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CHROMATOGRAPHIC SEPARATION OF PAPAIN EVALUATED BY IMMUNOCHEMICAL METHODS

LADISLAV FUKAL and JAN KÁŠ*
Department of Biochemistry and Microbiology, Institute of Chemical Technology, Suchbátarova 5, 166 28 Prague 6 (Czechoslovakia)
and
EDUARD PALUSKA
Institute of Hematology and Blood Transfusion, U nemocnice 1, 128 20 Prague 2 (Czechoslovakia)
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SUMMARY

The chromatographic separation of crude papain preparations on Sephadex G-50 (fine) enables pure papain to be obtained in a single step. Immunochemical techniques have been found to be very convenient for testing the purity of the individual chromatographic fractions. A general approach is presented that makes it possible to follow the course of the chromatographic purification of any immunogenic compound by simple qualitative immunochemical techniques that can be applied in any laboratory.

INTRODUCTION

Isolation of proteins is always accompanied by a requirement for the characterization of their purity. Different physico-chemical parameters are most frequently used as criteria of protein purity, *e.g.*, sedimentation coefficient, diffusion coefficient, partial specific volume, molar absorptivity and molecular weight. Protein characterization is often completed with the determination of its isoelectric point, amino acid composition, biological activity (*e.g.*, enzyme activity), etc.

The determination of most of these physico-chemical criteria is very exacting and hardly feasible in most laboratories. For routine control of protein isolation procedures different immunochemical methods (double immunodiffusion[1], immunoelectrophoresis[2], two-dimensional electrophoresis[3], etc.) seem to be very convenient, mainly owing to their relative simplicity and remarkable reliability.

In this paper we describe the application of immunochemical techniques to the testing of protein purity during the fractionation of a crude commercial papain preparation.

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EXPERIMENTAL

Materials

A crude papain preparation was obtained from Enzymase (Brussels, Belgium). Agarose and Sephadex G-50 (fine) were purchased from Pharmacia (Uppsala, Sweden), agar from Difco (Detroit, MI, U.S.A.) and CM-11 cellulose from Whatman (Maidstone, U.K.). The other chemicals used were supplied by Lachema (Brno, Czechoslovakia).

Preparation of antisera against crude and purified papain

Rabbits were injected in four places intramuscularly and subcutaneously with 0.5 mg of papain (either crude or purified) and again after 14 days. The third immunization followed 6 weeks later and the last after another 2 weeks. The third and fourth immunizations were performed with 1 mg of papain in the same manner as the preceding ones.

The formation of antibodies was tested by the double immunodiffusion method. At the end of immunization the rabbits were bled, the blood was left to coagulate and then centrifuged (3000 *g*, 10 min). The antiserum obtained was kept at -20°C.

Immunochemical methods

The conditions for double immunodiffusion (Ouchterlony) and immunoelectrophoresis were described elsewhere[1,2,4]. Two-dimensional (crossed) electrophoresis[3] was performed with the following modification: sample components were separated electrophoretically in an agarose gel layer (1% agarose in 0.02 *M* veronal buffer, pH 8.6) for 60–90 min at a potential gradient of 8–10 V cm^{-1}. Agarose (1%) with papain antiserum (1.25%) was then poured on to a glass pad in contact with a gel strip containing the electrophoretically separated sample. Immunoelectrophoresis was carried out in an upwards direction for the first separation (2 V cm^{-1}, 15–20 h, 15°C) in an LKB Multiphore device. The immunoelectropherograms were stained in the usual way[4].

Papain purification

For papain purification, two alternative methods were applied:

(I) Papain was isolated from the crude commercial preparation by fractional precipitation with ammonium sulphate and sodium chloride according Kimmel and Smith[5]. The papain preparation obtained was subsequently purified chromatographically on CM-cellulose by the method of Sluyterman[6].

(II) The crude papain preparation was directly fractionated by gel chromatography. Papain solution (2%) in 0.1 *M* acetate buffer (pH 4.8) was applied (1 ml) on the top of a Sephadex G-50 (fine) column (85 × 1.5 cm I.D.) and eluted with the same acetate buffer at an elution rate of 18 ml h^{-1}. The separation course was monitored by measurement of the absorbance at 280 nm, immunochemically and by assaying proteolytic activity.

