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The Role and Efficiency of Ammonium Sulphate Precipitation in Purification Process of Papain Crude Extract

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Abstract

It has been common to do fractionation (for example using ammonium sulphate as a precipitating agent) before doing a more sophisticated method for purification of a protein. The logic behind this is easy to understand, but in fact, the precipitation step often causes severe loss in yield and activity of the protein, making the whole purification effort too costly. In this work we evaluated the specific activity (thus, purification factor) and total activity (yield) during the purification process of papain from a crude extract using ion exchange chromatography (IEC), with and without prior fractionation using ammonium sulphate. Detail assays in each step were recorded and SDS-PAGE was also done to reveal the protein profile of the purification products.

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Nomenclature

μmol	micromol (mol x 10^{-6})
μg	microgram (gram x 10^{-6})
min	minute
ml	millilitre (litre x 10^{-3})

1. Introduction

The latex of *Carica papaya* is a rich source of the cysteine endopeptidases, including papain, glycylendopeptidase, chymopapain and caricain, which constitute more than 80% of the whole enzyme fraction[1]. Papain (EC 3.4.22.2) is a minor constituent (5–8%) among the papaya endopeptidases[1–3]. The enzyme is used widely as a meat tenderizer, and has also several other applications, e.g. for defibrinating wounds, treatment of edemas, shrink proofing of wool, etc. Purification of papain from papaya latex has traditionally been achieved by precipitation methods[4–6], however, the purified enzyme still remains contaminated with other proteases. An alternative purification strategy has involved the use of various chromatographic techniques including ion exchange, covalent, or affinity chromatography[1,7–10].

Initial observation showed that the fractionation process using ammonium sulphate actually frequently causes a quite significant loss of papain. In this work, we analyzed the role and the utility of doing fractionation using ammonium sulphate before subsequent ion exchange chromatography, in terms of specific activity (thus, purification factor) and total activity (yield).

2. Methods

2.1 Materials

The papaya fruit, *C. papaya* was grown locally in Surabaya, Indonesia and used as starting latex material. Polyacrylamide, bis-acrylamide, casein and ammonium sulphate were purchased from Sigma–Aldrich (St. Louis, USA), while other reagents were bought from Merck.

2.2. Isolation of latex from C. papaya

Fresh latex was collected from papaya fruit. Initially, four tosix longitudinal incisions were made on the unripe fruit using a stainless steel knife. The exuded latex was allowed to run down the fruit and drip into collecting devices attached around the trunk. Following collection, the latex was transferred to a plastic bottle and stored at −20°C.

2.3. Ammonium sulphate precipitation

The latex extract was centrifuged for 15 minutes at 2500 x *g*, at room temperature. The supernatant obtained was centrifuged at 5000 x *g* for 15 minutes at room temperature to obtain clean liquids. Proteins were sequentially precipitated from 50 mL of this crude extract by stepwise addition of solid ammonium sulphate with stirring at a certain degree of saturation, followed by incubation on ice for at least 2 h and centrifugation at 10,000 x *g* at 4°C for 15 minutes. The pellet obtained after each centrifugation was resuspended in max. 10 mL of buffer containing 10 mM phosphate, pH 7. Those steps above were done for 50, 60, 70 and 80% of ammonium sulphate saturation, respectively. Aliquots of precipitated fractions were analyzed for its protein concentration, enzyme activity and MW by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As comparison, part of the crude extract was also directly subjected to the same analysis.

2.4. Determination of protein concentration

The protein content in the samples during purification was determined by the Bradford method[11].

2.5. Protease activity determination

The reaction mixture contained 400 µl of casein substrate, 200 µl of enzyme extract and 700 µl of phosphate buffer, pH 6. The mixture was incubated at 65°C for 30 minutes and the reaction was stopped by adding 3 ml of 5% (v/v) trichloroacetic acid (TCA) and then cooled down to room temperature. The reaction mixture was centrifuged, and absorbance of the supernatant was measured at 275 nm. The reading was corrected for a blank in which the enzyme was added after addition of TCA. Tyrosine concentration was calculation based on a standard curve.

$$\text{Enzyme activity (mol/minute/ml)} = \frac{\text{mass of tyrosine per ml assay solution x V assay solution}}{\text{MW of tyrosine x incubation time x V enzyme extract}}$$

2.6. SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli[12] using 12% acrylamide. The samples were prepared in Tris/glycerol/β-mercaptoetanol loading buffer and placed in boiling water during 60s. Gels were stained with Coomassie-Blue R-250 and Brilliant Blue G (colloidal) concentrated by the method of Neuhoff[13].

2.7. Dialysis

Dialysis was performed for the precipitated fraction with the highest purification factor and yield. Compromise was taken in deciding the fraction to be dialyzed in case the two parameters were not in the same order of magnitude.

