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Chromatographic Behavior of mPEG-Papaya Proteinases Conjugates Examined on Ion-Exchange and Hydrophobic Gel Media

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ABSTRACT

The four cysteine proteinases, papain, chymopapain, caricain, and endoproteinase Gly-C were isolated and purified as the catalytically competent species from the commercially available latex of the tropical tree *Carica papaya* L. Their free thiol function (cysteine-25), which is essential for activity, was protected in the form of a mixed disulfide containing a 5 kDa polyethylene glycol (PEG) chain. The second (nonessential) free thiol function (cysteine-117) of chymopapain was blocked similarly. Caricain was also derivatized through acylation of its amino functions by PEG chains (average: 15 moles of PEG per mole of enzyme). The chromatographic behavior of these conjugates was examined on ion-exchange and hydrophobic gels and compared to the chromatographic behavior of the unpegylated proteinases. The results indicated that charge-shielding effects by PEG chain(s), surrounding the different proteinases, plays(play) a key role in the course of separation of pegylated and unpegylated species by ion-exchange chromatography. Similarly, PEG chain(s) is(are) able to

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mask hydrophobic regions on the surface of the proteinases. However, the affinity showed by PEG itself for the hydrophobic ligands immobilized on the matrix is the preponderant factor determining the behavior of the PEG-proteinases conjugates on Fractogel TSKButyl-650.

Index Entries: Papain; *Carica papaya;* cysteine-proteinases; polyethylene glycol; pegylation.

INTRODUCTION

Improved delivery and enhanced therapeutical potential of proteins result from their conjugation to polyethylene glycol (PEG) (for recent reviews, *see* refs. *1–5*). PEG-enzymes conjugates also show promising potentialities as catalysts for bioconversions, which may then be carried out even in pure typical organic solvents, such as benzene or trifluoroethylene (*5*).

Suppressing the immunogenicity of candidate therapeutical proteins as well as sustaining the activity of enzymes in solvents of low dielectric constant require, on the one hand, that the degree of modification by PEG chains be high and, on the other hand, that the covalent linkage between PEG and the protein be stable. It is therefore not surprising that most of the prepared conjugates at this point result from the chemical modification of the polypeptides amino functions. This approach inevitably leads to the obtention of complex mixtures of PEG-protein adducts (*6,7*) and thus suffers from the resulting impossibility to provide structurally well-defined PEG-protein conjugates.

More specific and better controlled modifications of proteins by PEG are readily achieved by using cysteine as the target amino acid; the thiol function being typically less abundant than the amino functions in proteins (*8–16*). This has also prompted some groups to produce mutant proteins containing cysteine residues in their amino acid sequences wherein they are normally absent (*14–16*). Furthermore, the thiol function is susceptible to be modified quite reversibly, e.g., in the form of mixed disulfides. Highly purified S-pegylthio derivatives of several plant cysteine proteinases including papain and caricain from *Carica papaya* L. and ananain from *Ananas comosus* have been obtained and characterized (*10,12,13*). The S,S′-dipegylthio derivative of chymopapain, another (dithiol) cysteine proteinase from *Carica papaya* latex was also purified to homogeneity. Crystals of native chymopapain obtained thereof, showed suitable for an accurate 3-D structure of this proteinase to be determined (*11*).

As part of program on structural studies of the cysteine-proteinases, we now report on the purification of endoproteinase Gly-C (EGC). Its successful purification was also achieved through the transient conversion of the enzyme into its S-pegylthio derivative. The cysteine proteinase EGC is of considerable interest because it greatly differs from the other

members of the papain superfamily. Also, because of its narrow substrate specificity, it has found application in protein sequencing (*17*).

By comparison of several well-defined PEG-papaya proteinases conjugates and their S-methylthio (or irreversibly oxidized) counterparts, we also examined to what extent PEG chain(s) was(were) able to affect some surface properties (apparent charge, hydrophobicity) of these enzymes. The results are discussed in the general context of the purification of PEG-protein conjugates.

Purification is of actuality in view of the regulatory criteria that are inevitably addressed to any novel pharmaceutical product including polymer-modified proteins for therapeutical use (*18*).

MATERIALS AND METHODS

Spray-dried papain was purchased from Enzymase International S.A. (B-1050, Brussels). This material is a rather complex mixture which not only contains papain *stricto sensu*, other cysteine-proteinase variants, and a chitinase as the main enzymes (*19*), but also nonpolypeptidic substances (around 60% on a dry weight basis) that need to be eliminated. On the other hand, since papaya proteinases are known to undergo thiol-dependent proteolysis as well as irreversible oxidation, these enzymes require to be protected (*17,19*). Both operations were performed as follows. Spray-dried papain (2.5 g from lot no 138–94) was dissolved in water (25 mL) and applied to a (3 × 15 cm) column of S-Sepharose Fast Flow pre-equilibrated in 50 m*M* sodium acetate buffer at pH 5.0 (all along this work, the molarities of acetate buffers always refer to the Na^+ concentrations). Most of the non-proteic material was eliminated by eluting (room temperature; 53.4 mL/h) with:

1. 50 m*M* sodium acetate buffer at pH 5.0 (start buffer; 50 mL);
2. 2.5 m*M* dithiothreitol (DTT) in the start buffer (25 mL);
3. the start buffer (50 mL); and
4. 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, as a whole, by pumping 2000 m*M* acetate buffer at pH 5.0 through the column, and were collected in the presence of 100 μmoles of methylmethanethiolsulfonate, which converted the cysteine-proteinases into their S-methylthio derivatives. After dialysis against water and lyophilization, 1 g of lyophilized powder was typically recovered. This material was used as the source of papaya enzymes in the course of this study.

