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Fractionation and purification of the thiol proteinases from papaya latex

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Abstract

Three cysteine proteinases, *i.e.* chymopapain, papaya proteinase IV and proteinase III, were purified to homogeneity from papaya latex using a combination of ion-exchange chromatography and hydrophobic interaction chromatography. During the purification procedure, the thiol-groups of the active center were reversibly blocked as mixed disulfides with 2-thiopyridone. Homogeneity was proved electrophoretically by native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE and rechromatography on a Mono S 5/5 column at pH 5.0.

1. Introduction

Four immunologically different cysteine proteinases have already been discovered in the latex of the papaya (*Carica papaya* L.) fruit: papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6), papaya proteinase III (EC 3.4.22.30), and papaya proteinase IV (EC 3.4.22.25). The recent discovery of papaya proteinase IV has again raised the question of the composition of papaya latex [1]. Papaya proteinase IV is a major contaminant in commercial, as well as in medical used chymopapain preparations and its physical characteristics closely resemble those of chymopapain. Probably much of the heterogeneity encountered in chymopapain preparations can be accounted for by the presence of papaya proteinase IV [2]. Besides chymopapain and papaya proteinase IV, papain [3] and papaya proteinase III [4] are the other well-characterized proteinases of papaya latex. However, the identification, nomenclature and the right number of molecular species of chymopapain still remain a problem.

Ion-exchange chromatography of papaya latex generally leads to the separation of two chymopapain pools, which can further be shown to contain several multiple species. Normally, the first eluted active compound from the cation-exchange column is called chymopapain A. It is followed by a second compound, called chymopapain B. Analysis of fresh papaya latex revealed that chymopapain B is probably the original enzyme in the latex and that the occurrence of different forms of chymopapain must be attributed to artefacts, resulting from the commercial processing of the latex [5]. Therefore, the name chymopapain B can be replaced by chymopapain. Also for papaya proteinase III possible heterogeneity has been reported [5–8].

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Jacquet *et al.* [9] have shown that blocking of the proteinases with 2,2'-dithiodipyridine (2-PDS) before analysis has a positive effect on the separation and the microheterogeneity arising from oxidation of the free thiol groups.

The main advantage of the method presented here is that all three enzymes can be purified using a single combination of columns. Moreover, it is an easy, rapid and convenient method that allows the purification of the enzymes in only one day.

2. Experimental

2.1. Materials

Commercial spray-dried latex was obtained from Enzymase International (Brussels, Belgium). Sigma (Brussels, Belgium) provided 2-PDS, methyl methanethiolsulphonate. Mono S 5/5, Hiload S-Sepharose HP 26/10, Clean Gel and Exel Gel were purchased from Pharmacia (Brussels, Belgium) and N^{α}-benzoyl-L-arginine-4-nitroanilide (L-BAPNA) from Merck (Brussels, Belgium). The Shodex HIC PH-814 column of Showa Denko was purchased from Millipore (Waters Chromatography Division, Brussels, Belgium). They also supplied the Waters 650 advanced protein purification system. All other reagents used were of the best quality available.

2.2. Preparation and chemical blocking of the protease mixture

A 1-g sample of spray-dried latex was dissolved in 10 ml 60 m*M* sodium acetate–15 m*M* acetic acid pH 5.0 containing 20 m*M* cysteine-HCl and 5 m*M* EDTA. The pH of this solution was adjusted to 5.0. After a 30-min incubation at room temperature, 30 mg of 2-PDS was added and this solution was dialysed against three changes of 500 ml of the same solution. The final volume of the latex solution was ± 13 ml.

2.3. Purfication

Cation-exchange chromatography at pH 5.0

A 10-ml volume of the chemically blocked protease mixture (see above) was applied to a cation-exchange column (Hiload S-Sepharose HP 26/10, 100 × 26 mm I.D.) which had been equilibrated with 0.120 *M* sodium acetate–0.030 *M* acetic acid buffer, pH 5.0. The proteins were eluted with a linear NaCl gradient (0.68 m*M*/ml) at a flow-rate of 5 ml/min. Fractions of 15 ml were collected. The proteins still bound on the column were eluted with 1 *M* NaCl. Active fractions were pooled, an excess of methyl methane thiolsulphonate (± 10 μl) was added and stored at 4°C until further analysis.

