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## The Value of Fungal Protease Inhibitors in Affinity Chromatography

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### 1. Introduction

Proteolytic enzymes (also known as proteases, proteinases or peptidases) offer a wide range of applications. They are routinely used in detergent, leather, food and pharmaceutical industries, as well as in medical and basic research. Therefore, effective isolation procedures are of great importance. The chapter describes the use of recently discovered protease inhibitors from basidiomycetes as affinity chromatography ligands for isolating proteases. Affinity columns with serine and cysteine protease inhibitors immobilized to the natural polymer Sepharose have been prepared, the chromatography procedure optimized and used for isolating proteases from various bacterial, plant and animal sources. The cysteine protease inhibitor macrocypin showed superior characteristics as a ligand, so was selected for immobilization to CIM (Convective Interaction Media) monolithic disks. Different immobilization chemistries and process conditions were optimized to determine the best conditions for high capacity and selectivity. A very effective method for isolating cysteine proteases was developed using affinity chromatography with the fungal cysteine protease inhibitor macrocypin immobilized to a CIM monolithic disk.

#### 1.1 Proteases and their applications

Proteases occur in all groups of organisms, including bacteria, archaea, protists, fungi, plants, animals and viruses. They are classified according to their catalytic type into aspartic, cysteine, glutamic, serine and threonine peptidases, based on the type of the amino acid residue at the active site, and metallopeptidases, that require a catalytic divalent metal ion within the active site. The MEROPS database (<http://merops.sanger.ac.uk>) further classifies peptidases according to their evolutionary relationships and encompasses information on all known proteases (Rawlings et al., 2010).

Proteases offer a wide range of biotechnological applications. Alkaline proteases are routinely used in the detergent industry. Proteases with elastolytic and keratinolytic activities have been used in the leather industry for dehairing and baiting skins and hides. They are used in the food industry in cheese making and baking, in preparing the various

protein hydrolysates used as flavour enhancers, in meat tenderization and in manufacturing protein-rich diets. In the pharmaceutical industry proteases have found uses as therapeutic agents as well as additives in preparations of slow-release dosage forms. In addition to industrial and medical applications, proteases have found use also in basic research, for example proteases with very selective peptide bond cleavage are used in protein sequencing and proteome analyses. Furthermore, unselective proteases are also used, for example proteinase K is used in nucleic acid isolation, and trypsin is widely used in animal cell culture maintenance (Kumar & Takagi, 1999; Østergaard & Olsen, 2010; Rao et al., 1998).

## 1.2 Protease inhibitors

Proteases play essential metabolic and regulatory functions in many biological processes, therefore the regulation of their activity is essential. Interaction with protease inhibitors constitutes a very important mechanism of protease regulation (Lopez-Otin & Bond, 2008; Rawlings et al., 2010). Protease inhibitors are either small molecules or proteins. They can be classified according to the source organism (microbial, fungal, plant, animal), according to their structure (primary and three-dimensional), or according to their inhibitory profile (broad-range, specific) and reaction mechanism (competitive, non-competitive, uncompetitive as well as reversible or irreversible). Commonly they are classified according to the class of protease they inhibit (for example: aspartic, cysteine or serine protease inhibitors) but a detailed classification of protein protease inhibitors based on their evolutionary relationship is available in the MEROPS database, together with a list of small molecule peptidase inhibitors (<http://merops.sanger.ac.uk/inhibitors/>). There are two general mechanisms by which protein inhibitors inhibit peptidases - irreversible "trapping" reaction, involving a conformational change of the inhibitor, and reversible tight-binding reactions, where the inhibitor forms a high-affinity interaction with the peptidase, most often at the active site (Christeller, 2005; Rawlings, 2010; Rawlings et al., 2004). The reversible protease inhibitors are used as ligands in affinity chromatography (Cuatrecasas et al., 1968).

### 1.2.1 Protease inhibitors of fungal origin

The only routinely used small molecule inhibitor of fungal origin is the irreversible inhibitor of cysteine proteases E-64, first isolated from the filamentous fungus *Aspergillus japonicus* (Hanada et al., 1978). Several other small molecule inhibitors with a broad inhibitory spectrum that are routinely used (e.g. pepstatin A, chymostatin, leupeptin, antipain, phosphoramidon, bestatin) were originally isolated from bacteria (Rawlings, 2010).

Information on protein protease inhibitors of fungal origin is limited. No protein metalloprotease inhibitors has been described, and only one family of aspartic protease inhibitors (family I34), which includes the highly specific inhibitor of the yeast proteinase A or saccharopepsin (Phylip et al., 2001). There are four families of serine protease inhibitors, namely the inhibitors of serine carboxypeptidase Y (family I51), *Aspergillus* elastase inhibitor family (I78), inhibitors of subtilisin-like proteases homologous to the subtilisin propeptide (family I9) and trypsin-specific protease inhibitors (family I66). Representatives of the latter two have been identified from higher fungi or mushrooms as well as from filamentous fungi (Rawlings, 2010). Furthermore, only three families of cysteine protease inhibitors have been described, namely mycocybins (families I48 and I85), that are unique to higher fungi, and a

cysteine protease inhibitor family (I79) with only one representative found in one plant pathogenic fungal species (Rawlings, 2010).

The serine protease inhibitor cnispin (family I66), identified in the mushroom *Clitocybe nebularis*, is a 16.4 kDa protein with acidic isoelectric point. It is a very stable protease inhibitor that resists short-term exposure to extremes of pH (between pH 2 and pH 11). It is a very strong inhibitor of trypsin (family S1) with  $K_i$  in the nanomolar range, and a weak inhibitor of chymotrypsin (family S1), with  $K_i$  in the micromolar range. Inhibition of kallikrein (family S1) and subtilisin (family S8) is very weak and other proteases are not inhibited (Avanzo et al., 2009).

Mycocypins are cysteine protease inhibitors unique to basidiomycete mushrooms and belong to two MEROPS families (I48 and I85). Family I48 is represented by cliticypin, identified in *Clitocybe nebularis* (Brzin et al., 2000; Renko et al., 2010; Sabotič et al., 2006) and family I85 by macrocypins identified from *Macrolepiota procera* (Renko et al., 2010; Sabotič et al., 2009b). These are small (16.8 to 20 kDa) and exceptionally stable proteins, exhibiting high thermal and broad pH stability (Galeša et al., 2004; Kidrič et al., 2002; Sabotič et al., 2009b). They have the  $\beta$ -trefoil fold, which is composed of a core six-stranded  $\beta$ -barrel surrounded by 11 loops (Fig. 1) that provide a versatile surface for the inhibition of several types of proteases (Renko et al., 2010). They are very strong inhibitors of papain-like peptidases (family C1), including papain, cathepsins L, V, S, and K in the low nanomolar range and cathepsins B and H with higher inhibition constants. A second inhibitory reactive site is involved in the inhibition of the cysteine protease asparaginyl endopeptidase (AEP, legumain), of family C13 by cliticypin, macrocypins 1 and 3, and in the inhibition of the serine protease trypsin (family S1) by macrocypin 4 (Renko et al., 2010; Sabotič et al., 2007a; Sabotič et al., 2009b).



Fig. 1. Three-dimensional structure of macrocypin 1. The two loops involved in inhibiting papain-like proteases (family C1) are indicated with #, and the loop involved in legumain (family C13) inhibition with \*.

### 1.3 Affinity chromatography

Affinity chromatography is a purification technique that exploits the unique biological properties of biomolecules to bind ligands specifically and reversibly (Cuatrecasas, 1970). It is exceptional in purification science as a tool for highly selective isolation of antigens (drugs, hormones, peptides, proteins, viruses, cell components), antibodies, enzymes, sugars, glycoproteins and glycolipids, immunoglobulins, nucleotide-binding and metal-binding peptides and proteins (Mallik & Hage, 2006). The use of specific interactions makes affinity chromatography a powerful tool in isolation of a particular substance from a complex mixture. In theory, it is capable of giving absolute purification from a complex mixture in a single process and it eliminates the need for ammonium sulphate precipitation, ion exchange, and gel filtration steps in an isolation protocol (Hermanson et al., 1992).

