

Review

Non-conventional affinity chromatography of serine proteinases and their inhibitors[★]

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From among a wide variety of protein purification techniques affinity chromatography has proved to be particularly effective for separation of proteolytic enzymes and their inhibitors. In this article, following a general description of affinity adsorbents used for purification of proteinases, we overview a simple separation procedure for some serine proteinases and their inhibitors by way of affinity chromatography in the presence of high NaCl concentration. It has been shown that some highly specific trypsin inhibitors exhibit also antichymotrypsin activity when high concentration of Na^+ but not K^+ or Li^+ ions are present in the reaction mixture. Taking advantage of this phenomenon the virgin forms of trypsin inhibitors from squash seeds, Kazal-type inhibitor from porcine pancreas and α_1 -proteinase inhibitor from human and sheep plasma, as an example, were separated using immobilized chymotrypsin or its inactive derivative methylchymotrypsin in the presence of 5 M NaCl.

Proteolytic enzymes play a regulatory role in the majority of fundamental processes ranging from protein synthesis to its degradation. Serine proteinases, for example, are recog-

nized as important factors in the control of multiple pathways associated with coagulation, fibrinolysis, connective tissue turnover, homeostasis, fertilization, complement activa-

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Abbreviations: BPTI, basic pancreatic trypsin inhibitor; CMTI, *Cucurbita maxima* trypsin inhibitor; CyPTI, *Cyclanthera pedata* trypsin inhibitor; HNE, human neutrophil elastase; STI, soybean trypsin inhibitor.

tion and inflammatory reaction. These enzymes are also involved in many diseases therefore, they are often targets for therapeutic intervention. Among substances that are able to selectively abrogate their action, serine proteinase inhibitors are of particular interest because they can be used either as drugs by themselves or may serve as a template for the design and development of highly specific drugs (Leung *et al.*, 2000). No wonder, then, that much work has been done to develop effective and simple methods for purification of both proteolytic enzymes and their inhibitors.

THE USE OF AFFINITY CHROMATOGRAPHY FOR PROTEINASE PURIFICATION

Affinity chromatography can certainly be considered as one of the most effective ways for protein separation and purification. In many cases this technique is fast, quite simple and very efficient. The method relies upon specific and reversible binding of a protein to a ligand, usually immobilized on an insoluble, porous and inert support. The method, due to its selectivity, has been commonly used in separation of proteins and peptides both naturally occurring, and obtained by chemical or genetic engineering. In particular, affinity chromatography has been found to be convenient for separation of proteolytic enzymes and their inhibitors.

In the case of protease purification, immobilized substrates or their analogues as well as different types of synthetic or naturally occurring inhibitors have been used. Theoretically, the best ligand for affinity separation of enzymes seems to be the substrate itself, in practice however, its use is generally precluded by the rapid turnover of the enzymatic reaction resulting in immediate conversion of the substrate into product after contact with the target enzyme. Nevertheless, covalently coupled bovine hemoglobin was employed as a suit-

able ligand for purification of alkaline protease from the larvae of *Spilosoma obliqua* (Anvar & Saleemuddin, 2002), and aspartic proteinases (cathepsin D) from bovine spleen (Turk *et al.*, 1977). It is worth noting that in the course of chromatography the hemoglobin undergoes enzymatic hydrolysis, so that in each consecutive run an enzyme molecules bind to immobilized products of hemoglobin degradation rather than to hemoglobin itself.

In a similar approach, trypsin, plasmin and chymotrypsin were purified on immobilized product-type ligands, e.g. a peptide with C-terminal L-arginine (Makriyannis & Clonis, 1997), derivatives of L-arginine (Ishi & Kasai, 1980) and L-lysine (Robinson *et al.*, 1971) and methylester of D-tryptophan (Cuatrecasas *et al.*, 1968).

Synthetic inhibitors of proteinases turned out to be very efficient ligands for many proteolytic enzymes. Immobilized ϵ -aminocaproyl-*p*-chlorobenzylamide · HCl was used to purify thrombin (Thompson & Davie, 1971) whereas benzamidine was employed for separation of human neutrophil elastase (Liau *et al.*, 1993) as well as trypsin, pronase E and trypsin-like protease from urine (Kanamori *et al.*, 1986). Besides, immobilized argininal semicarbazone-containing peptides, reversible inhibitors of trypsin and kallikrein, were found to be efficient ligands for serine proteinase purification (Basak *et al.*, 1990). The peptide Val-D-Leu-Pro-Phe-Phe-Val-D-Leu, an inhibitor of aspartic proteinases (Kučerová *et al.*, 1986), served for separation of gastricsin and pepsin from gastric juice.