Aldrich-Chemie (D-7924, Steinheim) provided DTT, 2,2′-dipyridyldisulfide, methylmethanethiolsulfonate, cysteamine hydrochloride, cetyltrimethylammonium bromide (CTAB), monomethoxypolyethylene glycol

(mPEG), glutaric anhydride, 4-dimethylamino pyridine (DMAP), and 1,3-diisopropylcarbodiimide.

Azocoll, N-α-benzoyl-DL-arginine p-nitroanilide (DL-BAPNA), lyophilized cells of *Micrococcus luteus*, N-acetyl-L-tryptophan ethyl ester, and N-acetyl-D-glucosamine were purchased from Sigma Chemical Co. (B-2880, Bornem). The chemicals for SDS-PAGE and the *Mr* protein standards were purchased from Bio-Rad Laboratories S.A. (B-9810, Nazareth Eke). Pharmacia Biotech. AB (S-75182, Uppsala) provided S-Sepharose Fast Flow and Sephadex G-25, and Merck (D-61, Darmstadt), Fractogel TSK Butyl-650 (M).

Fractionation of the Papaya Proteins Using Cation-Exchange Chromatography on S-Sepharose Fast Flow

The mixture of papaya proteins (1600 mg) was dissolved in 40 mL of 100 m*M* sodium acetate buffer at pH 5.0 (start buffer), dialyzed overnight at room temperature against 5 L of start buffer and applied to a (3 × 15 cm) column of S-Sepharose Fast Flow pre-equilibrated with the 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 56.8 mL/h. Elution was performed at room temperature and fractions of 14.2 mL were collected. Each individual chromatographic fraction (*see* Fig. 1) was analyzed 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-methylthio derivatives.

Conversion of Papain, Chymopapain, and Caricain into their S-pegylthio Derivatives

The S-Sepharose Fast Flow column provided the fractions containing respectively, papain and caricain as well as those enriched in chymopapain (*see* Fig. 1), which were used to convert the different proteinases into their S-pegylthio derivatives. This conversion was carried out as described in refs. *10–12*. The pegylated proteinases were, however, separated from their corresponding irreversibly oxidized species on the S-Sepharose Fast Flow (instead of the CM-Sephadex C-50) column. For that purpose, the experimental conditions described above to fractionate the papaya proteins were used except for some gradients: from 100–500 m*M* in the case of papain and from 300–800 m*M* in the case of caricain.

Fractionation of the Papaya Proteins Using Hydrophobic Interaction Chromatography (HIC) on Fractogel TSK Butyl-650

The mixture of papaya proteins (350 mg) was dissolved in 7.5 mL of 4*M* sodium acetate buffer (pH 5.0) and applied to a (1.6 × 15 cm) column

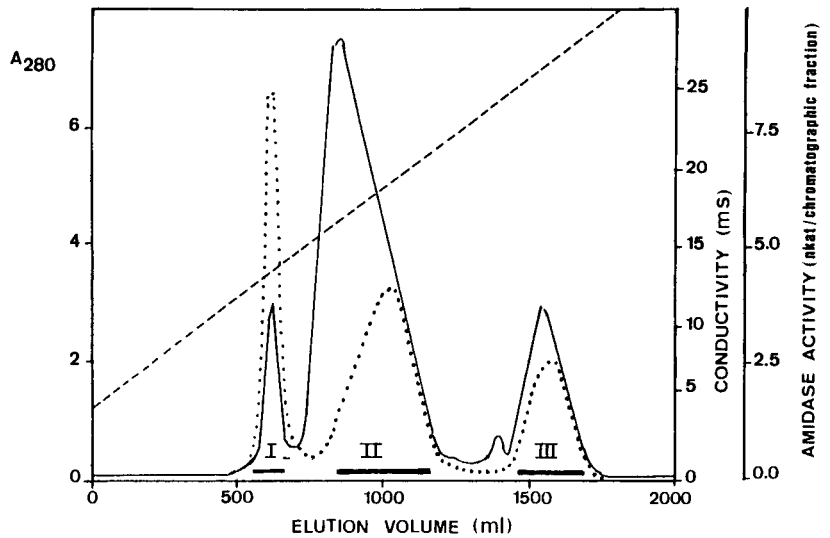


Fig. 1. Ion-exchange chromatography of the papaya enzymes on S-Sepharose Fast Flow. Column: 3 × 15 cm; fractions of 14.2 mL; flow rate: 56.8 mL/h; sample: the mixture of enzymes (1600 mg) present in the latex of *Carica papaya* L.; buffer: gradient 100–800 m*M* sodium acetate, pH 5.0, total volume: 2 L; room temperature. Each chromatographic fraction was analyzed by measurement of A_{280} (continuous trace), amidase activity (dots), and conductivity (broken trace). Several pools were constituted from the chromatographic fractions as indicated by the solid bars and used as the starting material to prepare the S-pegylthio derivatives of papain, chymopapain, and caricain (pools I to III respectively).

of Fractogel TSK Butyl-650 pre-equilibrated in 3*M* ammonium sulfate (start buffer).