Assay of proteolytic activity

Proteolytic activity in eluate was assayed with haemoglobin substrate[7] with

the following modification. To 1 ml of 2% denatured haemoglobin solution, 1 ml of 0.1 *M* phosphate buffer (pH 6.2) and 0.4 ml of eluate were added. The reaction was carried out at 40°C for 30 min, then stopped with 4 ml of 10% trichloroacetic acid. The precipitate formed was removed and the absorbance of filtrate was measured at 280 nm.

Disc electrophoresis

For the preparation of the separating and focusing polyacrylamide gels (PAAG), solutions A–F were prepared by dissolving the appropriate chemicals and by adjusting the total volume to 100 ml with distilled water in each instance: (A) 48 ml of 1 *M* hydrochloric acid + 36.6 g of Tris + 0.23 ml of TEMED (N,N,N′,N′-tetramethylethylenediamine); (B) 48 ml of 1 *M* hydrochloric acid + 5.98 g of Tris + 0.46 ml of TEMED; (C) 20 g of acrylamide + 0.735 g of N,N′-methylenebisacrylamide; (D) 10 g of acrylamide + 2.5 g of N,N′-methylenebisacrylamide; (E) 4 mg of riboflavin; and (F) 0.14 g of ammonium persulphate.

The separating gel was prepared by mixing solutions A, C and F and water in the proportions 1:2:4:1 and about 1.5 ml of this mixture was poured into glass tubes (fixed in a rubber stand) and covered with distilled water. When the polymerization was finished (in about 1 h), the water was removed and a gel layer of focusing gel (a mixture of solutions B, D and E and water in the proportions 1:2:1:4) was formed over the separating gel in the presence of a UV source. The electrophoresis was carried out in Tris–glycine buffer (pH 8.3) for 2.5 h at 5 mA per PAAG rod. Owing to the high isoelectric points of the separated proteins (>9.0), the separation occurred in the direction towards the cathode situated in the lower part of the electrophoretic device. The separated proteins were stained with amido black (0.5 g in 100 ml of 7% acetic acid) and the background was destained with 7% acetic acid.

RESULTS

Isolation and purification of papain

For the preparation of monospecific antiserum, pure papain preparation was needed as the antigen for the immunization. Papain was isolated from the commercial crude preparation by salt fractionation according to Kimmel and Smith[5]. The isolated papain showed, however, two distinct peaks after separation by cross-immunoelectrophoresis (Fig. 1a). Subsequent chromatographic purification of this preparation on CM-cellulose yielded a fraction (Fig. 2) giving on cross-immunoelectrophoresis a single peak (Fig. 1b). This pure papain was used for the preparation of monospecific antiserum, which enabled the papain purity to be proved after gel chromatography fractionation.

Fractionation of crude papain by gel chromatography

Crude papain contains several proteins, chymopapain, lysozyme, papain and peptidase being present in the highest concentrations[8]. These enzymes differ only slightly in their isoelectric points and molecular weights. For instance, under the described conditions of disc electrophoresis only two distinct protein bands were detected.

