Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 790 (2003) 229–238
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Fractionation and purification of the enzymes stored in the latex of *Carica papaya*

Mohamed Azarkan[a], Anouar El Moussaoui[a,b], Delphine van Wuytswinkel[a,b], Géraldine Dehon[b], Yvan Looze[b,*]

[a]*Laboratoire de Chimie Générale, Unité de Chimie des Protéines (CP 609), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, 808 Route de Lennik, B-1070 Brussels, Belgium*
[b]*Laboratoire de Chimie Générale, Unité de Chimie des Protéines (CP 206/4), Institut de Pharmacie, Université Libre de Bruxelles, Campus de la Plaine, Boulevard du Triomphe, B-1050 Brussels, Belgium*

Abstract

The latex of the tropical species *Carica papaya* is well known for being a rich source of the four cysteine endopeptidases papain, chymopapain, glycyl endopeptidase and caricain. Altogether, these enzymes are present in the laticifers at a concentration higher than 1 m*M*. The proteinases are synthesized as inactive precursors that convert into mature enzymes within 2 min after wounding the plant when the latex is abruptly expelled. Papaya latex also contains other enzymes as minor constituents. Several of these enzymes namely a class-II and a class-III chitinase, an inhibitor of serine proteinases and a glutaminyl cyclotransferase have already been purified up to apparent homogeneity and characterized. The presence of a β-1,3-glucanase and of a cystatin is also suspected but they have not yet been isolated. Purification of these papaya enzymes calls on the use of ion-exchange supports (such as SP-Sepharose Fast Flow) and hydrophobic supports [such as Fractogel TSK Butyl 650(M), Fractogel EMD Propyl 650(S) or Thiophilic gels]. The use of covalent or affinity gels is recommended to provide preparations of cysteine endopeptidases with a high free thiol content (ideally 1 mol of essential free thiol function per mol of enzyme). The selective grafting of activated methoxypoly(ethylene glycol) chains (with M_r of 5000) on the free thiol functions of the proteinases provides an interesting alternative to the use of covalent and affinity chromatographies especially in the case of enzymes such as chymopapain that contains, in its native state, two thiol functions.

Keywords: Reviews; *Carica papaya*; Latex; Enzymes; Papain; Lysozyme; Enzyme inhibitors

Contents

*Corresponding author. Tel.: +32-2-650-5101; fax: +32-2-650-5439.
E-mail address: ylooze@ulb.ac.be (Y. Looze).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(03)00084-9

1. Introduction

Carica papaya is widely cultivated in tropical and subtropical regions all around the world for its edible fruits and for the enzymes stored in its laticifers.

Laticifers are displayed in all aerial parts of the tree forming a dense network of articulated and anastomosing structures [1]. Damaging the papaya tree inevitably severs its laticifers, eliciting an abrupt release of latex. Papaya latex is a thixotropic fluid with a milky appearance that contains about 15% of dry matter. Forty percent of this dry matter is constituted by enzymes, mainly cysteine endopeptidases since altogether they account for more than 80% of the whole enzyme fraction. Assuming that the cysteine endopeptidases could circulate freely within the laticifers, their molar concentration has been calculated to be higher than 1 m*M* [2].

The papaya endopeptidases thus constitute a potential danger for the plant. They are however stored in the laticifers as inactive proforms [3–6] that rapidly convert into active mature enzymes after the release of latex from the plant [7,8]. This explains why these proforms have never been isolated from papaya latex.

It is because it contains several cysteine endopeptidases that the latex of *C. papaya* has found many applications in the food (e.g. as a meat tenderiser and for beer chill-proofing) and pharmaceutical industries [9–12 and references therein].

In the past, all the commercially available latex came from East Africa [from the province of Kivu in East Congo (ex-Zaire) and from Uganda, mainly]. Today, some production also arises from Central America, from India and from Vietnam. The chemical composition of these lattices differs somewhat from that produced in East Africa. This subject will not be discussed here. Hence, the economic interest of the papaya latex has prompted much research aimed at elucidating both the structures and the modes of action of the papaya endopeptidases.

Although papain (EC 3.4.22.2) is a minor constituent (about 8%) among the papaya endopeptidases, this enzyme, more easily purified, has been the most extensively studied. Its primary structure was fully elucidated in 1970 [13] and it was the second enzyme to be crystallized and to have its structure determined by X-ray methods [14]. The mechanistic geometry of its active site was reviewed for the first time in 1982 [15]. Today, its three-dimensional structure has ben determined at a resolution of 1.65 Å [16] and it is regarded as one of the archetypes in this family of enzymes. Joe R. Kimmel, Emil L. Smith and Jan Drenth have associated their names with the pioneering studies on papain.

The second papaya endopeptidase that has been purified up to apparent homogeneity has been caricain (EC 3.4.22.30) formerly known as papaya proteinase A or papaya proteinase Ω. The purification of caricain was greatly facilitated given that this protein is extremely basic (the letter omega, the last one in the greek alphabet, was associated with caricain to outline that this protein was surely the last one to be eluted from cation-exchange chromatographic supports). Its amino acid sequence and its crystallographic structure have both been determined [17,18]. Two similar but distinct cDNAs have been shown to code for caricain [4]. The deduced amino acid sequence of the shorter one is identical to the experimentally determined amino acid sequence of caricain (216 amino acids). The other differs at only one position (T 214 I) and, distinctively, contains a 19-amino acid-long C-terminal extension.

Interestingly, the presence of a papaya endopeptidase with the same isoelectric point (p*I*) as caricain but with a M_r of 28 000 (instead of 23 500 for caricain) has been reported [19]. The presence of such a molecular species called papaya proteinase β has since, however, not been confirmed.