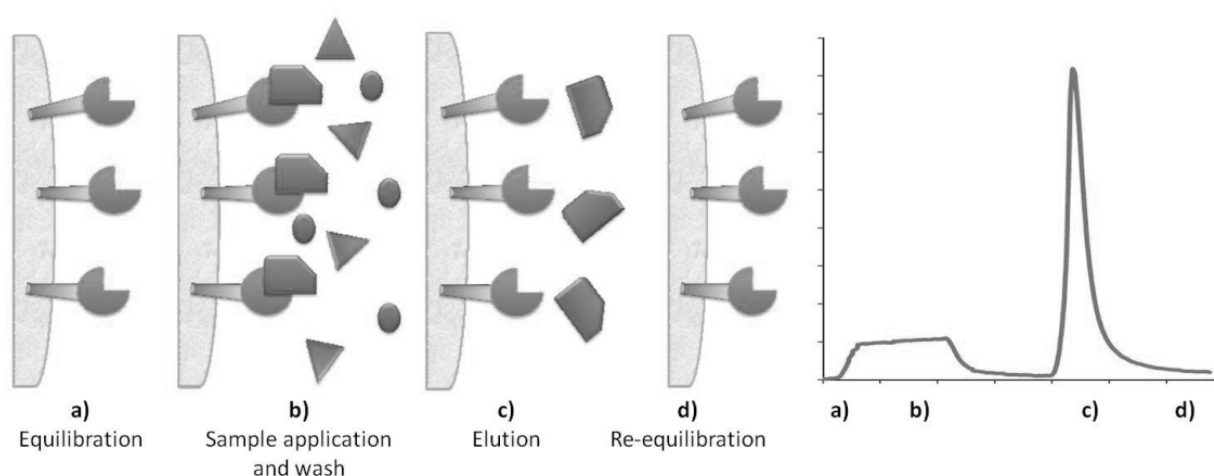


Fig. 2. Principle of affinity chromatography: a) Equilibration of the stationary phase in a binding buffer. b) Sample is applied to the column. Target molecules will bind specifically but reversibly to the ligand, non-specifically bound contaminants will pass through in the flow through during washing with binding buffer. c) The purified compound is released from the biospecific ligand by elution using specific competition or non-specific change in buffer composition. d) Stationary phase is re-equilibrated with a binding buffer. The different stages of affinity chromatography are represented in a typical chromatogram in the right panel.

In affinity chromatography one of the two interacting molecules, called the affinity ligand, is immobilized on a stationary phase, either by covalent immobilization or by physical adsorption (Tetala & van Beek, 2010). The ligand can consist of an immobilized sequence of DNA or RNA, a protein or enzyme, lectin, amino acids, immunoglobulin, a biomimetic dye, an enzyme substrate or inhibitor, or a small molecule (Hage, 2006; Healthcare, 2007). Target molecules to be purified are applied in a mobile phase known as the application buffer to the column containing insoluble polymer or gel. The buffer is generally chosen to allow optimal binding of the immobilized ligand to its target while other sample components pass through with little or no retention (Mallik & Hage, 2006). Interaction between the ligand and the target molecule in a sample can be the result of electrostatic or hydrophobic interactions, van der Waals' forces or hydrogen bonding. The retained target molecule is then eluted and several different methods are possible. If a ligand has a weak or moderate affinity for the target molecules, the elution can be performed under isocratic conditions, where



composition of the mobile phase remains unchanged during washing and elution. This technique is known as weak affinity chromatography. When a ligand has strong affinity to the target molecule, it can be released by changing the mobile phase or column conditions. For the most part, elution buffer differs from the application buffer in pH, ionic strength, temperature or polarity. This approach is known as nonspecific elution. Another possibility is biospecific elution, where a buffer with the same pH, ionic strength and polarity as the application buffer is used for elution but with a competing agent that can bind to either the retained target or the immobilized ligand (Tetala & van Beek, 2010).

### 1.3.1 Supports in affinity chromatography

The role of stationary phase or support in affinity chromatography is ligand immobilization (Hage, 2006). A support is any material to which a biospecific ligand may be covalently attached. Typically, the material to be used as an affinity support is insoluble in the system where the target molecule is found (Hermanson et al., 1992). The support main characteristics are: large specific area, high rigidity and suitable form of the particles, hydrophilic character and high permeability. The particle size and porosity are designed to maximize the surface area available for immobilization of ligand and consequently binding of target molecule. Supports in classical or low-performance affinity chromatography are usually non-rigid particles with large diameters like agarose, but also organic polymers like polyurethane or inorganic materials as large diameter silica particles (Hage, 2006). In column chromatography the adsorption rate is limited by slow particle diffusion for larger beads or low axial velocities and high pressure for smaller beads. The consequence is limited access for the biomolecules to small pores in the case of classical chromatographic supports. Good flow properties are desirable for rapid separations as they save considerable time during column equilibration, regeneration and cleaning (Champagne, 2007). The use of affinity supports with more rigid and effective materials that can be used in high performance liquid chromatography systems (HPLC) gives a technique known as high-performance affinity chromatography (Hage, 2006). In comparison with low pressure chromatography, HPLC is basically an improved form of the technique, where a mobile phase is forced through the column under high pressure instead of being allowed to drip through under gravity. Another improvement is an extremely sensitive detection system and complete automation of the process. Development of more resistant stationary phases has resulted in faster and better resolution and explains why HPLC became the most powerful and versatile form of affinity chromatography (Wilson & Walker, 2005).

Moreover, a chromatographic support should provide appropriate surface chemistry for the immobilization of affinity ligand and chemical stability for immobilization, adsorption, desorption and regeneration of the support itself (Champagne, 2007). Ability to withstand a wide range of thermal, mechanical, chemical and physical conditions and resistance to microbial and enzymatic cleavage are also desired (Tetala & van Beek, 2010). Different substances have been described and employed as affinity matrices to improve the separation and to overcome the limitations of particulate stationary phase.

#### 1.3.1.1 Natural supports

Natural polysaccharides, such as agarose and cellulose, possess highly ionic or carboxylate residues and they require processing before being suitable as affinity supports (Hermanson

et al., 1992). The most commonly used agarose is commercially available as a beaded form, known under the trademark Sepharose (GE Healthcare) and it has been further modified and developed to enhance properties required for an effective chromatographic material. It is an uncharged, hydrophilic matrix with an abundance of hydroxyl groups on the sugar residues which can be easily derivatized for covalent attachment of ligand (Healthcare, 2007). The primary structure of agarose consists of alternating residues of D-galactose and 3-anhydrogalactose. The secondary and tertiary structure forms a fabric with large accessible pores. The knitted porous structure of agarose is knotted at the juncture of the pores with strong hydrogen bonds. The large accessible pore structure of Sepharose yields affinity supports with sufficient capacity when the target molecule or ligand is a large protein or polysaccharide (Hermanson et al., 1992).

#### **1.3.1.2 Synthetic supports**

Synthetic supports, such as acrylamide derivatives, methacrylate derivatives, polystyrene and its derivatives and different membranes are produced by polymerization of functional monomers, to give matrices suitable for affinity-based separations. Commercially available synthetic materials have superior physical and chemical durability and can withstand the process of separation better than the natural soft gels. Synthetic supports are made with monomers that contain primary or secondary hydroxyl groups that maintain hydrophilicity and allow compatibility with most coupling methods (Hermanson et al., 1992).

#### **1.3.1.3 Monolithic supports**

To overcome the difficulties of slow mass transfer in different synthetic supports, a new generation of monolithic solid supports was introduced in affinity chromatography. Monolith is a name for the chromatographic stationary phase consisting of a single piece of highly porous material (Barut et al., 2008). They are prepared in various dimensions with agglomeration-type or fibrous microstructures. In recent years, the polymeric macroporous material, based on radical co-polymerization of glycidyl methacrylate and ethylene glycol dimethacrylate (GMA/EDMA) has been introduced under the trademark CIM® (Convective Interaction Media). There are several reasons for the popularity of monolithic supports in HPLC and affinity chromatography. The main advantage of CIM monolithic systems versus traditional chromatographic supports are much better mass transfer properties, ease of use, the ability to be manufactured with a wide range of pore sizes and shapes, simple scaling up and scaling down, and low back pressure even at very high volumetric flow rates (up to 10 column volumes/min) without the loss of efficiency and capacity (Podgornik & Štrancar, 2005). Monolithic supports can carry many specific ligands for affinity chromatography and the vast potential of monoliths for immobilization of affinity ligands has been recognized, resulting in an increase in experience in applying this technology to bioanalytical and biotechnological activities (Brne et al., 2009; Champagne, 2007; Švec et al., 2003).

#### **1.3.2 Support activation**

Support activation is the first step of ligand immobilization to a chromatographic support. It is a process of chemically modifying the support in a way to form a covalent bond with the ligand of choice. The reaction conditions and the proportion of the reagents will determine the number of ligand molecules that can be attached to the support surface (Wilson & Walker, 2005). The most common activation method for polysaccharide supports is with

Cyanogen Bromide (CNBr). At high pH CNBr introduces cyanate esters and imido carbonates into the matrix by reacting with the endogenous hydroxyl groups (Fig. 3). This activation works also with other synthetic polymers containing hydroxyl groups (Hermanson et al., 1992).