The elution was performed at 25°C using a flow rate of 60 mL/h. After loading the protein sample onto the column, the start buffer (100 mL) was pumped followed by: 2.5*M* ammonium sulfate (100 mL), 2*M* ammonium sulfate (200 mL), a linear gradient from 2 to 0*M* ammonium sulfate (total volume: 1.5 L), and water until the A_{280} reading returned to its baseline value.

Fractions of 15 mL were collected and analyzed 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-methylthio derivatives. The fractions that did not show amidase activity were further analyzed by measurement of the proteolytic activity against azocoll and of the lytic activity towards *Micrococcus luteus* cells.

Before each use, a 10 m*M* solution of CTAB (25 mL) was pumped through the column. Fractogel was then rinsed with water (100 mL) before re-equilibration with the start buffer.

An ammonium sulfate concentration higher than 2*M* was required to achieve the binding of all the papaya enzymes to Fractogel TSK Butyl-

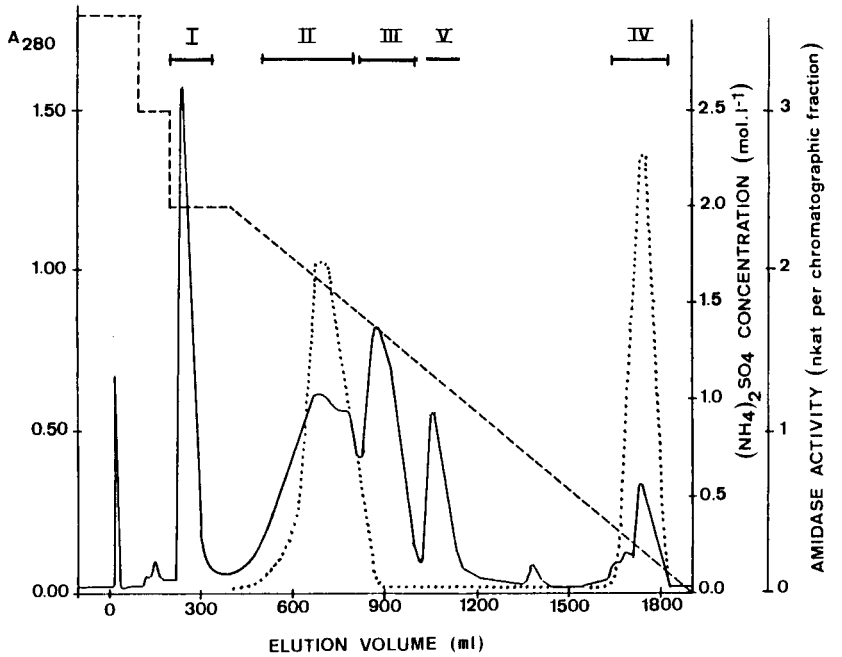


Fig. 2. Hydrophobic chromatography of the papaya enzymes on Fractogel TSK Butyl-650. Column: 1.6 × 15 cm; fractions of 15 mL; flow rate: 60 mL/h; sample: the mixture of enzymes (330 mg) present in the latex of *Carica papaya* L.; eluent gradient 3–0*M* ammonium sulfate; first eluent: 3*M* ammonium sulfate, 100 mL; second eluent: 2.5*M* ammonium sulfate, 100 mL; third eluent: 2*M* ammonium sulfate, 200 mL; fourth eluent: linear gradient 2–0*M* ammonium sulfate, 1500 mL; temperature: 25°C. Each chromatographic fraction was analyzed by measurement of A_{280} (continuous trace), amidase activity (dots), and ammonium sulfate concentration (broken trace) calculated from conductivity measurements.

650. Such high ammonium sulfate concentrations, however, led to protein precipitation. In marked contrast, sodium acetate buffer at pH 5.0 up to 4*M* did not affect the solubility of the papaya enzymes nor their ability to bind to the HIC adsorbent. Sodium acetate buffer (4*M*; pH 5.0) was thus used instead of 3*M* ammonium sulfate to prepare the solutions of the papaya enzymes for samples loading onto the HIC column.

Further Purification of EGC

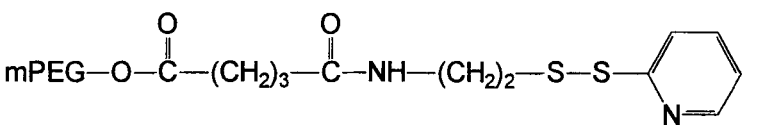
EGC was further purified by rechromatography on Fractogel TSK Butyl-650. For that purpose, the chromatographic fractions rich in this proteinase, isolated (pool III from Fig. 2) during the fractionation of the papaya proteins based on HIC, were pooled and concentrated by ultrafiltration (Amicon system; cut off of the membrane: 3 kDa) up to a volume of 20 mL. Solid ammonium sulfate was then added to this concentrated solution up to the appearance of a slight turbidity. The solution was then applied to the (1.6 × 15 cm) column of the gel pre-equilibrated in 1.75*M*

ammonium sulfate (start buffer). After loading the protein sample onto the column, the start buffer (80 mL) was pumped followed by a linear gradient from 1.75 to 1.00*M* ammonium sulfate (total volume: 1500 mL). Elution was performed at 25°C using a flow rate of 60 mL/h. Fractions of 15 mL were collected and analyzed by measurement of A_{280}. The chromatographic fractions which contained EGC (90% of the protein material engaged) were pooled, concentrated by ultrafiltration and dialyzed against 2 L of 100 m*M* sodium acetate buffer at pH 5.0. This preparation was used to obtain the S-pegylthio derivative of EGC.