Naturally occurring inhibitors are the best ligands for affinity purification of proteolytic enzymes. Some of them, however, are characterized by very low specificity which makes them "universal" affinity ligands allowing the isolation of proteases belonging to different families. An example of such an inhibitor is bacitracin, a branched cyclic dodecapeptide produced by *Bacillus licheniformis*, which was used for purification of metalloproteinases (Eijsink *et al.*, 1991), cysteine proteinases

(Irvine *et al.*, 1993) and serine proteinases (Rudenskaya *et al.*, 1995).

Another type of ligands includes group specific inhibitors, which are very selective and able to react exclusively with proteinases of a single class. An example of such an inhibitor is egg white cystatin, quite often used as an affinity ligand in the purification of cysteine proteinases from papain family (Tombaccini *et al.*, 2001). Pepstatin A is known to provide an efficient means for the purification of aspartic proteinases of animal as well as plant origin. This inhibitor facilitated, for example, purification of calf chymosin (Kobayashi & Murakami, 1978), aspartic proteinase from cucumber seeds (Wilimowska-Pelc *et al.*, 1983a) and from *Chlamydomonas reinhardtii* (Wilusz & Polanowski, 1985).

A number of specific inhibitors have been used for purification of serine proteinases. However, most of the naturally occurring protein inhibitors form inactive complexes with enzymes of different specificity. That creates a significant inconvenience, especially when an individual enzyme is to be separated from a mixture of several proteases, for example from pancreas or leukocyte extracts.

Bovine basic pancreatic trypsin inhibitor (BPTI) and inhibitors from legume seeds (Kunitz-type and Bowman-Birk-type) are the most often employed protein ligands in serine proteinase purification. BPTI inhibits several enzymes, among others trypsin, chymotrypsin and kallikrein, whereas the inhibitors from legume seeds, additionally, some pancreatic elastases. Taking advantage of even the small differences in their inhibition spectrum it is possible to separate, for example from pancreatic extract, first a mixture of trypsin and chymotrypsin on immobilized BPTI and then elastase with immobilized soybean inhibitor.

The best results in purification may be gained by using highly specific inhibitors. As

yet, only a limited number of inhibitors have been shown to possess such a property. Chicken ovomucoid, a selective inhibitor of trypsin, was used as early as in 1970 (Feinstein, 1970) for purification of this enzyme. In our laboratory several very specific trypsin inhibitors were purified from seeds of *Cucurbitaceae* family. These inhibitors, e.g. CMTI (*Cucurbita maxima* trypsin inhibitor), under standard conditions inhibit only trypsin and have neither antichymotrypsin nor antielastase activity, therefore they are very good ligands for selective purification of trypsin. A method utilizing sequential affinity chromatography on CMTI-, BPTI- and STI (soybean trypsin inhibitor)-Sephacrose columns allowed, due to the different specificities of the inhibitors, simultaneous separation of trypsin, chymotrypsin and elastase from chicken (Guyonnet *et al.*, 1999) and goose (Żelazko, 2000) pancreas.

The usefulness of an inhibitor in the purification procedure depends entirely on the association-dissociation rates of the ligand-protein complex, thus, carefully chosen conditions for adsorption and elution may allow for a selective recovery of the enzymes. Applying, for example, a pH gradient in the range 4.5–2.75 for elution of bovine trypsin from immobilized chicken ovomucoid, led to separation of the α - and β -forms (Robinson *et al.*, 1971). Similarly, selective elution of human leukocyte proteinases from immobilized BPTI affinity column enabled separation of cathepsin G from human neutrophil elastase (HNE) (Baugh & Travis, 1976).

Also, application of different ionic strengths gives a similar effect. Chromatography of bovine chymotrypsin on immobilized Bowman-Birk inhibitor resulted in separation of $A\alpha$ -chymotrypsin from the B-form, when 0.01 M HCl with and without 0.5 M NaCl, respectively, was used for elution (Wilimowska-Pelc *et al.*, 1983b).