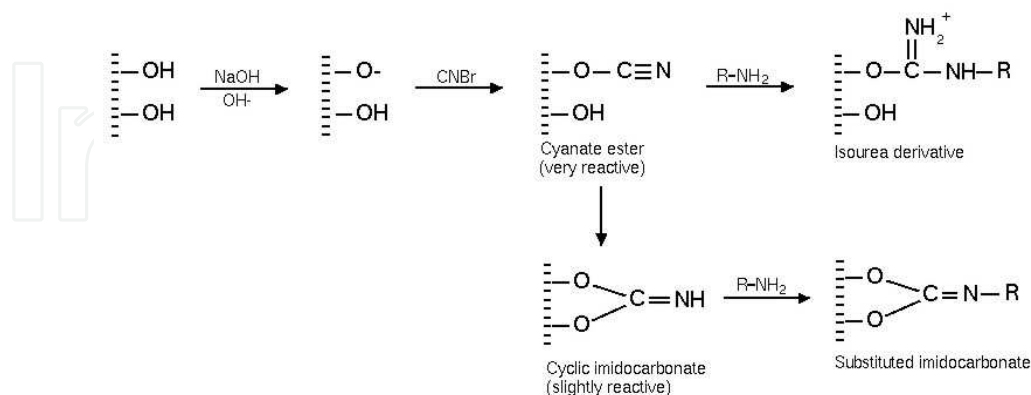


Fig. 3. Mechanism of activation of polysaccharide matrix by CNBr and subsequent coupling of amine-containing ligands (modified from Hermanson et al., 1992).

Alternative coupling procedures involve the use of bis-epoxides, N,N'-disubstituted carbodiimides, sulphonyl chloride, sodium periodate, N-hydroxysuccinimide esters and dichlorotriazines (Wilson & Walker, 2005). There are many different protocols for coupling a ligand to pre-activated supports and, as a consequence, it is easier to adjust the matrix and activation to suit the ligand than to adjust the ligand to suit the matrix and the activation. Moreover, many pre-activated matrices are commercially available.

### 1.3.3 Ligand and ligand immobilization

The ligand is a molecule that binds reversibly to a specific molecule, enabling separation by affinity chromatography. When a suitable ligand is available for the protein of interest, high selectivity, resolution and capacity of affinity chromatography are expected. Successful affinity separation requires a biospecific ligand that can be covalently attached to a solid support and retain its specific binding affinity for the target molecule after unbound material is washed away. Furthermore, the bond between the ligand and the target molecule must be reversible to allow the target molecule to be eluted in an active form. The most commonly used biological ligands are antibodies, antigens, inhibitors, substrates, cofactors and coenzymes, lectins, protein A and protein G, and, among non-biological ligands, tyrazine dyes and metal-chelates (Hermanson et al., 1992; Mallik & Hage, 2006; Turkova, 1993; Wilson & Walker, 2005).

The main criteria for the choice of an affinity ligand for target protein isolation are the functional groups of the ligand. To be immobilized, ligand must possess a functional group that will not be involved in the reversible binding of the ligand to the complementary target molecule, but which can be used to attach the ligand to the stationary support. The most common of such functional groups are  $-\text{NH}_2$ ,  $-\text{COOH}$ ,  $-\text{SH}$  and  $-\text{OH}$  (Wilson & Walker, 2005). In addition, the use of a long spacer arm is indispensable in the case of low-molecular weight affinity ligands, to provide accessibility to the binding site of the target molecule. For effective chromatography, an equilibrium dissociation constant,  $K_D$ , in the range of  $10^{-4}$  to  $10^{-6}$



<sup>8</sup> M in free solution is required for successful separation (Healthcare, 2007; Turkova, 1993). When the dissociation constant is outside this range, altering elution methods may however help to execute successful affinity chromatography (Healthcare, 2007).

Ligands in biospecific chromatography can be classified into two types, monospecific and group specific. Monospecific ligands are those that bind only one molecule; an example is monoclonal antibody to a protein. In this case, a monospecific ligand is expected to bind the target molecules with large association constant, while binding weakly or not at all, to others (Miller, 2005). Group specific ligands have an affinity for a group of related substances rather than for a single type of molecule. The specificity derives from the selectivity of the ligand and the use of selective elution conditions. For example, a lectin can be used for affinity purification of glycoproteins, polysaccharides and glycolipids with the same glyco-signature (Healthcare, 2007; Miller, 2005). The plant lectin isolated from jack bean (*Canavalia ensiformis*) Concanavalin A (ConA) is a routinely used group specific ligand (Healthcare, 2007).

Several approaches have been reported for placing ligands within supports for chromatography. Examples include various covalent immobilization methods, as well as biospecific adsorption, entrapment and cross-linking. Covalent binding is preferred among the other immobilization methods, because it prevents leaking and combines the high selectivity of the reaction with the chemical and mechanical properties of the support (Benčina et al., 2004).

Introducing a spacer arm minimizes the risk of steric interference such as binding between support and target molecule or low accessibility of the ligand. Spacer arms are low-molecular-weight molecules interposed between the ligand and the solid support. They usually consist of linear hydrocarbon chains with functionalities on both ends for easy coupling to the support and ligand. One end of the spacer is immobilized to the matrix using traditional immobilization chemistries, while the other end is connected to the ligand, using a secondary coupling procedure. The result is immobilized ligand that sticks out from the matrix by a distance equal to the length of spacer arm chosen, where the optimal length is up to 10 carbon atoms (Hermanson et al., 1992). In addition, ligands involved in interaction must be sufficiently distant from the solid support to minimize steric interference with the binding processes (Cuatrecasas et al., 1968; Hermanson et al., 1992). With rigid support materials, a spacer molecule may also provide greater flexibility, allowing the immobilized ligand to move into position to establish the correct binding orientation with a protein. Sometimes the chemical structure of a spacer arm is critical for the success of separation. It is also important to consider the hydrophilicity of the spacer molecule, as some spacers are purely hydrophobic, for example methylene groups; others are hydrophilic, possessing carbonyl or imido groups (Wilson & Walker, 2005). Several ready to use supports of agarose, cellulose and polyacrylamide with a variety of spacer arms and pre-attached ligands are commercially available.

#### 1.4 Affinity chromatography for purification of proteases

For the affinity chromatography of proteases different ligands from substrates or their analogues to various synthetic or naturally occurring inhibitors have been immobilized. Appropriate ligands for protease purification can be immobilized enzyme substrates

themselves, but in practice, substrates are immediately converted to product after contact with the target enzyme (Healthcare, 2007; Polanowski et al., 2003). Therefore, the best results in protease purification may be gained by using highly specific inhibitors.

Synthetic inhibitors present effective ligands for many proteases. The synthetic inhibitor para-aminobenzamidine is used as affinity ligand for trypsin, trypsin-like serine proteases and zymogens. Benzamidine-Sepharose is commercially available and frequently used for removing molecules from cell culture supernatants, bacterial lysates or serum (Healthcare, 2007). Bovine basic pancreatic trypsin inhibitor (BPTI), or aprotinin, and inhibitors from legume seeds, such as soybean trypsin inhibitor (SBTI), are frequently employed protein ligands for serine protease isolation (Hewlett, 1990; Polanowski et al., 2003). Group specific inhibitors, that are selective and able to react with a protease from single class, are also effective ligands for specific classes of proteases. Egg white cystatin C coupled to Sepharose allows selective isolation of cysteine proteases from tissue or cell extracts, biological fluids and culture media (Tombaccini et al., 2001). Pepstatin A has been employed as a universal means of purifying aspartic proteases from a variety of sources. An example is isolation of an aspartic protease family from wild growing basidiomycete *Clitocybe nebularis* (Sabotič et al., 2009a).

## 2. Materials and methods

The protease inhibitors from mushrooms, cnispin and macrocypin, were selected as examples of application of fungal protease inhibitors in affinity chromatography for isolation of proteases. Similar protocols can be followed for other protease inhibitors.

### 2.1 Preparation of ligands

Recombinant protein protease inhibitors cnispin from *Clitocybe nebularis* and macrocypin from *Macrolepiota procera* were heterologously expressed in *Escherichia coli* BL21(DE3), refolded from inclusion bodies and purified as previously described (Avanzo et al., 2009; Sabotič et al., 2009b).

### 2.2 Immobilization of ligands to the Sepharose matrix

Recombinant cnispin and macrocypin were immobilized to the CNBr-activated Sepharose (GE Healthcare) according to the manufacturer's recommendations. For each ligand a 15 ml Sepharose column was prepared. The inhibitory activity of the Sepharose immobilized protease inhibitors was confirmed by a trypsin inhibition assay for cnispin and papain inhibition assay for macrocypin as described (Avanzo et al., 2009; Sabotič et al., 2009b).

### 2.3 Immobilization of ligands to monolith supports

Convective Interaction Media (CIM) epoxy and CIM carbonyldiimidazole (CDI) disks with a diameter of 12 mm and thickness of 3 mm, equipped with a special cartridge, were obtained from BIA Separations (Ljubljana, Slovenia). The CIM epoxy monoliths are synthesized from glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) monomers in the presence of pore forming solvents dodecanol and cyclohexanol. The resulting rigid monolith bears epoxy groups. CDI monoliths are prepared from epoxy monoliths hydrolyzed to obtain

hydroxyl group, which are then treated with 1,1'-carbonyldiimidazole (Benčina et al., 2004). Macrocypin was immobilized on CIM monoliths using these two different activation chemistries and, additionally, to a monolith that contained short functional spacers (see 2.3.3).

