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Ion-exchange chromatography of proteins: modulation of selectivity by addition of organic solvents to mobile phase Application to single-step purification of a proteinase inhibitor from corn and study of the mechanism of selectivity modulation

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Abstract

Chromatography on a SP-Spherodex column was tried to purify pop corn inhibitor, a proteinase inhibitor from corn. Addition of organic solvent to mobile phase (acetonitrile, isopropanol or ethylene glycol) was tested as a method of improving selectivity. Thirty percent acetonitrile gave optimal results and allowed one to design a single-step purification procedure for the inhibitor. The efficacy of acetonitrile addition, most likely, is not due to an influence on eventual hydrophobic (or hydrophilic) interactions of the chromatographic support with proteins but to the induction of some subtle protein conformation changes of the proteins retained on the column. The effect of acetonitrile on protein conformation was studied by the sensitive technique of time-resolved fluorescence spectroscopy.

Keywords: Selectivity; Mobile phase composition; Preparative chromatography; Time-resolved fluorescence spectroscopy; Fluorescence spectroscopy; Proteins; Enzyme inhibitors; Pop corn inhibitor; Proteinase inhibitors

1. Introduction

It is well known that ion-exchange chromatography supports for purification of proteins are not interchangeable and that when working on protein purification problems it can be useful to evaluate different ion-exchange matrices. Different selectivities displayed by different ion-exchange matrices grafted with the same ion-exchange groups are most likely due to the interplay of true ion-exchange interactions and interactions with matrix itself. For example, some fully synthetic polymeric matrices are known to be prone to establish hydrophobic interactions with proteins [1], addition of organic solvent to the mobile phase is in this case an established method to cope with such interactions (if needed, since addition of hydrophobic interactions to coulombic interactions may be beneficial with regard

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to selectivity). Addition to mobile phase of organic solvent (to fairly high proportions) allows one to use cation-exchangers as hydrophilic interaction chromatography (HILIC) supports [2–5] a technique which is well established for separation of peptides and which was also applied to separation of proteins [6–8].

In this context, hoping to ameliorate selectivity of an ion-exchange column used for purification of a proteinase inhibitor from corn (pop corn inhibitor, PCI [7]), we tried to add organic solvents to the mobile phase. Favourable effects were observed as detailed in this report. Time-resolved fluorescence spectroscopy experiments suggest that the selectivity gain was probably due more to protein conformational changes than to a direct effect of organic solvents on hydrophobic or hydrophilic interactions with the chromatography support. Hence, probably, addition of organic solvents to mobile phases might be considered more generally as an useful tool to modulate selectivity of ion-exchange columns for protein purification.

Addition of organic solvents to the mobile phase allowed one to devise a practically one-step purification procedure for PCI on an ion exchanger. PCI which inhibits trypsin is an interesting reagent since it also inhibits Hageman Factor, one of the key enzymes of blood coagulation [9].

2. Experimental

2.1. Materials

Polenta, a traditional Italian crude corn flour, was obtained from Molino Boccardi (Morano Po MI, Italy). SP-Spherodex M was obtained from Biosepra (Gennevilliers, France). Chemicals were obtained from Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy). Chromatography columns were bought from Pharmacia (Uppsala, Sweden).

2.2. Preparation of corn extract [10]

Nine hundred g Polenta as obtained from the manufacturer were suspended in 3600 ml buffer A (10 m*M* NaCl in 5 m*M* sodium acetate buffer, pH 5.4). Seven hundred ml toluene was added and the suspension put on a magnetic stirrer overnight in a cold room. After letting the suspension sediment for about 20 min, the aqueous phase was aspirated with a vacuum line, and then filtered on Whatman 3MM paper.

2.3. Chromatographic experiments on a small scale

The composition of mobile phases used in this study was as follows: mobile phase A was 10 m*M* NaCl in 5 m*M* sodium acetate buffer, pH 5.4, mobile phase A′ was of the same composition as A but contained organic solvent (acetonitrile, isopropanol or ethylene glycol) at variable proportions (see Section 3.1). Mobile phase B was of the same composition as A but contained 200 m*M* NaCl. Mobile phase B′ was of the same composition as B but contained organic solvent at variable proportions. Mobile phase C contained 6 *M* urea, 2 *M* NaCl and 100 m*M* phosphate buffer, pH 7.5.

Small-scale experiments were performed using a HR5/10 Pharmacia column packed with SP-Spherodex to a height of 5.6 cm. Flow-rate was 0.6 ml/min. The column was equilibrated with mobile phase A and loaded with 32 ml of starting material prepared as described above, the column was then rinsed sequentially with mobile phases A and A′. Thereafter the column was developed with a 120 min linear gradient from mobile phase A′ to B′ (in each experiment organic solvent content of B′ was identical to that of A′: hence the column was developed with an increasing NaCl concentration gradient but the organic solvent content of mobile phase did not vary during the gradient). After gradient completion the column was washed with mobile phase C before being reequilibrated with mobile phase A for the next experiment.

All fractions collected during gradient development were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis performed in 15% acrylamide gels according to the Laemmli procedure [11]. Positions of peak maxima corresponding to the four major protein bands eluted during gradient development were established using gel scanning (using the Sebia Preference scanner – Issy les Moulinaux, France – or Sigma Gel from Jandel Scientific Software, Erkrath, Germany).