Full characterization of the other papaya endopeptidases has long been hampered by difficulties encountered in the course of their purification [20–23]. The revival in papaya latex enzyme research that took place during the last decade had to await the discovery of glycyl endopeptidase, also known as papaya proteinase IV [24] or chymopapain M [25] and the use of molecular biology techniques.

Five similar but distinct clones code for chymopapain (EC 3.4.22.6) [6], three among which chymo III, IV and V display C-terminal extensions containing nine amino acid residues that are missing in the experimentally determined amino acid sequences of chymopapain (both corresponding to chymo II) [26,27]. Chymopapain is unique among the papaya cysteine endopeptidases in that it has a free cysteine (at position 117, mature chymopapain numbering)

not involved in disulfide bonds or in the active site center. Two further chymopapain isoforms (III and IV) have yet a third free cysteine residue at position 88. This residue is a totally conserved tyrosine in all other papaya endopeptidases. Isolation from the latex of *C. papaya* of a molecular isoform of chymopapain with 3 mol of SH per mol of protein has been mentioned in only one report [28]. It seems that chymopapain isoform II is the sole one that is expressed in the papaya latex from East Africa. This assertion is supported by sequence works [26,27] as well as by X-ray crystallographic data [29].

In marked contrast to the three above mentioned papaya proteinases which exhibit a broad specificity [30], the specificity of glycyl endopeptidase (EC 3.4.22.25) is practically restricted to glycyl residues at position P_1 [31]. The reason is that the active site of glycyl endopeptidase is occluded due to the substitution of the highly conserved residues Gly 23 and Gly 65 by Glu and Arg, respectively [32].

The mature forms of the papaya proteinases generally contain from 212 to 218 amino acids. Their sequences exhibit a strong degree of homology [3–6].

Comparison of their X-ray structures also reveals that they adopt identical three-dimensional folds [16,18,29,32]. The substrate specificity of the papain family is mainly controlled by the S_2 subsite [33]. A close examination of their active site grooves that separate the two domains also shows that the specificity restrictions dictated by the S_2 subsites do not differ significantly among these four endopeptidases.

In addition to the above mentioned cysteine endopeptidases, several other enzymes are stored in the papaya laticifers. Some of these enzymes have been purified up to apparent homogeneity and further characterized. Those that fall in this category of papaya enzymes are a lysozyme [34–37], a class-II chitinase [38], a Kunitz-type inhibitor of serine proteinase [39,40] and a glutaminyl cyclotransferase (PQC, an enzyme that catalyzes the conversion of glutaminyl peptides into 5-oxoprolyl peptides with the concomitant liberation of ammonia) [41–44]. Others have not yet been isolated and are only known through their cDNA or through their enzymatic activity. This is the case of an endo-β-1,3-glucanase [45], of a cystatin [46] and of a lipase [47].

All these enzymes, including the cysteine endopeptidases, are thought to play a role within the defense mechanism of the plant [48]. As previously mentioned, the level of proteolytic activity in undamaged laticifers is rather low. Following release of latex, however, the concentration in active proteases increases rapidly to reach a maximum in less than 2 min after the laticifers have been injured [7,8]. Proteolytic cleavages rapidly leads to latex coagulation, a step that probably constitutes the primary defense mechanism since it leads to wound sealing and thus protects the plant from pathogen invasion.

Our laboratory has been engaged for about 15 years in the study of papaya enzymes and it is the purpose of the present review to comment on our experience in the field of the purification of these enzymes.

With the exception of papain and chymopapain that are commercially available (although not in pure forms), all the papaya enzymes have to be purified starting from papaya latex. Crude latex, obtained by sun-drying the whole papaya latex, is commercially available but presents the disadvantage of being, from a bacteriological point of view, highly contaminated. Spray-dried preparations, also commercially available, are somewhat more refined. Such preparations have been freed from any insoluble material and sterilized through membrane filtration. Lipase, being tightly associated with the particulate fraction of papaya latex, is normally absent from spray-dried latex. In some cases, a part of the fraction constituted by low molecular mass substances has also been eliminated through ultrafiltration resulting in preparations with higher enzymes content.

Whatever starting material is chosen, the free thiol functions, present in many papaya enzymes, should be blocked in order to protect them from air oxidation. This is particularly important in the case of the endopeptidases where cysteine 25 is essential to the catalytic activity [15]. Oxidation of these residues results in the loss of their proteolytic activities. Reaction of the free thiol functions with methylmethanethiol sulfonate, 2,2′-dipyridyl disulfide or tetrathionate ions [23,25] results in their conversion into mixed disulfide bonds (Fig. 1). Addition of low molecular mass thiols (e.g. dithiothreitol, cysteine) allows the regeneration of the free thiol functions when suited.