### 2.3.1 Immobilization on a CIM epoxy disk

Macrocypin was covalently immobilized to the original epoxy groups of the GMA/EDMA material. After conditioning the CIM epoxy disk with 0.5 M phosphate buffer pH 7.0 for 1 h, 2 ml of 2.5 mg/ml macrocypin in the same buffer was applied to the disk with a syringe. The monolithic disk was then incubated for 24 h at pH 7.0 at 45°C with gentle shaking. Afterwards, the monolith was washed for 1 h with water to remove unbound material, and then incubated for 1 h in 0.5 M H<sub>2</sub>SO<sub>4</sub> at 50°C to inactivate the remaining epoxy groups. The disk was then washed with the mobile phase 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0 for 1 h at 1 ml/min flow, and stored in the same buffer at 4°C.

### 2.3.2 Immobilization on a CIM CDI disk

Macrocypin was covalently immobilized to a CIM CDI disk. The disk was first washed with 0.5 M phosphate buffer pH 7 for 1 h, and then permeated with 2 ml of macrocypin at 2.5 mg/ml in the same phosphate buffer. Afterwards, the disk was incubated with macrocypin solution for 24 h at pH 7.0 at 45°C under gentle shaking. The disk was then rinsed with distilled water for 1 h at 1 ml/min flow and stored in 0.5 M phosphate buffer pH 7 at 4°C.

### 2.3.3 Immobilization on CIM disk by a short spacer arm

A CIM ethylene diamine (EDA) disk containing (free) amine groups was first incubated in 50 mM phosphate buffer, pH 8.0 for 1 h and then derivatized with 10% (v/v) glutaraldehyde solution in the same buffer overnight in the dark at room temperature and under gentle shaking. The disk was washed with 50 mM phosphate buffer, pH 8.0 to remove the reagent and then with 0.5 mM phosphate buffer, pH 3.0. To immobilize macrocypin, the derivatized disk was permeated with 1 ml of macrocypin (2.5 mg/ml in 50 mM phosphate buffer pH 3.0) and allowed to react for 24 h in the dark, at room temperature and under gentle shaking. The disk was then washed with 0.5 M phosphate buffer, pH 3.0 for 1 h. To reduce the formed Schiff's bases, the disk was additionally washed with 0.1 M sodium cyanoborohydride in 0.5 M phosphate buffer, pH 8.0, for 2 h. Free remaining groups were end-capped with 1 M monoethanolamine in phosphate buffer (0.5 M, pH 8.0) for 3 h. Finally, the disk was washed with distilled water for 1 h at 1 ml/min flow and stored in 0.01 M phosphate buffer at 4°C.

## 2.4 Protein extracts and enzymes

Crude protein extracts of papain-like cysteine proteases and legumain were prepared from plant (kiwi fruit and germinated bean seeds) and animal (pig kidney cortex) sources, and of the appropriate bacterial source for isolation of serine proteases. The above natural sources were selected based on previous experience and literature data as appropriate sources for target proteases. In addition, partially purified, commercially available bovine serine proteases and purified plant papain were used for characterization of the prepared affinity columns. The extracts were prepared in the appropriate binding buffers for direct application to the affinity chromatography column.

#### 2.4.1 Kiwi fruit

Kiwi fruits (*Actinidia deliciosa*), previously stored at -20°C, were thawed and homogenized in 0.1 M Na acetate buffer pH 6.5 with 0.1 % Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 0.3 M NaCl. Before application to the affinity column the crude protein extract was cleared by centrifugation for 15 min at 16000 g and 4°C.

#### 2.4.2 Germinated bean seeds

Bean seeds (*Phaseolus vulgaris* L.) were germinated aseptically at 28°C in the dark. After 3 days the cuticle was removed and the seeds homogenized at 4°C in 0.1 M Na acetate buffer, pH 6.5 supplemented with 0.5 M NaCl, 1.5 mM EDTA and 2 mM DTT using an ultraturax homogenizer (IKA-Labortechnik). The insoluble material was removed by centrifugation for 30 min at 12000 g and 4°C. Proteins in the supernatant were precipitated by ammonium sulphate at 80% saturation overnight at 4°C. The precipitate was collected by centrifugation (20 min, 16000 g), dissolved in 0.1 M Na acetate buffer, 0.5 M NaCl, pH 6.5, and dialyzed (7 kDa cut-off) against the same buffer. The extract was cleared by centrifugation (30 min, 16000 g) and applied to a Sephacryl S200 column (GE Healthcare) equilibrated with 0.1 M Na acetate, 0.5 M NaCl, pH 6.5 for size exclusion chromatography. Protein containing fractions (determined by measurement of absorbance at 280 nm) were pooled and concentrated by ultrafiltration (UM-10, Amicon).

#### 2.4.3 Pig kidney cortex

Cortical tissue was dissected from pig kidney and homogenized in 0.1 M phosphate buffer, pH 6 supplemented with 0.3 M NaCl, 60 mM EDTA and 15 mM DTT using an ultraturax homogenizer (IKA-Labortechnik). The insoluble material was removed by centrifugation at 4 °C for 20 min at 8000 g and the supernatant cleared by centrifugation for 20 min at 16000 g and 4°C.

#### 2.4.4 *Bacillus subtilis* culture supernatant

*Bacillus subtilis* was cultivated (0.5 % yeast extract, 1 % powdered milk) at 37°C and 220 rpm for 24 h. Bacteria were removed by centrifugation at 7000 g for 20 min at 4°C. Proteins in the culture supernatant were precipitated with ammonium sulphate at 80% saturation overnight at 4°C. After dialysis against 0.1 M Na acetate, 0.3 M NaCl, 1 mM EDTA, pH 6.5, the protein extract was cleared by centrifugation at 16000 g for 10 min at 4°C.

#### 2.4.5 Enzymes

Partially purified bovine trypsin and chymotrypsin mixture (Fluka) was dissolved at 1 mg/ml in 0.1 M Na acetate, 0.3 M NaCl, 1 mM EDTA, pH 6.5. Papain from *Carica papaya* (Sigma) was dissolved at 1 mg/ml in 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0.

### 2.5 Affinity chromatography

#### 2.5.1 Sepharose matrix affinity chromatography

A cnispin-affinity column was used to isolate subtilisin from the *B. subtilis* culture supernatant and trypsin from the partially purified bovine serine protease mixture.



Macrocypin-affinity chromatography was used for isolation of actinidin from kiwi fruit, legumain from germinated bean seeds and cysteine proteases from pig kidney cortex.

The Sepharose column was equilibrated with 10 volumes of the binding buffer appropriate for each protein extract (see 2.4). The protein sample was applied by gravity flow and the unbound proteins washed with the same binding buffer until the absorbance at 280 nm of the effluent approached zero. The bound proteins were eluted by a pH change using first 10 mM HCl and then 10 mM NaOH. Fractions were neutralized with 2 M Tris-HCl, pH 6.5, pooled and concentrated by ultrafiltration (UM-10, Amicon).

For isolation of cysteine proteases from germinated bean seeds and pig kidney cortex, an additional elution step was included before the pH change, namely the increased ionic strength using 0.7 M NaCl in a corresponding binding buffer.

### 2.5.2 CIM monoliths

Chromatographic experiments with papain were carried out with a high performance liquid chromatography system (Knauer). Papain (1 mg/ml in 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0) was pumped through the monolith coupled with macrocypin for 10 min at a flow rate of 1 ml/min. Bound proteins were eluted by a stepwise gradient, using 0.1 M glycine pH 2.0 at a flow rate of 1 ml/min. Fractions were collected in neutralization buffer 2 M Tris-HCl pH 8.0 to maintain papain stability and activity for further analyses.

The binding capacity of immobilized macrocypin was determined by pumping a 1 mg/ml papain solution in 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0 through the monolith for 10 min at a flow rate of 1 ml/min. During this experiment an early breakthrough of the papain was observed. However, when the experiment was continued and more sample was loaded onto the column, a substantial amount of papain was bound and eluted afterwards with 0.1 M glycine pH 2.0 and neutralized. The amount of papain bound was to a certain degree dependent on the amount of sample loaded. Based on this fact, we speculated that papain might exist in at least two different forms – one that is binding to the immobilized macrocypin and one that does not under the selected conditions. To obtain the papain form that binds to macrocypin, the eluted fraction was collected, desalted with PD-10 desalting columns (GE Healthcare) and re-applied to the monolithic disk. In this case, a typical breakthrough curve was obtained – the loaded papain bound to the column until a breakthrough was achieved. After that, the column was washed and the bound papain eluted in the same way as before. The dynamic binding capacity was calculated from this breakthrough curve at 50% breakthrough as previously described (Hage, 2006).

Legumain isolation experiments were performed on a fast protein liquid chromatography (FPLC) system (GE Healthcare). This technique is a type of high performance liquid chromatography, with lower operating pressure (1 – 2 MPa), adjusted for separation of amino acids, peptides and proteins (Wilson & Walker, 2005). The protein sample of germinated bean seeds (200 µl) was loaded onto a CIM epoxy disk, in a solution of 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0 - also used as mobile phase - at 0.2 ml/min. Bound proteins were eluted from disk with 0.1 M glycine, pH 2.0 and neutralized with 2 M Tris-HCl, pH 8.0 to prevent self-degradation.



