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# Affinity Chromatography as a Key Tool to Purify Protein Protease Inhibitors from Plants

Elizeu Antunes dos Santos, Adeliãna Silva de Oliveira, Luciana Maria Araújo Rabêlo, Adriana Ferreira Uchôa and Ana Heloneida Araújo Morais  
*Universidade Federal do Rio Grande do Norte  
Brazil*

## 1. Introduction

Several and distinct physiological processes in all the life forms are dependent on proteases, as processing and turnover of endogenous proteins, digestion of food proteins, regulation of formation and lysis of the clots, activation of apoptosis pathways, plant germination, sporulation, hormone activation, translocation through membranes, fertilization, control of immune response, cell differentiation and growing (Bode & Huber, 2000; Chou & Cai, 2006; Turk et al., 2000). Proteases are also involved in replication and propagation of infectious diseases, and the imbalance of their activity can cause important pathological disorders as inflammation, stroke, cancer and parasite infection (Chou & Cai, 2006; Johansson et al., 2002; Powers et al., 2002).

The principal naturally occurring control way of the proteases activity is achieved by the action of inhibitors of protein nature, which bind specifically and block proteases. Protease inhibitors (PIs) are found in all living organisms and are among the most intensively studied proteins. In plants they are widely distributed among different botanical families and have been found in reproductive organs, storage organs and vegetative tissues. They are synthesized constitutively in seeds or can be induced in tissue, as leaves, by the attack of herbivore or abiotic stress (Fan & Wu, 2005; Laskowski & Kato, 1980; Xavier-Filho, 1992). At least four PI families are known and can be distinguished based in their interaction with the protease class that they inhibit (Fan & Wu, 2005; Koiwa et al., 1997; Xavier-Filho, 1992). Plants PIs has received special attention because of their roles and potential biotechnological applications in agriculture as bioinsecticide, nematicidal, acaricidal, antifungal and antibacterial and in the biomedical field they are remarkable candidates in the production of drugs for human disease healing.

Purification of these inhibitors is a necessary and critical step in order to define their structural characteristics and binding specificity to the proteases. Isolate these from all other proteins that are present in the same biological source is a difficult task, since the PIs have a large molecular diversity. Nevertheless, due their specific and reversible binding capacity to the enzymes (without undergoing chemical change), PI purification can be greatly enhanced by the use of affinity chromatography techniques, where the binding agent are particular proteases (Gomes et al., 2005; Araújo et al., 2005; Oliveira et al., 2007a, 2007b).

## 2. Proteases

Proteases (EC<sup>1</sup> 3.4), also called peptidases, peptide hydrolase, and proteolytic enzymes, constitute a great group of enzymes that hydrolyze peptide bonds (Barrett, 1997; Rawlings et al., 2010). These enzymes are subdivided into two groups or class: exopeptidases (EC 3.4.11-19) and endopeptidases (EC 3.4.21-24 and EC 3.4.99). The first ones show capacity to hydrolyze amino acids from the N- or C-terminus while the second group cleaves the internal peptide bonds of polypeptides and, thus, are frequently assigned of proteinases<sup>2</sup> (Fan & Wu, 2005; Rawlings et al., 2010).

According to their catalytic mechanism and specificity, endopeptidases are classified in four major groups<sup>3</sup>: metalloproteases, aspartic proteases, cysteine and serine proteases (Fan & Wu, 2005; Rawlings et al., 2010), being the latter two classes better characterized (table 1).

<i>Class</i>	<i>Proteinases</i>	<i>Amino acid residue or metal in active site</i>	<i>Optimum pH</i>
Serine proteinase (EC 3.4.21)	trypsin, chymotrypsin, elastase, thrombin, cathepsins <sup>4</sup> A and G	Ser, His, Asp	7 - 9
Cysteine proteinase (EC 3.4.22)	papain, bromelain, cathepsins B, C, H, K, L, O, S and W	Cys	4 - 7
Aspartic proteinase (EC 3.4.23)	pepsin, cathepsins D and E, renin	Asp, Try	Below 5
Metalloproteinase (EC 3.4.24)	caboxipeptidases A and B, aminopeptidases, thermolysin	Metal ion (usually Zn)	7 - 9

Table 1. Proteinases classification (Modified from Fan & Wu, 2005).

### 2.1 Serine proteases

Serine proteases comprise a large group of peptidases characterized by presenting a catalytic serine residue. In fact, three residues (serine, histidine and aspartic acid) are essential in catalytic process. They work together to cleave the peptide bond of the substrate (Hedstrom, 2002) (see figure 1).

<sup>1</sup> The Enzyme Commission number (EC number) proposed by the Nomenclature Committee of the IUBMB (International Union of Biochemistry and Molecular Biology), is a numerical classification system for enzymes according to the nature of the chemical reactions they catalyze.

<sup>2</sup> In spite of the term "proteinase" be recommended as a synonymous with "endopeptidase", the name "protease" is also traditionally accepted to nominate all known endopeptidases or proteinases.

<sup>3</sup> For further details about classification of proteases see MEROPS (<http://merops.sanger.ac.uk/>), a database which constitutes a comprehensive and integrated information resource about peptidases and their protein inhibitors.

<sup>4</sup> Cathepsins are a group of proteases called by alphabetic letters in ascending order according to their order of discovery and can be grouped by to their mechanism of catalysis in serine, cysteine or aspartic proteases.

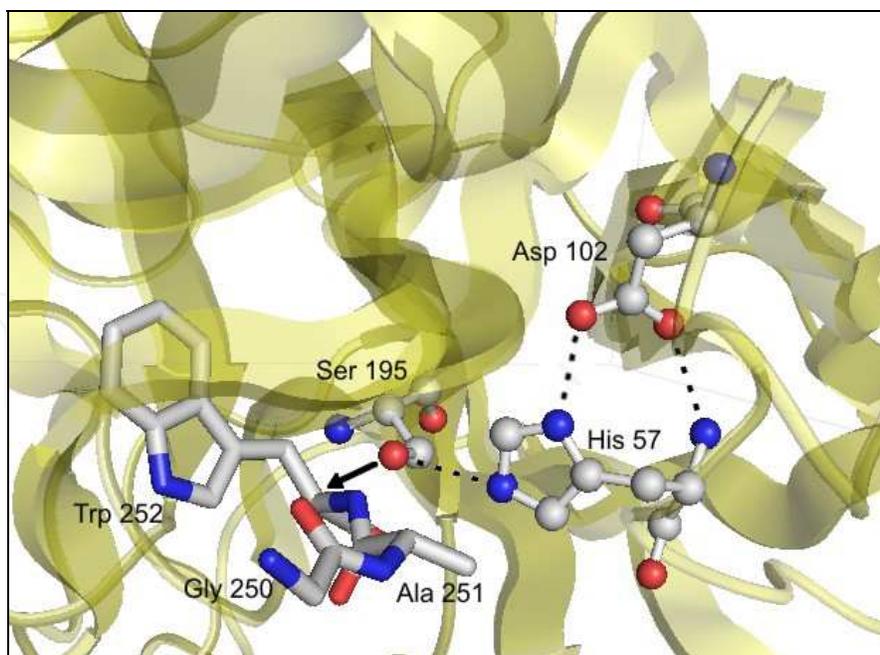


Fig. 1. The catalytic triad of the serine proteases.

Three-dimensional structure of bovine chymotrypsin (PDB code 8GCH) represented by transparent yellow ribbons. Catalytic triad (His 57, Asp 102 and Ser 195) is shown in ball-and-stick representation. The substrate (Gly-Ala-Trp tripeptide) is represented by sticks (note that the tryptophan lateral chain of the substrate is positioned in the recognize cleft of the chymotrypsin). Dotted line, hydrogen bonds. Arrow, cleavage site on the substrate. Figure made with PyMOL.

Trypsin, chymotrypsin and elastase are the largest and best studied serine proteases and they are involved in the protein digestion of the diet in animals, including human. They have the same three-dimensional structures and active site architecture, but differ in the substrate specificity: while trypsin cleaves peptide bonds on the C-terminal side of a positively-charged residue (Lys or Arg), except when it is followed by proline, chymotrypsin prefers large hydrophobic residues (Phe, Trp, Tyr) and elastase acts on small neutral amino acid residues (Ala, Gly, Val) (Hedstrom, 2002; Powers et al., 1977).

Other related serine proteases are implicated with blood coagulation process: plasmin, plasma kallikrein and clotting factors (X, XI, XII and thrombin) (Levi et al., 2006; 2010). Serine proteases such as proteinase 3, cathepsin G and, particularly, elastase from human leukocytes, play an important role in several inflammatory and pathologic processes (Liou & Campbell, 1995; Finlay, 1999; Shapiro, 2000). A distinct family of serine proteases includes subtilisin, the main protease secreted in the beginning of sporulation by the gram-positive bacterium, *Bacillus subtilis* (Power et al., 1986).

