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Note

Polyacrylamide gel electrophoresis and immunoblotting of native collagen molecules using the Phast system

Application to type XI collagen

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The collagens are a heterogeneous group of at least thirteen proteins (designated I–XIII). They provide stability to the structure of connective tissues, and are involved in morphogenesis, development, chemotaxis, platelet adhesion and aggregation and cell attachment. All collagen types have a triple-helical domain, comprised of three α-chains. The molecules are basic proteins, with measured isoelectric points ranging from 8.3 to 9.1 [1].

Electrophoretic separation of denatured collagen molecules is commonly carried out in alkaline media in the presence of sodium dodecylsulphate (SDS). However the separation of collagen molecules in their native state requires an acidic buffer system. In this paper, we report an original method to resolve collagen molecules by native polyacrylamide gel electrophoresis (PAGE) in a Phast System apparatus (Pharmacia, Uppsala, Sweden) using modifications in the buffer composition of precast PhastGel media and the reversed polarity electrode assembly. In addition, we describe the conditions for electrotransfer onto nitrocellulose of native and denatured collagen molecules that have been electrophoresed in the Phast System apparatus.

We have developed this technique in order to further characterize a polyclonal anti-type XI collagen antibody, which had been previously assayed by immunoblotting with denatured antigens only. We have looked for cross-reactivity with type II and V collagens, since it has been demonstrated that the α_3(XI) and α_1(II) chains are highly homologous, and that types V and XI are believed to be closely related collagens, both structurally and functionally (see ref. 2 for a review).

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EXPERIMENTAL

Electrophoresis

Native PAGE was performed on minigels (43 × 50 × 0.45 mm) in a Phast System apparatus with the reversed polarity electrode assembly. PhastGel homogeneous media (ultrathin gels, 7.5%, Pharmacia) were equilibrated for 30 min in 15 ml of 0.9 *M* acetic acid containing 6.0 *M* urea; the solution was renewed every 10 min. The effective concentration of polyacrylamide decreased during this treatment and was estimated as 5% after an incubation of 30 min.

Excess liquid was removed from the surface of the gel before placing it on the separation bed of the Phast System. Dry buffer strips (Pharmacia) were soaked in 0.9 *M* acetic acid for 30 min and placed in the buffer strip holder. Type II, V and XI collagens (Bioetica, Lyon, France) were dissolved to a final concentration of 2.0 mg/ml in 0.9 *M* acetic acid containing 6.0 *M* urea. An acidic buffer was necessary in order to retain the collagen solubility. Lower concentrations of urea (2.0 and 4.0 *M*) were found to give poorer results. A 1-μl volume of sample (corresponding to 2 μg) was loaded on the gel. Pyronin Y (Sigma, St. Louis, MO, U.S.A.) was added to each sample as tracking dye. The migration comprised three steps. The first step was done at 50 V, 4.0 mA and 0.5 W for 10 V h, the second one at 150 V, 4.0 mA and 1.0 W for 30 V h, and the third one at 200 V, 6.0 mA and 1.0 W for 135 V h. The overall separation lasted *ca.* 75 min. The first step of the electrophoretic migration was performed using a lower power in order to decrease the amount of the material remaining at the top of the gel.

The gels were stained for 20 min in a solution of 0.1% Coomassie Blue R250 (Merck, Darmstadt, F.R.G.) in 40% (v/v) methanol and 5% (v/v) acetic acid in water, and destained in 40% methanol and 5% acetic acid in water until the background had cleared. SDS-PAGE was performed on 7.5% PhastGel homogeneous media according to the protocol we have previously described [3].

Immunoblotting

Native collagen molecules were transferred to nitrocellulose (0.45 μm pore size, Schleicher and Schuell, Dassel, F.R.G.) in a semi-dry blotting apparatus (Biolyon, Dardilly, France) after removal of the rigid polyester backing of the gel with the LKB film remover (Pharmacia LKB, Bromma, Sweden). The proteins were blotted at 0.4 A for 25 min on to the membrane facing the cathode with 0.7% acetic acid as transfer buffer.

