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Thiol pegylation facilitates purification of chymopapain leading to diffraction studies at 1.4 Å resolution

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Abstract

Thiol pegylation of a protein may profoundly affect its chromatographic behavior on ion-exchange supports as a result of charge shielding effects induced by the presence of the polyethylene glycol (PEG) chain(s) at the surface of the polypeptide. When PEG chain(s) is(are) covalently bound via disulfide bonds, thiol pegylation is reversible and may be used in the context of purifying enzymes such as chymopapain, the dithiol proteinase from papaya latex, investigated here. Reaction of chymopapain with a dithiopyridyl poly(ethylene glycol) (PEG) reagent, possessing an extended spacer arm, followed by cation-exchange chromatography on S-Sepharose Fast Flow, afforded for the first time an homogeneous preparation of the native form of this proteinase. This constituted the key for obtaining highly diffracting crystals for chymopapain (as the protected S,S′-dimethylthio derivative) exhibiting diffraction spots visible up to a resolution of 1.4 Å.

Keywords: Thiol pegylation; X-Ray diffraction; Chymopapain; Enzymes; Poly(ethylene glycol)

1. Introduction

Obtaining highly purified chymopapain [EC 3.4.22.6], the prerequisite for the preparation of crystals suitable both for X-ray data collection and for the accurate three-dimensional structure determination, is not an easy task. This is first due to the presence, in the latex of the tropical species *Carica papaya* L. from which chymopapain is extracted, of several other enzymes with functional and/or physicochemical properties very similar to those of chymopapain. The purification is further complicated by the presence, in this enzyme, of two free thiol functions. These functions may undergo several post-translational as well as degrading chemical reactions leading to several coexisting molecular species.

Elimination of papain [EC 3.4.22.2], caricain [EC 3.4.22.30] and glutaminyl-peptide cyclotransferase [EC 2.3.2.5] generally constitutes the first step of the chymopapain purification process. This may be efficiently achieved through cation-exchange chromatography using (e.g. at pH 5.0) a linear gradient of increasing ionic strength [1–4]. Removal of glycyl endopeptidase [EC 3.4.22.25] may take advantage of the use of covalent chromatography supports which, unexpectedly however, do not bind glycyl endo-

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peptidase [5–7] while reversibly immobilizing chymopapain.

A more rationale procedure [2] exploits the remarkable resistance to acid denaturation exhibited by chymopapain and makes use of active-site directed affinity chromatography on immobilized alanylphenylalaninaldehyde semicarbazone. Acid-denatured glycyl endopeptidase does not bind to this support while chymopapain which resists acid denaturation (pH 1.5; 0°C; 90 min) does.

None of the covalent chromatographic supports nor even the active-site directed affinity support do however provide, as the single species, the molecular species of native chymopapain which contains two free thiol functions located at positions 25 (essential for the catalytic action of the proteinase) and 117 in its amino acid sequence [5,6]. Recently, it was estimated that the native species content in chymopapain preparations amounted to only about 25%. The remainder did contain chymopapain species in which at least one of the two SH functions is fully and irreversibly oxidized [8]. 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.

Covalent chromatography supports unable to immobilize fully oxidized chymopapain do provide the proteinase as an equimolar mixture of the three remaining molecular species. Such a mixture (incidentally obtained by us via a quite different route) showed its capacity to crystallize. It provided needle shaped crystals which diffracted up to 2.4 Å [8].

On the other hand, one may speculate that the use of active-site directed affinity gels [2] will also provide, at best, chymopapain as a mixture containing two molecular species. Such a route was thus not even explored by us. Speculating that high quality crystals of chymopapain could only be produced from a preparation which contains only one molecular species (preferentially the native form), we rather focused our attention on the S-pegylation technique, which previously showed promising, to reach this goal [8].

2. Experimental

A chymopapain fraction freed of papain, caricain and glutaminyl-peptide cyclotransferase was prepared by cation-exchange chromatography of commercially available spray-dried papain (Enzymase International, Brussels, Belgium) as described previously [8]. The chymopapain chromatographic pool was then submitted to S-pegylation as described elsewhere for the preparation of the mixture of S-pegylthio-papaya proteinases (see paragraph 2.3 in the rubric Materials and Methods of Ref. [9]). As part of this procedure, monomethoxypolyoxy-(ethylene glycol) (mPEG)-(glutaryl)–S–S–Py, described in Ref. [9], has been used in the present study as the thiol-disulfide interchange reagent.

