltons) were found in human pancreatic juice. Two trypsin isozymes differing slightly in mobility in SDS-PAGE have been characterized in detail earlier[16,17]. Using the chymotrypsin substrate S-2586, I observed one band in pancreatic juice migrating at 29 000 daltons, which corresponds to the molecular weight of human chymotrypsin, reported earlier[17]. The analysis of mucinous ovarian tumour cyst fluid with the S-2222 substrate revealed two proteolytic bands, a major one at 25 000 and a minor one at 28 000 daltons.

The sensitivity of the method depends on the hydrolysis rate of the chromogenic substrate used for a given protease. Using a temperature of 37°C and pH 8.0, a detection limit of 20 ng was obtained for commercial bovine trypsin after incubating for 40 min with S-2222 as a substrate. Lowering the temperature to 25°C decreased the substrate reaction but did not improve the sharpness of protease bands. Interestingly, by elevating the pH of the incubation media to 9.0, a faint amidolytic band was observed in cyst fluid migrating at 75 000 daltons that also bound labelled TATI. The identity of this proteolytic activity is unknown.

Immobilization of proteases on a nitrocellulose matrix offers several advantages. Once immobilized, proteases are stable for long periods of time, and they are accessible to different detection procedures and chemical reactions, *e.g.*, it is possible to activate the zymogen forms of proteases[9]. The binding of ^{125}I-labelled TATI to blotted proteases was also studied. Autoradiography of the bound TATI yielded a pattern of two bands identical with that obtained by the chromogenic substrate reaction in mucinous ovarian cyst fluid. Furthermore, inhibition of the proteolytic activ-

ity by TATI was used to demonstrate the inhibitor specificity of the bound protease.

Several *p*-nitroanilide substrates that allow sensitive and selective detection are now available for various proteases, for example plasminogen activators, plasmin, elastase and thrombin. If used as described here, they might provide useful alternatives to protein substrates for detection of proteolytic activity after SDS-PAGE.

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