THE USE OF AFFINITY CHROMATOGRAPHY FOR PURIFICATION OF PROTEINASE INHIBITORS

The usefulness of immobilized trypsin, α -chymotrypsin and kallikrein for purification of proteinase inhibitors was shown as early as in 1967 by Fritz *et al.* To date, thousands of papers have been published on this subject. In this paper discussion is limited to some selected problems which arose at different stages of our research on serine proteinase inhibitors. Due to its availability and simplicity trypsin immobilized on Sepharose has been a common tool in preparation of many protein inhibitors. The method was utilized, for example, for purification of BPTI from bovine lungs (Kassel & Marcinişzyn, 1971) and inhibitors from squash seeds (Polanowski *et al.*, 1980). The mixture of proteins subjected to affinity purification is applied onto the column under slightly basic conditions and elution of an inhibitor is accomplished at a pH below 3.0. In contrast to BPTI, which is recovered as a single, one chain molecule, the preparation of inhibitors from squash seeds consists not only of several isoinhibitors, but also of a mixture of their virgin and modified forms (peptide bond P₁-P'₁ hydrolyzed). This is so because some serine proteinase inhibitors can be specifically cleaved at the scissile bond by their target enzymes under acidic conditions. The hydrolysis of the reactive site peptide bond during dissociation of an enzyme-inhibitor complex considerably makes the purification more difficult particularly when several isoinhibitors, similar in terms of size and electric charge, are present in a solution. For instance a mixture of three squash trypsin inhibitors (CMTI I, CMTI III and CMTI IV), of which two consist of 29 and one of 31 amino-acid residues, upon chromatography on immobilized trypsin yields six inhibitory forms, three virgin and three modified. Due to their similarity in terms of activity, molecular size and

electric charge their final separation appeared to be extremely difficult.

Immobilized trypsin under acidic conditions is prone to split not only the reactive site peptide bond of the inhibitor being separated, but also hydrolyzes other bonds causing, e.g. inactivation of Kazal-type temporary inhibitors (Laskowski *et al.*, 1971). In this case applying the immobilized protease for purification of temporary inhibitors is practically useless. To avoid this inconvenience the catalytically inactive anhydrotrypsin (Ako *et al.*, 1972) have been used as ligands in affinity chromatography for isolation of virgin forms of inhibitors. The chemically modified enzyme, having of active site Ser195 altered to dehydroalanine, retain its ability to form stoichiometric complexes with inhibitors. Such modified enzyme was used for purification of virgin forms of secretory trypsin inhibitors from pancreas of turkey (Bogard & Laskowski, 1979), and of goose and duck (Wilimowska-Pelc *et al.*, 1996; 1999a), as well as from seeds of various species of *Cucurbitaceae* (Polanowski *et al.*, 1987). It should be pointed out however, that chemical modification of enzymes is a tedious multi-stage process involving conversion of an enzyme to its PMSF (phenylmethylsulfonyl fluoride)-derivative followed by dihydration and finally inactivation of unmodified molecules with TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone).

PURIFICATION OF PROTEINASES AND THEIR INHIBITORS IN THE PRESENCE OF HIGH SALT CONCENTRATION

The aforementioned results indicate that ionic strength significantly affects the interaction between some enzymes and inhibitors. Table 1 shows the influence of NaCl concentration on equilibrium association constants of selected inhibitors with some serine proteinases. The data indicate that only α -chy-

motrypsin and the chymotrypsin-like protease subtilisin Carlsberg are sensitive to high salt concentration. The activity of the remaining enzymes was almost unchanged. An increase of K_a for the interaction of an inhibitor with chymotrypsin and k_{cat}/K_m for substrate hydrolysis occurs only in the presence of Na^+ ions, while K^+ and Li^+ do not affect the activity of chymotrypsin (Wesołowska *et al.*, 2001).

The favorable effect of high ionic strength on the equilibrium association constant was taken

HNE with azurocidin on the second column. The selective conditions employed subsequently for recovery of the enzymes yielded cathepsin G, HNE and azurocidin separated from each other (Wątopek *et al.*, 1996).

The effect of high NaCl concentration on the association constant of some trypsin inhibitors with chymotrypsin has also been employed for separation of trypsin and chymotrypsin from extracts of porcine pancreas. A specific trypsin inhibitor from the seeds of squash (CMTI I) was

Table 1. The effect of high NaCl concentration on the association constants (K_a [M^{-1}]) for the interaction of selected trypsin inhibitors with some serine proteinases.