2.4. Large-scale purification of proteinase inhibitor from corn

PCI was purified from polenta extract using a SP-Spherodex column of 8 cm×2.5 cm I.D. Flow-rate was 25 ml/min.

The column was equilibrated with mobile phase A. Routinely, one litre clarified polenta extract was loaded, thereafter the column was rinsed with buffer A, then mobile phase was changed to buffer A′ containing 30% acetonitrile. After having passed 250 ml of this buffer, the mobile phase composition was changed to 40 m*M* NaCl–30% (v/v) acetonitrile in 5 m*M* sodium acetate buffer, pH 5.4 (buffer A′). Five hundred ml of this mobile phase was pumped to the column followed by buffer B and then buffer C.

Sample loading and all buffer changes were automatically performed using three-way valves mounted in series and controlled by a laboratory-made programmer.

PCI-containing fractions were pooled and buffer exchanged to 5 m*M* NH_4HCO_3 by diafiltration on a PM 10 membrane (Amicon Danvers, MA, USA) fitted in an Amicon modified pressure cell (acrylic plastic reservoir was changed for an acetonitrile resistant glass reservoir). PCI was then recovered in dry form by lyophilization.

2.5. Time-resolved fluorescence experiments

2.5.1. Preparation of samples

PCI used for the fluorescence experiments was obtained by the single-step purification procedure described above. Lyophilized powder was dissolved in 10 m*M* sodium acetate–20 m*M* NaCl buffer and in the same buffer containing 45% acetonitrile.

Fluorescence experiments were also conducted using as material a mixture containing 6.6, 21 and 27 apparent molecular mass bands which are prominent protein bands retained by the SP-Spherodex column together with PCI (see Section 3.2). Time-resolved fluorescence experiments were performed with this mixture prepared either in 10 m*M* sodium acetate–20 m*M* NaCl, pH 5.4 or in the same buffer containing acetonitrile at defined concentrations (see below). Absorbance peak eluted by buffer B from the preparative SP-Spherodex column was passed in 10 m*M* sodium acetate–20 m*M* NaCl buffer, pH 5.4 using a PM10 Amicon membrane (it was checked that electrophoretic band pattern was not changed by this treatment, this means that even X with M_r 6600 was retained by the PM10 membrane). The diafiltered protein mixture was then adjusted to desired acetonitrile concentration using 10 m*M* sodium acetate–20 m*M* NaCl buffer, pH 5.4 containing 90% acetonitrile.

2.5.2. Fluorescence measurements

It is possible to extract from time-resolved fluorescence experiments two kinds of data related to conformation of analyzed proteins: (i) evaluation of fluorescence lifetimes of the tryptophan side chains gives information on tryptophan environments hence a change of lifetime distribution may indicate, as will be discussed below, a change of protein conformation, (ii) anisotropy gives information on movement of the whole protein in solvent (hence on its hydrodynamic volume), but study of time resolved anisotropy variations gives insight into contributions from individual fluorophores, thus, information may be obtained on movements of protein domains and/or movements of fluorophores carried by different proteins in mixtures.

Fluorescence decays and fluorescence anisotropy decays were measured by the time-correlated single photon counting technique [12,13]. Details of the experimental set-up have been previously described [14]. The excitation light pulse source was a Ti:sapphire subpicosecond laser (Spectra-Physics) associated with a third harmonic generator tuned at 300 nm. The stability of the laser intensity was routinely better than 5% and the excitation peak position stability better than 10 ps. Fluorescence emission, detected through a monochromator (Jobin-Yvon H 10) was set at 350 nm ($\Delta\lambda$=16 nm).

The time scaling was 16 ps per channel and 2048 channels were in use. The anisotropy decay $Q(t)$ was extracted from the parallel Ivv(t) and perpendicular Ivh(t) polarized fluorescence decay components elicited by vertically polarized excitation. The polarized components were collected alternately by rotating the analyzer polarizer every 60 s over 30 periods. Corrections for the difference in monochromator transmissions of Ivv(t) and Ivh(t) were made. The corresponding experimental g-factor=Ivv/Ivh was

determined from *N*-acetyltryptophanamide solution measured under the same conditions.

2.5.3. Analysis and interpretation of data

After a ps vertically polarized excitation pulse of light, the expression of the decays of the two polarized components of fluorescence Ivv(t) and Ivv(t) are

$$\mathrm{Ivv}(t) = E(t) \otimes \int_0^\infty \int_0^\infty \beta(\tau,\theta) e^{-t/\tau} \left[1 + 2A_0 e^{-t/\theta}\right] \mathrm{d}\tau \mathrm{d}\theta$$

$$\mathrm{Ivh}(t) = E(t) \otimes \int_0^\infty \int_0^\infty \beta(\tau,\theta) e^{-t/\tau} \left[1 + 2A_0 e^{-t/\theta}\right] \mathrm{d}\tau \mathrm{d}\theta$$

where $E(t)$ is the measured instrumental response of the excitation pulse. $\otimes$ denotes a convolution product and $\beta(\tau,\theta)$ represents the number of chromophores having a lifetime τ and a rotational correlation time θ. We can reasonably assume that all tryptophans have the same initial anisotropy A_0.