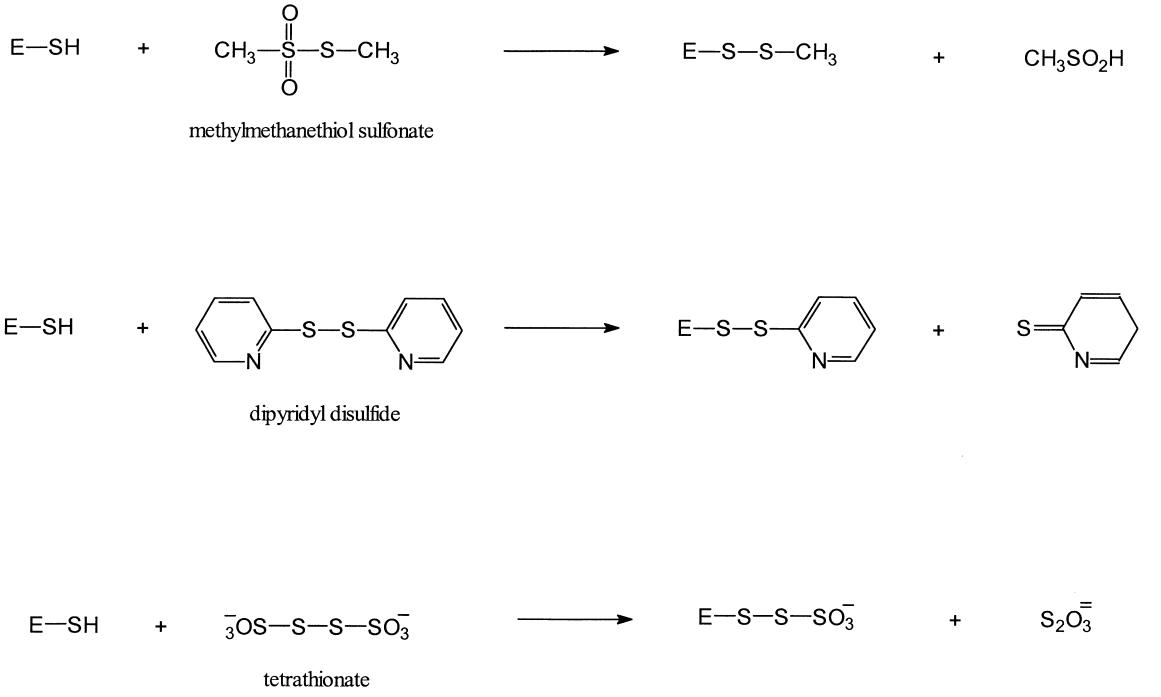

Fig. 1. Schematic representation of the conversion of the free thiol functions present in some papaya enzymes (E-SH) into mixed disulfide bonds using methylmethanethiol sulfonate, dipyridyl disulfide or sodium tetrathionate.

2. Fractionation of the papaya enzymes on cation-exchange supports

Chromatography on cation-exchange supports has traditionally been used as the first purification step of papaya enzymes [23,49–53]. The elution pattern obtained at pH 5.0 on SP-Sepharose Fast Flow (Pharmacia Biotechnology, Uppsala, Sweden) and shown in Fig. 2 illustrates well that fresh papaya latex is a complex mixture of enzymes. This elution profile is somewhat atypical since it was obtained starting from a fresh papaya latex preparation not submitted to spray-drying. Spray-drying (spray-dried latex, provided by Enzymase International, Brussels, Belgium) results in simplified elution profiles wherein several peaks (indicated by arrows in Fig. 2) either are no longer visible or have been considerably smoothed out.

Protein fractions from the cation-exchange supports are generally regrouped into four pools. The first pool amalgamates the fractions that do not bind at pH 5.0 to the chromatographic support. This pool contains very few proteins and no known enzymatic activity with the possible exception of glycosidases [54]. The second pool (denoted PAP in Fig. 2) contains papain as the main constituent and lysozyme as a contaminating species (the amidase activity of the four cysteine endopeptidases is measured spectrophotometrically at 410 nm following the release of *para*-nitroaniline from *N*-α-benzoyl-DL-arginine *p*-nitroanilide for papain, chymopapain and caricarin, and from *N*-acetyl-L-phenylalanylglycine 4-nitroanilide in the case of glycyl endopeptidase).

Lysozyme (denoted LYS in Fig. 4), identified through its bacteriolytic activity against *Micrococcus luteus* cells [suspended at 37 °C in 0.066 *M* phosphate buffer at pH 6.2; the decrease in the absorbance at 450 nm (initial value: 0.7) is followed as a function of time] [35,36] is also present in the third pool, mainly constituted by chymopapain and glycyl endopeptidase (respectively denoted CHP and GEP in Fig. 2) [23,50]. Papaya class-II chitinase denoted CHI in Fig. 4 (assessed fluorimetrically by its β-*N*-acetyl-D-glucosaminidase activity, measured at 50 °C

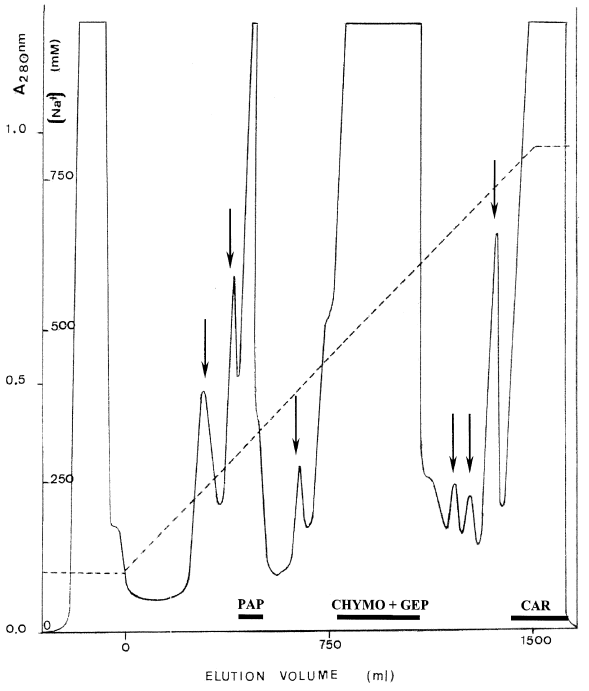

Fig. 2. Ion-exchange chromatography of the papaya enzymes on SP-Sepharose Fast Flow. Sample: 1330 mg of papaya enzymes derivatized with methylmethanethiol sulfonate; column: 30×2.6 cm I.D.; fractions of 15 ml; flow-rate: 60 ml/h; room temperature; eluting buffer: 100 m*M* (referring to Na^+ concentration) sodium acetate at pH 5.0 followed by a linear gradient 100–800 m*M* (total volume: 1500 ml) followed by 800 m*M*. Each chromatographic fraction was analysed by measurement of A_{280} and conductivity (continuous trace and broken line, respectively). Solid bars indicate the fractions that were pooled for further analysis.