Sephadex G-50 (fine) was found to be a very suitable chromatographic material

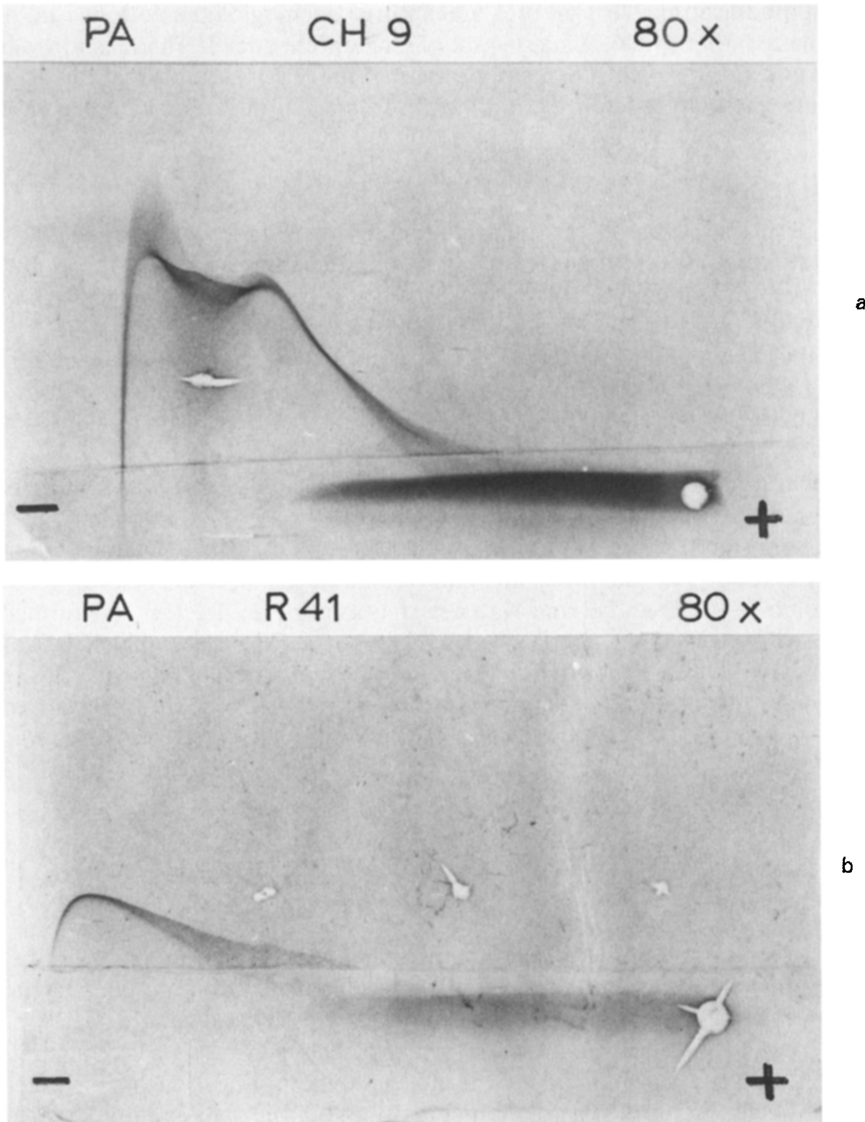

Fig. 1. Cross-immunoelectrophoresis of papain. (a) Preparation prepared by salt precipitation according to Kimmel and Smith[5]; (b) the above preparation further purified on CM-cellulose according to Sluyterman[6] (peak III of Fig. 2).

for crude papain fractionation. Typical chromatographic patterns of the crude papain separation on Sephadex G-50 (fine) are shown in Fig. 3. Fractions A–G were investigated by the method of double immunodiffusion in order to find the number and mutual positions of the precipitation lines which correspond to the individual antigens in the investigated sample (Fig. 4). Polyvalent papain antiserum was used for this purpose.

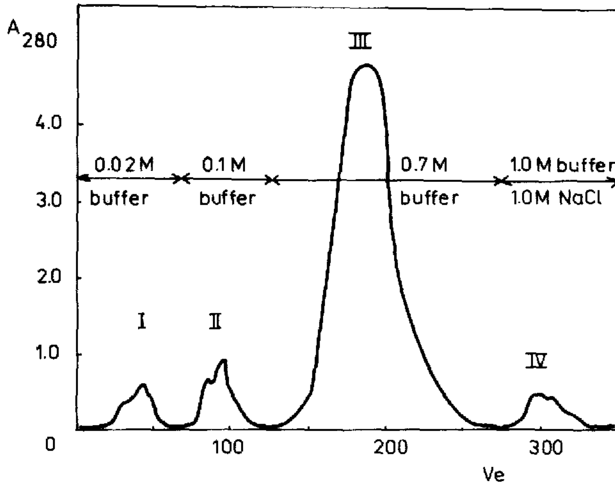

Fig. 2. Papain purification on CM-cellulose. Stepwise elution with acetate buffer (pH 5.0) at an elution rate of 50 ml h^{-1}; 90 mg of protein; column, 10 × 2.5 cm I.D.; peak III, purified papain; peaks I, II and IV, ballast proteins; V_e, elution volume (ml).

The fractions, characterized spectrophotometrically, were subjected to more detailed analysis by a simple technique of double immunodiffusion. For this method of evaluation the precipitation lines of standard crude papain were designated P1, P2 and P3 (corresponding to the unidentified antigens A1, A2 and A3, respectively) in the direction from the outer to the central well. On this basis, the precipitation lines of the individual chromatographic fractions, indicated in Fig. 3, may be characterized in the following way.

