ELSEVIER

Journal of Chromatography A, 724 (1996) 185–192

JOURNAL OF CHROMATOGRAPHY A

S-Pegylthiopapain, a versatile intermediate for the preparation of the fully active form of the cysteine proteinase archetype

Mohamed Azarkan[a], René T. Wintjens[b], Nicole Smolders[a], Michelle Nijs[a], Yvan Looze[a,*]

[a]*Protein Chemistry Unit, Faculty of Medicine, Free University of Brussels, Campus de la Plaine, Boulevard du Triomphe, B-1050 Brussels, Belgium*

[b]*Biological Macromolecule Conformation Unit, Faculty of Sciences, Free University of Brussels, Avenue P. Heger, B-1050 Brussels, Belgium*

Received 10 May 1995; revised 12 August 1995; accepted 17 August 1995

Abstract

Papain is widely used in several laboratories as a reference enzyme and, as such, needs to be highly pure. A one-step purification of the enzyme is described here starting from spray-dried papaya latex. After protection of the essential SH group with a monomethoxypolyethylene glycol derivative synthesised in our laboratory, the mixture of papaya enzymes is submitted to fractionation on S-Sepharose Fast Flow. This procedure leads to the isolation of the S-pegylthiopapain conjugate devoid of any contamination nor by the enzyme's irreversibly oxidized form nor by one of the other proteinases present in the original mixture. Thereafter, S-pegylthiopapain may be quite easily converted into its fully active form.

Keywords: S-Pegylthiopapain; Enzymes; Papain

1. Introduction

The latex of the tropical species *Carica papaya* L. is a rich source of proteinases used, since several decades, for food processing (beer clarification, meat tenderization etc.) on an industrial scale [1]. Its soluble fraction contains the proteinases papain, chymopapain (several forms?), caricain (formerly named papaya proteinase III and omega) and endo-proteinase Gly-C (also named chymopapain M or papaya proteinase IV) [2]. All belong to the family of cysteine proteinases and more precisely to the papain superfamily which encompasses a group of enzymes from quite various sources (plants, protozoa, insects,... and mammals). They display related amino-acid sequences and use a thiolate–imidazolium ion pair for their activity [3].

The medical interest raised by the papain superfamily of cysteine proteinases has also rapidly grown to include, today, several fields. Deregulated physiological conditions that lead to abnormal increase of the activity of the mammalian lysosomal cathepsins and the Ca^{2+}-dependent calpains are known to be associated with various disease states (see Refs. [4,5] and references therein). In addition, cysteine proteinases from some parasitic protozoa and helminths play an active role during the invasion of their hosts

* Corresponding author. Address for correspondence: Service de Chimie Général, Faculté de Médecine, Université Libre de Bruxelles, Campus de la Plaine, Boulevard du Triomphe, B-1050 Bruxelles, Belgium.

0021-9673/96/$15.00
SSDI 0021-9673(95)00910-8

[6]. Allergologists have also focused their attention on this superfamily of enzymes since they discovered that major allergens of some house dust mites (e.g. *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*) and of soya beans (*Glycine max*) have amino-acid sequences closely related to that of papain [7–9].

Physico-chemical and enzymatic properties of all these cysteine proteinases are most frequently investigated in the context of comparison with those of papain, generally recognized as their archetype.

As such, papain is thus widely used in many research laboratories. The enzyme may be purchased from several commercial sources. It may also be purified from papaya latex (see for example [10]). However, both alternatives actually suffer from several drawbacks. In this respect, it will be clear from the following discussion that the method described here offers several advantages.

2. Experimental

2.1. Materials

Aldrich-Chemie (Steinheim, Germany) provided dithiothreitol (DTT), 2,2′-dipyridyldisulfide, cysteamine hydrochloride, monomethoxypolyethylene glycol (mPEG), glutaric anhydride, 4-dimethylamino pyridine (DMAP), methylmethanethiolsulfonate and 1,3-diisopropylcarbodiimide. N-α-Benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) and N-acetyl-L-tryptophan ethyl ester were purchased from Sigma (Bornem, Belgium). The chemicals for SDS-PAGE and the molecular mass protein standards were purchased from Bio-Rad Laboratories (Nazareth Eke, Belgium). Pharmacia Biotechnol. (Uppsala, Sweden) provided S-Sepharose Fast Flow and Sephadex G-25 and Merck (Darmstadt, Germany) Fractogel TSK Butyl-650 (M). A 10 *M* aqueous solution of 3-mercapto-1,2-propanediol was obtained from Janssen Chimica (Beerse, Belgium). Spray-dried papain was purchased from Enzymase International (Brussels, Belgium). According to the manufacturer, this material is obtained through membrane technology (for filtering and sterilizing) from the crude latex freshly collected by tapping the fully-grown but immature fruits of the tropical species *Carica papaya* L.