using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as the substrate) which elutes between this third pool and the pool constituted by caricain (denoted CAR in Fig. 2) is generally regrouped with this latter one [38]. Glutaminyl cyclotransferase (denoted PQC in Fig. 4) and the inhibitor of serine proteinases (denoted PPI in Fig. 4) also elute in the CAR pool. Measurement of the PQC activity is based on the liberation of ammonia from L-glutamine *tert*.-butyl ester using the diagnostic kit provided by Sigma (St Louis, MO, USA). The activity of the inhibitor of serine proteinases is assessed through its ability to inhibit the amidase activity (*N*-α-benzoyl-DL-arginine *p*-nitroanilide as the substrate) of bovine pancreatic trypsin.

Each individual enzyme is located in one single pool with the exception, as previously mentioned, of lysozyme. The distribution of the latter into two pools is in agreement with the observation by Howard and Glazer of the existence of several forms of lysozyme separable on Amberlite CG-50 (Aldrich, Steinheim, Germany), another cation-exchange chromatographic support [35,36].

Papaya cystatin constitutes the other noteworthy exception. The papaya cystatin-encoding cDNA has been sequenced [46]. The predicted amino acid sequence revealed a single polypeptide chain that contains 98 amino acids (M_r=11 131). The absence of a signal peptide and of putative glycosylation site(s) in the predicted sequence strongly suggests that papaya cystatin is located in the cytoplasm of the laticifers where it could possibly prevent undesirable vessel damage by endogenous proteinases. When the latex is expelled, papaya cystatin comes in contact and forms tight complexes with the endopeptidases resulting in the distribution of cystatin within the three chromatographic pools containing the proteinases. As shown in Fig. 3, papaya cystatin

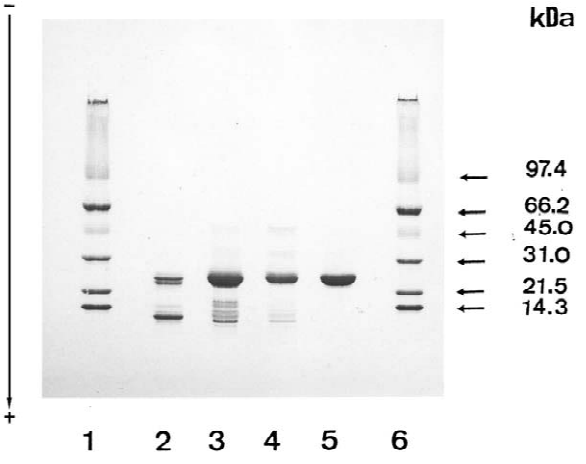

Fig. 3. SDS–PAGE of the different chromatographic pools obtained after chromatography of the papaya enzymes on SP-Sepharose Fast Flow. Experiments were carried out on slab gels using the Mini-Protean II cell (Bio-Rad Labs) as described previously [41]. Lanes: 1 and 6, molecular mass standards (kDa) including (from top to bottom) phosphatase B (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5) and lysozyme (14.3); 2, the papain (PAP) pool; 3, the chymopapain (CHP) pool; 4, the caricain (CAR) pool and 5, the glycyl endopeptidase (GEP) pool from the Fractogel TSK Butyl 650(M).

is visible on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Examination of Fig. 3 shows that papaya cystatin is the main contaminant of papain and of chymopapain while it bound less strongly to caricain and to glycyl endopeptidase.

Such an observation is in agreement with the measured dissociation constants between papaya cystatin and the papaya endopeptidases (K_d of 0.39, 0.43, 1.49 and 3.44 n*M*, respectively for papain, chymopapain, caricain and glycyl endopeptidase) [46].

3. Fractionation of the papaya enzymes on hydrophobic supports

Hydrophobic supports have disclosed their extreme usefulness in the context of the purification of papaya enzymes [38,41,53,55]. Three such supports have been evaluated, namely: Phenyl Sepharose [53] (Pharmacia Biotechnology), Fractogel TSK Butyl 650(M) [38,41,55] and Fractogel EMD Propyl 650(S) (both from Merck, Darmstadt, Germany) (results reported here for the first time).

Despite some success encountered with Phenyl Sepharose on an analytical scale (HPLC experiments), the scaling-up of hydrophobic interaction chromatography (HIC) with this kind of gel caused problems [53]. This is not the case with the other examined hydrophobic supports.