Serine proteases similar to those from mammals, specially with respect to the optimum pH, are found as predominant digestive enzymes in a wide variety of insects, as Thysanura (Zinkler & Polzer, 1992), Orthoptera (Lam et al., 1999, 2000), Hymenoptera (Schumaker et al., 1993), Diptera, (Silva et al., 2006), Lepidoptera (Bernardi et al., 1996; Gatehouse et al., 1999; Novillo et al., 1997) and Hemiptera (Colebatch et al., 2001). Trypsin-like proteases were also found in Coleopteran insects, although be known that these insects have an acid

intestinal fluid (Alarcon et al., 2002; Franco et al., 2004; Girard et al., 1998; Oliveira-Neto et al., 2004; Purcell et al., 1992; Zhu & Baker, 1999, 2000).

## 2.2 Cysteine proteases

Cysteine proteases present a catalytic dyad composed by cysteine and histidine, which work the same way as serine and histidine of the serine proteases to cleave peptide bonds (Fan & Wu, 2005). In all the live organisms, most cysteine proteases show catalytic activity in an optimum range slightly acid of pH (4.0-6.5) and are represented by proteins with molecular mass around 21-30 kDa (Rawlings & Barret, 1994; Turk et al., 2000).

This class of proteinases comprises several plant proteases, such as papain (the most studied), bromelain, actinidin, chymopapain, ficin and caricain. Plant proteases are involved in protein processing (activation, maturation, degradation) in virtually all aspect of the physiology and development of plant (Brzin & Kidric, 1995; Grudkowska & Zagdanska, 2004; Salas et al., 2008). Other important cysteine proteases include most of the lysosomal cathepsins, the calpains (a cytosolic "calcium-activated neutral protease"), caspases (essential for apoptosis process) (Fan et al., 2005; Xu & Chye, 1999) and several viral and parasite proteases (Fan & Wu, 2005; Otto & Schirmeister, 1997; Rawlings et al., 2010; Turk et al., 2000).

Cysteine proteases represent the main larval digestive enzymes of several pest insects of the coleoptera order, such as the bruchid bean weevil (*Acanthoscelides obtectus*), mexican bean weevil (*Zabrotes subfasciatus*) and cowpea bruchid (*Callosobruchus maculatus*) (Lemos et al., 1990; Silva et al., 1991, 2001; Xavier-Filho et al., 1989), but also species of chrysomelidae (Liu et al., 2004, Cristofolletti et al., 2005) and curculionidae (Cristofolletti et al., 2005). Some hemipterans also present cysteine proteases (Rahbé et al., 2003; Cristofolletti et al., 2005)

## 2.3 Aspartic proteases

Aspartic (or aspartyl) proteases were so named because their catalytic mechanism involves residues of aspartic acid from their active site. These enzymes work at acidic (or neutral) pH and their specificity is typically for peptide bonds between two hydrophobic amino acid residues (Simões & Faro, 2004). Aspartic proteases are widely distributed in animals, yeast, virus and plants, performing various functions (Fan & Wu, 2005; Pearl, 1987). In vertebrates, these enzymes act in the digestion of dietary protein (pepsin and chymosin) and lysosomal protein (cathepsins D and E) and regulation of blood pressure (renin). HIV1-protease, essential for the life-cycle of HIV, is another example of aspartic protease (Brik & Wong, 2003).

## 2.4 Metalloproteases

Metalloproteases constitute a large family of proteases occurring in bacteria, fungi and animals (including man), which require metal ion for catalysis (Fan & Wu, 2005). Most metalloproteases posses zinc in the catalytic site, coordinated via three amino acids residues (among histidin, glutamate, aspartate, lysine and arginine) (Gomis-Ruth et al., 1994; Stocker & Bode, 1995). Many important biologic events are exercised by metalloproteases. Caboxipeptidase A and aminopeptidases are typical metalloproteases involved in peptide digestion (Rosenberg et al., 1975). The group of the matrix metalloproteases (MMPs) is

responsible for differentiation and remodeling of the extracellular matrix (ECM), in addition to the cleavage of protein receptors and ligands. Because of their ability to hydrolyze articular cartilage, these enzymes are associated with arthritis and rheumatism (Zhen et al., 2008). Membrane metalloproteases are also related to cancer and inflammation (Seals & Courtneidge, 2003).

### 3. Protease inhibitors

#### 3.1 Definition, occurrence and distribution

Protease inhibitors (PIs) are proteins naturally occurring in living organisms and able to inhibit and so, control the activity of proteases. They are ubiquitous proteins, occurring in animals, microorganisms and plants. In this former, they constitute one of the more abundant classes of proteins, being founded in reproductive, vegetative and storage organs. Seeds and tubercles contain about 10% of their total protein as PI (Brzin & Kidrič, 1995; Mandal et al., 2002; Ussuf et al., 2001).

Protease inhibitors were identified, isolated and purified from different monocotyledon and dicotyledonous plant species. Among monocots, investigations were directed particularly to plants of the grass (Poaceae) family, as rice, barley, corn, wheat, rye and sorghum as main representants. Among dicotyledons, the Solanaceae family, represented by tomato, potato and tobacco, and legume (Fabaceae) family, represented by beans, soybeans and peas, have received special attention. However, other families were studied on a minor scale, for example, Moraceae, Araceae and Caricaceae families (Brzin & Kidrič, 1995; Schuler et al., 1998).

In different plant tissues and organs were detected, isolated and purified proteinase inhibitors, as in fruit pulp (Araújo et al., 2004), pollen (Rogers et al., 1993), floral buds, seedlings (Lim et al., 1996), apples peel (Ryan et al., 1998), string (Misaka et al., 1996), latex (Monti et al., 2004), roots, stems, leaves, fruits (Brzin & Kidrič, 1995) and particularly in tubers (Huang et al., 2008; Valueva et al., 1997, 1998, 1999) and seeds (Araújo et al., 2005; Bhattacharyya et al., 2007, 2009; Cavalcanti et al., 2002; Gomes et al., 2005; Macedo et al., 2004; Macedo et al., 2007; Oliveira et al., 2007a, b).

The expression of these inhibitors varies according to the maturation stage and tissue location. The levels of these inhibitors in plants are variable and depend on the stage of maturation, tissue location, time of harvest and storage, as also the variety of plant, with possible co-existence of different classes of inhibitors as well as a variety of isoforms in a single tissue or organ (Bhattacharyya et al., 2007, 2009; Brzin & Kidrič, 1995; Ryan, 1990).

#### 3.2 Classification of protease inhibitors

PIs are primarily classified based on the class of protease that they inhibit, thus four main inhibitor families have been established: serine protease inhibitors, cysteine protease inhibitors, metalloprotease inhibitors and aspartyl protease inhibitors (Koiwa et al., 1997; Ryan, 1990). Compared with the serine and cysteine protease inhibitors, studies directed for purification, characterization and biotechnological use of aspartic protease and metalloprotease inhibitors from plants are still very few. For this reason and due to the limited space it will be discussed only the two first families.

### 3.2.1 Serine protease inhibitors

Inhibitors of serine protease in plants are grouped into subfamilies based on their molecular weight, structural similarity, presence of cysteine residues and disulfide content (Brzin & Kidrič, 1995; Koiwa et al., 1997; Ryan, 1990). Thus, it was established at least eight inhibitor subfamilies: Bowman-Birk, Kunitz, Potato I, Potato II, Cucurbit, Cereal, Thaumatin-like and Ragi A1 (Koiwa et al., 1997; Ryan, 1990). Despite the variety of the subfamily of serine protease inhibitors, the most studied inhibitors are Kunitz and Bowman-Birk groups (Koiwa et al., 1997; Lawrence & Koundal, 2002; Ussuf et al., 2001). Studies are mainly directed to those found in seeds of legume subfamilies (Norioka et al., 1988).

**Inhibitors of the Bowman-Birk subfamily** found in monocotyledons and dicotyledons were grouped into three classes of proteins based on molecular weight, number of reactive sites<sup>5</sup>, cysteine residues and disulfide bridges. In monocots, Bowman-Birk inhibitors are divided into two classes: the first consisting of inhibitors of approximately 8 kDa possessing a single reactive site ("single-headed") and five disulfide bridges and the second consisting of inhibitors of about 16 kDa, composed of about 180 amino acid residues, with two reactive sites (dual head inhibitors or "double-headed") and ten disulfide bonds. Inhibitors of 16 kDa are composed of two domains, each of 8 kDa with high identity, similar to the 8 kDa inhibitors. The presence of these two domains of 8 kDa was explained due to gene duplication events, leading to this group of inhibitors (Qi et al., 2005). Mello and colleagues (2003) reported the presence of Bowman-Birk inhibitors consisting of about 250 amino acids residues, distributed in three areas of 8 kDa, with high identity. In this same study, these authors, analyzing the amino acid sequences of various Bowman-Birk inhibitors from monocotyledons, consisting of 8 kDa, observed that some members of this family of inhibitors found in corn and cane sugar are glycoproteins. In dicotyledons, Bowman-Birk inhibitors have a molecular mass of 8 kDa. They are generally composed of about 104 amino acid residues including 14 cysteine residues involved in seven disulfide bridges, having two reactive sites (Mello et al., 2003; Qi et al., 2005). The comparison between different members of this family showed that the first reactive site is located in the N-terminal region and is more conserved than the second reactive site, located in C-terminal region of the molecule (Prakash et al., 1996; Wu & Whitaker, 1991). These inhibitors can interact simultaneously and independently with two serine proteases, not necessarily identical, i.e., with two molecules of trypsin or a trypsin and a chymotrypsin molecule (Mello et al., 2003; Qi et al., 2005). The first inhibitor that gave rise to this protein subfamily was purified from soybean seeds and consisted of a single chain protein with 71 amino acid residues and two kinetically independent reactive sites, one for trypsin (Lysine16-Serine17) and the other for chymotrypsin (Leucine 44-Serine 45) (Odani & Ikenaka, 1973a, b).