Total fluorescence intensity decay $I(t)$ is obtained by summing the parallel and twice the corrected perpendicular components which are the experimental data effectively measured and stored.

$$I(t) = \mathrm{Ivv}(t) + 2g\mathrm{Ivh}(t) = E(t) \otimes \int_0^\infty \alpha(\tau) e^{-t/\tau} \mathrm{d}\tau$$

The pre-exponential factor $\alpha(\tau)$ describes the fluorescence lifetime distribution. $\alpha(\tau)$ is proportional to the concentration of chromophore having lifetime τ:

$$\alpha(\tau) = \int_0^\infty \beta(\tau,\theta) \mathrm{d}\theta$$

Lifetime distribution may be conveniently extracted by deconvolution of the experimental $I(t)$ decay curve.

The fluorescence anisotropy is defined as a quotient:

$$Q(t) = \frac{\mathrm{Ivv}(t) - g\mathrm{Ivh}(t)}{\mathrm{Ivv}(t) + 2g\mathrm{Ivh}(t)}$$

In the simple case of a chromophore rigidly linked to a rotating sphere, the anisotropy function is given by:

$$Q(t) = A_0 e^{-t/\theta} \quad \text{with} \quad \theta = \eta\nu/kT$$

where ν is the volume of the hydrated molecule, η the viscosity of the solvent, k is the Boltzman constant and T the absolute temperature. The hydrodynamic volume of proteins is not only dependent on the molecular mass but also on the hydration layer thickness on the surface. So the experimental correlation time does not vary linearly with the molecular mass.

If the molecule is non-spherical [15] or if there are any internal flexibilities in the structure [16] the anisotropy is given by:

$$Q(t) = \sum \rho_j e^{-t/\theta_j} \quad \text{with} \quad \sum_j \rho_j = A_0$$

If we suppose that there is some internal flexibility in the macromecule, movement of the flexible domain can be modeled as a rotation in a cone of semi angle ω (wobbling in cone model). $Q(t)$ is then given by:

$$Q(t) = \rho_1 e^{-t/\theta_1} + \rho_2 e^{-t/\theta_2}$$

where the first term describes the internal motion and the second the overall rotation of the macromolecule characterized by a correlation time ρ_2. The semiangle ω is related to the pre-exponential coefficients ρ_1 and ρ_2 by the relation [17]

$$\cos^2 \omega(1 + \cos \omega)^2/4 = \rho_2/(\rho_1 + \rho_2)$$

Analysis of the total fluorescence intensity $I(t)$ was performed using the PULSE5 program (MaxEnt Solutions, Cambridge, UK) based on the quantified maximum entropy method, QMEM [18,19].

The method does not use a χ^2 statistic or entropy itself as selectors to pick out the ''best'' of a family of distributions but the most probable of a complete family of solutions. This allows error bars to be calculated [18,19]. A complete description of the data analysis method has been previously described [18]. In practice, 100 equally spaced values on the τ scale, between 0.2 and 15 ns were used.

2.6. Modeling of the PCI three-dimensional structure

The three-dimensional structure of a trypsin inhib-

itor extracted from *Eleusine coracana* seeds which is highly similar (66% sequence identity) has been established by nuclear magnetic resonance (NMR) [20]. The coordinates which are available from the Protein Data Bank (PDB identifier 1BIP) were used to build a model of the PCI structure using the SwissModel facilities [21].

3. Results and discussion

3.1. Small-scale experiments, addition of organic solvent to mobile phase modifies column selectivity

Fig. 1 shows the recorder trace obtained in one small-scale chromatographic experiment and analysis through gel electrophoresis of collected fractions. The gels show that essentially four major protein bands were eluted during gradient development of the SP-Spherodex column. The band with a molecular mass (M_r) of 14 400 corresponds to PCI. The other bands with M_r of, respectively 6600, 21 000 and 27 000 correspond to proteins of unknown function which will be referred to as X, Y and Z later in the text.

Addition of organic solvent to mobile phase has been tried with the hope of obtaining more satisfactory resolution between these four proteins since resolution was clearly not adequate when the column was developed with gradient between A and B buffer