## 2.6 Protein characterization

### 2.6.1 SDS-PAGE

Eluted proteins were separated on 12 % polyacrylamide gels using Low Molecular Weight (LMW) markers of 14.4 - 97 kDa (GE Healthcare). Proteins were visualized with Coomassie Brilliant Blue R-250 or silver staining, as appropriate, following the standard protocols.

### 2.6.2 Immunoblot analysis

Proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) in a tank transfer system (Bio-Rad). The membrane was blocked for 1h in blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5 % milk powder) and then incubated with anti-recombinant macrocypin serum (dilution 1:10000) overnight at 4°C. After washing at room temperature five times for 15 min in washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween 20) the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Dianova) secondary antibodies (dilution 1:20000) for 1 h at room temperature. The membrane was washed as above and the chemiluminescence detection performed with Lumi-Light<sup>PLUS</sup> (Roche).

### 2.6.3 Protein sequencing

For N-terminal sequence analysis, proteins separated by SDS-PAGE were electro-transferred to an Immobilon-P membrane (Millipore) as described above, and visualized by Coomassie staining. Bands were excised and sequencing was performed on a Procise 492A Automated Sequencing System (Applied Biosystems). Alternatively, bands excised from a Coomassie Brilliant Blue stained SDS-PAGE were, after in-gel digestion with trypsin, analysed by mass spectrometry using electrospray ionization (LC-MS-MS) on MSD Trap XCT Ultra (Agilent).

### 2.6.4 Enzyme activity assays

The substrate Suc-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (Suc-Ala-Ala-Pro-Phe-AMC) (Bachem) was used for the analysis of serine protease activity. Different amounts of samples were mixed with the buffer (0.1 M phosphate buffer, pH 8) and the reaction initiated by the addition of Suc-Ala-Ala-Pro-Phe-AMC substrate (30 µM). After 15 min incubation at 37°C the reaction was stopped by the addition of HCl and product formation monitored on a Luminescence Spectrometer LS30 (Perkin Elmer) using 370 nm for excitation and 460 nm for emission.

The substrate Z-Phe-Arg-AMC (Bachem) was used for measuring papain-like cysteine protease activity in a fluorimetric assay. Different amounts of samples were mixed with the buffer (0.1 M Na phosphate, pH 6.5, 3 mM DTT, 2 mM EDTA) and the reaction initiated with the addition of the substrate to a final concentration of 100 µM and incubated for 10 min at 37 °C. The reaction was stopped with iodoacetic acid and the release of the fluorescent product monitored at 370 nm excitation and 460 nm emission using Luminescence Spectrometer LS30 (Perkin Elmer).

The substrate Z-Ala-Ala-Asn-AMC (Bachem) was used for analysis of legumain activity. Different amounts of samples were mixed with the buffer (0.1 M phosphate-citrate buffer,

pH 5.8, 0.1 % w/v CHAPS, 1 mM EDTA, 3 mM DTT), the reaction was initiated by the addition of the substrate (50  $\mu$ M) and incubated for 15 min at 37°C. The reaction was stopped by the addition of iodoacetic acid and product formation (370/460 nm) monitored on a Luminescence Spectrometer LS30 (Perkin Elmer).

### 2.6.5 Zymogram analysis

Gelatin zymography was performed as described (Sabotič et al., 2007b) using developing solution at pH 8.0 (100 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl<sub>2</sub>) to analyse the general proteolytic activity of serine and cysteine proteases in samples. Following Coomassie Brilliant Blue staining the proteolytic activities are seen as white bands on a dark background.

Native PAGE, followed by detection of proteolytic activity using fluorescent substrate Z-Phe-Arg-AMC, was used for analysis of proteolytic activity of mammalian papain-like cysteine proteases as described (Budič et al., 2009).

## 3. Results and discussion

### 3.1 Cnispin-affinity chromatography for isolation of serine proteases

The serine protease inhibitor cnispin was used for the isolation of secreted proteases of *Bacillus subtilis*, which are mainly of the serine catalytic type. Bacterial secreted alkaline proteases are an indispensable detergent additive (Rao et al., 1998). Since their expression increases during the stationary growth phase (Priest, 1977), proteolytic activity in the culture supernatant was monitored for 24 h (Fig. 4a) to determine the optimal time of harvest. The culture supernatant after 24 h incubation that showed the highest proteolytic activity was used for precipitation of secreted proteins. They were applied to cnispin-affinity chromatography and unbound proteins washed off, followed by elution of the bound proteins by a change in pH (from pH 6.5 during application to pH 2 for elution). SDS-PAGE analysis revealed three bands in the pooled eluted fractions (23, 24 and 27 kDa), which also showed proteolytic activity on a gelatin zymogram (Fig. 4b). Furthermore, the specific activity against the chromogenic substrate Suc-Ala-Ala-Pro-Phe-AMC increased in the bound fractions relative to the unbound and applied sample (Fig. 4c). This activity was abolished by chymostatin, a broad-range inhibitor of serine proteases of families S1 (e.g. trypsin and chymotrypsin) and S8 (e.g. subtilisin).

Commercially obtained partially purified bovine trypsin mixture was further purified using the cnispin-affinity chromatography. Highly purified trypsin is for example used in protein digestion preceding mass spectrometry analyses. Cnispin is a very strong inhibitor of trypsin and a weak inhibitor of chymotrypsin, therefore trypsin purification was expected. The trypsin mixture (1 mg/ml) was applied to the column and bound proteins were eluted by lowering the pH. SDS-PAGE analysis revealed that a less contaminated trypsin was obtained with this additional step, seen as a predominant band at 23 kDa, which corresponds to the theoretical molecular mass of the bovine trypsin (Fig. 5).

Although cnispin affinity chromatography was successful in purifying serine proteases from the bacterial culture supernatant and the high trypsin specificity of cnispin enabled purification of trypsin from a mixture of similar enzymes, the amount of isolated proteases

was very small. This indicates either a low capacity for protease binding to the immobilized inhibitor or ineffective elution of the protease from it and makes the described cnispin-affinity chromatography useful mainly for laboratory or analytical scale experiments.

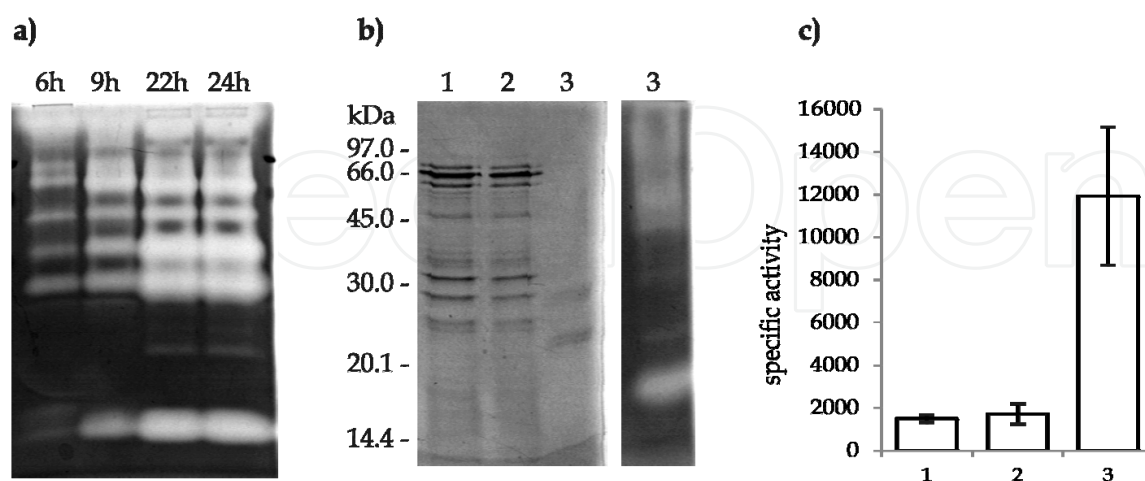


Fig. 4. Purification of secreted *B. subtilis* proteases by cnispin-affinity chromatography. (a) Gelatin zymogram of the *B. subtilis* culture supernatant collected at different times after inoculation. (b) SDS-PAGE (left) and gelatin zymogram (right) analysis of serine proteases isolated from the *B. subtilis* culture supernatant by cnispin-affinity chromatography. Lane 1, the applied precipitated secreted proteins; lane 2, unbound proteins; lane 3, eluted bound proteins. (c) Specific activity with standard deviation measured against the subtilisin substrate Suc-Ala-Ala-Pro-Phe-AMC. Sample numbers correspond to lanes in panel (b).