2.2. Synthesis and characterization of mPEG(glutaryl)-S-SPy

This mPEG derivative was prepared by reacting glutaryl mPEG [11] and 2′-aminoethyl-2-pyridyldisulfide [12] in the presence of 1,3-diisopropyl-carbodiimide according to standard procedures.

It was first grossly purified by precipitation in diethyl ether. Aliquots (~250 mg) were then chromatographed on a (150×2.6 cm I.D.) column of Sephadex G-25 (eluent: H_2O) to remove any residual traces of UV-absorbing materials (2,2′-dipyridyldisulfide, 2-thiopyridone) susceptible to interfere with the spectrophotometric determination of mPEG(glutaryl)-S-Spy ($\epsilon_{281}=4850\ M^{-1}\ cm^{-1}$) [12].

2.3. Preparation of the mixture of S-pegylthiopapaya proteinases

Spray-dried papain (2.7 g from lot No. 001-94) was dissolved in water (40 ml) and applied to a (15×3 cm) column of S-Sepharose Fast Flow pre-equilibrated in a 50 m*M* sodium acetate buffer at pH 5.0 (throughout this work, the molarities of acetate buffers always refer to the Na^+ concentrations). Most of the proteolytically inactive material was eliminated by eluting (room temperature, 56.6 ml/h) with (1) 50 m*M* sodium acetate buffer at pH 5.0 (start buffer: 50 ml), (2) 2.5 m*M* DTT in the start buffer (25 ml), and (3) 100 m*M* acetate buffer at pH 5.0 until the A_{280} reading returned to its baseline value (about 100 ml). The papaya proteins were then eluted with 2 *M* acetate buffer pH 5.0 and collected in the presence of 40 μmoles of mPEG(glutaryl)-S-Spy. The reaction mixture was dialysed against H_2O and then against 100 m*M* acetate buffer pH 5.0 prior to fractionation.

2.4. Purification of S-pegylthiopapain

The dialysed solution containing the mixture of S-pegylthiopapaya proteinases was applied to a (15×

3 cm) column of S-Sepharose Fast Flow preequilibrated with a 100 m*M* acetate buffer at pH 5.0 (start buffer). After loading the protein sample onto the column, a linear elution gradient (total volume: 2 l) from 100 to 800 m*M* sodium acetate buffer at pH 5.0 was then applied at a flow-rate of 56.6 ml/h. Elution was performed at room temperature and fractions of 14.15 ml were collected. Each individual chromatographic fraction was analysed by measurement of A_{280}, conductivity and catalytic activity towards DL-BAPNA in the presence of 2.5 m*M* DTT to regenerate the active proteinases from their S-pegylthio derivatives. The chromatographic fractions which contained S-pegylthiopapain (see Fig. 2) were pooled, concentrated by ultrafiltration (Amicon; molecular mass cut-off of the membrane=3000), dialysed against H_2O and lyophilised.

2.5. Hydrophobic interaction chromatography (HIC) on Fractogel TSK Butyl-650

The protein sample under examination was dissolved in 4 *M* sodium acetate buffer pH 7.0 and applied to a (15×1.6 cm) column of Fractogel TSK Butyl-650 preequilibrated in 2 *M* ammonium sulfate (start eluent). Elution was performed at 25°C using a flow-rate of 60 ml/h. After loading the protein sample onto the column, the start eluent (200 ml) was pumped followed by a linear gradient from 2 to 0 *M* ammonium sulfate (total volume: 1500 ml). Fractions of 15 ml were collected and analysed by measurement of A_{280} and conductivity.