2.8. Cation Exchange Chromatography

Aliquots of protein dissolved in 0.4 M sodium acetate buffer, pH 5.0, were applied to a CM-cellulose column (1.5 x 20 cm) equilibrated with the same buffer. The material was eluted using a discontinuous gradient (0.4 to 1.0 M) of sodium acetate buffer, pH 5.0. The peaks obtained were pooled and dialyzed against 1 mM EDTA at 4°C with three successive changes of 10 h each. After dialysis, they were lyophilized and then submitted to a gel electrophoresis.

3. Results and Discussion

This work began with determination of the optimum pH for the protease activity assay of the papain extract. It is shown in Table 1 that papain from the papaya latex used in this experiments worked best as protease at pH 6, as expected[14].

Table 1. Optimization of pH for protease activity assay at 65°C

pH	Protein (µg/ml)	Activity (µmol/min/ml)
5	14250	56.9
6		**123.5**
7		72.5
8		68.3

That pH was used for the rest of the work. Part of the crude extract was than fractionated using the ammonium sulphate precipitation method. The pellet resulting from each precipitation step was redissolved in the phosphate buffer, pH 6 and analyzed for protein concentration and protease activity. At this step, the majority of protease was found in the pellet of the precipitation at 60% saturation of ammonium sulphate, giving not only the highest specific activity (thus, purification factor), but also the highest yield among all fractions (Table 2).

Table 2. Monitoring of fractionation using ammonium sulphate

Step	Activity (μmol/min/ml)	Specific Activity (μmol/min/ml/μg)	Purification Factor	Volume (ml)	Total Activity (μmol/min)	Yield (%)
crude extract	123.5	0.0087	1.00	50	6175	100.0
50	35.45	0.0025	0.29	6	212	3.4
60	**279.9**	**0.0196**	**2.27**	**10**	**2799**	**45.3**
70	205.59	0.0144	1.66	2	411	6.7
80	153.9	0.0107	1.24	4	612	9.9
dialysis	227.5	0.0160	1.84	10.3	2344	38.0

The papain solution from the precipitation at 60% saturation of ammonium sulphate was then subjected to a dialysis process to remove the ammonium sulphate, followed by subsequent cation exchange chromatography (IEC), from which 30 fractions of eluate were collected (each fraction was subjected to determination of protein concentration and protease activity). From those, fraction numbers 16 up to 21 shown the best purity and yield, and therefore, were harvested and combined together. The same IEC procedure and condition was applied directly to the crude extract, from where 42 fractions were collected. The difference in fractions number (thus, elution volume) needed for the IEC of both condition above is still unclear, but probably comes from the higher loading capacity since the crude extract contained higher protein and higher papain concentration. The comparison of the end result is shown in Table 3. Basically, the result from the IEC process of crude extract without prior fractionation seems to be better, both seen from the purification factor (specific activity value) and yield (total activity) achieved. We also try to picture the result by doing SDS-PAGE as shown in the Fig. 1. No significant difference was observed in term of band profile of the final purification result from both conditions, rather the IEC without prior fractionation gave a bit more intense papain band, confirming the higher activity and higher concentration of protein as given from the previous assays.

Table 3. Comparison of IEC with and without prior fractionation

Parameters	WITH prior amm. sulphate fractionation	WITHOUT amm. sulphate fractionation
Harvested Fraction Number	16-21	20-36
Activity, in average (μmol/min/ml)	0.503	1.547
Protein concentration, in average(μg/ml)	100.2	252.7
Specific Activity, in average (μmol/min/ml/μg)	**0.0050**	**0.0061**
Total Activity (μmol/min)	176.1	657.5
Initial Total Activity (μmol/min)	2799	6175
Yield (%)	**6.3**	**10.7**

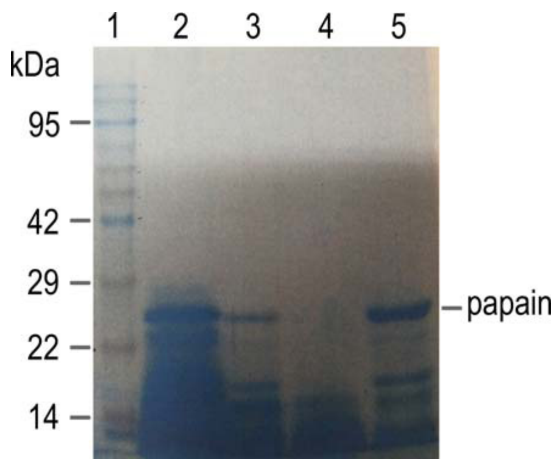

Fig. 1. Coomassie blue stained SDS-PAGE of papain, lane-1: protein marker; lane-2: papain crude extract, lane-3: fraction harvested from IEC with prior fractionation, lane-4: supernatant from the ammonium sulphate precipitation at 80% saturation; lane-5: fraction harvested from IEC without prior fractionation.

4. Conclusions

The result from the IEC process of crude extract without prior fractionation was better, both seen from the purification factor (specific activity value) and yield (total activity) achieved. No significant difference was able to be observed in term of band profile of final purification result from both condition as shown by SDS-PAGE gels, rather the IEC without prior fractionation gave a bit more intense papain band, confirming the higher activity and higher concentration of protein as given from the previous assays.

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