The association constants were determined in 0.1 M Tris/HCl, 0.02 M CaCl_2 , 0.005% Triton X-100, pH 8.3 with appropriate NaCl concentration at 22°C.

ENZYME	NaCl conc. [M]	INHIBITOR				
		LUTI ^{a)}	BPTI ^{b),c)}	CMTI I ^{d)}	CyPTI V ^{e)}	CyPTI VII ^{e)}
Bovine α -chymotrypsin (EC 3.4.21.1)	0	5.0×10^5	2.0×10^7	4.0×10^4	6.4×10^4	1.7×10^6
	3	6.6×10^7	5.8×10^9	2.8×10^7	3.0×10^7	1.3×10^9
Subtilisin Carlsberg (EC 3.4.21.62)	0	4.5×10^6	3.4×10^2	nd	nd	nd
	3	1.3×10^9	1.1×10^3	nd	nd	nd
Human leukocyte elastase (EC 3.4.21.37)	0.5	9.1×10^4	1.1×10^6	nd	nd	nd
	3	2.9×10^5	2.0×10^7	nd	nd	nd
Porcine pancreatic elastase (EC 3.4.21.36)	0	8.6×10^3	4.6×10^3	nd	nd	nd
	3	2.9×10^4	1.6×10^4	nd	nd	nd

a) Lorenc-Kubis *et al.*, 2001; b) Wesołowska *et al.*, 2001; c) Gonciarz, 1996; d) Jakimowicz, 1996; e) Kowalska, 2002. LUTI, *Linum usitatissimum* trypsin inhibitor; BPTI, basic pancreatic trypsin inhibitor; CMTI, *Cucurbita maxima* trypsin inhibitor; CyPTI, *Cyclantera pedata* trypsin inhibitor.

advantage of in developing a non-conventional method of purification of some serine proteinases and their inhibitors in the presence of high salt concentration. The method was used among others, for separation of cathepsin G from other neutrophil proteinases. Advantageous was the fact that cathepsin G in the presence of at least 1 M NaCl forms a complex with the specific trypsin inhibitor CMTI I. Loading of the neutrophil granules homogenate under slightly basic conditions in the presence of 1 M NaCl on a tandem of CMTI I- and BPTI-Sepharose columns resulted in adsorption of cathepsin G on the first, and of

used as a ligand. First, applying of the pancreatic extract on a column with immobilized CMTI under slightly basic conditions, resulted in adsorption of trypsin which was removed at pH 3.0. Then the trypsin free effluent, upon adjusting it with NaCl to 5 M concentration was loaded to the same column equilibrated with 5 M NaCl. The bound chymotrypsin could be recovered with water.

The data of our studies indicated that some inhibitors, although specific for trypsin are able to inhibit also chymotrypsin and the chymotrypsin-like enzymes in the presence of high salt concentration. This salt effect was

applied for the purification of trypsin inhibitors from the seeds of *Cucurbita ficifolia* and *C. pepo* as well as Kazal-type inhibitor from porcine pancreas, by affinity chromatography on immobilized chymotrypsin in the presence of 5 M NaCl (Wilimowska-Pelc *et al.*, 1999b). The inhibitors were adsorbed onto the column under slightly basic conditions in the presence of 5 M NaCl and after washing out of all

immobilized chymotrypsin in the presence of 5 M NaCl, could be eluted in two sets. The first set, comprising 5 of the 7 adsorbed trypsin inhibitors, was recovered with water, the remaining two inhibitors required acidic conditions for dissociation and were eluted with 0.01 M HCl. Figure 1 shows the amino-acid sequences and equilibrium association constants with bovine β -trypsin and α -chymo-

		K_a [M^{-1}] β -trypsin	K_a [M^{-1}] α -chymotrypsin
CyPTI V	RICPRILM E CK A DSDCLA Q CICQ E SGFCG	$> 10^{11}$	6.35×10^4
CyPTI VII	ARICPRILM K CK K DSDCLA E CICQ E HGFCG	$> 10^{11}$	1.69×10^6

Figure 1. Amino-acid sequences of representative trypsin inhibitors from *Cyclanthera pedata* and equilibrium association constants for their interaction with bovine β -trypsin and α -chymotrypsin.