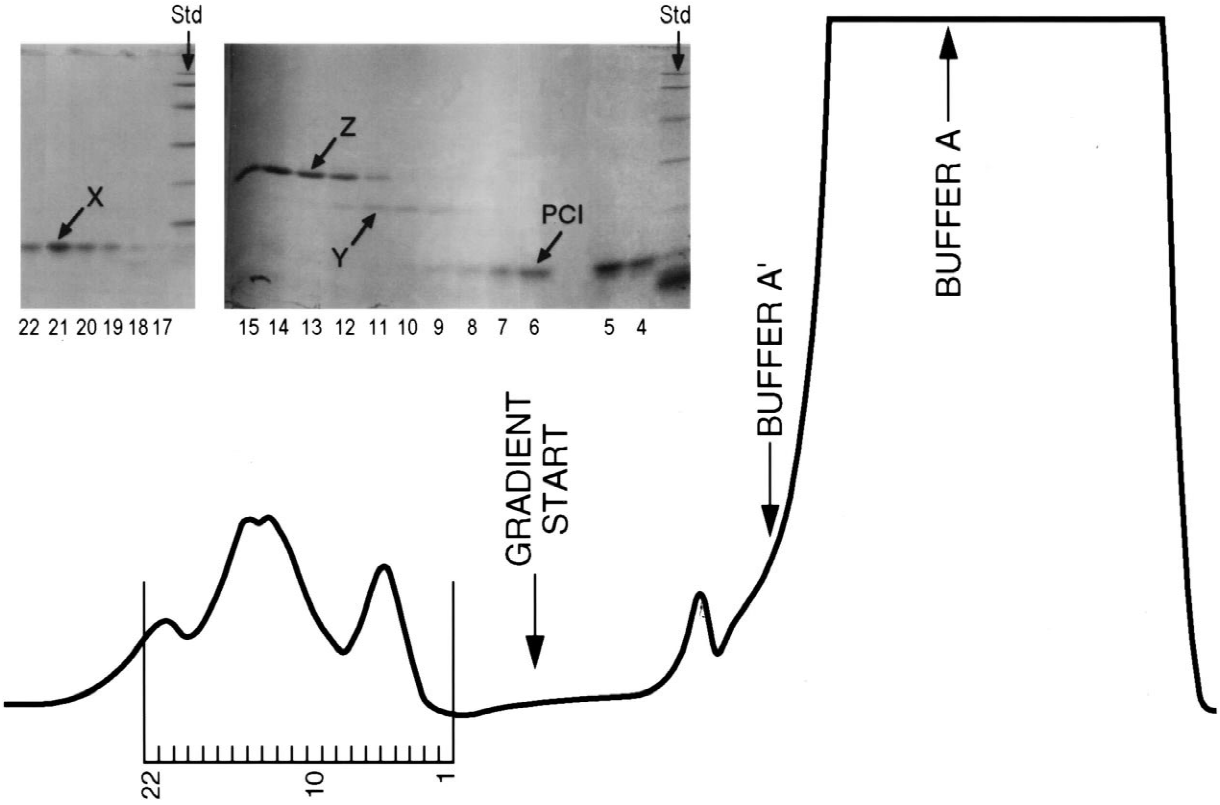

Fig. 1. Small-scale chromatography of polenta extract on SP-Spherodex. SP-Spherodex was packed to a height of 5.6 cm in a 0.5 cm internal diameter column. Support was equilibrated in 5 m*M* sodium acetate buffer, pH 5.4 containing 10 m*M* NaCl (buffer A). After extract loading and further rinsing with buffer A the same buffer but containing acetonitrile (buffer A′) [acetonitrile content was 20% (v/v) for the chromatogram shown] was passed through the column, and then developed with a linear gradient between buffer A′ and buffer B′ (of same composition as A′ but containing 200 m*M* NaCl). Selected fractions have been analyzed by SDS–PAGE performed in 15% gels (insert). Fraction numbers are indicated at the bottom of the gel and of the recorder trace. Lanes marked Std contain standard proteins (M_r values are from top to bottom 94 000, 67 000, 43 000, 30 000, 20 100, 14 400 and 6600).

(i.e., without organic solvent). Calculated NaCl concentrations at peak maxima for individual proteins at the tested organic solvent concentrations are given in Fig. 2.

Fig. 2 shows that addition of ethylene glycol does not appreciably change NaCl concentrations corresponding to peak maxima for individual proteins. Addition of increasing proportions of isopropanol to mobile phase induces practically an earlier elution for all four protein bands (that is elution in the gradient at a lower NaCl concentration).

NaCl concentration at peak maxima for X and PCI diminished steadily with increasing concentrations of acetonitrile in mobile phases used to mix the gradient. By contrast, the NaCl concentrations necessary to elute Y and Z increased when concentrations of acetonitrile added to mobile phases was increased. This differential effect of acetonitrile addition suggested that adequate resolution of PCI from other proteins on a preparative scale might be obtained by adding this solvent to mobile phase. Results of the large-scale purification procedure will be described below.

Besides their obvious practical interest, results obtained with acetonitrile were in themselves somewhat puzzling. If the primary effect of acetonitrile addition to mobile phase was depression of hydrophobic interactions between proteins and stationary phase, one should assume that earlier elution would be obtained for those proteins retained both by ionic and hydrophobic interactions and that the retention times of those proteins retained mostly by ionic interactions would not be appreciably modified or, at most, moderately reduced due to the effect of dielectric constant reduction on coulombic interactions: in fact, both earlier and delayed elution were observed.