**Kunitz inhibitors subfamily** are proteins with molecular weights ranging from 18 to 26 kDa, constituted by approximately 180 amino acid residues, having a low content of cysteine residues and involved in one or two disulfide bridges (Krauchenco et al., 2004; Pando et al., 2001). Members of this subfamily have been found in a variety of botanical families, however most research has been directed to inhibitors of the three legume subfamilies (Fabaceae family). Inhibitors of the Papilionoideae and Caesalpinioideae subfamilies usually have a polypeptide chain while inhibitors from the Mimosoideae

<sup>5</sup> The reactive (inhibitory) site corresponds to the region of the inhibitor that binds with the enzyme.

subfamily consist of two polypeptide chains joined by disulfide bridges, being characterized as dimeric proteins (Batista et al., 1996; Norioka et al., 1988). The presence of carbohydrates is a common feature for inhibitors of the Kunitz family; however, there are few reports on the structural aspects. The inhibitor purified from jackfruit seeds (*Artocarpus integrifolia*) was characterized as a glycoprotein member of the Kunitz subfamily presenting in its structure units of galactose, glucose, mannose, fucose, xylose, glucosamine and uronic acid (Bhat & Pattabiraman, 1989). The presence of mannose, xylose, fucose and other sugars were also detected in the structure of Kunitz inhibitor purified from the papaya latex (*Carica papaya*), called PPI (Odani et al., 1996). The presence of sugars was also detected as a constituent in structure of other Kunitz inhibitors, including the inhibitor purified from *Bauhinia rufa* (Sumikawa et al., 2006) and from seeds of *Swartzia pickelli* (Cavalcanti et al., 2002). However, the role of carbohydrates in inhibitors structure has not been clarified. In general, these inhibitors have only one reactive site. Because of this structural feature, they are known as one head inhibitors ("single headed") (Ryan, 1990). On the other hand, few representants of this family were characterized as inhibitors that have two sites for two different enzymes (Migliolo et al., 2010; Valueva et al., 1999; Bösterling & Quast, 1981). Arginine and Lysine residues are part of reactive sites of the inhibitors (Iwanaga et al., 2005). This inhibitor subfamily has the ability to differentially inhibit serine proteases from various sources as trypsin and chymotrypsin responsible for digestion in mammalian and insects, enzymes of the blood clotting, among others (Oliva et al., 2000; Batista et al., 1996) (Table 2).

### 3.2.2 Cysteine proteases inhibitors

Cysteine protease inhibitors from plants are grouped into a single well-characterized protein family, commonly called plant cystatins<sup>6</sup> or phytocystatins (Brzin & Kidrik, 1995; Margis et al., 1998). These proteins exhibit the ability to suppress catalytic activity of cysteine proteases members, including papain family, calpains, clostripains, streptococcal cysteine protease and viral cysteine proteases or caspases, also called apopains (Abe et al., 1994; Brzin

Source	Family	M	Aa	Db	pI	Pc	Specificity	Reference
<i>Alocasia macrorrhiza</i>	A	19.7	184	2			T, Q	Argall et al., 1994
<i>Brassica juncea</i>	B	20	178	2			T	Mandal et al., 2002
<i>Carica papaya</i> *	C	24	184	2			T, Q	Odani et al., 1996
<i>Terminalia arjuna</i>	Cb	21.5					T, Q	Rai et al., 2008
<i>Putranjiva roxburghii</i>	Eu	34				1	T	Chaudhary et al., 2008
<i>Bauhinia bauhinoides</i>	L-Ce	20	154	0	6.9	1	T, Q, K	Oliva et al., 2001
<i>B. rufa</i> *	L-Ce	20	144	1	4.6	1	PE	Sumikawa et al., 2006
<i>B. variegata</i>	L-Ce	18.5	167	2	4.8	1	T	Di Ciero et al., 1998
<i>Caesalpinia bonduc</i>	L-Ce	20		2		2	T, Q	Bhattacharyya et al., 2007

<sup>6</sup> Cystatin from cysteine protease inhibitor.

Source	Family	M	Aa	Db	pI	Pc	Specificity	Reference
<i>C. echinata</i>	L-Ce	20				1	T, K, Pl, XII, X	Cruz-Silva et al., 2004
<i>Delonix regia</i>	L-Ce	22	185	2		1	T, K	Pando et al., 2001
<i>Schizolobium parahyba</i>	L-Ce	20	169	2	4.5	1	Q	Souza et al., 1995, 2000
<i>Swartzia pickelli</i> *	L-Ce	20	174	1		1	T, Q, K, Pl	Cavalcanti et al., 2002
<i>Acacia confusa</i>	L-M	19.4	176	2		2	T, Q	Hung et al, 1994
<i>Adenanthera pavonina</i>	L-M	20	176	2		2	T, Q, P	Macedo et al., 2004
<i>Albizia kalkora</i>	L-M	19.7		2		2	T	Zhou et al., 2008
<i>Calliandra selloi</i>	L-M	20		1	4.0	2	T, Q, K	Yoshizaki et al., 2007
<i>Dimorphandra mollis</i>	L-M	23				1	T, K, Pl	Mello et al., 2001
<i>Entada scandens</i>	L-M	19.7		1	7.4	1	T	Lingaraju & Gowda, 2008
<i>Enterolobium contortisiliquum</i>	L-M	22	174	2		2	T, Q, XII, K, Pl	Batista et al., 1996
<i>I. laurina</i>	L-M	20	180	1		1	T	Macedo et al., 2007
<i>Leucaena leucocephala</i>	L-M	20	174	2		2	T, Q, K, Pl, XII	Oliva et al., 2000
<i>Plathymenia foliolosa</i>	L-M	19					T, Q	Ramos et al., 2008, 2009
<i>Crotalaria pallida</i>	L-P	32.5				2	T, Q, PE, P	Gomes et al., 2005
<i>C. paulina</i>	L-P	20	177		4.0	1	T, Q	Pando et al., 1999
<i>Erythrina acanthocarpa</i>	L-P	17.4	163	2			T, Q	Joubert, 1982
<i>Glycine max</i>	L-P	21	181	2		1	T, Q, X, K	Kim et al., 1985
<i>Psophocarpus tetragonolobus</i>	L-P	20.2	183	2		1	Q	Kortt, 1980
<i>Artocarpus integrifolia</i>	M	26				1	T, Q	Bhat and Pattabiraman, 1989
<i>Murraya koenigii</i>	R	27				1	T	Shee and Sharma, 2007
<i>Solanum tuberosum</i>	S	21	186	2	6.3	2	T, Q, NE	Valueva et al., 1997, 1998
<i>S. tuberosum</i>	S	21	186	2	5.2	2	T, Q	Valueva et al., 1997, 1998

Table 2. Serine proteases inhibitors (Kunitz)

M, Moraceae; R, Rutaceae; B, Brassicaceae; A, Araceae; C, Caricaceae; S, Solanaceae; L-Ce, Leguminosae-Caesalpinioideae; L-MI, Leguminosae-Mimosoideae; L-P, Leguminosae-Papilionoideae; Cb, Combretaceae; Eu, Euphorbiaceae, S, Sapindaceae; MM, molecular mass (kDa); Aa, amino acid; Db, disulfide bonds; pI, isoelectric point; Pc, polypeptide chain number; T, pancreatic trypsin; Q, pancreatic quimotrypsin; PE, pancreatic elastase; P, papain; Pl, plasmin; K, human plasma kallikrein; XII, factor XIIa; X, factor Xa; NE, human neutrofil elastase; (\*) glycosylated inhibitors.

& Kidrik, 1995; Brzin et al., 1998; Fernandes et al., 1993; Gaddour et al., 2001; Margis et al., 1998; Pernas et al., 1998) with different affinity degrees. Phytocystatins comprise a polypeptide chain devoid of disulfide bridges. In general, they are small molecules that have molecular weights ranging from 7.5 kDa, as the phytocystatin purified from pumpkin seeds (Levleva et al., 1997), or the inhibitor purified from soybean seeds (*Glycine max*) with approximately 26 kDa (Misaka et al., 1996) (Table 3). However, some representants of this family have higher molecular masses, like the phytocystatins purified from potato tuber (*Solanum tuberosum*), with approximately 85 kDa (Waldron et al., 1993), tomato leaves (*Lycopersicon esculentum*), with similar molecular mass (Jacinto et al., 1998; Wu & Haard, 2000) and sunflower seeds (*Helianthus annuus*) with a molecular mass of 32 kDa (Kouzuma et al., 2000).