Hydrophobic interaction chromatography

To 5 ml of the pooled fractions of the ion-exchange chromatography, 5 ml of 3 *M* $(NH_4)_2SO_4$ was added. This solution was applied on a hydrophobic interaction column (Shodex HIC PH-814, 75 × 7.5 mm I.D.) which had been equilibrated with 100 m*M* KH_2PO_4, pH 7.0 containing 1.8 *M* $(NH_4)_2SO_4$. Approximately 200 m*M* KH_2PO_4 was adjusted to pH 7.0 with NaOH and diluted to 100 m*M*. The proteins were eluted using a linear $(NH_4)_2SO_4$ gradient from 1.8 *M* to 0.0 *M* $(NH_4)_2SO_4$ during 20 min at a flow-rate of 1 ml/min. Fractions of 1.2 ml were collected. If larger amounts of material were required, several identical runs were performed, with collection into a single set of tubes.

2.4. Rechromatography on Mono S at pH 5.0

A 500-μl volume of the two-fold diluted fractions after hydrophobic interaction chromatography was applied to a Mono S 5/5 column (50 × 5 mm I.D.) which was equilibrated with 150 m*M* acetate buffer pH 5.0. The proteins were eluted using a linear gradient (31.25 m*M*/ml) at a flow-rate of 1 ml/min.

2.5. Polyacrylamide gel electrophoresis

PAGE

Electrophoresis was carried out according to the method of Gabriel [10]. Modifications were made to use pre-casted and dried Clean Gels. The gels (11 × 25 × 0.5 mm) were rehydrated with a 0.072 *M* KOH–0.012 *M* acetic acid buffer,

pH 4.3 according to the prescriptions of the manufacturer. The separation gels were 10% acrylamide and the stacking gels were 5% with 25 sample wells of 17 μl. The buffers in both electrode chambers were replaced by electrode wicks, soaked with 0.36 *M* β-alanine–0.14 *M* acetic acid buffer, pH 4.5. Separation was towards the cathode and methylene blue was used as a dye marker. Gels were immersed for 30 min in a fixing solution that contained ethanol–acetic acid–water (40:10:50, v/v). After fixation, silver staining was carried out according to the instructions of the suppliers. Electrophoresis was carried out at 5 mA during 10 min and continued at 20 mA until the dye marker had moved ± 70 mm.

SDS-PAGE

Electrophoresis on Exel Gel (8 to 18% polyacrylamide) gradient gels and subsequent silver staining were carried out following the instructions of the suppliers.

2.6. Enzyme assay for amidase activity

For assays of the hydrolysis of L-BAPNA, 200 μl of each sample were added to 800 μl of 0.03 *M* citric acid–0.138 *M* disodium phosphate buffer, pH 6.4 containing 100 m*M* of cysteine-HCl and 1 m*M* EDTA. The enzyme was allowed to activate at 37°C for 5 min before the reaction was started by addition of 750 μl substrate solution. The substrate solution is prepared by adding 2 ml DMSO to 50 mg L-BAPNA. This mixture is warmed in warm, but not boiling water, until completely dissolved. Subsequently, this solution is rapidly diluted 1:50 in 0.03 *M* citrate–phosphate buffer, pH 6.4 at room temperature. The solution has to be protected from light. After 30 min, the reaction is stopped by addition of 250 μl of 60% (v/v) acetic acid. Released 4-nitroaniline was determined by measurement of the absorbance at 410 nm (A_{410}). One unit of activity (nkat) corresponds to the release of 1 nmol of 4-nitroaniline ($\epsilon = 8800$ $M\ cm^{-1}$) per second from L-BAPNA at pH 6.4 and 37°C.

2.7. Determination of protein concentrations

During the purification, protein was measured as A_{280} assuming a specific absorbance ($A^{1\%}_{1cm}$) of 20.0. For the determination of the concentration of the purified papaya proteinases, published $A^{1\%}_{1cm}$ values were used: 25.0 for papain, 18.3 for chymopapain and papaya proteinase III [11], and 16.5 for papaya proteinase IV [1].