Fig. 4 compares the chromatographic behavior of the papaya enzymes on Fractogel TSK Butyl 650(M), on Fractogel EMD Propyl 650(S) and on a thiophilic support [56] obtained by successively treating BioGel 0.5 m (Bio-Rad Labs, Hercules, CA, USA) with divinylsulfone and 2-mercaptoethanol. This gel, called T-gel, obviously also behaves, at

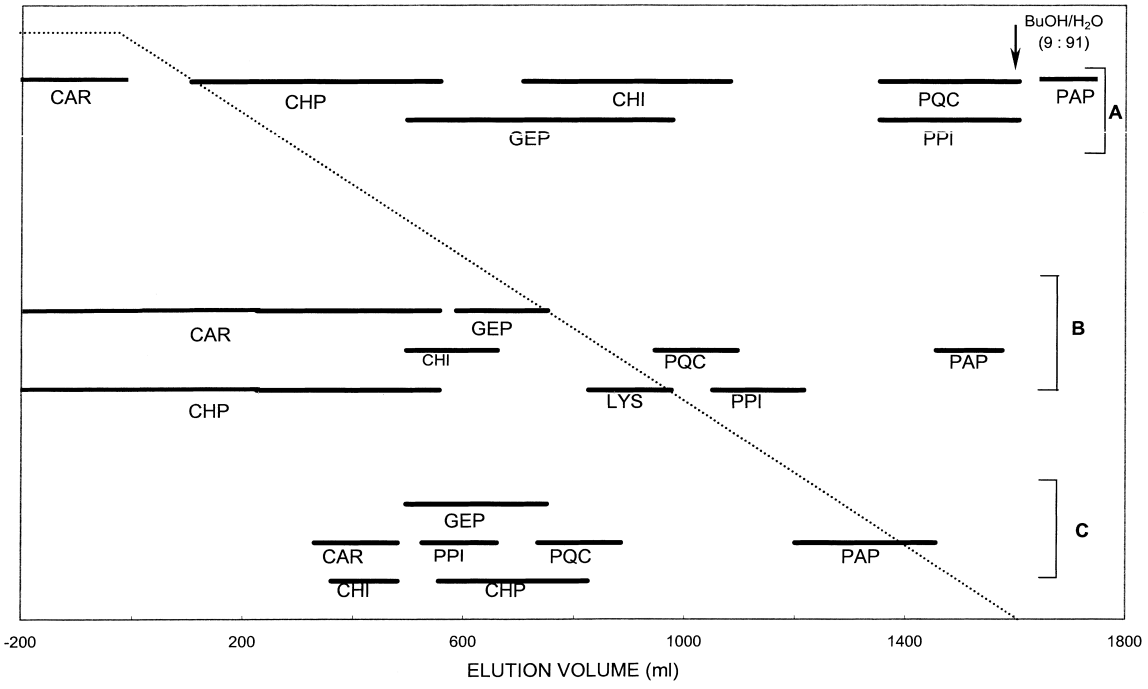

Fig. 4. Chromatographic behavior of the individual papaya enzymes on Fractogel TSK Butyl 650(M) (A), Fractogel EMD Propyl 650(S) (B) and on the Thiophilic gel (C). Column: 12×3.2 cm I.D.; fractions of 15 ml; flow-rate: 60 ml/h; room temperature; eluting solutions: 2 *M* ammonium sulfate followed by a linear gradient (total volume: 1600 ml) from 2 to 0 *M* ammonium sulfate, followed by water and finally by 9% *n*-butanol in water. Each chromatographic fraction was analysed by measurement of A_{280} and conductivity (broken line). Solid bars indicate the elution volumes of the different papaya enzymes.

least partly, as a hydrophobic support. In all cases, elution of the bound proteins proceeds through the application of a linear gradient decreasing in ionic strength from 2 to 0 *M* $(NH_4)_2SO_4$.

As shown in Fig. 4, among the three examined supports, Fractogel TSK Butyl 650(M) is the one that fractionates the papaya enzymes over the widest range of ionic strength. Caricain does not bind to this support even in the presence of 2 *M* $(NH_4)_2SO_4$ while, on the other hand, elution of papain which fails even in the absence of salts, requires the presence of 9% *n*-butanol.

Combining two chromatographies successively on SP-Sepharose Fast Flow and on Fractogel TSK Butyl 650(M) provides, in a nearly homogeneous form, all the papaya enzymes with the exception of the glutaminyl cyclotransferase and the inhibitor of serine proteinases which coelute on both gels. Their separation is however readily achieved through affinity chromatography on immobilized trypsin [39] or through chromatography on the thiophilic gel (Fig. 4). Passage of the glutaminyl cyclotransferase preparation through the T-gel also removes a proteinase, visible with difficulty on SDS–PAGE gels, but readily detectable by its amidase activity on fluorescent substrates [41]. Fractogel EMD Propyl 650(S) and T-gel are less efficient than Fractogel TSK Butyl 650(M) leading to highly contaminated preparations of chymopapain, glycyl endopeptidase, caricain and class-II chitinase.

4. Covalent and affinity chromatography of papaya endopeptidases

As judged by native and SDS–PAGE experiments, the combination of chromatographies on SP-Sepharose Fast Flow followed by Fractogel TSK Butyl 650(M) provides the four individual endopeptidases in a homogeneous form. From 30 to 50% of the proteinases in the starting papaya latex are however irreversibly oxidized, their cysteine residues being converted into cysteic acid [14]. As a consequence, each proteinase preparation is a mixture of irreversibly and reversibly (e.g. in the form of a *S*-thiomethyl derivative) oxidized endopeptidase. Several chromatographic supports (Table 1) have been designed with the aim of separating the fully active endopeptidases from the irreversibly oxidized ones. The first successful attempt was realized in 1970 on an agarose organomercurial support and resulted in a papain preparation titrating 1 mol of SH per mol of enzyme [57]. Chymopapain and caricain also bind to the organomercurial gel [23]. Other commercially available covalent chromatographic supports include immobilized glutathione-*S*–*S*-(2′-pyridyl) and immobilized 2-hydroxypropyl-*S*–*S*-(2′-pyridyl) [25]. Both chromatographic supports are able to specifically react with fully active papain [25], chymopapain [58,59] and caricain [23]. Glycyl endopeptidase, on the other hand, does not bind at all to the organomercurial gel [23] nor to the electrophilic sites of the