Phytocystatins possess three regions quite conserved, interacting with their target proteases: a central motif consisting of Gln-X-Val-X-Gly, where X represents any amino acid residue, a dipeptide usually formed by a proline and a tryptophan near C-terminus and a glycine

Source	Family	MM (kDa)	Aa	pI	Specificity	Reference
<i>Helianthus annuus</i>	A	9	83	5.6	P, Fi, CH, CB, CL	Kouzuma et al., 1996
<i>Helianthus annuus</i>	A	11	100	9.5	P, Fi, CH, CB, CL	Kouzuma et al., 1996
<i>Daucus carota</i>	Ap	18	133		P	Ojima et al., 1997
<i>Carica papaya</i>	Cp	11.3	99	5.1	P, Qp, Car, PIV	Song et al., 1995
<i>Curcubita maxima</i>	Cu	7.5		6.0	P, F	Levleva et al., 1997
<i>Castanea sativa</i>	F	11.2	102		P, Fi, Qp, CB,	Pernas et al., 1998
<i>Oryza sativa</i>	G	11.8	102	5.3	P, CH, CB, CL	Abe et al., 1987
<i>Oryza sativa</i>	G	11.9	107		P, CH, CB, CL	Kondo et al., 1990
<i>Zea mays</i>	G	18	135		P, CB, CH, CL	Abe et al., 1992; 1994
<i>Zea mays</i>	G	9.2	85	5.2	P, Fi, Qp, CB, CH	Abe & Whitaker, 1988
<i>Persea americana</i>	L	11.3	100		P	Kimura et al., 1995
<i>Vigna unguiculata</i>	Le	10.7	97		P	Fernandes et al., 1993
<i>Phaseolus lunatus</i>	Le	14		5.5	P, CB, CH, CL	Brzin et al., 1998
<i>Glycine max</i>	Le	26	245		P	Misaka et al., 1996
<i>Glycine max</i>	Le	12	100		P	Zhao et al., 1996
<i>Glycine max</i>	Le	11	92		P	Zhao et al., 1996
<i>Hordeum vulgare</i>	Le				P, Fi, Qp	Gaddour et al., 2001
<i>Chelidonium majus</i>	Pa	10	90	9.3	P, CL, CH	Rogelj et al., 1998
<i>Malus domestica</i>	R	11.3	83		P, Fi, B	Ryan et al., 1998
<i>Fragaria x ananassa</i>	R	23.1	206		P, CH, CB	Martinez et al., 2005

Table 3. Biochemical characterization of Phytocystatins

MM, molecular mass; Aa, amino acid number; pI, Isoelectric point; A, Asteraceae; Ap, Apiaceae; G, Gramineae (Poaceae); L, Lauraceae; L-Ce, Leguminosae-Caesalpinioideae (Fabaceae); S, Solanaceae; F, Fagaceae; Ca, Caryophyllaceae; Cp, Caricaceae; Cu, Curcubitaceae; Pa, Papaveraceae; R, Rosaceae; S, Solanaceae; An, ananain; Ac, actinidin; Cr, cruzipain; CG, Cathepsin G; CB, Cathepsin B; CH, Cathepsin H; PPE, porcine pancreatic elastase; HNE, human neutrofil elastase; B, Bromelain; Qp, Quimopapain; Fi, Ficin; Car, Caricain; PIV, papaya proteinase IV; PIII, papaya proteinase III; P, papain.

residue located near the N-terminal region, known as flexible region of phytocystatins (Kondo et al., 1990; Stubbs et al., 1990; Margis et al., 1998). The three interaction regions can be found as tandem domains along protein structure. Thus, phytocystatins can be grouped into two distinct classes: one comprising phytocystatins of low molecular weight, with a single cystatin domain (Abe et al., 1987, 1992; Fernandes et al., 1993; Pernas et al., 1998); other class comprising phytocystatins of high molecular mass, with multiple cystatin domains, that is, with three interaction regions occurring repeatedly in primary structure of the inhibitor (Bolter, 1993; Diop et al., 2004; Kouzuma et al., 2000), and in this latter case, they are called multicystatins. Multicystatins from potato tuber and tomato leaves possess eight domains that can interact simultaneously with eight cysteine proteases (Jacinto et al., 1998; Waldron et al., 1993; Wu & Haard, 2000), however, sunflower multicystatin has three domains (Kouzuma et al., 2000) and the one from bean-to-string (*V. unguiculata*) has two cystatin domains (Diop et al., 2004).

### 3.3 PI inhibition mechanisms

In general terms, all the PIs bind to their specific protease preventing access of the substrate to the active site. For some PIs, the docking occurs directly in the protease active site, while for others the binding takes place in a neighborhood of the catalytic centre but leading to its steric hindrance (Krowarsch et al., 2003).

The majority of the known PIs, specially the serine protease inhibitors, interact with the enzyme catalytic sites in a "canonical" manner, similar to the enzyme-substrate interaction, via an exposed reactive site loop of conserved conformation (Bode & Huber, 2000) (figure 2). A well known member of this group of classic inhibitors is the Kunitz-type trypsin inhibitor

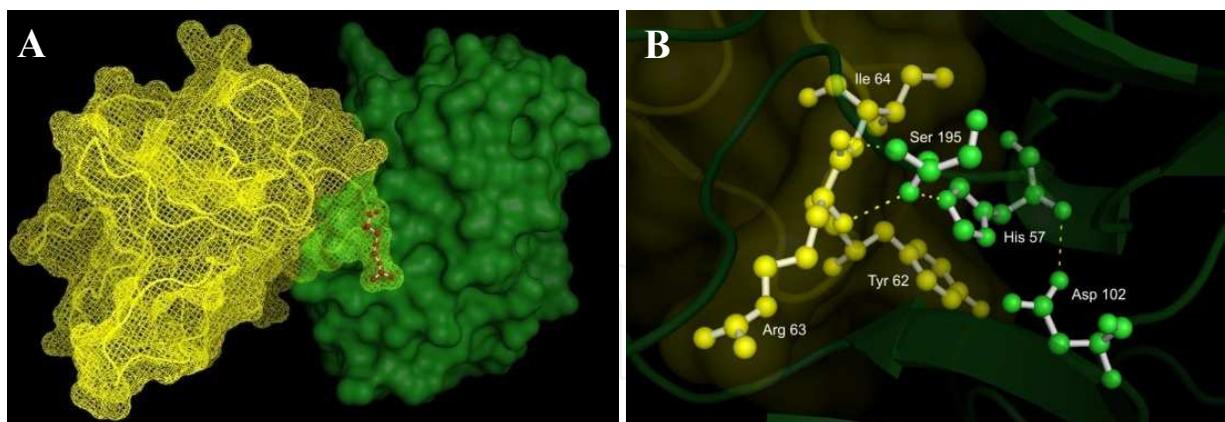


Fig. 2. Stereo diagram showing the interaction between porcine pancreatic trypsin and the soybean trypsin inhibitor (SKTI).

(A) SKTI is shown in yellow ribbon and mesh representation and trypsin in transparent dark green surface. The light green intersection represents the interface between the reactive site and the catalytic site, with the Arg 63 side chain (red ball and stick representation) of the inhibitor protruding into the specificity cleft of the trypsin. (B) Detail of the interaction interface of the trypsin catalytic triad (green ball and stick) and the reactive site loop residues of the SKTI (yellow ball and stick). Dotted line, hydrogen bonds. Figure made with PyMOL (PDB code 1AVW).

from soybean (SKTI, SBTI or STI), a potent trypsin inhibitor but that also inhibits in a lesser extent the chymotrypsin (De Vonis Bidlingmeyer et al., 1972) and plasmin (Nanninga & Guest, 1964). The SKTI is a 21.5 kDa non-glycosylated protein containing 181 amino acid residues in a single polypeptide chain crosslinked by two disulfide bridges. Its reactive site loop possess an arginine residue (Arg 63) whose side chain fits into the specificity pocket of the trypsin, while its carbonyl carbon makes contact with the serine (Ser 195) of the active site without, however, suffering catalysis and thus it blocks the enzymatic action (Song & Suh, 1998; Macedo et al., 2007, 2011; Krowarsch et al., 2003) (figure 2B). One network of hydrogen bonds between inhibitor and enzyme is also formed outside of the reactive site to stabilize the complex (Song & Suh, 1998).

This canonical conformation of the reactive loop characterizes a mechanism of competitive inhibition and is also found in the trypsin inhibitors from *Erythrina caffra* seeds and *Psophocarpus tetragonolobus* chymotrypsin inhibitor (Song & Suh, 1998; Krauchenco et al., 2003). Trypsin inhibitors, such as from *Swartzia pickelii*, can present also glutamine residue in the reactive site (Cavalcanti et al., 2002). Specific inhibitor for chymotrypsin, in general, possess leucine residue in its reactive site (Kimura et al., 1993; Dattagrupta et al., 1996) and in the *Bauhinia rufa* elastase inhibitor one valine was identified in the reactive site (Sumikawa et al., 2006).