Fraction A. The precipitate P2, compared with the standard, is shifted towards

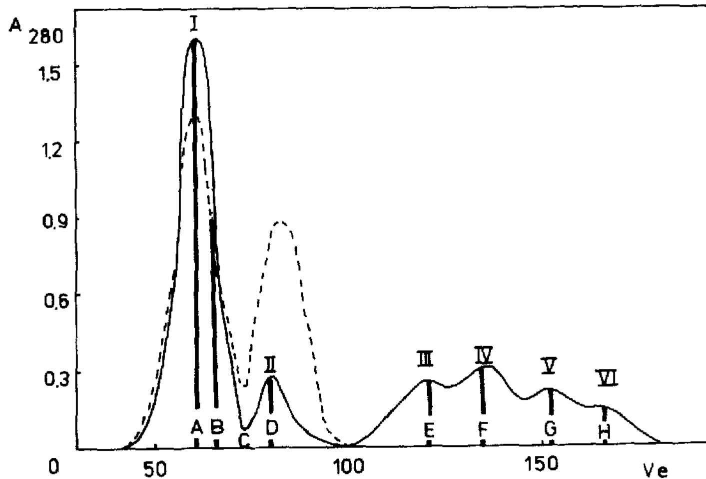

Fig. 3. Crude papain fractionation on Sephadex G-50 (fine). —— Monitoring of proteins at 280 nm. – – –, Proteolytic activity measured on haemoglobin substrate; I–VI, peaks of proteins and peptides; A–H, designation of fractions (full lines) used for the immunochemical evaluation; V_e, elution volume (ml).

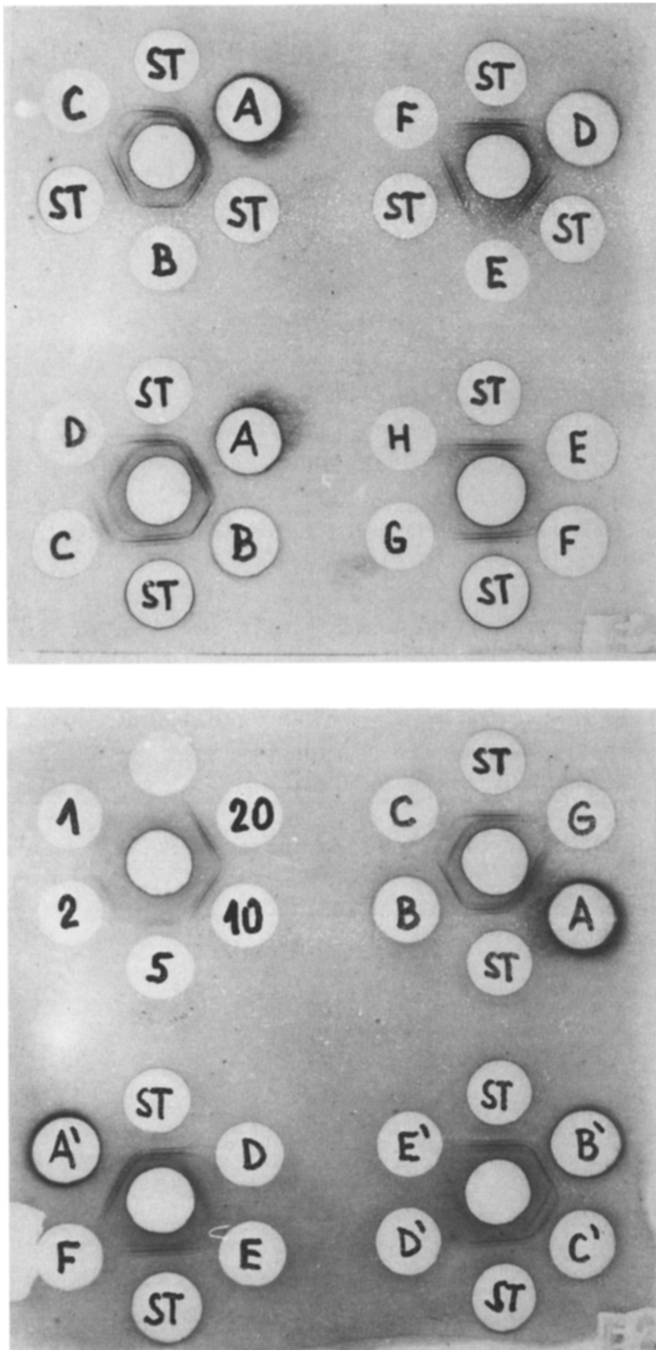

Fig. 4. Evaluation of chromatographic fractions by double immunodiffusion. ST, standard (100 mg of crude papain per 100 ml); A–H, fractions obtained during crude papain separation on Sephadex G-50 (see Fig. 3); A′–H′, samples from the second chromatographic run; 1, 2, 5, 10 and 20, different dilutions of papain standard (1–20 mg per 100 ml).