Worth noting is that SP-Spherodex which is based

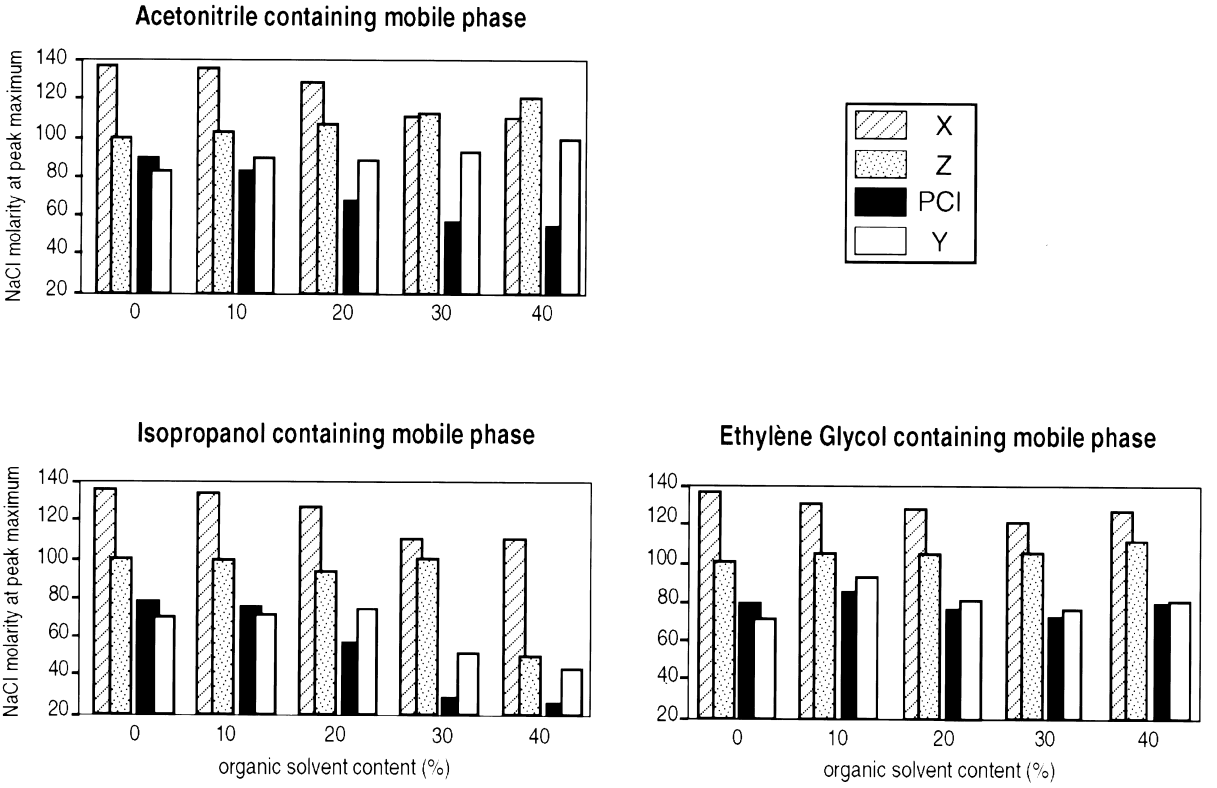

Fig. 2. Positions of peak maxima of PCI, X, Y and Z proteins obtained by developing SP-Spherodex column with gradients from buffer A′ to B′. Buffers A′ and B′ contained organic solvent at proportions indicated on the abscissa axis (see caption to Fig. 1 Section 2.3 for other details). NaCl molarities which were calculated from gradient curve are reported on the ordinate axis.

on a dextran-covered silica cannot probably make significant hydrophobic interactions with the solutes, ion-exchange groups are sulfate groups directly grafted to dextran polymer without any spacer arm, and the only source for hydrophobic interactions might come from the agent used for reticulation of the dextran covering on the silica surface (probably butane diol diglycidyl ether). It has been demonstrated that cation exchangers could be used as supports for hydrophilic interaction chromatography [3,4,6–8], as a matter of fact these supports may exhibit a mixed ionic and hydrophilic separation mechanism. It has been shown, with amino acids and peptides, that augmentation of organic solvent proportion in mobile phase increased k' values of species chromatographed on HILIC support [2]. Again, since proteins had opposite behaviours (Y and Z were eluted later, X and PCI eluted earlier after addition of acetonitrile) one cannot argue that effect of organic solvent was to modulate hypothetical hydrophilic interactions between solutes and stationary phase.

Hence, to explain the experimental observation it was hypothesized that organic solvent effect was primarily mediated through an effect on protein conformation. This hypothesis was tested using the sensitive technique of time-resolved fluorescence spectroscopy results of which are detailed below.

3.2. Time-resolved fluorescence experiments

Among the four protein bands which were eluted during ionic strength gradient only PCI was easily obtained in pure form (see below). Since PCI contained four tryptophan residues, time-resolved fluorescence spectroscopy seemed to be an efficient method to assess modifications of its conformation. No information was available on the nature and amino acid composition of the other proteins (X, Y and Z) but satisfactory fluorescence signals were generated by excitation at 300 nm (at this latter wavelength tryptophan residues are selectively excited). Worth emphasizing is the fact that time-resolved fluorescence spectroscopy allowed one to probe conformational variations of proteins present in a mixture.

As expected all fluorescence decays were multi-exponential. That is not surprising since even single tryptophan protein have often a multi-exponential fluorescence decay [22].

For PCI in buffer without acetonitrile, fluorescence lifetime distribution displays four peaks ranging from 0.1 ns to 5 ns. In a 45% acetonitrile mixture the lifetimes distribution showed three peaks ranging from 0.66 ns to 5.7 ns. (Fig. 3, upper panel): in fact the shortest lifetime component observed without acetonitrile disappeared on the addition of acetonitrile and the three other peaks were shifted to longer values.

Addition of acetonitrile decreases the polarity of the medium and may affect the lifetimes of tryptophan residues as long as they are directly exposed to the solvent. It seems likely that it is the case for PCI tryptophan residues as judged from the observed shifts of three of the peaks.