It would be interesting to note that there are Kunitz-type inhibitors able to inhibit proteases belonging to different mechanistic classes. Protease inhibitor purified from *Prosopis juliflora* seeds (PjTKI) presents a competitive inhibition mechanism directly interacting between its reactive site (Arg 64) and the catalytic site in target trypsin (Ser 195). Moreover, PjTKI also possesses an inhibitory activity against papain and a cysteine protease present in the digestive system of several phytophagous insect-pests (Oliveira et al., 2002; Franco et al., 2002). This bifunctional property was also observed for the Kunitz inhibitor from *Adenantha pavonina* seeds (ApTKI). ApTKI was a strong non-competitive inhibitor of trypsin and moderate noncompetitive inhibitor to papain. Different from PjTKI, that was incapable of simultaneous inhibition of trypsin and papain, the interaction sites of the ApTKI did not overlap, and it formed a ternary complex that was observed through *in vitro* and *in silico* methods (Macedo et al., 2004; Prabhu & Pattabiraman, 1980; Migliolo et al., 2010).

Non-canonical mechanisms of inhibition are common to other classes of PI. A typical case is the inhibition of papain-like cysteine protease by the cystatins, which interact with the enzyme surface subsites adjacent to the active site, blocking it without direct contact with the catalytic groups (Stubbs et al., 1990; Bode & Huber, 2000; Turk et al., 2000).

### 3.4 Biological function of protease inhibitors

Protease inhibitors of plant origin are known for many years and their participation in a variety of endogenous and exogenous events continue to be the subject of much research. Regulation of endogenous proteases, storage function and defensive role against predators and pathogens are the principal proposed biological functions for plant PIs (Lawrence & Koundal, 2002). The proposed role for protease inhibitors as storage proteins was first suggested by Pusztai (1972). Plant storage proteins function as a store of nitrogen, carbon and sulfur and they are so considered when are deposited in tissues of reserves in

concentrations higher than 5% of the total protein content (Shewry, 2003). In seeds, tubers and other plant tissue reserves, PIs are found from 1 to 10%, being one of the major storage proteins (Mandal et al., 2002; Brik & Wong, 2003; Shewry, 2003). Besides their storage function, by providing nitrogen and sulphur required during germination, other functional roles have been assigned for the trypsin inhibitors, such as regulating endogenous plant proteases to prevent precocious germination, inhibiting trypsin during passage through the animal's gut, thus helping in seed dispersal, and protecting plants against pests and diseases (Derbyshire et al 1976; Laskowski & Kato, 1980; Shewry, 2003). Confirmation of the role of PIs in plants is primarily based on *in vitro* experiments using insect gut proteases as well as the efficiency of these inhibitors on a variety of proteases from pathogens such as fungi, viruses, mites and nematodes (Fosket, 1994; Dunaevsky et al., 2005; Fan & Wu, 2005). The second line of evidence that strengthens the role of these molecules as defense compounds of plants results from the fact that these inhibitors interfere with normal growth and development of microorganisms when added to artificial diet system (Araújo et al., 2005; Gomes et al., 2005). Another line of evidence is the induction level of these molecules following mechanical injury or another biotic or abiotic stress. And finally, the increased resistance to insects and pathogens in plants transformed with genes expressing PIs (Kiggundu et al. 2010; Masoud, 1993).

### 3.5 Biotechnological/pharmacological applications of PI

Despite the mechanisms to control proteases, loss of proteolytic control is observed in a wide range of diseases. Indeed, increased proteolysis has been shown to underpin various pathological processes and, as a result, PIs have emerged as a class of highly promising chemotherapeutic agents (Scott & Taggart, 2010). Thus, many inhibitors can be strong therapeutic candidates for treating diseases, such as cancer, fungal, parasitic and neurological disorders, inflammatory, immune, respiratory and cardiovascular diseases (Leung et al., 2000).

There is evidence that inhibitors suppress various stages of carcinogenesis, including initiation, promotion and progression. Although many PIs have the ability to prevent carcinogenic processes, the most potent are those with activity antichymotrypsin (Kennedy, 1998; Zhang et al., 2007) and it is not yet known how these inhibitors work suppressing carcinogenesis. It is known that there may be multiple inhibition pathways of carcinogenesis, e.g., preventing the release of superoxide radicals and hydrogen peroxide by polymorphonuclear leukocytes or other cell types, stimulated by tumor promoting agents (Kennedy, 1998). Recent studies indicate that Bowman-Birk type inhibitors are potential candidates for inhibition of carcinogenic activity (Sessa & Wolf, 2001).

Because PIs naturally inhibit a diversity of proteases from plants pathogens, their genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programmes (Lawrence & Koundal, 2002).

## 4. Purification of PIs using affinity chromatography (on protease-matrix)

### 4.1 General advantages of affinity chromatography on protease-matrix

Affinity chromatography is a very efficient technique capable of purifying proteins based on reversible interactions between a protein and a specific ligand coupled to a chromatographic

matrix. The affinity chromatography is usually used as one of the last steps in the purification process of PIs and combines advantages as great time-saving and high capacity of selection and concentration of the target protein from a complex mixture of contaminating substances in a large sample volume (Cuatrecasas, 1970). The binding property of the PIs with enzymes (figure 2) has been exploited for the affinity purification of inhibitors from various sources, especially for those of plant origin.

#### 4.2 Coupling

The first step to carry out the purification of the inhibitor of interest by affinity chromatography is to immobilize its corresponding enzyme in a coupling gel. The gel substance (commonly based on a polysaccharide) should exhibit mechanical and chemical stability to the coupling and elution conditions, minimal nonspecific interaction with proteins and form a loose porous network which allows the free flow of large molecules. Cross-linked dextran (Sephadex<sup>7</sup>) and, better yet, derivatives of agarose (Sephacrose<sup>8</sup>) are polysaccharides that have many of these features and therefore are widely used as coupling gel (Cuatrecasas, 1970). Many studies have reported the purification of PIs by using a specific protease immobilized by covalent bonding to agarose resins (CNBR-activated Sepharose 4B) and chitosan (Xi et al., 2005), that have low non-specific adsorption of proteins. The coupling of protease using cyanogen halides (CNBr-activated Sepharose) is well described and consists in linking primary amino groups directly in pre-activated matrices (figure 3). The establishment of multipoint connections provides greater stability to the immobilized enzyme. However, it is important that these multipoint connections do not interfere with the enzyme binding site and thus compromise the effectiveness of the interaction of the inhibitor with the protease. The resins of CNBr-activated Sepharose 4B type are stable in a wide range of pH (2-11) and have a good range of ligand coupling.

#### 4.3 Sample application and washing

After coupling the protease of choice with the gel and before applying the mixture sample containing target PIs, the matrix must be packaged in a column and pre-equilibrated in binding buffer. Usually, Sepharose gels containing the immobilized enzyme are equilibrated with 2-3 volumes buffers containing NaCl at concentrations ranging from 0.1-0.5 M and pH is in the same range of optimal pH activity of the enzyme.

It is not possible to predict a single optimum flow rate for loading the sample in an affinity chromatography because the degree of interaction between ligands is widely variable. The binding is favored by the use of a very low flow rates in the sample loading, especially for weak affinity systems. In extreme cases of very weak affinity may be useful to stop the flow after loading the sample or to re-apply it. It is therefore interesting to test the best flow rate to each case, and it is not recommended flow rates higher than 10 mL/cm<sup>2</sup>/h.

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<sup>7</sup> Sephadex (from separation Pharmacia dextran) is a trademark of GE Healthcare (formerly: Pharmacia).

<sup>8</sup> Sepharose (from separation Pharmacia agarose) is a trademark of GE Healthcare.

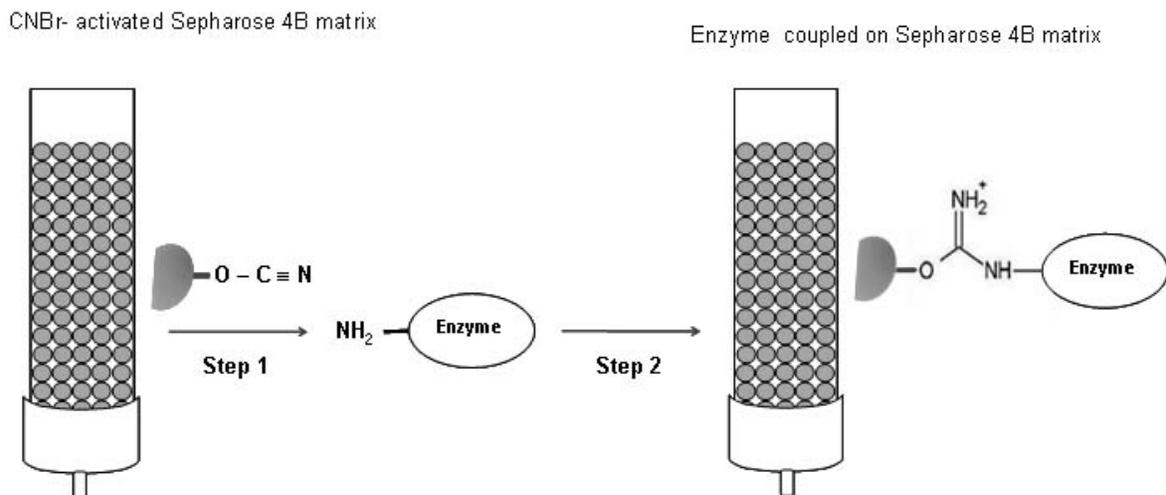


Fig. 3. Coupling the enzyme to CNBr-activated Sepharose 4B.