Inhibitors were purified by affinity chromatography on immobilized chymotrypsin and recovered under different conditions. CyPTI V represents the inhibitors eluting with water whereas CyPTI VII those eluting with 0.01 M HCl. The amino-acid residues different in the two inhibitors are printed in bold.

ballast proteins, the inhibitors could be released either with water or a buffer of low ionic strength. In contrast to trypsin, the use of immobilized chymotrypsin in the presence of 5 M NaCl for purification of those inhibitors allowed to obtain them in their virgin forms (reactive site peptide bond intact). Moreover this technique enables purifying the intact Kazal-type inhibitor from porcine pancreas.

It is worth noting that under standard conditions complexes of serine proteinase-inhibitors dissociate at pH below 3.0 whereas, in the presence of 5 M NaCl, inhibitors from *C. ficifolia* and *C. pepo*, even under acidic conditions (pH < 3.0), do not dissociate from the ligand. This feature creates possibility of removing nonspecifically bound proteins by washing them out both under neutral and acid conditions in the presence of 5 M NaCl followed by eluting the target protein with a solution of low ionic strength.

In contradistinction to that the inhibitors from *Cyclanthera pedata*, belonging to the same squash inhibitor family, when bound to

trypsin of two representative inhibitors of both sets. CyPTI V is an example of the inhibitors removable from the ligand with water and CyPTI VII represents those eluting with 0.01 M HCl (Kowalska, 2002). Both inhibitors form very strong complexes with bovine β -trypsin. The inhibitors eluting under acidic conditions are more basic proteins and their association constants with bovine α -chymotrypsin exceed about 30-fold those of the inhibitors removable with water. This distinction may be the reason of their different affinity for chymotrypsin. The data indicate that the differences in the association constants of inhibitors with chymotrypsin can be turned to account in their separation. Employing the described method all trypsin inhibitors, comprising of 29 to 30 amino-acid residues, present in the extract were purified in virgin forms.

It should be emphasized that the described method is suitable for purification of only such inhibitors which are not susceptible to degradation by chymotrypsin in the presence of high salt. An attempt to purify, for example,

trypsin inhibitor from flax seeds failed because the enzyme, under the condition used, totally degraded it (Kowalska, 2002). Taking that into account, we utilized for affinity chromatography, the catalytically inactive methylchymotrypsin. The technique takes advantage of the fact that chymotrypsin methylated at the position His57 to 3-methyl histidine loses its catalytic activity while retaining the capacity to form complexes with inhibitors (Ryan & Feeney, 1975). In contrast to trypsin modification, the method of chemical modification of His57 in chymotrypsin is very simple and efficient. Under specified conditions, strong chymotrypsin inhibitors interact with the modified enzyme at low ionic strength, whereas weak inhibitors require the presence of high salt concentration for adsorption. Moreover, inhibitors specific for trypsin can also be separated on the same immobilized enzyme but only in the presence of 5 M NaCl. The bound inhibitors can be selectively recovered from the column using either elution with decreasing salt concentration or a combination of salt concentration and pH gradient.

Employing this method we purified the above mentioned trypsin inhibitor from flax seeds LUTI (*Linum usitatissimum* trypsin inhibitor) (Lorenc-Kubis *et al.*, 2001) and α_1 -antiproteinase from human and ovine plasma (Grybel & Wilusz, 2003). For the purification of the latter immobilized active proteinases are useless since with α_1 -antiproteinase they form practically irreversible complexes. To overcome this problem, Drechsel *et al.* (1984) employed immobilized anhydrochymotrypsin for purification of α_1 -antiproteinase and for desorption they used another inhibitor of chymotrypsin, namely chymostatin. Economic considerations however, militate against the use of chymostatin in large-scale purification of α_1 -antiproteinase for therapeutic purposes due to the high cost of this substance. In our procedure, the albumin fraction of plasma is applied to immobilized methylchymotrypsin in the presence of 5 M NaCl and after washing

out of balast protein with a buffer containing 5 M NaCl the inhibitor is eluted with water. It was proved that this simple technique can be usable for separation of α_1 -proteinase inhibitor from the serum of animals and from the milk of transgenic livestock.

Application of immobilized chymotrypsin and methylchymotrypsin in the presence of high salt concentration for affinity chromatography seems to be a very promising technique for purification of serine proteinase inhibitors in their virgin forms as well as those belonging to the serpin family.

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