Table 1
Covalent and affinity supports used in the context of the purification of the papaya proteinases titrating 1 mol of essential thiol group per mol of proteinase

Ligand	Bound endopeptidase(s)
Phenyl-Hg^+ CH_3COO^-	Papain [57], chymopapain [23] caricain [23]
Glutathione-*S*–*S*-(2-pyridyl)	Papain [58], chymopapain [23,59] caricain [23]
2-Hydroxypropyl-*S*–*S*-(2′-pyridyl)	Papain and glycyl endopeptidase [25]
S–SO_2–R	Papain [61]
Gly–Gly–Tyr(Bz)–Arg	Papain [62], caricain [63]
Gly–Phe–NH–CH_2–CN	Papain, glycyl endopeptidase [24]
Epoxysuccinyl-L-Leu-amino-4-guanidinobutane	Papain [64]
(Kunitz type)-soybean-trypsin-inhibitor	Caricain [65]
Chicken-cystatin	Papain [66]

Sepharose glutathione-2-pyridyl disulfide [60]. Fully active glycyl endopeptidase however reacts with immobilized 2-hydroxypropyl-*S*–*S*-(2′-pyridyl) [25]. Immobilization of alkyl thiolsulfonate groups also provides a means to obtain fully active papain [61]. It is expected that such gels would also be able to react with the three other papaya endopeptidases provided that, in the case of glycyl endopeptidase, the alkyl group on the thiolsulfonate function is not too bulky.

Immobilization of the tetrapeptide Gly–Gly–Tyr(Bz)–Arg similarly results in the binding of fully active papain [62] and caricain [63] while the irreversibly oxidized enzymes are not retained. Surprisingly, an affinity column consisting of this tetrapeptide attached to Sepharose binds caricain at an ionic strength (400 m*M*) significantly higher than the one at which papain is bound. Bound caricain is released by deionized water suggesting an important contribution of hydrophobic interactions between subsite S_2 of this enzyme and the side chain of Tyr(Bz).

Glycyl endopeptidase has been discovered, quite fortuitously, in the course of experiments aimed at testing new ligands for the affinity purification of cysteine proteinases. One such new ligand, namely Gly–Phe–aminoacetonitrile, linked to CH-Sepharose 4B is able to selectively retain both papain and glycyl endopeptidase while chymopapain and caricain pass straight through the gel [24]. Papain and glycyl endopeptidase, after their elution, titrate, respectively 0.84 and 0.95 mol of thiol function per mol of enzyme.

trans-(Epoxysuccinyl)-L-leucylamino-4-guanidinobutane (E-64), isolated from *Aspergillus japonicus*, is another inhibitor of papain, chymopapain and caricain but not of glycyl endopeptidase [24]. E-64 is an irreversible inhibitor being capable through its epoxy group to react with the essential thiol function of the proteinases [64]. Attachment of the inhibitor to thiopropyl Sepharose through its epoxy group results in the loss of its irreversible inhibition activity but does not affect the specificity of interaction to bind cysteine proteinases [65]. Papain that has served as a model cysteine endopeptidase was fully active after its elution.

Binding of caricain and of papain, respectively to an immobilized Kunitz-type trypsin inhibitor from soybean and to chicken cystatin has also been reported [66,67].

5. Further purification of chymopapain through the *S*-thiol poly(ethylene glycol) derivatization technique

The sole isoform of chymopapain that has been isolated so far from the latex of *C. papaya* contains two free thiol functions located at cysteinyl residues 25 (essential for the catalytic competence of the proteinase) and 117 in its amino acid sequence [26,27]. Since about 50% of the thiol functions in papaya latex are irreversibly oxidized, it may be estimated that the native species (with two free thiol functions) content in chymopapain preparations amounts to only 25%. The remainder does contain chymopapain species in which at least one of the two SH functions is fully and irreversibly oxidized. Thus, chymopapain may be viewed as an equimolar mixture of four molecular species according to the oxidation state of the sulfur atoms of the two cysteinyl residues. Both cysteines 25 and 117 are located on the protein surface [29] and are thus freely accessible to covalent chromatographic supports. This explain why such supports, unable to immobilize fully oxidized chymopapain, do provide the proteinase as an equimolar mixture of the three remaining molecular species. On the other hand, the use of active-site directed affinity gels does also provide chymopapain, at best, as a mixture containing two molecular species differing by the oxidation state of the sulfur atom of cysteine 117.

Several monomethoxypoly(ethylene glycol) (Aldrich) derivatives (mPEG), such as the one shown below, capable of reacting with thiol functions of proteins have been synthesized in our laboratory in order to covalently graft mPEG chains with molecular mass of about 5000 on thiol-containing enzymes [68–71].

CH_3–(O–CH_2–CH_2)$_n$–spacer arm–*S*–*S*–(2′-pyridyl)

Modification of chymopapain leads to a mixture of differently grafted enzymes. About 25% of the

chymopapain preparation contain two mPEG chains, 50% only one mPEG chain and the remainder 25% are unmodified, as expected [68].