Disappearance of the shortest lifetime component

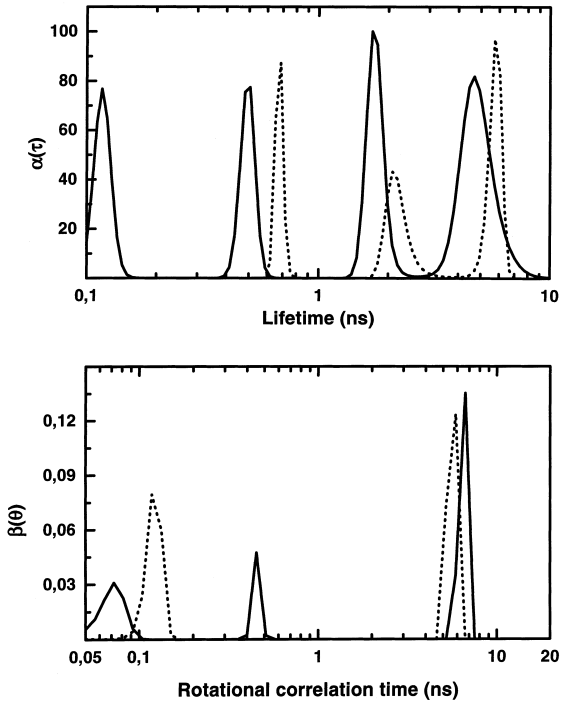

Fig. 3. Time-resolved fluorescence spectroscopy of PCI. Upper panel: distributions of fluorescence tryptophan lifetimes of PCI dissolved in 10 m*M* sodium acetate–20 m*M* NaCl, pH 5.4 (continuous line) or in same buffer containing 45% acetonitrile (dotted line). Lower panel: distributions of rotational correlation times of PCI dissolved in 10 m*M* sodium acetate–20 m*M* NaCl, pH 5.4 (continuous line) or in same buffer containing 45% acetonitrile (dotted line).

might be explained by (i) a faster dynamic quenching of tryptophan fluorescence which made it undetectable or (ii) by an effect of acetonitrile on protein structure inducing static quenching of this tryptophan. The increase of the steady-state fluorescence observed on addition of acetonitrile (not shown) favours the first hypothesis.

Analysis of the fluorescence anisotropy decays are shown in the lower panel of Fig. 3. The data were analyzed in terms of rotational correlation time distribution. Numerical analysis found three correlation times for the protein dissolved in buffer without acetonitrile:

The fastest correlation time (70 ps) might correspond to some local flexibility but we cannot be sure of its authenticity since the corresponding accuracy was poor. Most probably the intermediate correlation time (0.45 ns) might be due to a segment of protein free to move locally [23]. From the amplitude of this component, and assuming that movement is best described by the wobbling in a cone model, a cone semi-angle of 24° was calculated. The longest correlation time (6.5 ns) corresponds to the overall Brownian motion of protein. The apparent molecular mass of PCI appears to be 14 400 on SDS gel while it is 13 600 g/mol if computed from gene sequence data [24] and analysis of mature protein *N*-terminus by Edman chemistry [25]. A theoretical correlation time for a spherical hydrated protein with molecular mass of 13 600 g/mole is 6.9 ns [26]. This value is consistent with our result.

In the 45% acetonitrile mixture the flexibilities pattern corresponding to the 70 ps and 0.45 ns correlation times in buffer without acetonitrile (see above) simplified to a single motion (0.12 ns). Its large amplitude corresponds to a semi-angle of 36°. The longest correlation time decreased from 6.5 ns (without acetonitrile) to 5.6 ns. Since the viscosity of the solvent is practically not affected by addition of acetonitrile (1.03 cP), the observed decrease of the correlation time may be explained by a reduction of protein hydrodynamic volume. The five disulfide bridges makes it unlikely that the results are explained by the appearance of new domain flexibilities. In fact the probable existence of an unstructured C terminal peptide (see Section 3.3) might explain the decrease of correlation times of both the fast movement (from 0.45 ns to 0.12 ns) discussed above and of slow movement (from 6.6 to 5.6 ns).

For the mixture of the three other proteins (X, Y and Z) the fluorescence lifetimes distribution displays four peaks ranging from 0.2 ns to 6 ns (Fig. 4, upper panel). The pattern of the distribution is not strongly modified upon addition of 45% acetonitrile. The first lifetime peak around 0.3 ns is shifted to 0.2 ns while the three other peaks are shifted to longer values. That can be explained by a faster dynamic quenching of tryptophan residues by the surrounding environment and the decrease of the solvent polarity due to the 45% acetonitrile increasing the fluorescence lifetime in the three other peaks.

The fluorescence anisotropy decays of the protein mixture evidence again a strong effect of acetonitrile on the rotational dynamics of the fluorescent proteins (Fig. 4, lower panel). Without acetonitrile the correlation time distribution displays three components: the longest, above 100 ns, corresponds to a molecu-