The volume of the sample loaded is not critical, since the principle of the technique is the affinity, however for interactions with weak affinity it recommended to apply it in a small volume (about 5% of bed volume). In order to remove the unbound substances (and prior to the elution), the column should be washed with about 10 volumes of the starting buffer. The figure 4 presents a schematic view of the steps of an affinity chromatography.

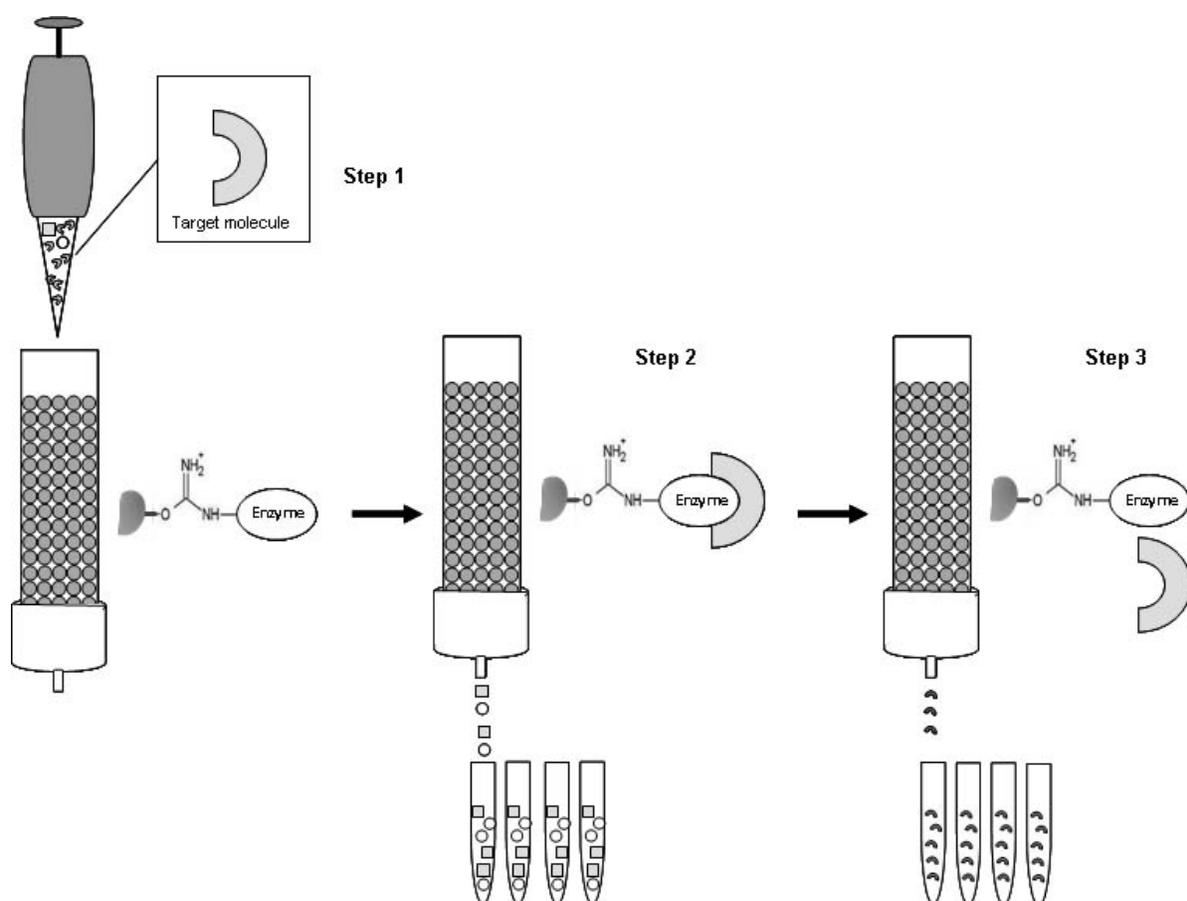


Fig. 4. Steps of a typical affinity chromatography on protease-matrix.

Step 1, application of the sample; Step 2, binding of target molecules and washing of the unbound substances; Step 3, Elution of the target molecules.

#### 4.4 Elution

Methods of elution of target protein from the column can be selective (using a competitive ligand) and non-selective (changing the pH, ionic strength or polarity).

Some cysteine proteinase inhibitor has been isolated by affinity chromatography using immobilized papain. Systemin-inducible papain inhibitor from *Lycopersicon esculentum* leaves (Jacinto et al., 1998) was eluted as using 50 mM  $K_3PO_4$  buffer, pH 11.5, containing 0.5 M NaCl and 10% glycerol (Anastasi et al., 1983). A cysteine proteinase inhibitor from chestnut seeds, *Castanea sativa*, named Cystatin CsC, retained in the affinity column on carboxymethylated-papain NHS-Sepharose was eluted of the column using 0.2 M trisodium phosphate buffer, pH 11.5, containing 0.5 M NaCl, then immediately neutralized using 2 M tris-HCl, pH 7.5, and desalted (Pernas et al., 1998). Solution of 10 mM NaOH was used for the recovery of chelidocystatin, cystatin from mature *Chelidonium majus* plants of the papain Sepharose affinity chromatography (Rogelj et al., 1998). Papain inhibitor of the immature fruit from *Malus domestica* was eluted of papain affinity column with solution of 50 mM HCl and immediately neutralised with 3 M Tris-HCl buffer, pH 10 (Ryan et al., 1998).

Protein inhibitors of serine proteases retained in protease trypsin-and chymotrypsin-Sepharose affinity column are generally recovered from resin by the use of fixed concentration of HCl. Trypsin is the enzyme most often used as a ligand for the purification of PIs by affinity. As example, Macedo et al. (2011) purified a trypsin inhibitor from *Sapindus saponaria* seeds (SSTI) using a trypsin-Sepharose column, where the inhibitor was eluted with 0.01 M HCl. Another inhibitor purified by the method of elution with fixed concentration of HCl was the inhibitor purified from *Adenanthera pavonina* seeds (APTI), using 0.1 M HCl (Macedo et al., 2004, 2011). Trypsin inhibitor of *Cocculus hirsutus* leaf (ChTI) was recouped of the affinity column with 0.2 N HCl, pH 3.0 (Bhattacharyya et al., 2009). HCl concentration of 0.015 M was efficient for removal from *Cycas siamensis* seeds inhibitor (Konarev et al., 2008). The mixture of 0.2 M Gly-HCl buffer in pH 3.0 containing 0.5 M NaCl was effective for the recovery of a Kunitz trypsin inhibitor of *Entada scandens* seeds (ESTI) bound the affinity chromatography on trypsin-Sepharose (Lingaraju & Gowda, 2008). Solution of 1 mM HCl was necessary to remove the trypsin inhibitors of *Pithecellobium dumosum* (Oliveira et al., 2007a, b; 2009) and *Crotalaria pallida* seeds (Gomes et al., 2005).

Purification of inhibitors from different classes of proteolytic enzymes using few purification steps and enzyme immobilization techniques has been used intensively for decades. Four competitive Kunitz-type trypsin inhibitors (JB1, JB2, JB3 and JB4) were purified from *Pithecellobium dumosum* by TCA precipitation, affinity chromatography on immobilized trypsin-Sepharose and reverse phase HPLC using Vydac C-18 column seeds with  $K_i$  values of 3.56, 1.65,  $2.88 \times 10^{-8}$  M and  $5.70 \times 10^{-10}$  M (Oliveira et al., 2007). The percentage inhibition of JB1, JB2 and JB3 on papain varied between 32.93 to 48.82% and was indicative of its bifunctionality with exception of JB4 that inhibited this activity in 9.9%. The papain inhibition by JB1 and JB2 were noncompetitive type and the  $K_i$ -values were  $7.6 \times 10^{-7}$  and  $5.1 \times 10^{-7}$  M, respectively. Among these a highly purified Kunitz-type inhibitors denominated PdKI (JB1) was isolated by affinity chromatography on trypsin-Sepharose column and HPLC (Figure 5) (Oliveira et al., 2007a).