2.6. Conversion of S-pegylthio- into S-methylthiopapain

Papain was first regenerated from S-pegylthiopapain (2 μmol) dissolved in a 20 m*M* phosphate buffer pH 7.5 (10 ml) in the presence of 2.5 m*M* DTT for 15 min at room temperature. Methylmethanethiolsulfonate (60 μmol) was then added and the reaction mixture was applied to a (10×0.8 cm) column of S-Sepharose Fast Flow preequilibrated in a 100 m*M* acetate buffer pH 5.0 (start buffer). The column was eluted (flow-rate: 56.6 ml/h; room temperature) with the start buffer until the A_{280} reading returned to its baseline value. S-Methylthiopapain was then eluted by pumping 2000 m*M* acetate buffer pH 5.0 through the column, dialysed against water and lyophilised.

2.7. Measurement of amidase activity

The amidase activity of papain, chymopapain and caricain was measured using DL-BAPNA as the substrate. Each test tube (total volume: 2 ml) contained 10% DMSO, 1 m*M* substrate, 2.5 m*M* DTT, 1 m*M* EDTA and up to 2 μM proteinase in a buffer containing citrate, borate and phosphate (100 m*M* each) at pH 6.8 [12].

The proteinase was preincubated at 37°C in the buffer in the presence of DTT and EDTA for 15 min before the reaction was started by adding the substrate (prepared as a 10 m*M* stock solution in DMSO). The reaction proceeded at 37°C and was stopped by adding 500 μl of 50% acetic acid. The release of 4-nitroaniline was determined spectrophotometrically using $\epsilon_{410}=8800\ M^{-1}\,cm^{-1}$ [13]. One unit of activity (nkat) is the amount of proteinase that hydrolysed one nmole of substrate per second under the above-cited conditions.

2.8. SDS-PAGE

Experiments were carried out on slab gels using the mini protean II cell (Bio-Rad Laboratories). The resolving gels (pH 8.8, 12% acrylamide) were run at a constant voltage (200 V) and prepared according to Laemmli [14]. The stacking gels consisted of 4% polyacrylamide (pH 6.8). The upper and lower chambers contained Tris–glycine buffer (pH 8.3) with 0.1% SDS. The separation was toward the anode and bromophenol blue was used as the tracking dye. Before loading onto the gel, the protein solutions were diluted with a buffer containing 25% Tris-HCl 1 *M*, pH 6.8, 4% SDS, 23% glycerol and 1% bromophenol blue and boiled for 4 min. The gels were stained with 0.25% Coomassie Blue R250 dissolved in the washing solution. The latter was used also for destaining and contained MeOH, HOAc and H_2O (9:2:9).

The molecular mass standards were: lysozyme (14 300), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 200) and phosphorylase B (97 400).

2.9. Analytical methods

The conductivities (mS) were measured with a radiometer conductivity meter CD M3 equipped with a radiometer measurement cell type CDC 314. The mS values were measured at constant temperature. Papain concentrations were determined spectrophotometrically at 279 nm using the $A_{1\ mg/ml}$ value of 2.42 calculated from the amino-acid sequence data [15,16]. Absorbances were measured with a Varian DMS 300 spectrophotometer.

3. Results and discussion

The isolation of papain (M_r=23 406; pI=8.8) from the commercially available latex of *Carica papaya* L. involves the removal of the two other monothiol proteinases caricain (M_r=23 289; pI=11.4) and EGC (M_r=23 313; pI=10.6), of the dithiolproteinase chymopapain (M_r=23 657; pI=10.4) [17] and of papaya lysozyme (M_r ~28 000), a basic protein which contains no free thiol function [18]. The use of chromatographic procedures [10,19] is even required when crystalline preparations of papain are used as the starting material. Such preparations indeed are contaminated with chymopapain [20].

However, even homogeneous chromatographic preparations of papain obtained at this stage of purification may contain a high proportion (typically 50%) of inactive material resulting from oxidation. Air oxidation of the papaya proteinases takes place as soon as the latex is collected and results into irreversible conversion of the essential SH group into SO_nH with n=1, 2 or 3. Large proportions of inactivatable material such as those found in papain preparations may seriously handicap some studies on the enzyme (e.g. detailed kinetic).

Removal of irreversibly oxidized papain is thus required and generally performed by covalent or affinity chromatography [21]. Ideally, the fully active enzyme should also be further derivatized (e.g. as mercaptides or as mixed disulfides with low molecular mass thiol-containing compounds) to protect it from inactivation upon storage.

Few commercial preparations, if any, do indeed meet the two criteria by providing an homogeneous and stable enzyme. On the other hand, obtaining a papain preparation which contains a high fraction (0.85–0.95) of (regenerable) fully active enzyme such as provided by conventional methods (a combination of ion-exchange and covalent or affinity chromatography) is time-consuming.