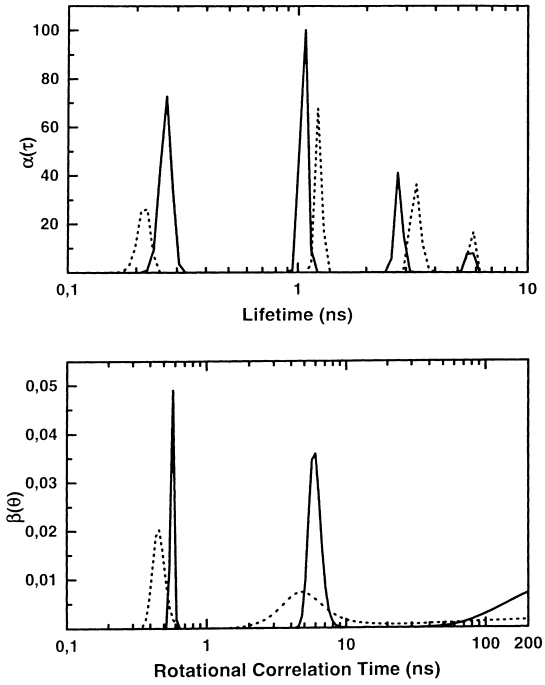

Fig. 4. Time-resolved fluorescence spectroscopy of the X, Y and Z protein mixture. Upper panel: distributions of fluorescence tryptophan lifetimes of proteins mixture dissolved in 10 m*M* sodium acetate–20 m*M* NaCl, pH 5.4 (continuous line) or in same buffer containing 45% acetonitrile (dotted line). Lower panel: distributions of rotational correlation times of proteins mixture dissolved in 10 m*M* sodium acetate–20 m*M* NaCl, pH 5.4 (continuous line) or in same buffer containing 45% acetonitrile (dotted line).

lar mass greater than M_r 90 000 and might be assigned to associated or aggregated proteins. The intermediate peak with a correlation time of about 6 ns cannot correspond to proteins with M_r 6600, 21 000 or 27 000. The theoretical molecular mass of a globular protein with an overwhole rotation of 6 ns would be 12 000 g/mole. Hence we can hypothesize that this observed correlation time of 6 ns might be due to either dimerization of the M_r 6600 band or to the presence of a large internal flexibility in the Y and Z proteins.

The first peak around 0.5 ns indicates an internal flexibility of a small part of the polypeptide chain. The corresponding cone semi-angle can be estimated at 26°.

In presence of 45% acetonitrile the long rotational correlation time peak almost disappears suggesting dissociation of postulated aggregates. The intermediate peak is broadened and its top is shifted at a lower value. The theoretical correlation time for a M_r 6600 molecule is 3.1 ns. The broadening of this peak might be explained by a partial dissociation of the hypothesized M_r 13 200 dimer (formed by association of two M_r 6600 subunits, see above). An alternative explanation might be the increase of the ''hinge bending'' in a molecule.

The fast sub-ns motion becomes faster in 45% acetonitrile with a ''wobbling in cone'' of 49° a fact which is consistent with an increase of the dynamic quenching detected from the fluorescence lifetime.

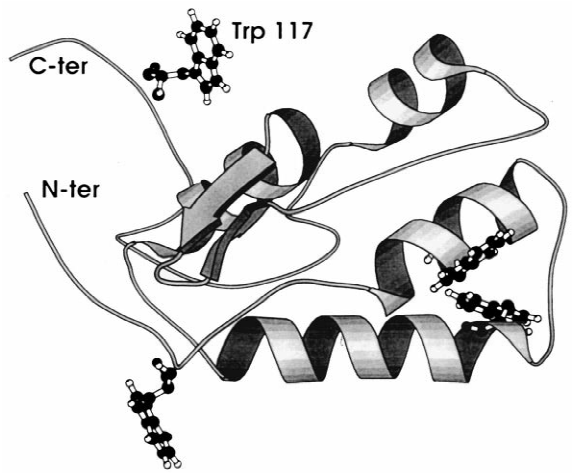

Fig. 5. Ribbon drawing of PCI model using Molscript [27] showing the tryptophan residues.

3.3. Prediction of PCI three-dimensional structure

PCI sequence was established by Edman sequencing of purified protein and of fragments thereof [25] and its gene was cloned [24]. PCI contains four tryptophan residues. A model of the three-dimensional structure of PCI is shown in Fig. 5, it predicts that PCI contains five disulfide bonds. It is most likely that C terminal amino acids of PCI are completely unstructured as they are in *Eleusine coracana* inhibitor [20] (coordinates of this protein were used to build the PCI three-dimensional structural model, see Section 2.6). the probably unstructured C terminal domain contains a tryptophan residue at position 117.

3.4. Large-scale purification of proteinase inhibitor from corn

A record of the absorbance monitor output is shown in Fig. 6 as well as electrophoresis of pooled fractions. Thirty mg pure PCI are produced in one chromatographic run in less than 2 h. This procedure which is automated allowed one to easily produce several hundreds of milligrams of this valuable protein with a minimum amount of labor.

4. General comments and conclusion

Our results show that selectivity of a separation procedure can be enhanced using acetonitrile as an additive to mobile phase which, in a subtle way, can modify the protein's three-dimensional structure, at least in regions which are not constrained in regular secondary structures.

Denaturants have been used for long for some separations, e.g., the classical method for globin chain separation by ion-exchange chromatography in the presence of 8 *M* urea [28,29]. In this case urea was necessary first to maintain solubility of denatured chains. Urea at lower concentrations might probably be used in some cases to modulate selectivity of some chromatographic procedures, for example elution position of *Arthrobacter* xylose isomerase from a DEAE Sephacel column was shown to depend on oligomeric state of the protein which itself was considerably influenced by urea concentration added to mobile phase [30]. It has been