Electrotransfer of the denatured molecules, from the SDS polyacrylamide gels on to the nitrocellulose sheet facing the anode, was completed in only 8 min. The anodic buffer was 25 m*M* Tris, 192 m*M* glycine (pH 8.3) containing 20% (v/v) methanol, and the cathodic buffer was 25 m*M* Tris, 192 m*M* glycine (pH 8.3) containing 0.1% (w/v) SDS.

The different steps of immunodetection are only briefly summarized here, since a more detailed protocol has already been reported [4]. After transfer, the nitro-

cellulose membrane was incubated for 1 h at 37°C to prevent non-specific binding, and probed overnight with the polyclonal anti-type XI antibody raised in rabbit [5], diluted 1:50 in phosphate-buffered saline–3% bovine serum albumin. Immunodetection was achieved using a biotinylated secondary antibody (Vector Labs., Burlingame, CA, U.S.A.) and a preformed avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit, Vector Labs.). 4-Chloro-1-naphthol was added as colour substrate.

RESULTS AND DISCUSSION

In native-PAGE human type II and V collagens migrated as a single band (Fig. 1d and b) corresponding to the whole molecule. No difference was observed in the migration pattern for bovine and human type II collagens (Fig. 1c and d). Nevertheless, a second band appeared in bovine type II collagen and might correspond to a non-collagenous contaminant, since it was not recognized by anti-type II (data not shown).

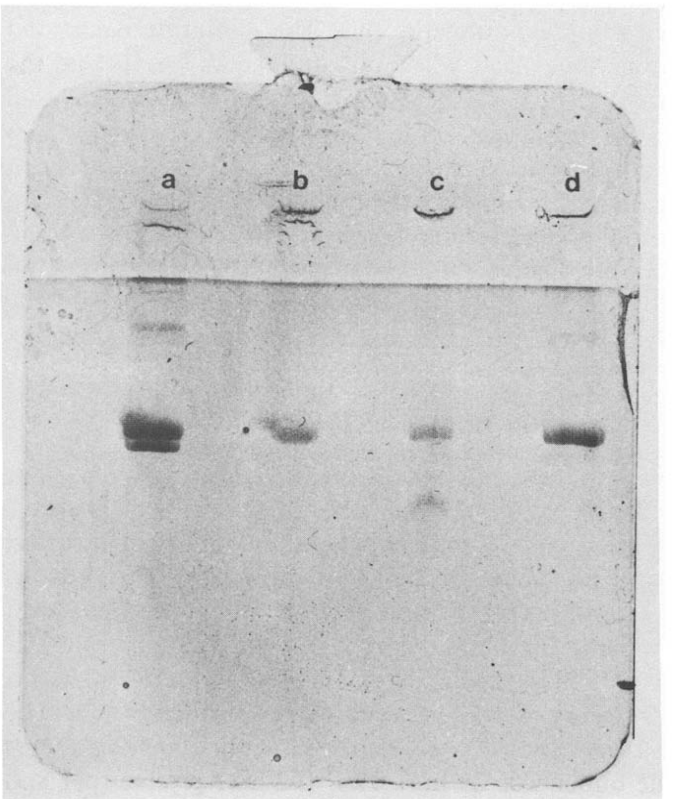

Fig. 1. Native-PAGE of collagen samples. PhastGel homogeneous medium (5%) run for 75 min and stained with Coomassie Blue. Lanes: a, bovine type XI collagen; b, human type V collagen; c, bovine type II collagen; d, human type II collagen.

Native type XI collagen appeared as a doublet of two closely spaced bands (Fig. 1, lane a), which were both recognized by anti-type XI collagen antibody (Fig 2A, lane a). This result raises the possible existence of two molecular species for type XI collagen, which have not been previously reported. No cross-reactivity was found neither with native bovine and human type II, nor with native type V collagen. On the other hand, the antibody reacted strongly with the denatured α_1(XI) and α_2(XI) chains, and also with the denatured α_1(V) chain (Fig. 2B, lanes a and b). No reaction was detected with the denatured bovine or human α_1(II) chains.

The antibody, raised in rabbits with native bovine type XI collagen as antigen, reacted with native and denatured type XI. Its reactivities against the denatured α_1(XI) and α_2(XI) chains appeared to be similar, whereas a rabbit anti-chicken type XI antibody has been reported to recognize mainly the α_2(XI) chain and in a lesser extent the α_1(XI) chain [6].