When grafted on proteins, mPEG chain(s) exert(s) charge shielding effects resulting in modifications of their behavior on ion-exchangers. Chromatography of the mixture of modified chymopapains on e.g. SP-Sepharose Fast Flow provides a nice separation of chymopapain that contains two mPEG chains from the species that contain only one mPEG chain and from the underivatized enzyme. These two molecular species elute from the gel in the order of decreasing mPEG chain content. The unmodified enzyme is completely inactive, as expected, while the specific activity (using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as the substrate) of the molecular species containing two mPEG chains is twofold higher than that with only one mPEG chain attached. *S*,*S*′-Dimethylthio-chymopapain, prepared from the *S*,*S*′-diPEGylthio-chymopapain conjugate, crystallized in the monoclinic space group C_2. Diffraction spots, up to a resolution of 1.4 Å, were visible. Previously, using a chymopapain preparation, where the Cys 117 exists as a mixture of sulfonic acid and thiomethyl derivatives, crystals diffracting up to only 2.4 Å resolution could be obtained [29].

Grafting mPEG chains on papain and caricain has also allowed to obtain these endopeptidases with a free thiol content of 1 mol per mol of enzyme [69–71] providing an interesting alternative to covalent and affinity chromatographies.

Acknowledgements

The authors gratefully acknowledge Janine Ralet for her skilled technical assistance.

References

[1] I. Roth, I. Claunsitzer, Acta Bot. Venez. 7 (1972) 187.
[2] K.A. Oberg, J.M. Ruysschaert, M. Azarkan, N. Smolders, S. Zerhouni, R. Wintjens, A. Amrani, Y. Looze, Eur. J. Biochem. 258 (1998) 214.
[3] L.W. Cohen, V.M. Coghlan, L.C. Dihel, Gene 48 (1986) 219.
[4] D.F. Revell, N.J. Cummings, K.C. Baker, M.E. Collins, M.A.J. Taylor, I.G. Sumner, R.W. Pickersgill, I.F. Connerton, P.W. Goodenough, Gene 127 (1993) 221.
[5] K.C. Baker, M.A.J. Taylor, N.J. Cummings, M.A. Tunon, K.A. Worboys, I.F. Connerton, Protein Eng. 9 (1996) 525.
[6] M.A.J. Taylor, M. Al-seikh, D.F. Revell, I.G. Sumner, I.F. Connerton, Plant Sci. 145 (1999) 41.
[7] L.G. Silva, O. Garcia, M.T.P. Lopes, C.E. Salas, Braz. J. Med. Biol. Res. 30 (1997) 615.
[8] V. Moutim, L.G. Silva, M.T.P. Lopes, G. Wilson Fernandes, C.E. Salas, Plant Sci. 142 (1999) 115.
[9] K. Iizuka, T. Aishima, J. Food Agric. 80 (2000) 1413.
[10] F.X. Jin, K. Toda, Biotechnol. Lett. 10 (1988) 221.
[11] J.W. Simmons, E.J. Nordby, A.G. Hadjipavlou, Eur. Spine J. 10 (2001) 192.
[12] J. Leipner, R. Saller, Drugs 59 (2000) 769.
[13] R.E.J. Mitchel, I.M. Chaiken, E.L. Smith, J. Biol. Chem. 245 (1970) 3485.
[14] J. Drenth, J.N. Jansonius, R. Koekoek, H.M. Swen, B.G. Wolthers, Nature 218 (1968) 929.
[15] L. Polgar, P. Haslasz, Biochem. J. 207 (1982) 1.
[16] I.G. Kamphuis, K.H. Kalk, M.B.A. Swarte, J. Drenth, J. Mol. Biol. 179 (1984) 233.
[17] T. Dubois, T. Kleinschmidt, A.G. Schnek, Y. Looze, G. Braunitzer, Biol. Chem. Hoppe-Seyler 369 (1988) 741.
[18] R.W. Pickersgill, P. Rizkallah, G.W. Harris, P.W. Goodenough, Acta Crystallogr. B 47 (1991) 766.
[19] P.W. Goodenough, J. Owen, Phytochemistry 26 (1987) 75.
[20] K. Brocklehurst, E. Salih, Biochem. J. 213 (1983) 559.
[21] L. Polgar, Biochem. J. 221 (1984) 555.
[22] A.J. Barrett, D.J. Buttle, Biochem. J. 228 (1985) 527.
[23] T. Dubois, A. Jacquet, A.G. Schnek, Y. Looze, Biol. Chem. Hoppe-Seyler 369 (1988) 733.
[24] D.J. Buttle, A.A. Kembhavi, S.L. Sharp, R.E. Shute, D.H. Rich, A.J. Barrett, Biochem. J. 261 (1989) 469.
[25] M.P. Thomas, C. Verma, S.M. Boyd, K. Brocklehurst, Biochem. J. (1995) 306.
[26] A. Jacquet, T. Kleinschmidt, A.G. Schnek, Y. Looze, G. Braunitzer, Biol. Chem. Hoppe-Seyler 370 (1989) 425.
[27] D.C. Watson, M. Yaguchi, K.R. Lynn, Biochem. J. 266 (1990) 75.
[28] I. Korodi, B. Asboth, L. Polgar, Biochemistry 25 (1986) 6895.
[29] D. Maes, J. Bouckaert, F. Poortmans, L. Wyns, Y. Looze, Biochemistry 35 (1996) 16292.
[30] A. Jacquet, T. Kleinschmidt, T. Dubois, A.G. Schnek, Y. Looze, G. Braunitzer, Biol. Chem. Hoppe-Seyler 370 (1989) 819.
[31] D.J. Buttle, Methods Enzymol. 244 (1994) 539.
[32] B.P. O'Hara, A.M. Hemmings, D.J. Buttle, L.H. Pearl, Biochemistry 34 (1995) 13190.
[33] J. Drenth, K.H. Kalk, H.M. Swen, Biochemistry 15 (1976) 3731.
[34] E.L. Smith, J.R. Kimmel, D.M. Brown, E.O.P. Thompson, J. Biol. Chem. 215 (1955) 67.
[35] J.B. Howard, A.N. Glazer, J. Biol. Chem. 242 (1967) 5715.