Recent work from this laboratory has demonstrated that the free thiol group(s) of papain [22], chymopapain [23] and caricain [24] was (were) susceptible to chemical modification with a mPEG derivative similar to the mPEG(glutaryl)-S-SPy synthesized and used here as shown in Fig. 1. By grafting a PEG chain (M_r=5000), several new properties are conferred to these proteinases including a lower ability to bind to cation-exchange gels. This property was exploited to separate and purify the S-pegylthio derivatives of the individual proteinases from their unmodified (oxidized) molecular species. For that purpose, each individual proteinase was first purified by ion-exchange chromatography before being submitted to S-pegylation. Thus, in this context, the covalent attachment of mPEG was exploited only to eliminate irreversibly inactivated (see Fig. 1) forms of the proteinases.

One of the purposes of the present study consisted in examining the feasibility of purifying the S-pegylthioproteinases directly from the mixture of papaya enzymes. This strategy was shown to be particularly successful in the case of S-pegylthiopapain. This prompted us to further characterize this conjugate and to compare the S-pegyl group to others derived from low-molecular-mass thiol-containing compounds for its ability to protect papain from inactivation upon storage.

3.1. Fractionation of the S-pegylthio derivatives of the papaya proteinases after pegylation of the mixture of papaya enzymes

This fractionation was carried out on a S-Sepharose Fast Flow column preequilibrated at pH 5.0 with a 100 mM sodium acetate buffer and eluted with a linear gradient from 100 to 800 mM sodium acetate buffer. These experimental conditions were quite similar to those used previously [22–24] although S-Sepharose Fast Flow was used instead of CM-Sephadex C-50.

Examination of the elution profiles, such as the

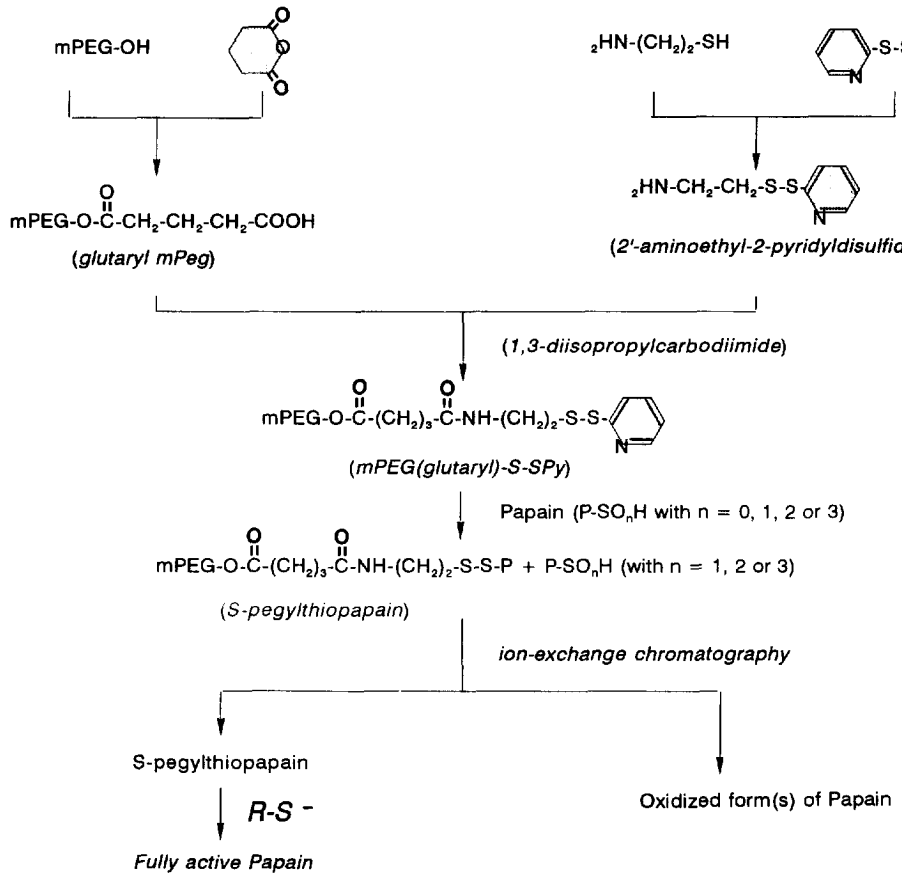

Fig. 1. Preparation and conjugation of mPEG(glutaryl)-S-SPy to papain.