3. Results and discussion

3.1. Effect of the chemical blocking of the thiol groups in the active center with 2-PDS

The blocking of the thiol groups in the active center with 2-PDS before analysis of the papaya proteinases was used for the first time by Jacquet *et al.* [9]. They observed a remarkable simplification in the elution pattern of these proteins on Sephadex CM-50. Three peaks eluted from their column: the first eluting peak contained papain, the second chymopapain, and the third papaya proteinase III. Chymopapain eluted under these conditions as a single peak. This is in sharp contrast with our observations. Fig. 1 shows that blocking with 2-PDS has the opposite effect on the elution pattern of chymopapain. Instead of one peak, three almost separated peaks can be observed in the chymopapain fraction of which two peaks revealed activity against L-BAPNA. The separation of these compounds on Mono S 5/5 at pH 5.0 is better with blocking than without blocking. These results support the argument of Jacquet *et al.* [9] that the microheterogeneity of chymopapain is due to the different forms of the thiol groups during chromatographic analysis.

3.2. Purification

The choice of ion-exchange chromatography as the first step in our purification scheme is obvious. It is a well-known procedure, which even can be improved by inhibition of the proteinases with 2-PDS. To minimize the total number of unit operations, a combination of techniques based on the different surface prop-

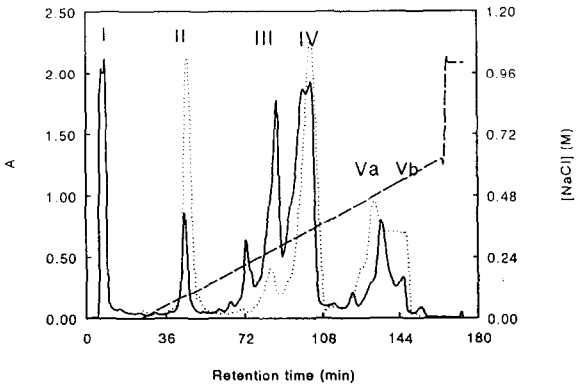

Fig. 1. Ion-exchange chromatography of the chemically blocked papaya proteinases on a HiLoad S-Sepharose HP 26/10 column Column: 100 × 26 mm I.D.; fraction volume: 14.4 ml; flow-rate: 8 ml/min; buffer: 150 m*M* sodium acetate buffer pH 5.0; elution with a linear gradient of sodium chloride from 0 to 625 m*M* in 140 min. (—): A_{280}; (· · · ·): A_{410} (amidase activity against L-BAPNA); (– – –): sodium chloride gradient. Fraction I: unbound material; fraction II: papain; fraction III: mixture of chymopapain A (degradation product) and lysozyme; fraction IV: mixture of chymopapain and papaya proteinase IV; fraction Va and Vb: papaya proteinase III.

erties of the compounds to be separated, is chosen; *e.g.* ion-exchange chromatography and hydrophobic interaction chromatography. Fig. 1 shows the pattern obtained after cation exchange at pH 5.0. We can observe 3 regions with high enzyme activity against L-BAPNA: the papain (fraction II), chymopapain (fraction IV), and papaya proteinase III (fraction Va and Vb) fractions. The chymopapain fraction is well separated from another fraction in which the activity is low (fraction III). This fraction is normally called the chymopapain A fraction. The form of the peaks indicates that neither chymopapain, nor papaya proteinase III is pure. Therefore, further purification is necessary and which can be performed with hydrophobic interaction chromatography. We had to replace 2-PDS with methyl methanethiolsulphonate after the first step of our purification to avoid the problem of hydrophobic interaction between the matrix and the inhibitor. We also divided the papaya proteinase III fraction in two different fractions of which the first eluting fraction contained papaya proteinase III with contaminants (Fig. 2b), while the second