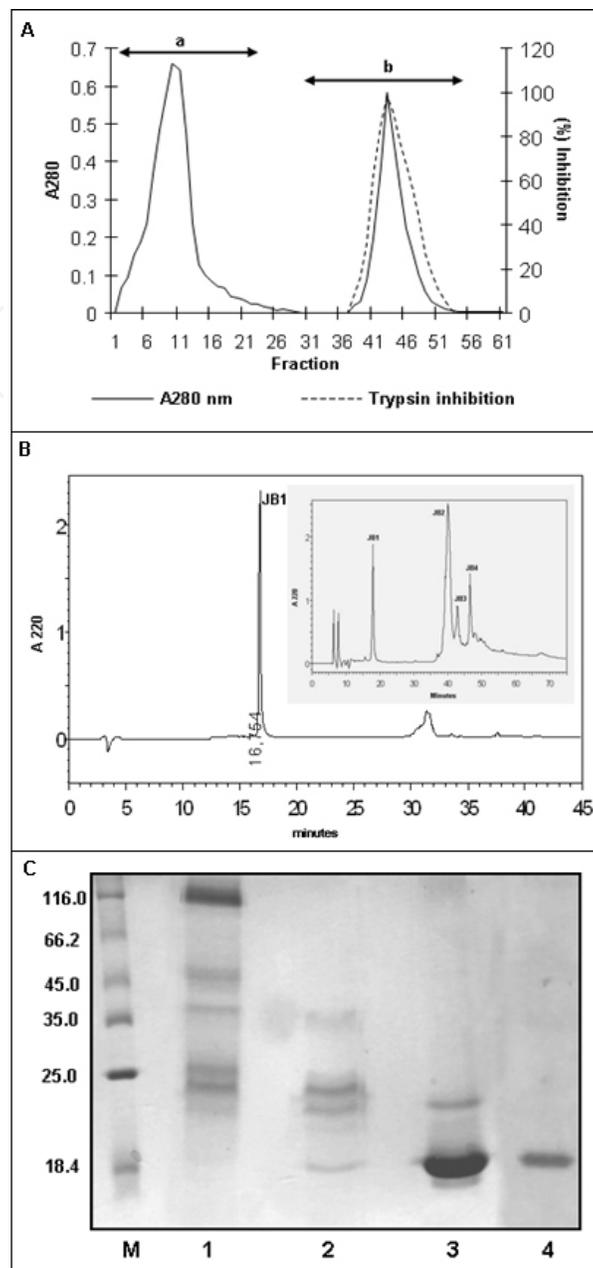


Fig. 5. (A) Chromatographic profile of Inhibitor of *Pithecellobium dumosum* seeds on Trypsin-Sepharose. Column (10 cm X 1.5 cm) was equilibrated with 50 mM Tris-HCl, pH 7.5 buffer and the retained proteins were eluted with 1 mM HCl. Fractions obtained were assayed against trypsin. (B) Elution profile on HPLC (Vydac C-18) column. The fractions obtained from Trypsin-Sepharose column were separated by semi-preparative reverse-phase HPLC column at a flow rate of 9 ml/min. Insets: the purified protein was then again subjected to analytical reverse-phase HPLC column at a flow rate of 1 ml/min. Both were eluted using a gradient of solvent B (60% acetonitrile in 0.1% TFA) in solvent A (0.1% TFA/H<sub>2</sub>O), and monitored at 220 nm. (C) SDS-PAGE (15%) of PdKI from *P. dumosum* seeds, stained with silver nitrate. (M) Protein molecular weight markers:  $\alpha$ -galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp981 (25 kDa),  $\alpha$ -lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). (1) Crude extract; (2) fraction treated with TCA; (3) Trypsin-Sepharose retained peak; (4) PdKI.

Although most studies describe elution of proteins retained on affinity column using a fixed molarity of HCl, however, it is interesting to test the elution of adsorbed proteins with solutions containing different molar concentrations of HCl, similar to stepwise (step by step). This procediment is important because high molar HCl concentrations may be unnecessary. For example, Figure 6A shows the elution profile of chymotrypsin-Sepharose column of a protein sample fractionated with ammonium sulfate at 30-60% saturation, which exhibited inhibitory activity to chymotrypsin. The column was equilibrated with Tris-HCl 0.05 M, pH 7.5. Non-adsorbed proteins were eluted with the same equilibration buffer and proteins adsorbed to matrix were eluted with 5 mM HCl. This experiment also used 10 and 100 mM HCl. However, only the concentration of 5 mM HCl was necessary for elution of total active proteins. In addition, inhibitors bind to immobilized enzyme linked to Sepharose affinity in different degrees. For example, Figure 6B shows elution profile of another sample containing high inhibitory activity against chymotrypsin. Three different molar concentrations of HCl were tested for elution of adsorbed proteins. The adsorbed proteins were differentially eluted with two concentrations of HCl. Proteins presenting lower affinity for matrix were eluted with 5 mM HCl, while proteins with higher affinity for matrix were only eluted with 100 mM HCl. No elution was observed when 10 mM HCl was used.

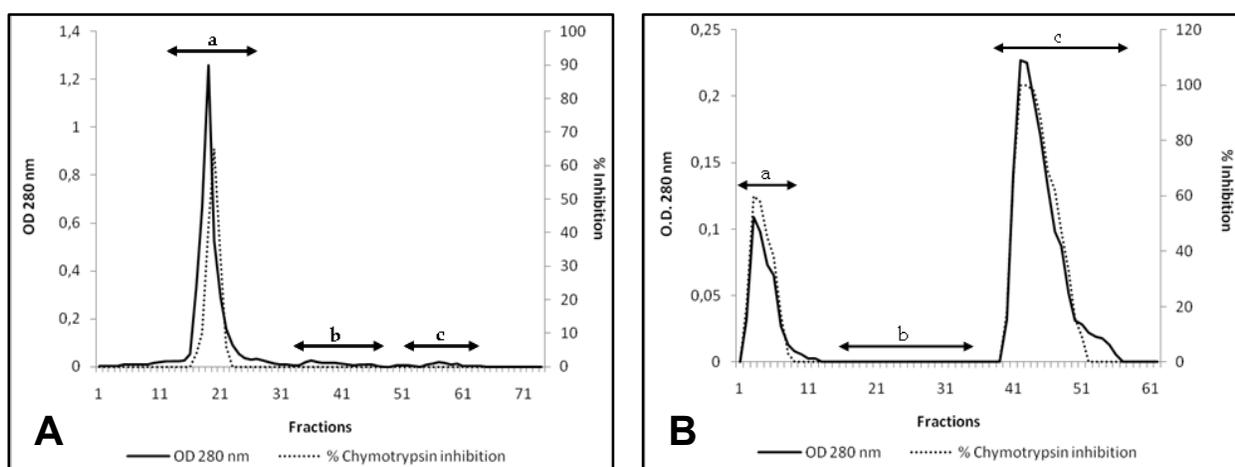


Fig. 6. Chromatographic profiles of different samples of inhibitors (A, B) from legume seeds on Chymotrypsin-Sepharose affinity column.

Elution Conditions: a: 5 mM; b, 10 mM and c, 100 mM HCl.

Finally, affinity column are also useful to exclude contaminants from protein samples or delete unwanted activities. To retrieve specific inhibition activity for chymotrypsin in a sample showing predominant trypsin inhibitory activity against chymotrypsin, it was necessary to test binding ability on a trypsin-Sepharose column to exclude trypsin inhibitory activity in the sample (Figure 7). Thus, different concentrations of the sample were applied and inhibitory activity was monitored. Figure 7A shows a chromatographic profile in affinity column when 35 mg of protein was applied on a trypsin-Sepharose column after balanced with equilibration buffer. Inhibition tests showed that both fractions, non-adsorbed and adsorbed, inhibitory activity toward trypsin was 100% and inhibitory activity for chymotrypsin was determined only for non-adsorbed fractions. Following this procedure, 21 mg protein from the same sample was applied in trypsin-Sepharose column (Figure 7B). Inhibition assays showed that trypsin inhibition for non-adsorbed fractions

were much lower than before (about 49% inhibition), compared to that obtained in adsorbed fractions and when compared to Figure 7A. However, the use of about 7 mg protein resulted in adsorption of total protein responsible for inhibitory activity when linked to trypsin immobilized on chromatographic matrix. Inhibitory activity against chymotrypsin was determined only in non-adsorbed fractions (Figure 7C). Fractions containing only inhibitory activity against chymotrypsin were pooled and applied to chymotrypsin-Sepharose affinity column, which all inhibitory activity against chymotrypsin was recovered free of any activity against trypsin (Figure 7D). This procedure allowed the elimination of total inhibitory activity for trypsin and total recovery of inhibitory activity to chymotrypsin present in sample.