[36] J.B. Howard, A.N. Glazer, J. Biol. Chem. 244 (1969) 1399.
[37] F.W. Dahlquist, C.L. Borders Jr., G. Jacobson, M.A. Raftery, Biochemistry 8 (1969) 694.
[38] M. Azarkan, A. Amrani, M. Nijs, A. Vandermeers, S. Zerhouni, N. Smolders, Y. Looze, Phytochemistry 46 (1997) 1319.
[39] S. Odani, Y. Yokokawa, H. Takeda, S. Abe, S. Odani, Eur. J. Biochem. 241 (1996) 77.
[40] A. Shimazaki, Y. Makino, K. Omichi, S. Odani, S. Hase, J. Biochem. 125 (1999) 560.
[41] S. Zerhouni, A. Amrani, M. Nijs, N. Smolders, M. Azarkan, J. Vincentelli, Y. Looze, Biochim. Biophys. Acta 1387 (1998) 275.
[42] M.Y. Gololobov, I. Song, W. Wang, R.C. Bateman Jr., Arch. Biochem. Biophys. 309 (1994) 300.
[43] M.Y. Gololobov, W. Wang, R.C. Bateman Jr., Biol. Chem. Hoppe-Seyler 377 (1996) 395.
[44] S.W. Dahl, C. Slaughter, C. Lauritzen, R.C. Bateman Jr., Protein Expr. Purif. 20 (2000) 27.
[45] G. Wilson, Biochem. Soc. Trans. 2 (1974) 1115.
[46] I. Song, M. Taylor, K. Baker, R.C. Bateman Jr., Gene 162 (1995) 221.
[47] R. Giordani, A. Moulin, R. Verger, Phytochemistry 30 (1991) 1069.
[48] A. El Moussaoui, M. Nijs, C. Paul, R. Wintjens, J. Vincentelli, M. Azarkan, Y. Looze, Cell Mol. Life Sci. 58 (2000) 556.
[49] P. Schack, Compt. Rend. Trav. Lab. Carlsberg 36 (1967) 67.
[50] B.S. Baines, K. Brocklehurst, J. Protein Chem. 1 (1982) 119.
[51] I.U. Khan, L. Polgar, Biochim. Biophys. Acta 760 (1983) 350.
[52] S. Zucker, D.J. Buttle, M.J.H. Nicklin, A.J. Barrett, Biochim. Biophys. Acta 828 (1985) 196.
[53] P.M. Dekeyser, S. De Smedt, J. Demeester, A. Lauwers, J. Chromatogr. 656 (1994) 203.
[54] C. Desplanque, G. Petiau-de Vries, D. Baeyens-Volant, Arch. Physiol. Biochem. (2002) in press.
[55] M. Azarkan, M. Nijs, N. Smolders, C. Guermant, J. Vincentelli, Y. Looze, Appl. Biochem. Biotechnol. 60 (1996) 167.
[56] T.W. Hutchens, J. Porath, Biochemistry 26 (1987) 7199.
[57] L.A. Sluyterman, J. Wijdenes, Biochim. Biophys. Acta 200 (1970) 593.
[58] K. Brocklehurst, J. Carlsson, M.P.J. Kierstan, E.M. Crook, Biochem. J. 133 (1973) 573.
[59] B.S. Baines, K. Brocklehurst, P.R. Carey, M. Jarvis, E. Salih, A.C. Storer, Biochem. J. 233 (1986) 119.
[60] M.P. Thomas, C.M. Topham, D. Kowlessur, G.W. Mellor, E.W. Thomas, D. Whitford, K. Broklehurst, Biochem. J. 300 (1994) 805.
[61] F. Batistaviera, M. Barbieri, K. Ovsejevi, C. Manta, J. Carlsson, Appl. Biochem. Biotechnol. 31 (1991) 175.
[62] S. Blumberg, I. Schechter, A. Berger, Eur. J. Biochem. 15 (1970) 97.
[63] P. Schack, N.C. Kaarsholm, Biochem. J. 219 (1984) 727.
[64] E. Govrin, A. Levine, Protein Expr. Purif. 15 (1999) 247.
[65] A. Albeck, S. Kliper, Biochem. J. 322 (1997) 879.
[66] T.A. Valueva, L.I. Grigoryeva, L.T. Fu, N.A. Potapenko, T.Y. Kovalievich, V.C. Nyan, V.V. Mosolov, Bioorg. Khim. 20 (1994) 162.
[67] C. Dolenc, B. Turk, J. Kos, V. Turk, FEBS Lett. 392 (1996) 277.
[68] M. Azarkan, D. Maes, J. Bouckaert, M.H.D. Thi, L. Wyns, Y. Looze, J. Chromatogr. 749 (1994) 69.
[69] C. Paul, J. Vincentelli, J. Brygier, T. Musu, D. Baeyens-Volant, C. Guermant, Y. Looze, Phytochemistry 35 (1994) 1413.
[70] M. Azarkan, R.T. Wintjens, N. Smolders, M. Nijs, Y. Looze, J. Chromatogr. 724 (1996) 185.
[71] T. Musu, M. Azarkan, J. Brygier, C. Paul, J. Vincentelli, D. Baeyens-Volant, C. Guermant, M. Nijs, Y. Looze, Appl. Biochem. Biotechnol. 56 (1996) 243.