The reaction mixture resulting from the S-pegylation step was fractionated on a S-Sepharose Fast Flow column (Pharmacia Biotechnology, Uppsala, Sweden) (details are given in the legend of Fig. 1). Elution was performed at room temperature and each chromatographic fraction (14.2 ml) was analysed by measurements of A_{280}, conductivity and catalytic activity towards N-α-benzoyl-DL-arginine-p-nitroanilide [9] in the presence of 2.5 mM dithiotreitol (Aldrich-Chemie, Steinheim, Germany) to regenerate the active proteinases from their S-pegylthio derivatives. The fractions which were devoid of amidase activity towards this first synthetic substrate were further assayed for their ability to liberate the chromophore p-nitroaniline (ϵ_{410}=8800 M^{-1} cm^{-1}, [10]) from Gly-p-nitroanilide (Fluka, Buchs, Switzerland) and from Boc-L-Phe-Gly-p-nitroanilide. This later putative substrate was synthesized by reacting Boc-L-Phe (10 mmol; Fluka) and Gly-p-nitroanilide (10 mmol) in dry methylene chloride (200 ml; Aldrich) for 20 h at room temperature in the presence of 1,3-diisopropylcarbodiimide (11 mmol; Aldrich). After reduction of the volume of the reaction mixture (to 50 ml), the insoluble material was collected by filtration, washed with cold methylene chloride and recrystallized from ethanol. As expected by reference to similar p-nitroanilides [10], the UV absorption spectrum of Boc-L-Phe-Gly-p-nitroanilide was characterized by λ_{max} and ϵ_{313} values of 313 nm and 15 000 M^{-1} cm^{-1} (solvent: acetonitrile) respectively.

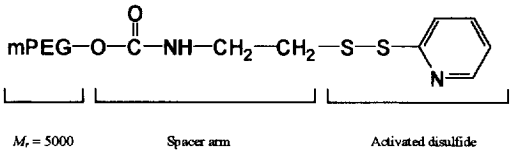
SDS-PAGE experiments were carried out on slab gels prepared according to Laemmli [11]. Chymopapain concentrations were determined spectrophotometrically using a calculated ϵ_{278} value of 43 900 M^{-1} cm^{-1} [5,12].

Prior to the crystallization experiments, the

protecting mPEGthio chains were removed from the double pegylated chymopapain and substituted by methylthio groups as described in [8]. Crystals were grown at 4°C by vapour diffusion of a 10 μl hanging drop, composed of 5 μl of 6 mg ml^{-1} (water) chymopapain and 5 μl of bottom solution (containing 2 *M* sodium acetate and 0.1 *M* sodium citrate; pH 7.6). Plate shaped crystals appeared with a typical dimension of 0.6×0.25×0.06 mm and were mounted in 0.7 mm capillaries, with a small quantity of bottom solution at one end to prevent dehydration. The crystals were exposed to X-rays from a rotating anode source operated at 45 kV and 98 mA at the CuKα edge.

3. Results and discussion

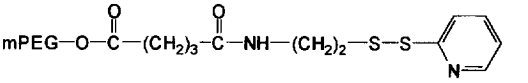
Several mPEG derivatives such as:



have recently been synthesized in order to covalently graft mPEG chains on thiol-containing enzymes [8,9,13–15]. In the course of the characterization of the reaction between I and an equimolar mixture of active and irreversibly oxidized forms of papain, we realized that the mPEG–papain conjugate could be completely separated from the oxidized (and thus unpegylated) papain fraction on ion-exchange supports. Since the mPEG grafting did not change the isoelectric point of the protein, but modified its behavior on an ion-exchanger, the PEG chains must have charge shielding effects [13].

In any case, this prompted us to examine the possibilities offered by this technique in the context of the purification of the native form of chymopapain [8]. It was observed that reduced glycyl endopeptidase showed a total lack of reactivity towards I and thus, from a chromatographic standpoint, behaved similarly to its oxidized forms as well as to the various fully oxidized molecular species of chymopapain. On the other hand, Cys-117 of chymopapain was freely accessible for reaction with I while Cys-25 reacted only slowly. Finally, we noted that the dipegylthio derivative of chymopapain and the monopegylthio derivatives of this proteinase showed some tendency to differently bind to and elute from CM-Sephadex C-50 supports even if the resulting separation efficiency was rather poor.