Synthesis of mPEG(Glu)-SS-Py, a mPEG-Derivative Suitable to S-Pegylate EGC

The mPEG-SS-Py derivative that was used previously to prepare the conjugates of various cysteine-proteinases (*10–13*) did not react with EGC (*11*).

Introducing spacer arms between the mPEG and the cysteaminyl moieties of mPEG-SS-Py, led to the obtention of PEG derivatives able to also modify EGC (unpublished observations). One such a functionalized PEG, used here, has the following structure:



For its obtention, glutaryl-mPEG was first synthesized by reacting mPEG (5 kDa) and glutaric anhydride in dry pyridine (4 h; 50°C) in the presence of DMAP (*20*). It was then reacted with 2′-aminoethyl-2-pyridyldisulfide (*11*) in dry methylene chloride in the presence of 1,3-diisopropylcarbodiimide.

mPEG(Glu)-SS-Py was purified by precipitation in diethyl ether and molecular sieving (Sephadex G-25; eluent: H_2O) to remove any residual trace of 2,2′-dipyridyl disulfide and/or of 2-thiopyridone whose presence would affect the spectrophotometric determination of this mPEG derivative.

Preparation of Caricain-N-15mPEG

This N-acylated caricain conjugate, which contains 15 moles of mPEG chains (5 kDa each) covalently bound per mole of enzyme, was obtained, as previously described (*21*) after reaction of the S-methylthio derivative of the proteinase with mPEG-N-succinimide carbonate (3 moles of functionalized mPEG per mole of amino function). After removal of the protecting S-methylthio group, caricain-N-15mPEG was fully active towards low molecular weight substrates, such as DL-BAPNA or benzoyloxycarbonyl-phenylalanyl-L-arginyl-7-(4-methyl)coumarylamide but devoid of any proteolytic activity towards protein substrates (*21*).

Enzymes Assays

The amidase activity of papain, chymopapain, and caricain was measured using DL-BAPNA as the substrate (*19*). Each test tube (total volume: 2 mL) contained 10% DMSO, 1 m*M* of substrate, 2.5 m*M* of DTT, 1 m*M* of EDTA, and up to 2 μM of proteinase in a buffer containing citrate, borate and phosphate (100 m*M* each) at pH 6.8. The enzyme 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 (from 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-nitroanilide was determined spectrophotometrically using $\epsilon_{410} = 8800 M^{-1} \cdot cm^{-1}$. One unit of activity (nkat) is the amount of proteinase that hydrolyzes one nmol of substrate per second under the above-cited conditions.

EGC is characterized by a narrow substrate specificity, which is largely restricted to cleavage of peptide bonds the Gly at P1 (*22*). EGC is thus inactive towards DL-BAPNA but readily dissolved azocoll, a dye-impregnated hide powder preparation. The presence of this proteinase was thus assessed in some chromatographic fractions by their inability to liberate p-nitroanilide from DL-BAPNA and their ability to release soluble dye from the suspension of azocoll (1 mg/mL) in a buffer (pH 6.8) containing phosphate, citrate, and borate (100 m*M* each) and DTT (2.5 m*M*) (qualitative test).

The bacteriolytic activity of papaya lysozyme was measured using *Micrococcus luteus* cells as the substrate as in ref. *23*.

Molecular Weight Determinations

The molecular weights (M_r) of the mPEG-proteinases conjugates were determined from semilogarithmic plots of M_r vs migration distances on SDS-PAGE performed as described in ref. *10*. The M_r standards used were: hen egg white lysozyme (14,300 M_r), soybean trypsin inhibitor (21,500 M_r), carbonic anhydrase (31,000 M_r), ovalbumin (45,000 M_r), bovine serum albumin (66,200 M_r), and phosphorylase B (97,400 M_r).

Fluorescence Measurements

Fluorescence was measured at 25°C with a Shimadzu RF-5001-PC spectrofluorimeter. Excitation was at 290 nm and emission spectra were scanned from 270 to 450 nm. Emission and excitation band widths were 5.0 and 1.5 nm respectively.

Analytical Methods

The conductivities (mS) were measured with a Radiometer Conductivity meter CDM3 equipped with a Radiometer measurement cell type CDC314. The mS values were measured at constant temperature. The proteinase concentrations were measured spectrophotometrically at 279 nm

(Varian DMS 300 spectrophotometer) using as the extinction coefficients those that were calculated (*24*) from the corresponding amino acid sequences (*25–28*). This led to A_{279} (1 mg/mL) values of 2.42, 1.91, 1.69, and 1.82 respectively, for papain, chymopapain, caricain, and EGC.