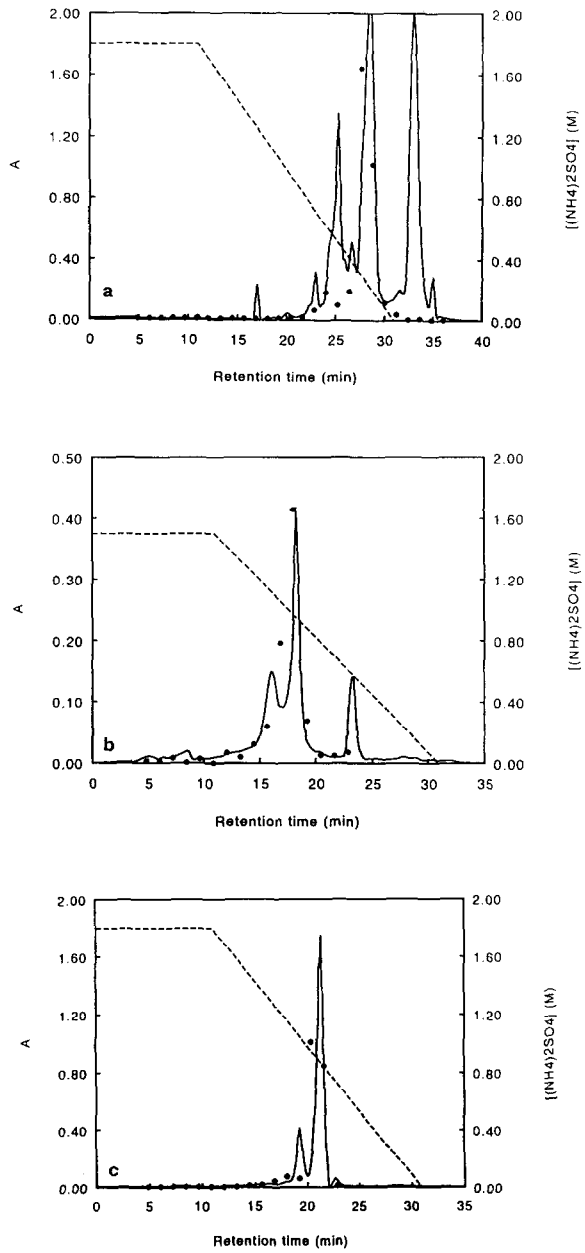

Fig. 2. Hydrophobic interaction chromatography of chymopapain (IV) and papaya proteinase III (Va and Vb) on a Shodex HIC PH-814 column. Column: 75 × 8 mm I.D.; fraction voluem: 1.2 ml; flow-rate: 1 ml/min; buffer: 20 m*M* phosphate buffer pH 7.0, containing 1.8 *M* ammonium sulphate. Elution: linear gradient from 1.8 to 0 *M* ammonium sulphate in 20 min; (—): A_{280}; (- -): ammonium sulphate gradient; (●): A_{410} (amidase activity against L-BAPNA).(a) Chymopapain, (b) papaya proteinase III with contaminants, and (c) papaya proteinase III almost without contaminants.

fraction contained almost pure papaya proteinase III (Fig. 2c). Using a linear gradient, a good separation was obtained between the papaya proteinase III and its contaminants (Fig. 2c). However, it was not possible to purify papaya proteinase III from fraction Va because of the presence of impurities with almost identical physical properties.

Also the chymopapain fraction could be further purified on a hydrophobic interaction column (Fig. 2a). This fraction contains at least two different proteinases, namely chymopapain (1) and papaya proteinase IV (2). Chymopapain can be easily differentiated from papaya proteinase IV since this enzyme shows proteolytic activity, but no amidase activity against L-BAPNA.

The only drawback of this method is the difficulty to purify large amounts of enzyme. Scaling-up the hydrophobic interaction chromatography step on HiLoad Phenyl Sepharose HP caused problems. The resolution between chymopapain and papaya proteinase IV was not satisfactory on this type of column. However, this column could be used for the purification of large amounts of papaya proteinase III.

3.3. Purity of the proteinases

Determination of the true composition of latex has been a problem for many years because of the inavailability of a technique that can separate compounds that are very much alike. Native electrophoresis (Fig. 3) and SDS-PAGE (results not shown) of the different proteinases in the latex show that the papaya proteinase IV and chymopapain have practically the same electrophoretic properties. This is why it took so long before papaya proteinase IV was discovered. A possible way to differentiate between chymopapain and papaya proteinase IV is rechromatography on a Mono S 5/5 column at pH 5.0. Table 1 shows that the elution times of chymopapain and papaya proteinase IV differ and thus rechromatography can be used for determination of protein purity. Fig. 4. shows that all three enzymes are almost pure after rechromatography at pH 5.0. Finally, pure chymopapain, papaya proteinase III, and papaya proteinase IV can be