one displayed in Fig. 2, indicated that at least nine different molecular species resulted from the modification of the papaya enzymes with mPEG-(glutaryl)-S-SPy. Most of them (with the exception of pools I and V) were only partially resolved and only four did contain active material against DL-BAPNA. This active material was pooled into the three different fractions I, II and IV as indicated by the solid bars in Fig. 2. The different pools I to V were then analysed by SDS-PAGE. The results from this study, shown in Fig. 3, indicated that, with the exception of pool I (see lane 2), all the chromatographic fractions separated on the S-Sepharose Fast Flow column were heterogeneous. In particular, they all contained the low-molecular-mass contaminants present in the mixture of proteinases (see lane 7) before ion-exchange chromatography.

The material constituting pool I, which was unambiguously identified as S-pegylthiopapain, attracted our attention because of its apparent homogeneity. As shown in Fig. 3, this material indeed migrated on the SDS-PAGE gels as an unique band to which an apparent M_r of 31 000 was attributed. In both regions corresponding to M_r=40 000 (where S,S'-dipegylthiochymopapain migrated, see lanes 4 and 7 and Ref. [23]) and M_r=24 000 (where native papain migrated, see Ref. [22]), the gels were transparent. Examination of Fig. 2 already showed that this material eluted from the S-Sepharose Fast Flow as a symmetrical peak, well-separated from the material constituting pool II. Furthermore, the elution profiles obtained by measurement of A_{280} or by measurement of regenerated amidase activity superimposed quite well.

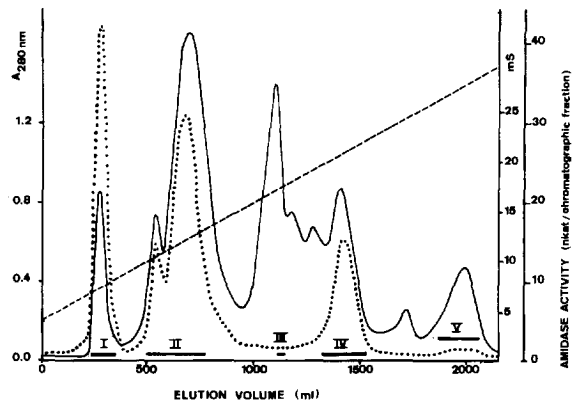

Fig. 2. Purification of S-pegylthiopapain by ion-exchange chromatography on S-Sepharose Fast Flow. Column: 15×3 cm; fractions of 14.15 ml; flow-rate: 56.6 ml/h; sample: mixture of the enzymes (~1 g) present in the latex of *Carica papaya* and modified with mPEG(glutaryl)-S-SPy; eluting buffer: gradient 100–800 m*M* sodium acetate, pH 5.0, total volume 2.5 l; room temperature. Each chromatographic fraction was analysed by measurements of A_{280} (continuous trace), amidase activity (dots) and conductivity (broken trace). Several pools (roman letters numbering) were made up from the chromatographic fractions, as indicated by the solid bars, for further analysis.

The specific amidase activity regenerated from pool I typically amounted to 6.20±0.15 nkat/mg which compares favourably with the 5.89 nkat/mg value measured previously [22].

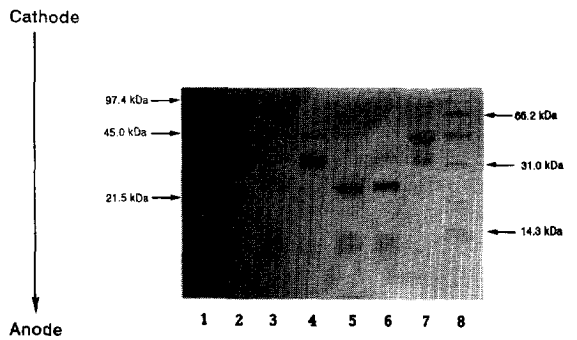

Fig. 3. SDS-PAGE. Lanes 1 and 8: the SDS molecular mass markers; lanes 2–6: pools I, III, II, IV and V, respectively (see Fig. 2); and lane 7: the mixture of enzymes present in the latex of *Carica papaya* after their conversion into S-pegylthio derivatives and before being submitted to ion-exchange chromatography on S-Sepharose Fast Flow (Fig. 2). Details are given in Section 2.