RESULTS AND DISCUSSION

The latex of the tropical species *Carica papaya* L. is a rich source of the cysteine proteinases, papain, chymopapain (one or several forms?), caricain (formerly named papaya proteinase III or omega), and EGC (also named papaya proteinase IV or chymopapain M) (*17,25–29*).

Papain (M_r: 23,406 Da; pI: 8.8) and caricain (M_r: 23,289 Da; pI: 11.4) (*29*) are readily purified by cation-exchange chromatography at pH 5.0 (*17,19,30*). Upon increasing the ionic strength of the eluent, these proteinases elute, as expected from their pI values, respectively on both sides of a main chromatographic fraction which contains, as an unresolved mixture, chymopapain (M_r: 23,657 Da; pI: 10.4), EGC (M_r: 23,313 Da; pI: 10.6) (*31*), and papaya chitinase (M_r: 28 kDa) (*23*).

This is shown in Fig. 1, which illustrates a typical elution pattern of the papaya enzymes on a S-Sepharose Fast Flow column.

After their elution from the cation-exchange columns, both papain and caricain fractions are still heterogeneous. They indeed contain mixtures of irreversibly (the thiol function of Cys-25 is converted into $-SO_nH$ with n = 1, 2, or 3) and reversibly (S-methylthio derivative) oxidized forms of the proteinases. Air oxidation of the proteinases takes place as soon as the papaya latex is collected and proceeds all along the refining process. In the resulting commercially available spray-dried powder, the percentage of irreversibly oxidized cysteine proteinases typically represents around 50% out of the total proteinase content (*10–12*).

Substituting the protecting S-methylthio group of papain (*10*), caricain (*12*), and ananain (*13*) by a S-pegylthio group with a mPEG chain of 5 kDa greatly affected some physicochemical properties of these enzymes. This provided the basis for their chromatographic separation from the irreversibly oxidized forms on Sephadex G-50 or by ion-exchange chromatography on CM-Sephadex C-50. This technique also proved to be successful to extract and purify to homogeneity the catalytically competent form of chymopapain from the mixture of chymopapain (dithiol proteinase), EGC (monothiol proteinase), and lysozyme (which does not contain accessible free thiol function) (*11*), which constituted the main fraction from ion-exchange chromatography (*see* e.g., Fig. 1).

Fractionation of the Papaya Enzymes on Fractogel TSK Butyl-650

The further purification of the papaya proteinases as provided by the ion-exchange columns has been generally obtained after having made use

of covalent and/or affinity chromatography (*17,19,29*). One group, however, has attempted to realize this objective with the use of HIC on a phenyl-Sepharose column (*32*). Despite some success encountered on an analytical scale, this group concluded that this method suffered from some drawbacks. On the one hand, the resolution between chymopapain and EGC was not satisfactory and, on the other hand, the scaling-up of HIC on phenyl-Sepharose caused problems. This prompted us to evaluate other ligands (octyl and butyl) for their ability to fractionate the papaya enzymes as well as for their capability to discriminate the papaya proteinases from their S-pegylthio derivatives. This preliminary study led to the selection of butyl as the hydrophobic ligand. As shown in Fig. 2, Fractogel TSK Butyl-650 fractionated the mixture of papaya enzymes into several distinct chromatographic fractions as a function of the concentration (from 2 to 0*M*) of ammonium sulfate used as the eluent. These fractions were identified as follows: Fraction I, which showed a specific amidase activity of 1.035 nkat/mg, contained 13% of the total A_{280} units, which were applied at the top of the fractogel column and submitted to HIC. This material, mostly (90%), was constituted by caricain as unambiguously shown by its elution profile on the S-Sepharose Fast Flow column and comparison with that displayed in Fig. 1.

The Minor Fraction IV was easily identified as papain in view of its high (4.62 nkat/mg) specific amidase activity (a characteristic that is also visible by examining the elution profile displayed in Fig. 1).

Fraction III was identified as EGC since it was made up of material devoid of amidase activity (BAPNA as the substrate), but proteolytically active toward azocoll.

All the remaining amidase activity was eluted from the Fractogel column at the same position as the protein material constituting Fraction II identified, on the basis of this criteria, as chymopapain. Finally, Fraction V was tentatively identified as papaya lysozyme since it was devoid of any measurable activity toward both BAPNA and azacoll. Furthermore, its fluorescence emission spectrum (λmax: 336 nm; quantum yield: 0.040 assuming a quantum yield value of 0.13 for N-acetyl-L-tryptophan ethyl ester) was identical to that reported for authentic papaya lysozyme (*23*) and, as expected, independent of pH within the range 3 to 8. On the other hand, the addition of N-acetyl-D-glucosamine (final concentration: 100 m*M*) caused changes in the fluorescence emission spectrum of Fraction V, which showed (at pH 5.0) a 7.7% increase in the intensity.

Unfortunately, attempts to demonstrate that Fraction V (with an apparent M_r of 28 kDa, result not shown) was able to catalyze the lysis of *Micrococcus luteus* cells were inconclusive. Caricain, chymopapain and EGC eluted from the Fractogel TSK Butyl column in the same order as from the phenyl-Sepharose column (*32*) and from the octyl-Sepharose column (results not shown). As compared to these three proteinases, papain bound much more strongly to Fractogel TSK Butyl. Such an observation is rather surprising taking into account the similitude shown by the 3-D structures of papain (*33*) and caricain (*34*), and those expected

for chymopapain (35) and EGC (17). As a consequence, we thus examined the possibility for Fractogel TSK Butyl to fractionate the papaya proteinases according to a different mechanism than that based on hydrophobic interactions.