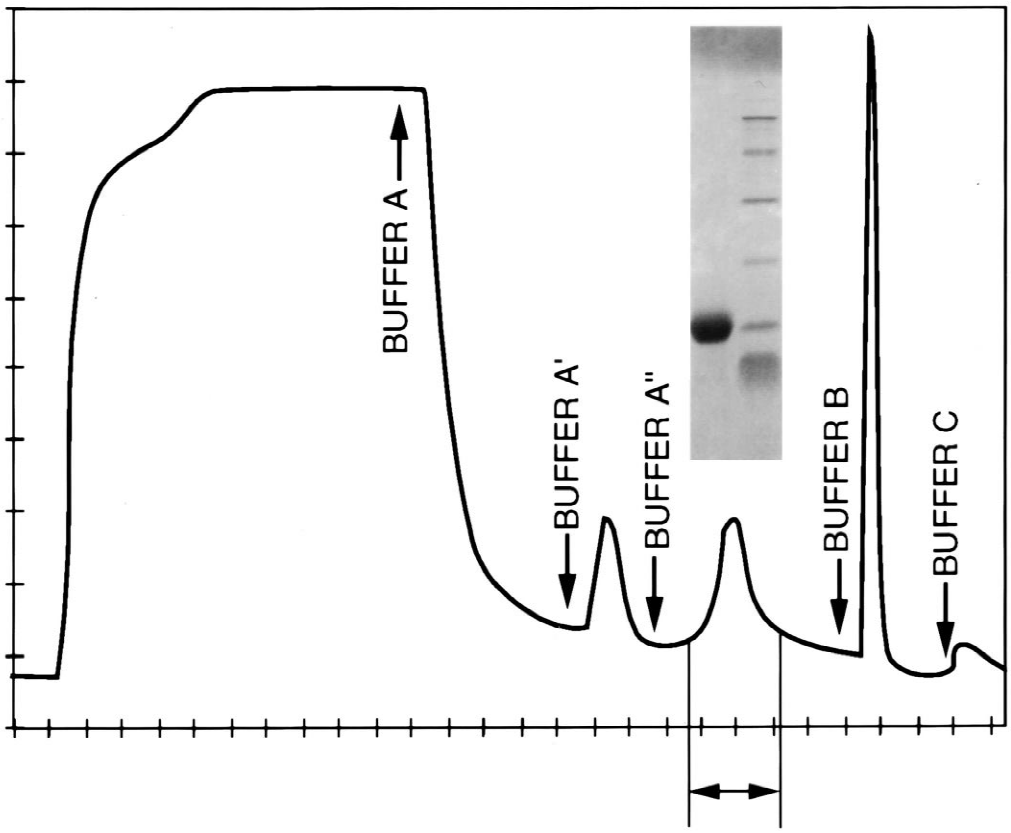

Fig. 6. Large-scale purification of proteinase inhibitor from corn. PCI was purified from polenta extract using a 8 cm×2.5 cm I.D. SP-Spherodex column. Flow-rate was 25 ml/min. Column was equilibrated in 5 m*M* sodium acetate buffer, pH 5.4 containing 10 m*M* NaCl (buffer A). After extract loading, column was passed (vertical arrow) in same buffer but containing 30% (v/v) acetonitrile (arrow, buffer A′) then mobile phase composition was changed (arrow, buffer A″) to 40 m*M* NaCl–30% (v/v) acetonitrile in 5 m*M* sodium acetate buffer, pH 5.4. The column was then developed (vertical arrows) by buffer B (of same composition as A but containing 200 m*M* NaCl) followed by buffer C, 6 *M* urea and 2 *M* NaCl in 100 m*M* phosphate buffer, pH 7.5. Double headed arrows show how were pooled buffer A″ eluted PCI containing fractions. Insert shows SDS–PAGE of one aliquot of pooled PCI containing fractions (left lane), and of standard proteins (M_r values are from top to bottom 94 000, 67 000, 43 000, 30 000, 20 100, 14 400 and 6600).

stressed by Shaltiel that some paradoxical observations of proteins elution from hydrophobic columns through an increase of ionic strength might be probably mediated by the onset of conformational modifications [31].

At first when we tried to add organic solvent to mobile phase to ameliorate selectivity we thought that changes, if any, would be mediated by some effect on supposed hydrophobic or hydrophilic interactions of proteins with support. Time-resolved experiments were used to positively demonstrate that in fact acetonitrile modifies conformations of the target protein, PCI, and also of contaminating unwanted proteins, the overall result being adequate separation. It is worth noting that this effect was obtained only with acetonitrile, the effect of ethylene glycol on chromatographic behaviour was modest. Retention of all proteins on SP-Spherodex decreased with increasing concentrations of isopropanol, a fact which might correspond to decreased coulombic interactions related to the decrease of dielectric constant.

The effect of acetonitrile on PCI conformation is likely to be fully reversible, this protein has been

successfully purified in the past using reversed-phase liquid chromatography (RP-LC) as one of two steps [10,32]: hence protein may be exposed without harmful effects to higher acetonitrile concentrations than used for the present work and also to contact with RP-LC stationary phase (which is known to participate to protein conformational changes observed during RP-LC [33]). Obviously before to use acetonitrile as a way to modulate selectivity through an effect on protein conformation it might be in some cases wise to check if it does not induce irreversible effects on target protein structure and function.

This report describes the beneficial effects of acetonitrile addition to mobile phase in ion-exchange chromatography. Since such effects are likely to be due to protein conformational changes they might as well occur in other chromatography modes, where usage of organic solvents is not traditional, like e.g., affinity chromatography or immobilized metal affinity chromatography.

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