3.2. Further demonstration of the homogeneity of the S-pegylthiopapain preparation as provided by examination of elution patterns on Fractogel TSK Butyl-650

Fractogel TSK Butyl-650 separates proteins in solution as a result of their varying degrees of hydrophobic interaction with the butyl ligands attached to the gel matrix. Papain, caricain, EGC and chymopapain (as their S-methylthio derivatives) greatly differ in their ability to bind to Fractogel TSK Butyl-650 as shown in Fig. 4. It was also observed, for each of the four individual papaya proteinases, that irreversible oxidation of the thiol group(s) did not affect its elution pattern as compared to that of the S-methylthio protected enzyme (not shown). In marked contrast, substituting the

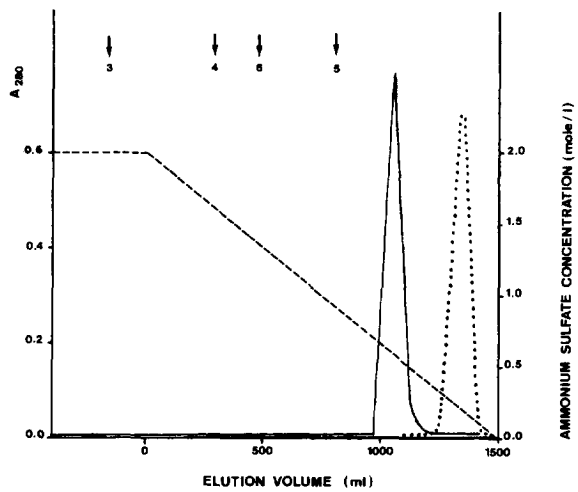

Fig. 4. Elution pattern of S-pegylthiopapain on Fractogel TSK Butyl-650 (HIC). Column: 15×1.6 cm; fractions of 15 ml; flow-rate: 60 ml/h; sample 1: the S-pegylthiopapain as provided by the material constituting pool I of Fig. 2; sample 2: S-methylthiopapain prepared from sample 1; sample 3: S-methylthiocaricain prepared according to Ref. [24]; samples 4 and 5: S,S′-dimethylthio- and S,S′-dipegylthiochymopapain prepared according to Ref. [23]; sample 6: S-methylthioEGC (unpublished results). The elution of samples 1 and 2 was monitored by measurements of A_{280} (continuous trace and dots, respectively). The positions of the peak maxima of the other samples are indicated by arrows; eluent: concentration gradient (calculated from conductivity measurements: broken trace) from 2 to 0 *M* ammonium sulfate; temperature 25°C.

S-methylthio group(s) by the S-pegylthio group(s) increased the hydrophobicity of caricain, chymopapain and EGC while it decreased that of papain (see Fig. 4).

From the examination of the results displayed in Fig. 4, it may be concluded that the combination of S-pegylation on the one hand, and of the use of Fractogel TSK Butyl-650 as the chromatographic support on the other hand, is a powerful tool for evaluating the homogeneity of papaya proteinase preparations. If one applies this criterion, one observes that the S-pegylthiopapain preparation, as provided by pool I from Fig. 2 shows no sign of heterogeneity due to e.g. the presence of residual irreversibly oxidized papain species. Furthermore, the S-methylthiopapain preparation obtained from pool I also appeared quite homogeneous and did not contain residual traces nor of pegylated species nor of any other proteinase.

4. Concluding comments

The general concept of protecting cysteine proteinases from air oxidation and/or from autolysis through chemical modification has been widely used since the pioneer studies by Smith et al. [25]. Occasionally, the chromatographic separations of cysteine proteinases were also observed to be best achieved after treatment of the mixture with thiol-modifying reagents [10,26,27]. However, only very recently the use of chemical modification was introduced as both a protection and separation tool [12,22–24].

In this perspective, S-pegylthio groups (with a PEG chain of molecular mass around 5000) showed promising. This is clearly further illustrated in the present communication which describes the one-step (chromatographic) purification of papain from the complex mixture of papaya proteinases.

Acknowledgments

Professor S. Wodak is gratefully acknowledged for given us access to computers and software in her laboratory. Enzymase is acknowledged for having generously supplied spray-dried papain.