Our anti-type XI collagen antibody did not cross-react with type II collagen, either native or denatured. The only cross-reactivity we detected was with the denatured α_1(V) chain, although the possibility that the reaction arises from a contamination of the placental type V preparation with α_1(XI) cannot be ruled out, since α_1(XI) has been detected in several non-cartilagenous tissues [7] (M.

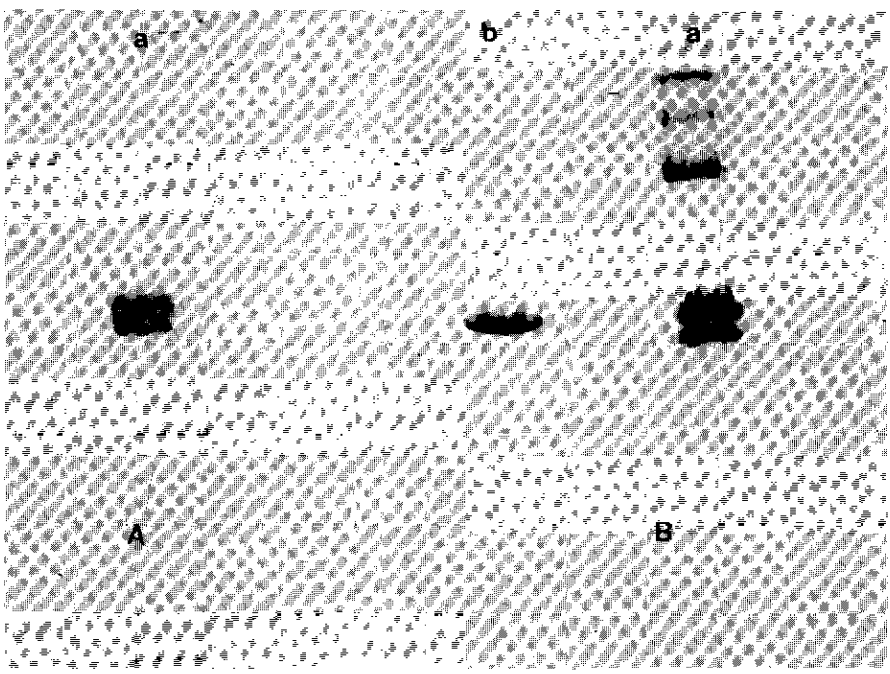

Fig. 2. Immunoblots performed after native-PAGE (A) and SDS-PAGE (B) of collagens, and probed with a polyclonal anti-bovine type XI antibody diluted 1:50 in PBS-BSA. Lanes: a, bovine-type XI collagen; b, human type V collagen.

Van der Rest and M. F. Champliaud, personal communication). No comparison can be made on this point with the anti-chicken type XI antibody, since it has not been tested with type V collagen [6].

The method we report here for the native-PAGE and the immunoblotting of native collagen molecules, using the Phast System apparatus, presents several advantages over the protocol described for large gels [8]. The migration time is decreased four-fold, and the gel preparation and polymerization steps are avoided by using precast PhastGels, which require only 30 min of equilibration to acidic pH before electrophoresis is started. For large gels, equilibration takes place during a prerun of at least 90 min [8].

Electrotransfer of the native collagen molecules onto nitrocellulose required a longer time than the transfer of denatured molecules (25 min *vs.* 8 min). Native collagen molecules have a molecular mass of 285 000, whereas the molecular mass of the denatured chains is 95 000. Since large and small molecules do not transfer at the same rate [9], the differences we have observed for the transfer time probably arise from the difference in molecular mass.

However, in both cases the transfer from PhastGels is faster than from large gels. After SDS-PAGE, denatured collagen molecules are transferred in 8 min from PhastGels and in 60 min from large gels [8] whereas the transfer of native collagens requires 25 min in our conditions instead of 16 h for large gels [8].

In conclusion, the rapid electrophoretic migration of native antigens, a short transfer time and the miniaturization of the sample and probe volumes make characterization of anti-collagen antibodies with native antigens easier, allowing a rapid screening of antibody specificity against native collagens. This additional information regarding antibody cross-reactivity will improve the accuracy of the data obtained by immunological techniques and will be useful for the characterization of circulating collagen antibodies after injection of collagen implants or in a number of other clinical situations.

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