Examination of the Effect of 2-butanol on the Conformation of the Papaya Proteinases

In this context, Fink and Gwyn have reported that concentrations of 2-butanol (or other derivatives of butane such as 1-butanol, 2-butanone, . . .) higher than 0.1 m*M* caused a time-dependent irreversible inactivation of papain. Inactivation of the proteinase was associated with a loss of the accessibility of its free thiol function and caused a change (detectable by fluorescence or UV spectroscopy) in the conformation of the enzyme (36).

According to the manufacturer, the butyloxy group content of Fractogel TSK Butyl-650 is 1.0 ± 0.1 mmol/mL. Thus, according to Fink and Gwyn (36) it may reasonably be expected that the interaction of active papain with this hydrophobic support should be accompanied by a complete loss of activity. Obviously, this is not the case since the different proteinases including papain were eluted from the Fractogel column as reactivable enzymes. In all cases (except for EGC), the eluted proteinases had specific amidase activities that agreed well with those expected at this step of purification for partially irreversibly oxidized enzymes.

On the other hand, one may also suspect that the observed elution pattern from the Fractogel TSK Butyl-650 column should not be representative of that of fully native enzymes, but rather that of conformationally altered proteinases. The possibility that some reversible conformational transition took place during elution was thus also examined. For that purpose, 15 μ*M* of fully active papain or of caricain-15-mPEG, S-methylthiopapain, S-PegylthioEGC, or S,S′-dimethylthio chymopapain were incubated at 25°C for 2 h in the presence or the absence of 6.4 m*M* of 2-butanol in a 100 m*M* phosphate buffer at pH 6.8 containing 5 m*M* EDTA (incubation buffer). Fluorescence emission spectra (λexcitation: 290 nm) were then measured at 25°C after appropriate dilution of the samples in the incubation buffer containing or not 6.4 m*M* 2-butanol.

It was observed that the presence of 2-butanol did solely affect the fluorescence emission spectra (7% increase in intensity at 340 nm) of unprotected papain.

The proteinase samples (with the exception of S-pegylthioEGC) were also diluted in the incubation buffer containing 2.5 m*M* DTT but no 2-butanol and amidase activities were measured within 10 min after dilution. All the samples were observed to be active with the exception of (initially) unprotected papain for which no measurable activity could be shown.

Thus, modification of the free thiol function of papain, as the mixed disulfide with methylmercaptan fully protected the enzyme against inactivation and modification of its 3-D structure by 2-butanol. It was also concluded from all these results that the elution patterns from the Fractogel

TSK Butyl-650 such as those shown in Fig. 2 and 4 were essentially governed by hydrophobic interactions.

Preparation of S-pegylthioEGC

An interesting starting material for the preparation of S-pegylthioEGC is provided by Fraction III from Fig. 2. For that purpose, this chromatographic fraction was first submitted to rechromatography on Fractogel TSK Butyl-650 (for the experimental details, *see* Materials and Methods section) in order to eliminate any residual trace of chymopapain (easily identified by its amidase activity). In the course of this step, elimination of chymopapain is important since indeed two out of the four molecular species of chymopapain contain one mole of free thiol (the other thiol function being irreversibly oxidized) per mole of this proteinase. In contrast, the elimination of residual lysozyme is less important; this enzyme being characterized by the absence of accessible free thiol group (*23*), is not susceptible to be modified by mPEG(Glu)-SS-Py.

Modification by mPEG(Glu)-SS-Py (1.05 mole of mPEG derivative per mole of EGC) of DTT-activated EGC (5 mg/mL; prepared as previously described for the other cysteine-proteinases investigated here, *see* refs. *10–13*) proceeded at pH 5.0 in a 2000 m*M* sodium acetate buffer. Progress of the reaction was monitored spectrophotometrically at 343 nm (which measured the release of 2-thiopyridone).

When the reaction was completed (around 15 min), aliquots of the reaction mixture were removed and submitted to fractionation either on S-Sepharose Fast Flow (Fig. 3) or on Fractogel TSK Butyl-650 (Fig. 4).

Fractionation of the S-pegylthio- from the Irreversibly Oxidized Forms of the Proteinases by Ion-Exchange Chromatography on S-Sepharose Fast Flow

The elution volume (at pH 5.0) from ion-exchange columns of the S-methylthio derivative of a cysteine-proteinase has been observed on many occasions to be identical to that of the irreversibly oxidized forms (*11,13,19 see* also Fig. 1).

On the other hand, although substitution of the S-methylthio moiety by the S-pegylthio group does not modify the net charge of the conjugate, it afforded the possibility to nicely separate, by ion-exchange chromatography on CM-Sephadex C-50, the S-pegylthio derivative from the irreversibly oxidized forms of the different proteinases investigated so far (*10–13*). Such a repeated observation thus raised the question of the mechanism sustaining separations on CM-Sephadex C-50. In the absence of any evidence showing that the conformation of the proteinase is indeed affected by the presence of PEG tail(s), the possibility that some molecular sieving contributed to the ion-exchange based fractionation has been evoked (*10*).