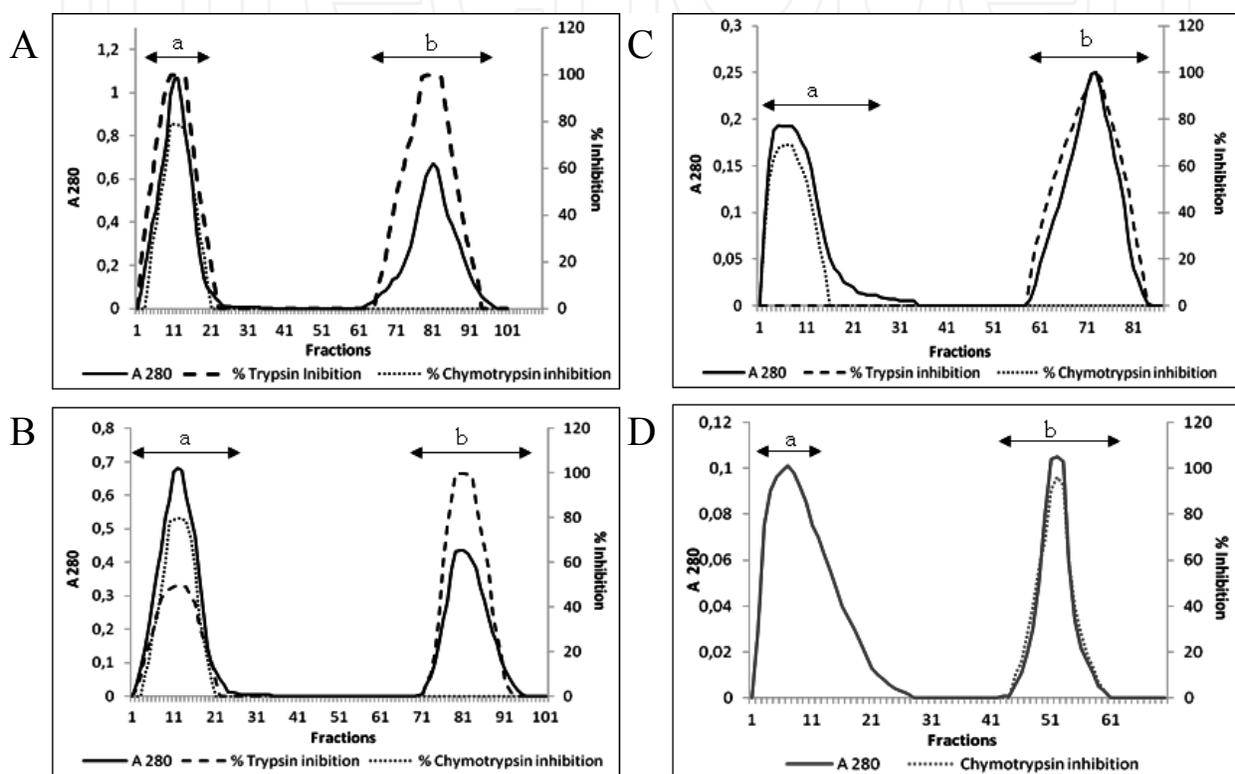


Fig. 7. Chromatographic profile of trypsin and chymotrypsin inhibitors in affinity columns. Seed extracts were loaded onto trypsin-Sepharose 4B (A-C) or chymotrypsin-Sepharose 4B (D) columns equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Adsorbed proteins were eluted with 5 mM HCl: (3A) load 35 mg proteins, (3B) 21 mg (3C) 7 mg (3D) 3 mg, respectively. It was collected 2 mL/fraction and monitored at  $A = \lambda_{280 \text{ nm}}$ .

#### 4.5 Detection (inhibitory activity)

Trypsin and chymotrypsin from bovine pancreas are serine protease more used *in vitro* assays for determination of the presence of inhibitory activity by the crude extracts of several origins, by accompaniment of inhibitory activity during all the isolation process, as well as to characterize inhibitor purified, including determination of dissociation constant ( $K_i$ ), formation of inhibitor-serine protease complex and studies about stability of the inhibitory activity (Mello et al., 2001; Macedo et al., 2002, 2003, 2007; Pando et al., 1999; Gomes et al., 2005; Araujo et al., 2005; Oliveira et al., 2002, 2007a,b, 2009). Mature trypsin is composed for 223 amino acid residues with His57, Asp102 and Ser195 residues forming its catalytic triad (figure 2). Trypsin is 24 kDa distributed in a single chain polypeptide cross-

linked by 6 disulfide bridges. Trypsin hydrolyzes specifically peptides on the carboxyl side of Lys and Arg amino acid residues (Walsh, 1970) in substrate protein such as azocasein (Xavier-Filho et al., 1989) or synthetic substrate as BAEE, benzoyl L-arginine ethyl ester (Avineri-Goldman et al., 1967; Delaage & Ladunski, 1968); TAME, p-toluenesulfonyl-L-arginine methyl ester (Bhattacharyy et al., 2006, 2007, 2009); tosyl-L-arginine methyl ester and BApNA, Na-benzoyl-L-arginine p-nitroanilide (Erlanger et al., 1961; Gomes et al., 2005; Araujo et al., 20005; Oliveira et al., 2007, 2009; Migliolo et al., 2010).

Chymotrypsin from bovine pancreas is a protein of 25 kDa, pI of 8.7, consisting of 241 amino acid residues composed by three peptide chains (A, B and C chain with 13, 131 and 97 residues, respectively), joined by disulfide bridges that shows capacity to hydrolyze peptide bonds on the C-terminal side of tyrosine, phenylalanine, tryptophan, leucine as well as methionine, isoleucine, serine, threonine, valine, histidine, glycine, and alanine (Appel, 1986; Ui, 1971). Hydrolyze of azocasein (Xavier-Filho et al., 1989) and release of p-nitroanilide from amide synthetic substrate (BTpNA, N-Benzoyl-L-tyrosine p-nitroanilide; N-Succinyl-L-phenylalanine-p-nitroanilide) are commonly used to assay chymotrypsin inhibitory activity (Nakahata et al., 2006; Macedo et al., 2007; Mello et al., 2001; Pando et al., 1999) as well as the hydrolyze of ester linkages of BTEE, N-Benzoyl-L-tyrosine ethyl ester (Bhattacharyy et al., 2006, 2007, 2009).

Both enzymes (Trypsin and chymotrypsin) are soluble, stabilized and can be stored in 1 mM HCl solution for 1 year at -20°C. The addition of calcium into of enzyme solution beyond stabilizing enzymes prevents the process of autolysis (Sipos & Merkel, 1970). The formation of p-nitroaniline (bright yellow) from amide substrate (BApNA, BTpNA) by trypsin and chymotrypsin is monitored at 405-410 nm (Nakahata et al., 2006; Macedo et al., 2007; Mello et al., 2001; Pando et al., 1999; Oliveira et al., 2007, 2009; Oliva et al., 1996).

The hydrolysis of azocasein (casein with 23.6 kDa conjugated to an azo-dye) is a procedure very used for the determination of proteolytic enzymatic activities that act in pH above of 5.0 as serine and cysteine proteases. Azocasein solution precipitates in pH below of 4.5. In general, in azocasein hydrolysis assays, reaction is stopped by the addition of trichloroacetic acid solution resulting in the formation of colored (red-orange) soluble components which are measured at (absorption maximum) 440 nm (Charney & Tomarelli, 1947).

## 5. Conclusions

The process of purification of biomolecules includes a combination of separation techniques, such as: extraction, fractionation by precipitation and chromatographies addressing different properties. The type and amount of technique will depend on the nature and characteristics of the molecule of interest, as well as the degree of purity desired in the final product. Among the chromatographic techniques, the affinity chromatography, by which specific biological properties can be exploited, stands out for its high purification capacity. For example, affinity chromatography on columns containing immobilized enzymes provides an efficient and rapid process of isolation and purification of protease inhibitors from different sources. This procedure provides advantages as high enrichment of inhibitor fraction, reduction of purification steps due to high binding specificity of the protein immobilized on a chromatographic matrix and purification of different protease inhibitors in the same fraction by differential elution of material retained on chromatographic matrix

using different conditions to destabilize the adsorption between the complex enzyme-inhibitor. Protease inhibitors purified in this way can be evaluated for their use in agriculture and pharmaceutical industry. To analyze the application of protease inhibitors in these sectors are needed bioassays that require a lot of protein for development of dose-response curves. So the affinity chromatography represents a powerful tool for the enrichment of these proteins and enables the rapid achievement of these for analysis of activities like: bioinsecticide, bionematicide, bactericidal, anti-inflammatory, anticoagulant, and antitumor among others. The use of affinity chromatography on an industrial scale could also facilitate the achievement of these molecules for their use.

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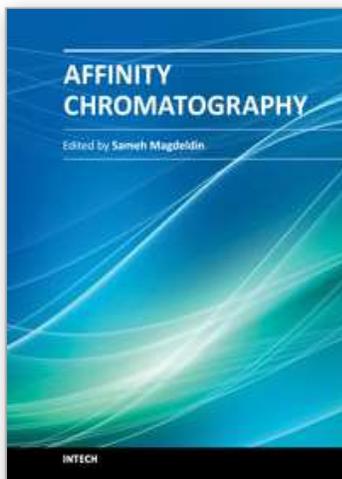
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### **Affinity Chromatography**

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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

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