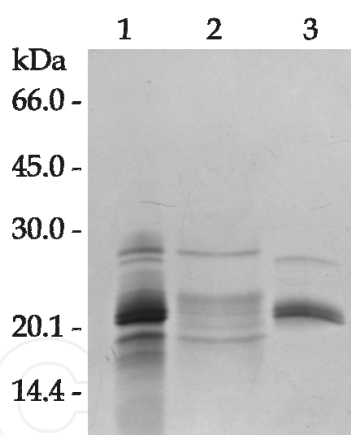


Fig. 5. Trypsin purification with cnispin-affinity chromatography. SDS-PAGE analysis of the trypsin mixture further purified by cnispin-affinity chromatography. Lane 1, the applied trypsin mixture; lane 2, unbound proteins; lane 3, eluted bound proteins.

### 3.2 Macrocypin-affinity chromatography for isolation of cysteine proteases

The cysteine protease inhibitor macrocypin 1 from *Macrolepiota procera* inhibits two families of cysteine proteases, utilizing different reactive loops (Fig. 1), namely papain-like proteases (family C1) and legumain (family C13). To determine whether macrocypin-affinity chromatography would be applicable for isolation of these proteases from plant and animal sources, we tested kiwi fruits as a source of papain-like protease actinidin, germinated bean

seeds as a source of plant legumain and pig kidney cortex as a source of both papain-like cysteine proteases and legumain.

Actinidin (also called actinidain) is a papain-like cysteine protease abundant in kiwi fruits (*Actinidia deliciosa*). Several applications of actinidin have been considered, such as in meat tenderization (Aminlari et al., 2009), in cheese making (Lo Piero et al., 2011) and for isolation of various cell types from human and animal tissues (Mostafaie et al., 2008).

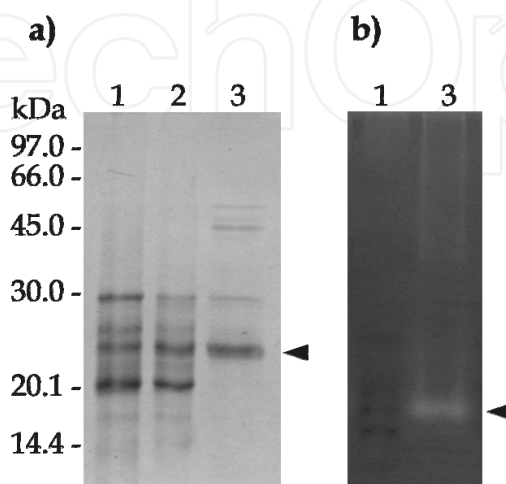


Fig. 6. Partial purification of actinidin from kiwi fruit using macrocypin-affinity chromatography. SDS-PAGE (a) and gelatine zymogram (b) analysis of actinidin purification by macrocypin affinity chromatography. Lane 1, kiwi fruit extract; lane 2, unbound proteins; lane 3, eluted bound proteins.

Macrocypin-affinity chromatography was used as a one-step purification procedure for actinidin from crude protein extracts of kiwi fruits, where a change in pH (from pH 6.5 during application to pH 11 for elution) was used for elution of bound proteins. SDS-PAGE analysis revealed a partial purification of actinidin, as a 24 kDa band corresponding to a mature actinidin was predominant (Fig. 6a). The presence of mature actinidin in the 24 kDa band was confirmed by N-terminal sequencing ( $\text{H}_2\text{N-LPSYVDW RSA}$ ) and zymogram analysis confirmed its proteolytic activity (Fig. 6b).

Legumain-like proteases (also called vacuolar-processing enzymes) are abundant in seeds of leguminous plants and, in kidney bean seeds (*Phaseolus vulgaris*) legumain performs controlled hydrolysis of storage protein phaseolin during and after germination (Senyuk et al., 1998). A protein extract of germinating bean seeds was first purified by size exclusion chromatography and then applied to the macrocypin-affinity chromatography. Three different means of elution were performed to determine the optimal purification procedure, namely higher ionic strength (0.7 M NaCl in binding buffer), and elution by low pH (10 mM HCl) and high pH (10 mM NaOH). Purification of legumain was achieved and confirmed by mass spectrometry, where it was present in the 48 kDa band (Fig. 7a, arrow) that corresponds to the mature enzyme, in addition to phaseolin (47.5 kDa), which was probably retained on the column because of its abundance in the extract. The activity, measured against the legumain substrate Suc-Ala-Ala-Asn-AMC, in the applied sample, flow-through and eluted fractions confirmed the purification of legumain with elution by lowering the buffer pH (Fig. 7b). The change of ionic strength was not sufficient to elute the protease, while high pH causes its denaturation.

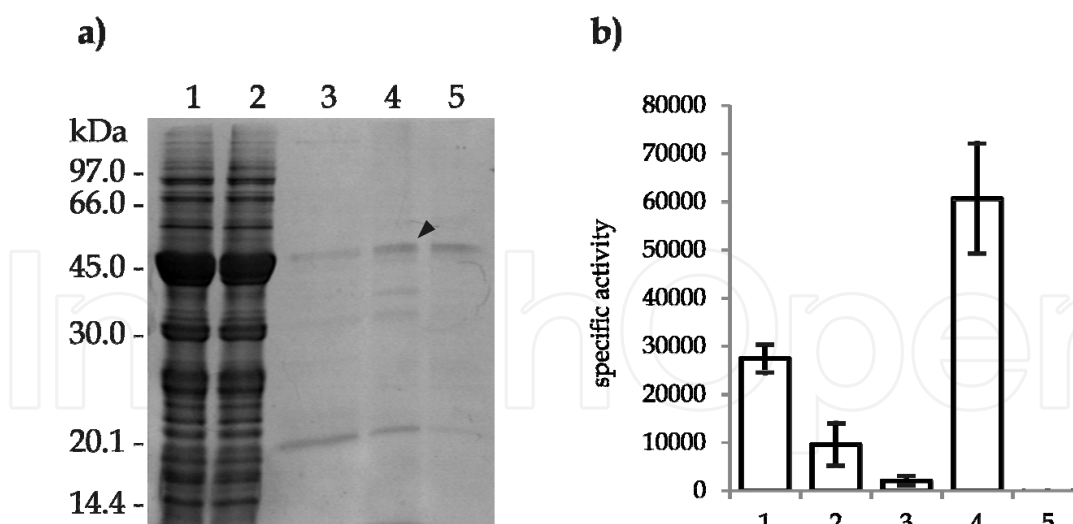


Fig. 7. Legumain purification from germinated bean seeds. (a) SDS-PAGE analysis of the macrocypin-affinity chromatography process. Lane 1, the applied crude protein extract; lane 2, unbound proteins; lane 3, bound proteins eluted by high ionic strength (0.7 M NaCl); lane 4, bound proteins eluted by low pH (10 mM HCl); lane 5 bound proteins eluted by high pH (10 mM NaOH). (b) Specific activity with standard deviation measured against the legumain specific substrate Z-Ala-Ala-Asn-AMC. Sample numbers correspond to lanes in panel (a).

Since isolation of plant legumain was successful with the macrocypin-affinity column, we wanted to confirm its applicability for animal legumain as well. Mammalian legumain is most abundant in kidneys (Chen et al., 1997), therefore, pig kidney cortex was used as a source material. Since many papain-like proteases are also present in mammalian tissues, their co-purification on the macrocypin-affinity column was expected. Two versions of the elution procedure were employed. First, for purification of papain-like proteases, the bound proteins were eluted (experiment A) by a change of pH only (from pH 6.0 in the application buffer to pH 12 in elution). Secondly, for purification of legumain and papain-like proteases (experiment B), elution with higher ionic strength (0.7 M NaCl in application buffer) was followed by low (10 mM HCl) and high pH (10 mM NaOH) elution. Papain-like proteases were isolated by both procedures, as seen by zymogram analysis (Fig. 8, lanes A3 and B4), however their specific activity was not increased (Fig. 8c, white bars). On the contrary, legumain isolation was, as with plant legumain, achieved optimally by elution with low pH, resulting in higher specific activity (Fig. 8c, black bars). However, as seen in the SDS-PAGE analysis (Fig. 8a), further purification steps would be required to obtain pure enzyme.

We have shown the applicability of macrocypin-affinity chromatography for isolation of cysteine proteases from plant and animal sources. The affinity chromatography purification procedure would have to be complemented by one other purification step to obtain a purified protein. Furthermore, the capacity of the macrocypin-affinity column was very low, possibly due to steric hindrance to protease binding to the immobilized inhibitor, making the protease inhibitor immobilized to the Sepharose matrix applicable only on the laboratory or analytical scale. Both of these issues were addressed by development of macrocypin-affinity chromatography on monolithic support.