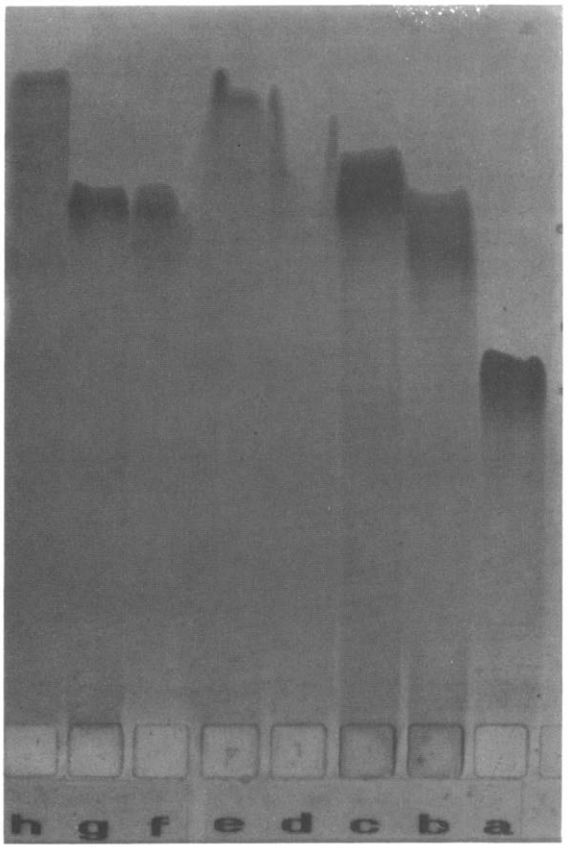

Fig. 3. Native PAGE electrophoresis of the thiol-proteinases from *Carica papaya* latex at various stages of their purification. For experimental details, see Experimental section. (a) Fraction II, containing papain, (b) fraction III, containing lysozyme and chymopapain A, (c) fraction IV, containing chymopapain and papaya proteinase IV, (d, e) fraction Va and Vb, containing papaya proteinase III, (f, g and h) chymopapain, papaya proteinase IV and papaya proteinase III, respectively, after hydrophobic interaction chromatographies.

Table 1
Mean retention times of the purified enzymes on Mono S 5/5 pH 5.0[a]

Enzyme	Retention time (mean ± S.D., $n = 3$) (min)
Chymopapain	19.33 ± 0.09
Papaya proteinase III	26.47 ± 0.08
Papaya proteinase IV	18.2 ± 0.1

[a]For chromatographic conditions see Experimental.

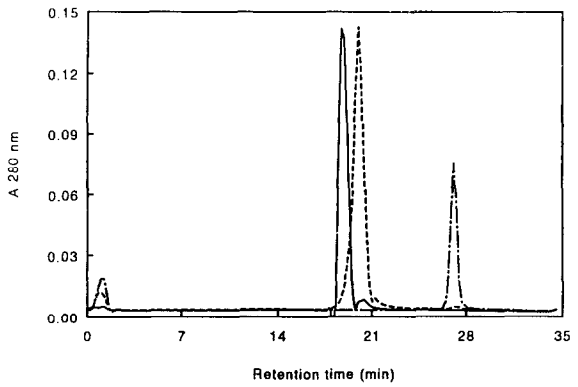

Fig. 4. Rechromatography of the purified enzymes on a Mono S 5/5 column Column: 50 × 5 mm I.D.; flow-rate: 1 ml/min; buffer: 150 m*M* sodium acetate buffer pH 5.0; elution with a linear gradient of sodium chloride from 0 to 625 m*M* in 20 min. (—): Papaya proteinase IV; (- -): chymoapapain; (- · · -): papaya proteinase III.

obtained by affinity chromatography on Activated Thiol Sepharose CL 4B [12,13].

3.4. Stability of the pure enzymes

Once pure enzymes are obtained, the next question is how to maintain the activity. Using inhibitors, the enzymes can be stored for up to 2–3 months in solution with only a small decrease in activity. If it is necessary to store the products for longer periods, they have to be lyofilized. No results on the stability of the purified enzymes after lyofilization are available at present, but will be published soon.

4. Conclusion

We have shown that it is possible to purify chymopapain, papaya proteinase III, and papaya proteinase IV to homogeneity from papaya latex using a single combination of columns. We have also shown that the microheterogeneity of chymopapain during chromatographic analysis is mainly due to the presence of papaya proteinase IV and not to the presence of different forms of the thiol group. Further purification at the level of the active center of the enzymes can be achieved by adding an affinity chromatography step to our purification scheme.

5. References

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