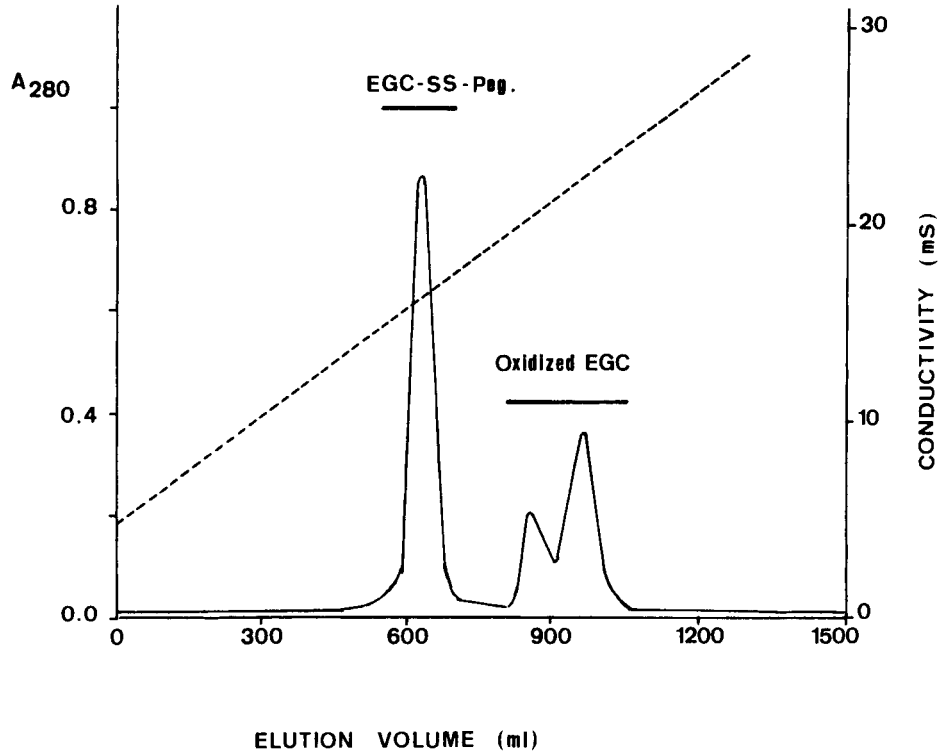


Fig. 3. Fractionation of S-pegylthioEGC and irreversibly oxidized EGC on S-Sepharose Fast Flow. Column: 3 × 15 cm; fractions of 15.35 mL; flow rate: 61.4 mL/h; sample: a mixture of S-pegylthioEGC and of irreversibly oxidized EGC (2.70 μmoles); buffer: gradient 100–800 m*M* sodium acetate, pH 5.0, total volume: 1500 mL; room temperature. Each chromatographic fraction was analyzed by measurement of A_{280} (continuous trace) and conductivity (broken trace).

This possibility was examined here by using S-Sepharose Fast Flow instead of CM-Sephadex C-50. According to the supplier, both ion-exchangers have a nearly identical capacity (around 170–250 μmoles/mL). However, while mPEG 5000 is excluded from Sephadex G-50 (*12*), Sepharose Fast Flow exchangers have an exclusion limit of approx 4000 kDa. Thus, if molecular sieving importantly contributed to separations on CM-Sephadex C-50, then, it is expected that, under similar experimental conditions, pegylated and unpegylated mixtures of proteinases would be badly separated on S-Sepharose Fast Flow.

Examination of Fig. 3, which displays a typical elution pattern on S-Sepharose Fast Flow of a mixture of unpegylated and S-pegylthioEGC, shows that this is not the case. Separations were also obtained in the cases of papain, chymopapain, and caricain as well [not shown].

S-pegylthioEGC, after purification by ion-exchange chromatography on S-Sepharose Fast Flow, showed on SDS-PAGE, a unique band with an