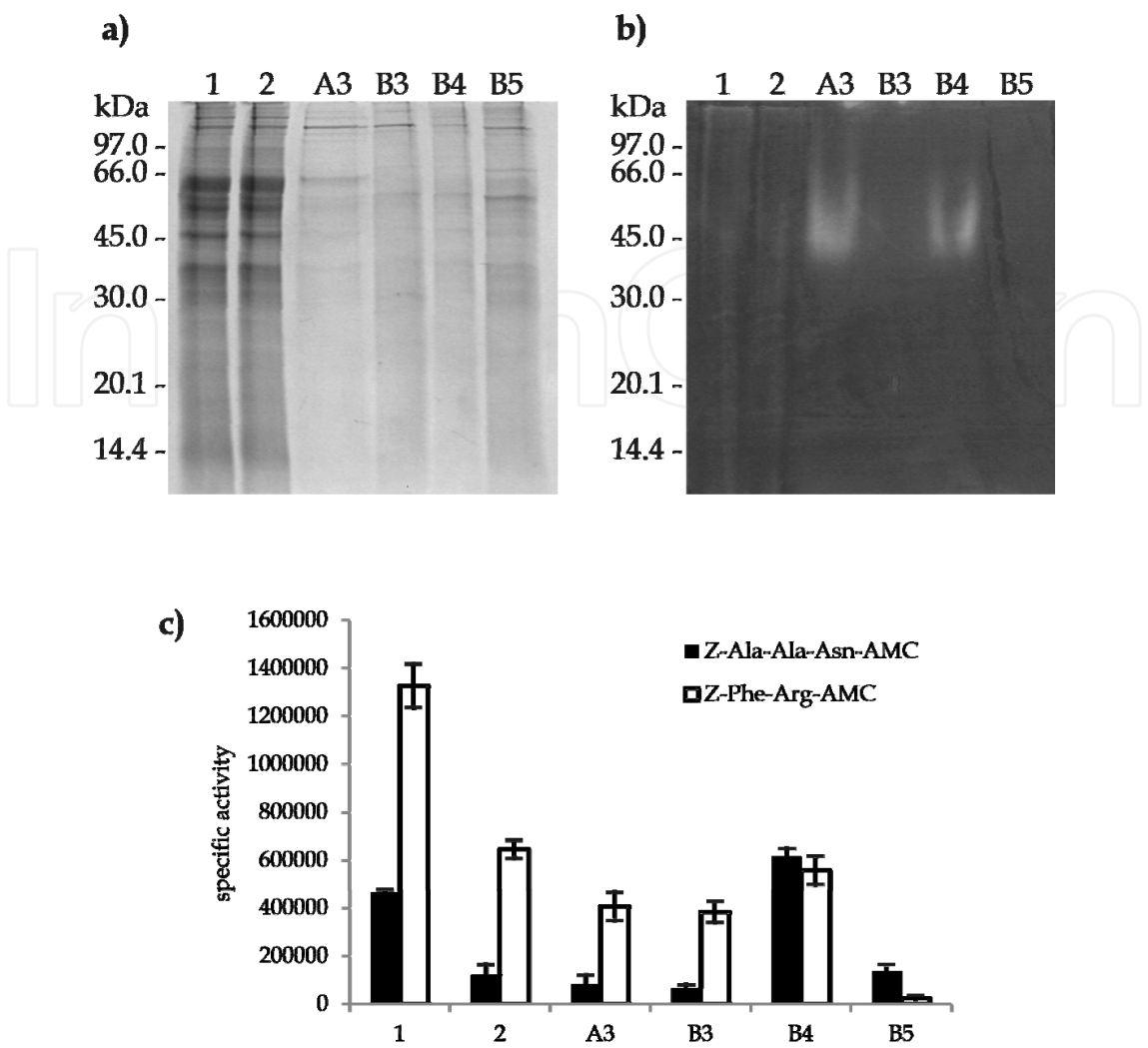


Fig. 8. Purification of cysteine proteases from pig kidney cortex using macrocypin-affinity chromatography. (a) SDS-PAGE analysis of applied, washed and eluted fractions: lane 1, the applied crude protein extract; lane 2, unbound proteins; lane A3, bound proteins eluted by high pH in experiment A; lane B3, bound proteins eluted by high ionic strength (0.7 M NaCl); lane B4, bound proteins eluted by low pH (10 mM HCl); and lane B5 bound proteins eluted by high pH (10 mM NaOH) in experiment B. (b) Zymogram analysis with the substrate Z-Phe-Arg-AMC for detection of papain-like cysteine protease activities in applied, washed and eluted fractions. Lane numbers correspond to those in panel (a). (c) Specific activity with standard deviation measured against the legumain specific substrate Z-Ala-Ala-Asn-AMC and papain-like protease substrate Z-Phe-Arg-AMC. Sample numbers correspond to lanes in panels (a) and (b).

### 3.3 Optimization of macrocypin-affinity chromatography on monolithic disk support

Three monolithic disks (CIM epoxy, CIM CDI and CIM with glutaraldehyde spacer arm) were used as supports for macrocypin immobilization (Fig. 9). Covalent binding of macrocypin to solid monolithic support was verified with immunoblot analysis (Fig. 10b). Results indicated that macrocypin immobilization was stable and macrocypin was not leaking from the support during experiments in all three versions of prepared disks.

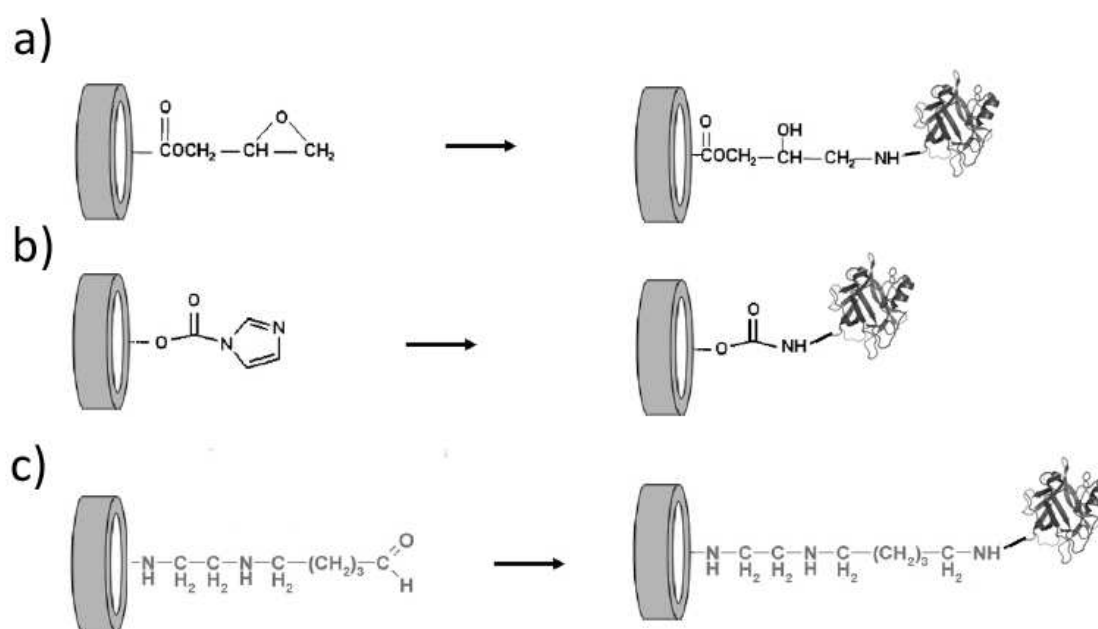


Fig. 9. Macrocypin immobilization on a: (a) CIM epoxy disk; (b) CIM CDI disk; (c) CIM with glutaraldehyde spacer arm.

### 3.3.1 Immobilization on CIM epoxy disk

The majority of the immobilizations on glycidyl methacrylate monolith supports were performed via epoxy groups, since they are formed *in situ* during the polymerization process and thus readily available for chemical modification. Macrocypin was immobilized on a CIM epoxy disk (Fig. 9a) as described in 2.3.1. Macrocypin solution was syringed through the disk to completely fill all the monolith pores. pH 7.0 was used as the optimal pH for coupling reaction between the epoxy groups and amino residues of macrocypin. A solution of 0.5 M  $\text{H}_2\text{SO}_4$  at  $50^\circ\text{C}$  was employed for end-capping the remaining free epoxy groups and preventing side reactions. To analyze the CIM epoxy macrocypin affinity disk, 10 mg of crude papain solution was applied at 1 ml/min and eluted at lower pH. SDS-PAGE analysis (Fig. 10a) of eluted samples revealed more concentrated papain (23.4 kDa) in flow-through (Fig. 10a, lane C2) than from elution (Fig. 10a, lane C3).

