Isolation of Highly Active Papain from Dried Papaya Latex

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Summary: Papain, the cysteine proteinase, from dried papaya latex was purified by ammonium sulphate fractionation followed by agarose mercurial chromatography. The enzyme was further purified by covalent chromatography on thiol-Sepharose column in order to obtain 95-100% pure form. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis with or without sodium dodecyl sulphate. The enzyme had a molecular weight of 23,400 as determined by SDS-polyacrylamide gel electrophoresis. The reactions with 2,2-dipyridyl disulphide and dithiobisnitrobenzoic acid showed that highly active papain contains one-SH group per enzyme molecule.

Introduction

The latex of the fruit of the tropical fruit tree Carica papaya contains several cysteine proteinases like papain (EC 3.4.22.2) chymopapain A and B (collectively designated as EC 3.4. 22.6) and papaya peptidases A and B [1]. Papain is the most important cysteine proteinases for pharmaceui tcal preparations. Many pharmaceuitcal uses of papain have been reported by Flynn [2]. These include external treatment of hard tissues, wart and scar tissue removal, skin cleaning treatment and inclusion of tooth paste. It has also been used for the preparation of tyrosine derivative [3] and immunoglobulin samples for intravenous injections [4]. Since the papaya latex contains considerable quantity of papain a study on its isolation based on affinity chromatography and covalent chromatoghraphy was undertaken.

Experimental

Materials

Papaya latex type I was obtained from Sigma Chemical Co., activated thiol-Sepharose, Sepharose 4B and Sephadex G-25 from Pharmacia Fine Chemicals. Cyanogenbromide and 5,5-dithiobis 2-nitrobenzoic acid (DTNB) were obtained from Fluka AG. Aldrich Chemical Co., supplied 2,2-dipyridyl disulphide (PDS) and p-aminophenyl mercuric acetate. N-benzyloxy-carbonylglycine p-nitro phenyl ester from Cyclo Chemical CO., Acetonitrile was a product of J.T.Baker Chemicals. Coomassie brilliant blue R-250 and G-250 were obtained from BDH Chemical Ltd. The other reagents used were of analytical grade.

Method

Assay Procedures

The activity of proteinase was measured with N-benzyloxycarbonylglycine p-nitrophenyl ester as described by Asboth and Polgar [5]. The reaction was followed at 340 nm on a Carry 118C spectrophotometer at 25°C in 0.1M acetate buffer, pH 5.5 containing 3.3% acetonitrile, 1mM EDTA and 0.1mM N-

benzyloxycarbonylglycine p-nitrophenyl ester. When the assay was carried out in the presence of cysteine (20ul of 0.2M cysteine-HCl was added to 3 ml above reaction mixture), the nonenzymatic hydrolysis of the substrate augmented. Correction for the nonenzymatic hydrolysis was always made, when its rate was higher than 3% of the enzymatic rate. Specific activity is expressed as u mol p-nitrophenol released/min/mg protein under the above conditions. The molar extinction coefficient of 6670 M^{-1} cm^{-1} was used for p-nitrophenol.

The protein concentration was calculated by using A 1% =25.0. When UV-absorbing materials were present, the Bradford method [6] was used.

Purification and concentration of papain on agarose-mercurial column.

Agarose mercurial column involves the use of p-aminophenyl mercuric acetate attached to Sepharose 4B. Agarose-mercurial was prepared according to Sluyterman and Widjenes [7]. 12.0 ml sample containing 430 mg protein was applied to an agarose mercurial column (1 x 10 cm) at a flow rate of 18 ml/h.Fractions of 4 ml were collected. The column was washed with 0.05M acetate buffer, pH 5.0 containing 1mM EDTA. The bound thiol enzyme was eluted with 0.05 M acetate buffer pH 5.0 containing 0.5mM $HgCl_2$. The enzyme can be stored as mercuripapain to prevent autolysis and can be activated with cysteine before use.