We thus designed II, [mPEG(glutaryl)–S–S–Py], as an analogue of I wherein the length of the spacer arm was increased



speculating that this change should reduce problems of steric hindrance origin and increase the chemical reactivity of the activated disulfide moiety as a result [9].

Derivatization by II of a mixture of chymopapain and glycyl endopeptidase indeed successfully provided an homogeneous preparation of S,S′-dipegylthio–chymopapain as the first eluting fraction from a S-Sepharose Fast Flow column (see Fig. 1 below).

SDS-PAGE experiments showed that this first eluting protein material migrated on the 12.5% slab gels (not shown) as a unique band corresponding to M_r 40 000. No bands corresponding to M_r 31 000 (single pegylated molecular species of papaya proteinases) or to M_r 24 000–26 000 (underivatized papaya proteinases) were visible even when the gels were overloaded. Inversely, the band corresponding to M_r 40 000 was not visible when protein material arising from the various other chromatographic fractions shown in Fig. 1 were applied on the gels.

Active site titration of the S,S′-dipegylthio–chymopapain preparation using iodoacetate (as the titrant) in the presence of 2.5 m*M* dithiothreitol [16] showed that the addition of 1.01 (±0.02) mol of titrant per mol of chymopapain abolished the amidase activity of the enzyme confirming the homogeneity of the preparation. Interestingly, this result was obtained whether N-α-benzoyl-DL-arginine-*p*-nitroanilide or Boc-L-Phe-Gly-*p*-nitroanilide was used as the substrate. Since glycyl endopeptidase which actively hydrolyses Boc-L-Phe-Gly-*p*-nitroanilide (unpublished results) is not rapidly inactivated by iodoacetate [17], our results demonstrate that the mPEG–chymopapain conjugate is not at all contaminated by glycyl endopeptidase.

S,S′-Dimethylthio-chymopapain, prepared from the S,S′-dipegylthio–chymopapain conjugate [8]

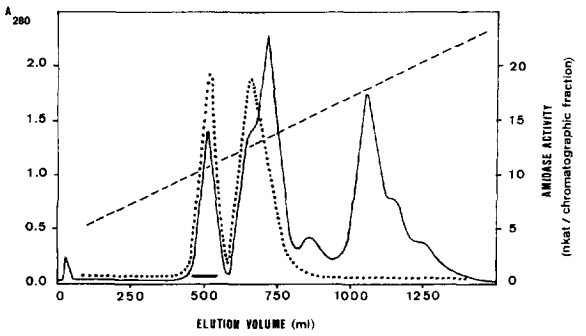


Fig. 1. Purification of S,S′-dipegylthio–chymopapain by cation-exchange chromatography on S-Sepharose Fast Flow. Sample: 400 mg of protein material obtained after derivatization of a mixture of chymopapain, glycyl endopeptidase and papaya chitinase [8] with mPEG (M_r 5000)-(glutaryl)–S–S–Py carried out as described in [9]: column, 15×3.5 cm I.D.; fractions of 14.2 ml; flow-rate, 56.8 ml/h; room temperature; eluting buffer, gradient 100–800 mM (refering to Na^+ concentration) sodium acetate at pH 5.0; total volume, 2.5 l. Each chromatographic fraction was analysed by measurements of A_{280} (continuous trace), amidase activity using N-α-benzoyl-DL-arginine-p-nitroanilide as the substrate (dots) and conductivity (broken trace). The solid bar indicates the fractions containing the dipegylthio–chymopapain conjugate which were pooled for further analysis.

crystallized in the monoclinic space group C_2 with a=145.18 (10), b=32.35 (7), c=47.42 (6) Å and β=98.37 (7)°. Diffraction spots, up to a resolution of, 1.4 Å were visible. A data set to 1.7 Å was collected. The knowledge of a high resolution X-ray structure for chymopapain will be helpful for the understanding of some aspects of the structure–activity relationship within this important family of proteinases [18–23].

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