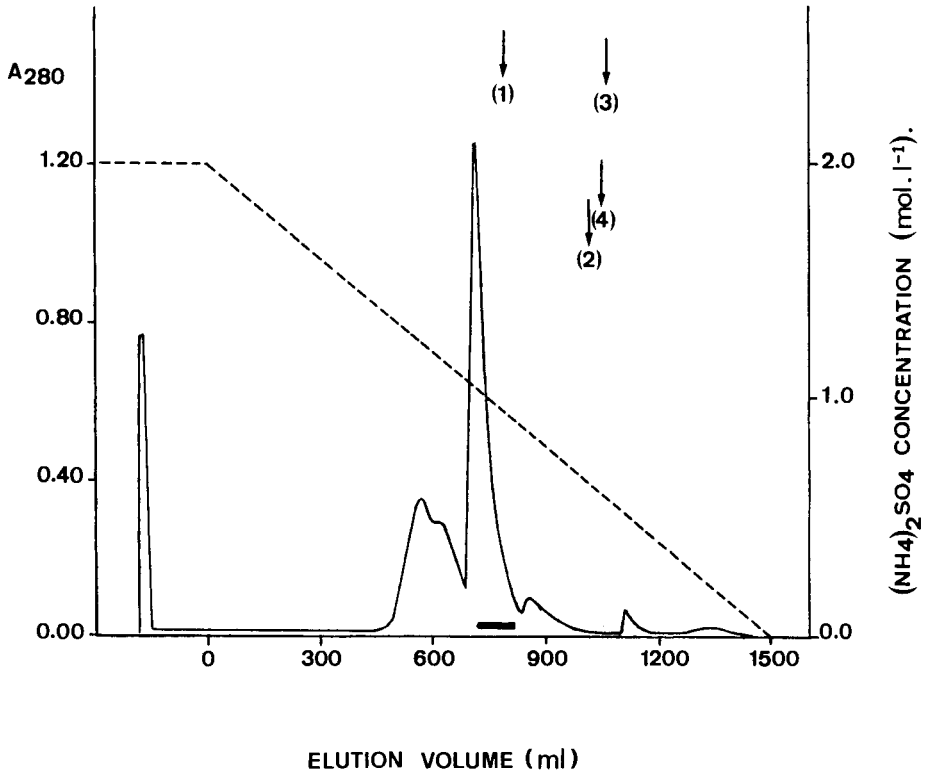


Fig. 4. Hydrophobic chromatography of mPEG-proteinase conjugates, irreversibly oxidized EGC and mPEG(Glu)-SS-Py on Fractogel TSK Butyl-650. Column: 1.6 × 15 cm; fractions of 15 mL; flow rate: 60 mL/h; sample: the mixture of S-pegylthioEGC and irreversibly oxidized EGC (2.80 μmoles); eluent: linear gradient (calculated from conductivity measurements, broken line) from 2 to 0*M* ammonium sulfate; temperature: 25°C. The elution was monitored by measurement of A_{280} (continuous trace); the positions of the peak maxima of the other samples are indicated by arrows, (1): S,S'-dipegylthiochymopapain, (2): mPEG(Glu)-SS-Py, (3): caricain-N-15mPEG, and (4): S-pegylthipapain.

apparent M_r around 31 kDa. The gel was transparent in the region corresponding to apparent M_r = 40 kDa (where the diPEG-substituted chymopapain species migrated) providing an additional argument showing the absence of chymopapain in the preparation of S-pegylthio EGC (results not shown).

Elution Profiles of Some Pegylated Species of Papaya Proteinases on Fractogel TSK Butyl-650

HIC separates substances as a result of their varying degrees of hydrophobic interaction with the hydrophobic groups of the gel matrix. Among several commercially available hydrophobic ligands, as already discussed above, the butyl group was selected for providing the best fractionation of the papaya proteinases based upon HIC. On the other hand,

Fractogel media have been found more suitable (less nonspecific interactions) than others (e.g., Superdex) for the molecular weight determination of PEGs (*37*). Fractogel TSK Butyl-650 was therefore chosen as the HIC medium to examine the behavior of the pegylated species of papaya proteinases investigated here.

The results of this study, displayed in Fig. 4, strongly suggest that the PEG chains within the conjugates are mainly responsible for their binding to the butyl ligands of Fractogel TSK Butyl-650. Some minor contribution of the proteinase moieties, however, still manifests. Examination of Fig. 4 indeed shows that all the mPEG-proteinases adducts (whatever the proteinase under consideration) were eluted from the Fractogel within a narrow range of ammonium sulfate concentrations: from 1.0 to 0.7*M* while the parent unmodified proteinases were eluted all along the range 2 to 0*M* (*see* Fig. 2). Thus, the degree of modification (1, 2, or 15 moles of PEG per mole of proteinase) had only a limited effect on the elution volumes of the conjugates.

On the other hand, S-pegylthiopapain eluted together with caricain-15-mPEG and later than S-pegylthioEGC. This observation clearly shows that the unique PEG chain grafted on papain was not able to completely mask the more pronounced hydrophobic character of this proteinase. From a practical point of view, it should be noted that the use of Fractogel TSK Butyl-650 as a chromatographic support afforded excellent separations of PEG-proteinase conjugates from the unmodified species of the enzymes. As compared to ion-exchange chromatography, better resolutions were obtained in the cases of caricain and chymopapain while similar separations were observed in the case of papain. For EGC, ion-exchange chromatography should, however, be preferred to HIC (compare Fig. 3 and 4).

CONCLUSION

The work presented in this paper further demonstrates the general usefulness of S-pegylthio derivatives of cysteine-proteinases in order to purify the catalytically competent species of these enzymes. The choice of EGC to examine here this point was dictated by previous observations which suggested that this proteinase greatly differed from most of the other members of the papain superfamily examined so far.

The final success of the pegylation technique requires that the free thiol function of the proteinase be accessible to the mPEG reagent. It also depends upon the importance of the changes induced by the grafted PEG chain on the protein surface.

Important charge shielding effects induced by the attachment of PEG chain(s) was(were) observed for the four papaya proteinases investigated. Their pegylated forms could thus be properly separated from their irreversibly oxidized species by ion-exchange chromatography.

This work has also shown that in cases such as where charge shielding effects would not be important enough, a valuable alternative to ion-exchange chromatography is provided by HIC. Obtaining homogeneous preparations of well-defined mPEG-protein adducts (such as the S-pegylthio proteinases described in this work) is an important step which allows to examine in detail the structural organization of such conjugates.

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