Covalent chromatography on thiol-Sepharose column

Mercuripapain obtained from agarose -mercurial chromatography was activated by the addition of 0.1 volume of 0.2M cysteine hydrochloride adjusted to pH 6.0 with 1N NaOH before use. After incubation for 10 minutes thiol enzyme was gel filtered on Sephadex G-25 with equilibration buffer (0.05 M acetate buffer pH 5.0 containing 1 mM EDTA and 0.3 M NaCl).

The gel filtered enzyme was applied to an activated thiol-Sepharose 4B column (Sepharose-glutathione-2-pyridyl disulphide conjugate) washed with the equilibration buffer. The chromatography was performed at room temperature. Fractions of 5 ml were collected at a flow rate of 20 ml/h. The protein not bound to the column was washed with the equilibration buffer until the absorbance of the effluent decreased below 0.05 units measured at 280 nm as well as at 343 nm, characteristic of the absorbance of 2-thiopyridone librated on protein binding. Elution of enzyme was effected by washing the column with 10 mM cysteine first in equilibration buffer pH 5.0 (one column volume) then in 0.05 M phosphate buffer pH 8.0 containing 1 mM EDTA and 0.3 M NaCl. Enzymatically active peak was concentrated to about one fourth volume by ultrafiltration through a UM-10 membrane (Amicon). One tenth volume of 0.2M cysteine, pH 6.0 was added and after 15 min. incubation the fraction was gel filtered on Sephadex G-25 column. The gel filtered enzyme was applied to agarose mercurial column and the protein was collected in the mercuric salt form as described previously. After each run the thiol-Sepharose column was activated or regenerated with PDS by methods described in [8].

Thiol group determination

The number of thiol group in papain was determined with PDS [9] and DTNB [10]. The molar extinction coefficients

used were 7480 at pH 8.0 and 7700 at pH 4.0 with PDS measured at 343 nm [11] and 14150 with DTNB at 412 nm [12].

Gel-electrophoresis

The acidic β-alanine system [13] was used with 7.5% polyacrylamide gels. The electrophoresis was carried out at 5°C for the first 30 min at 2 mA/tube then 3 h at 3 mA/tube. Staining was performed according to Blakesley and Boezi [14].

Molecular weight determination

The molecular weight of papain was determined by SDS-polyacrylamide gel electrophoresis 4-26% gradient slab gel method. Pharmacia molecular weight markers ranging from 17,200 to 83,000 were used as standard proteins.

Results and Discussion

Dried papaya latex (Sigma 6 grams) was ground in a mortar and extracted with 60 ml of 0.02 M cysteine pH 6.0 at room temperature with occasional stirring. After 30 minutes, the pH of the suspension ws adjusted to pH 9.0 by adding 1N NaOH with stirring for 5 minutes and was centrifuged at 6000 x g for 30 minutes. The supernatant 60 ml was collected and the precipitate was re-extracted with 30 ml of 0.02 M cysteine adjusted to pH 9.0 immediately before use, centrifuged and the two supernatents were combined. The remaining pellet was discarded.

The extract was saturated with ammonium sulphate (277 g/1 extract) to 45%. The solution was stirred until the salt was dissolved. Then it was allowed to stand for one hour at 4°C and centrifuged at 6000 x g for 30 minutes at 4°C in a Beckman Model J-12B centrifuge. The supernatent was saved for the preparation of chymopapain and papaya peptidases.

The precipitate obtained after saturation to 45% with ammonium sulphate was dissolved in 50 ml of 1 mM EDTA (Protein concentration 10-15 mg/ml) and adjusted to 40% saturated (243 g/l) with ammonium sulphate. After standing 1 hour at 4°C, the suspension was centrifuged at 6000 x g for 30 minutes at 4°C. The precipitate was dissolved in 30 ml 0.1 M phosphate buffer pH 7.5 containing 20 mM cysteine and 1 mM EDTA. Solid NaCl 3 grams was then added with stirring for 15 minutes and the precipitate was isolated with centrifugation as above. The precipitation with NaCl was repeated two times.