the central well, whereas the precipitate P3 is situated at the level of P1. This means that the concentration of antigen A2 in this fraction is higher than in the standard (*i.e.*, in the original preparation) and, in contrast, the concentration of A3 is lower. The mutual comparison of the positions of the precipitation lines (corresponding to the antigens of crude papain) with the results of gel chromatography (*i.e.*, the components are eluted in order of decreasing molecular weight) led to the conclusion that antigen A2 has a higher molecular weight than A3.

Fraction B. The concentrations of all three detected antigens are lower than in fraction A (the distances of the precipitation lines from the central well are longer).

Fraction C. The concentration of antigen A3 remains virtually unchanged whereas the concentration of A2 decreases and that of A1 is near to the detection limit. It can be also concluded that the molecular weight of A1 is higher than that of A3, for the same reasons as outlined above.

Fraction D. The concentration of antigen A3 increases considerably, whereas the concentrations of A1 and A2 decrease to very low levels. On the basis of the positions of the precipitation lines, found for the various concentrations of the antigens (in the standard papain solution), it can be concluded that the concentration of A2 in this fraction is about 100 times lower than in the standard and, in contrast, the concentration of A3 is about 100 times higher. Antigen A1 was not detected at all. The concentration of antigen A2 in fraction D is about 10,000 times lower than in fraction A.

Fractions E, F, G and H. These fractions do not contain detectable amounts of any antigen tested.

Proteolytic activity assay demonstrates that the proteolytic activity is concentrated in the first two peaks (I, II) recorded spectrophotometrically, and hence papain may be located in either one or both of these peaks.

The immunochemical method of double immunodiffusion showed that the first peak (I) contains several compounds, although it looks symmetrical. In contrast, peak II (fraction D) contains antigen A3 with only minor contamination from A2. These findings were verified by immunoelectrophoresis.

The results in Fig. 5 support the finding obtained by double immunodiffusion. It was proved that peak I (fraction A) contains several antigenic compounds (in this instance four, compared with three found by double immunodiffusion). The smaller number of antigens showed by double immunodiffusion can be explained by the lower differentiating ability of this technique comparing with immunoelectrophoresis. According to the results of immunoelectrophoresis (Fig. 5), fraction D (peak II) contains only one antigenic compound and it is remarkably pure considering the one-step separation of crude papain.

The application of monovalent papain antiserum indicated that the antigen A3, highly concentrated in peak II (fraction D), corresponds to papain. Fractionation of crude papain on Sephadex G-50 (fine) appeared to be a very effective procedure, making it possible to obtain a pure papain preparation if the column is not overloaded.

The application of immunochemical techniques to the evaluation of chromatographic separations represent a universal approach that is applicable to all kinds of chromatographic separations in which immunogenic compounds (*e.g.*, any protein) are involved. The only critical step in the use of this approach is the preparation

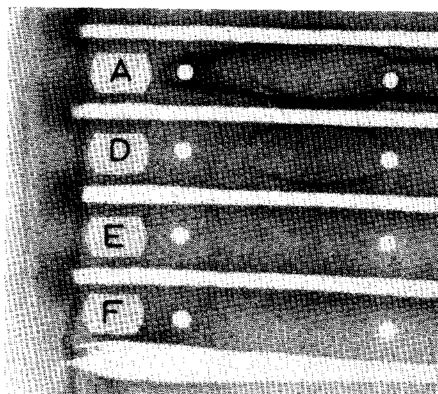

Fig. 5. Immunoelectrophoresis of fractions obtained after crude papain separation on Sephadex G-50. A, D–F, chromatographic fractions (see Fig. 3).

of a suitable antiserum, if it is not commercially available. Immunochemical methods, owing to their specificity, are so reliable that they can often replace much more expensive techniques.

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