References

[1] J.C. Caygill, Enzyme Microb. Technol., 1 (1979) 233–242.
[2] D.J. Buttle, A.A. Kembhavi, S.L. Sharp, R.E. Shute, D.H. Rich and A.J. Barrett, Biochem. J., 261 (1989) 469–476.
[3] P.J. Berti and A.C. Storer, J. Mol. Biol., 246 (1995) 273–283.
[4] K.K.W. Wang and P.W. Yuen, Trends Pharmacol. Sci., 15 (1994) 412–419.
[5] B.J. Gaur-Salin, P. Lachance, C. Plouffe, A.C. Storer and R. Menard, J. Med. Chem., 36 (1993) 720–725.
[6] J.H. Mc Kerrow, E. Sun, P.J. Rosenthal and J. Bauvier, Ann. Rev. Microbiol., 47 (1993) 821–853.
[7] K.Y. Chua, G.A. Stewart, W.R. Thomas, R.J. Simpson, R.J. Dilworth, T.M. Plozza and R.J. Turner, J. Exp. Med., 167 (1988) 175–182.
[8] N.A. Kent, M.R. Hill, J.N. Keen, P.W.H. Holland and B.J. Hart, Int. Arch. Allergy Immunol., 99 (1992) 150–152.
[9] T. Ogawa, H. Tsuji, N. Bando, K. Kitamura, Y.L. Zhu, H. Hirano and K. Nishikawa, Biosci. Biotechnol. Biochem., 57 (1993) 1030–1033.
[10] M.P. Thomas, C.M. Topham, D. Kowlessur, G.W. Mellor, E.W. Thomas, D. Whitford and K. Brocklehurst, Biochem. J., 300 (1994) 805–820.
[11] S.I. Wie, C.W. Wie, W.Y. Lee, L.G. Filion, A.H. Sehon and E. Åkerblom, Int. Arch. Allergy Appl. Immun., 64 (1981) 84–99.
[12] T. Musu, J. Brygier, J. Vincentelli, C. Guermant, C. Paul, D. Baeyens-Volant and Y. Looze, Int. J. Bio Chromatogr., 1 (1994) 17–27.
[13] B.F. Erlanger, N. Kokowsky and W. Cohen, Arch. Biochem. Biophys., 95 (1961) 271–278.
[14] U.K. Laemmli, Nature, 227 (1970) 680–685.
[15] L.W. Cohen, V.M. Coghlan and L.C. Dihel, Gene, 48 (1986) 219–227.
[16] S.C. Gill and P.H. von Hippel, Anal. Biochem., 18 (1989) 319–326.
[17] I.G. Sumner, G.W. Harris, M.A.J. Taylor, R.W. Pickersgill, A.J. Owen and P.W. Goodenough, Eur. J. Biochem., 214 (1993) 129–134.
[18] P. Bernasconi, R. Locher, P.E. Pilet, J. Jollès and P. Jollès, Biochim. Biophys. Acta, 915 (1987) 254–260.
[19] P.M. Dekeyser, S. De Smedt, J. Demmeester and A. Lauwers, J. Chromatogr. A, 656 (1994) 203–208.
[20] B.S. Barnes and K. Brocklehurst, Biochem. J., 173 (1978) 345–347.
[21] D.J. Buttle, Methods Enzymol., 244 (1994) 639–648.
[22] C. Paul, J. Vincentelli, J. Brygier, T. Musu, D. Baeyens-Volant, C. Guermant and Y. Looze, Phytochemistry, 35 (1994) 1413–1417.
[23] J. Vincentelli, J. Bauckaert, A. Jacquet, C. Paul, M.H.D. Thi, F. Poortmans, L. Wijns and Y. Looze, Int. J. Bio Chromatogr., (1994) in press.

[24] T. Musu, M. Azarkan, J. Brygier, C. Paul, J. Vincentelli, D. Baeyens-Volant, C. Guermant, M. Nijs and Y. Looze, Biotechnol. Appl. Biochem., (1995) in press.
[25] E.L. Smith, J.R. Kimmel, D.M. Brown and E.O.P. Thompson, J. Biol. Chem., 215 (1955) 67–89.
[26] T. Dubois, A. Jacquet, A.G. Schnek and Y. Looze, Biol. Chem. Hoppe-Seyler, 369 (1988) 733–740.
[27] A.D. Napper, S.P. Bennett, M. Borowski, M.B. Holdridge, M.J.C. Leonard, E.E. Rogers, Y. Duan, R.A. Laursen, B. Reinhold and S.L. Shames, Biochem. J., 301 (1994) 727–735.