The white precipitate was dissolved in 30 ml of 0.05 M acetate buffer, pH 5.0 containing 20 mM cysteine and 1 mM EDTA and incubated at room temperature for 30 minutes. After incubation it was applied to Sephadex G-25 (2 x 20 cm) column, equilibrated with 0.05 M acetate buffer, pH 5.0 containing 1 mM EDTA. The gel filtered enzyme was applied to an agarose -mercurial column (1 x 10 cm) and eluted with 0.5 mM $HgCl_2$ in 0.05 M acetate buffer pH 5.0.

This procedure usually yields about 200-250 mg of papain of which about 65% was a mixture of active papain and papain-L-cysteine mixed disulphide which produces active papain in reducing media [15]. The other 35% was irreversibly oxidised papain, at least some of which is the sulphinic acid papain [16].

Pure papain 95-100% was obtained from thiol Sepharose column chromatography in which a column of Sepharose-glutathione-2-pyridyl disulphide

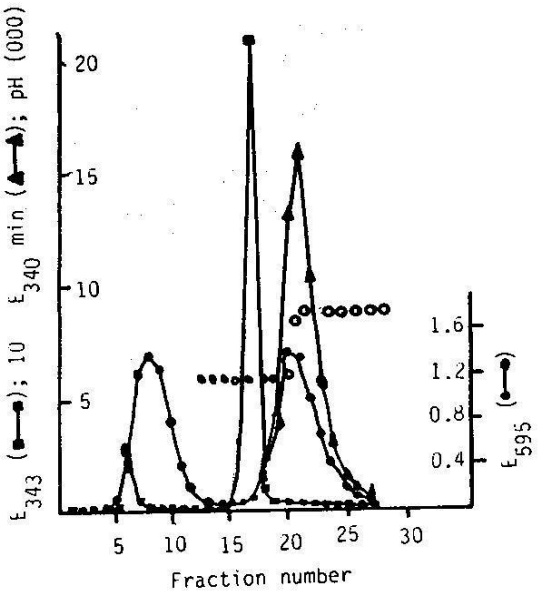

Fig. 1: Chromatography of papain on thiol-Sepharose column (1.5 x 12 cm). Papain prepared from papaya latex was used. E_{343} is chracterisitc of 2-thiopyridone; E_{595} represents protein content as determined by the method of Bradford. Enzymatic activity was measured at 340 nm.

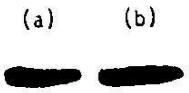

Fig.2: Polyacrylamide disc gel electrophoresis pattern of papain (a) after covalent chromatography (b) authentic sample of papain. The electrophoresis was carried out in the β-alanine system.

forms a disulphide bond with the active site of thiol of papain with the release of 2-thiopyridone. The active papain was eluted with cysteine (Fig. 1).

The enzyme was identified as papain and its homogeneity was checked on polyacrylamide disc gel electrophoresis and SDS slab gel electrophoresis (Fig. 2). It behaves in a fashion identical with that of an authentic sample of papain. Its molecular weight was

Table-1 Increase of thiol content of papain on thiol-Sepharose chromatography.

	Mole of thiol group per mole of enzyme		
	DTNB pH 8.0	PDS pH 8.0	PDS pH 4.0
After Ammonium sulphate fractionation. Before chromatography.	0.3 ± 0.05	0.35 ± 0.07	0.32 ± 0.06
After agarose-mercurial chromatography	0.81 ± 0.05	0.7 ± 0.05	0.72 ± 0.07
After thiol-Sepharose chromatography	1.00 ± 0.07	1.05 ± 0.06	1.00 ± 0.05

determined as 23,400 ± 200. This value agrees well with that found by [1] from spray dried papaya latex.

The amount of free - SH groups in papain was determined with DTNB and PDS (Tabl-1). Measurements with DTNB and PDS at pH 8.2 yield the total free-SH groups, whereas PDS at pH 3.8 determines the active site-SH groups. The - SH groups content was calculated for the same molecular weight, 23,400. Table-1 shows that highly active papain contains one SH group per enzyme molecule measured either at pH 8.2 or pH 3.8, which is similar to that reported for papain by Shipton and Brocklehurst [17].

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