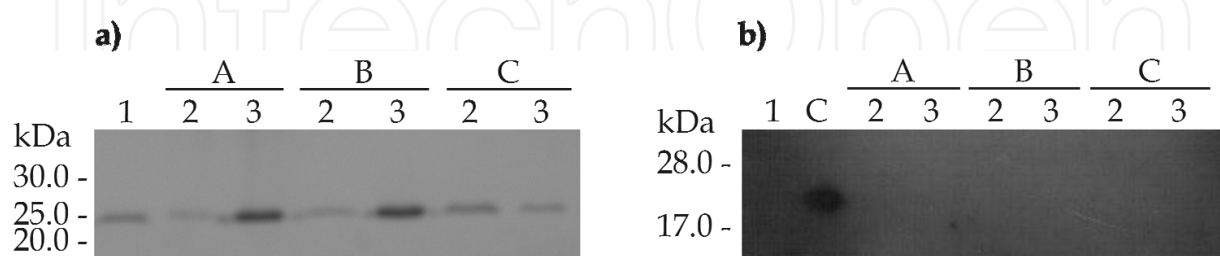


Fig. 10. Papain purification with monolith macrocypin-affinity chromatography. (a) SDS-PAGE analysis of papain purification on (A) CIM disk with glutaraldehyde spacer arm, (B) CIM CDI disk and (C) CIM epoxy disk; lane 1, the applied papain; lane 2, unbound papain; lane 3, eluted bound papain. (b) Immunoblot analysis of the same samples as in panel (a); polyclonal anti-macrocypin antibodies were used and immunoreactive bands visualized by chemiluminescent detection; lane C, macrocypin as positive control.

Although this method of immobilization was easily achieved, the active site of macrocypin was probably not accessible to the binding sites on papain, therefore macrocypin immobilization to CIM epoxy disk did not demonstrate the desired characteristics. Chemical modification of the epoxy groups to imidazole carbamate groups is one alternative to overcome the limitations of the epoxy method, so a macrocypin CIM CDI disk was prepared.

### 3.3.2 Immobilization on a CIM CDI disk

Imidazole carbamate groups that react with N-nucleophiles give an N-alkyl carbamate linkage, resulting from the reaction between hydroxyl groups obtained by hydrolysis of epoxy groups and 1,1'-carbonyldiimidazole (Hermanson et al., 1992). This activated support was used in macrocypin immobilization by means of nucleophilic substitution between the activated sites and primary amines on the protein, resulting in a stable amide linkage (Fig. 9b). This method is faster than the epoxy method and involves fewer steps (Mallik & Hage, 2006). In addition, the remaining free imidazole groups of the supports are rapidly self-deactivated after the immobilization process in aqueous solution, forming the original hydroxyl groups and releasing CO<sub>2</sub> and imidazole (Benčina et al., 2004; Nicoli et al., 2008).

To assess the performance of the CIM CDI macrocypin affinity disk, 10 mg of papain solution was loaded in 10 min to the disk and bound papain was eluted at low pH. SDS-PAGE analysis (Fig. 10a, lanes B) revealed better separation than with the CIM epoxy disk for the same amount of papain.

The CIM CDI disk demonstrated sufficient accessibility for protease to immobilized inhibitor, probably due to different steric orientation of the macrocypin on the support.

### 3.3.3 Immobilization on a CIM disk with spacer arm

Even better accessibility for the protease active site was achieved with the introduction of a spacer arm, which removes the inhibitor from the solid phase surface and minimizes steric interference during binding (Cuatrecasas, 1970). Polymer aldehyde groups formed after activation allow fast covalent binding of amino group bearing ligands under mild conditions, with elimination of water as the only side product (Ponomareva et al., 2010). The glutaraldehyde spacer (Fig. 9c) presumably provides greater flexibility, allowing the macrocypin to move into the right position to establish the correct binding orientation with protein. Affinity chromatography with 10 mg of papain solution was accomplished in 10 min and bound papain was eluted by lowering the pH. Compared with CDI and the epoxy immobilized disk, glutaraldehyde spacer revealed superior properties for papain separation, as seen on SDS-PAGE analysis (Fig. 10a, lanes A).

Macrocypin was also covalently immobilized on CIM disks with three different spacer arms: Ethylenediamine, 1,6-diaminohexane, and 1,4-butanediol diglycidyl ether. However, improved binding characteristics for papain were not observed (not shown).

### 3.4 Determination of binding capacities of CIM disks

The binding capacity of papain to macrocypin immobilized via glutaraldehyde and imidazole carbamate groups was found to be higher than to macrocypin immobilized via epoxy groups. Dynamic binding capacities were determined by measuring the

breakthrough curve (Fig. 11). For CIM epoxy disk, the capacity was 0.34 mg/ml, for CIM CDI disk 5.1 mg/ml and for CIM disk with glutaraldehyde spacer arm 9.2 mg/ml.

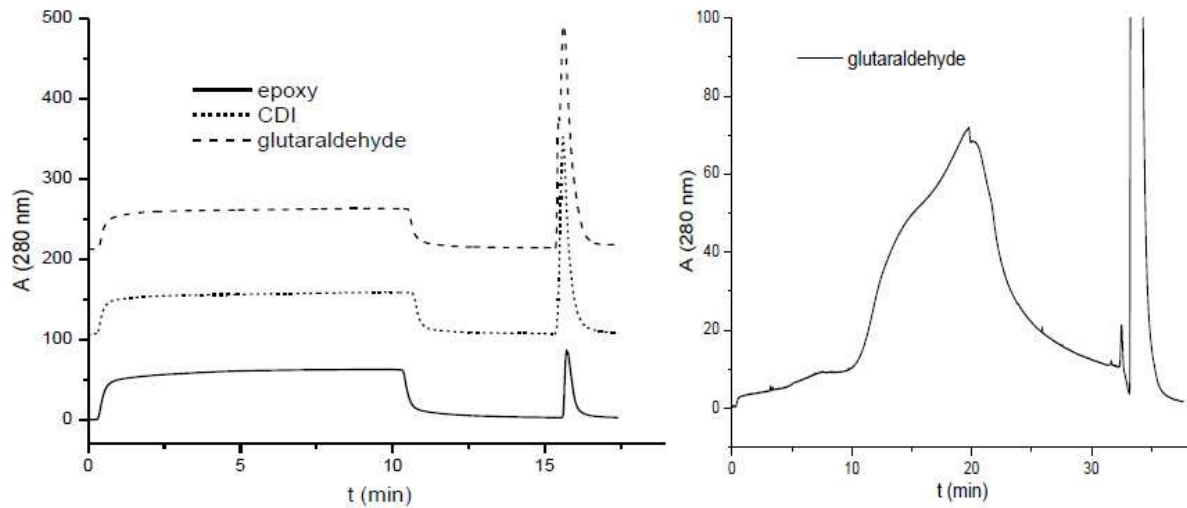


Fig. 11. Comparison of papain separations on CIM epoxy, CIM CDI and CIM with glutaraldehyde spacer arm (left). Chromatographic conditions: flow rate 1 ml/min; concentration of papain 1 mg/ml in 0.1 M Tris-HCl, 0.6 M NaCl, pH 7.0; elution with 0.1 M glycine pH 2.0; detection wavelength 280 nm. Eluted bound proteins were collected together and applied again to the disks to determine the 50% breakthrough. (right) An example of the breakthrough curve with papain bound to the CIM disk with macrocypin immobilized via a glutaraldehyde spacer arm.

**3.5 Monolith macrocypin-affinity chromatography for isolation of plant cysteine protease**

To explore the possibility of wider applications of macrocypin affinity disks, legumain from a crude protein extract of germinated bean seeds (*Phaseolus vulgaris*) was subjected to purification.

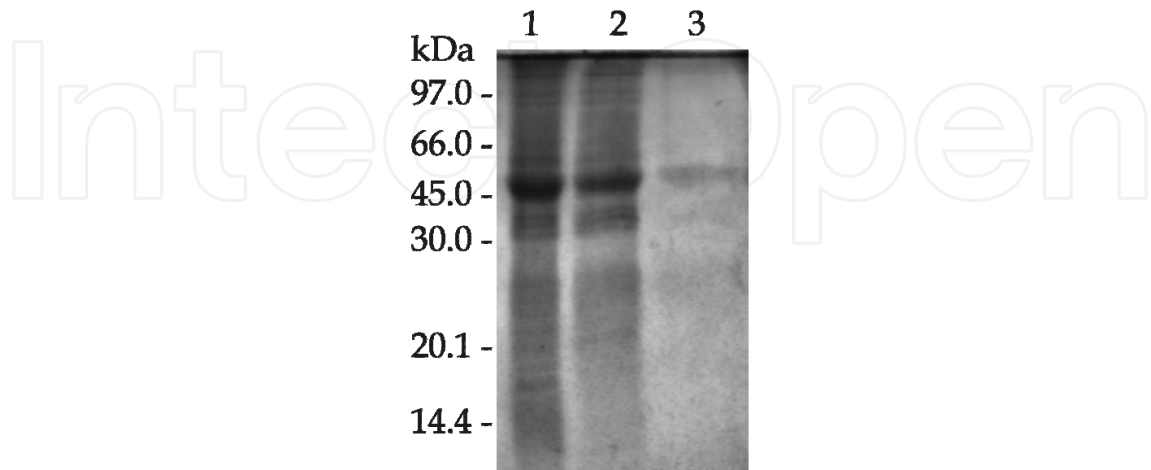


Fig. 12. SDS-PAGE analysis of legumain purification from germinated bean seeds with a CIM epoxy monolith disk. Lane 1, the applied crude protein extract; lane 2, unbound proteins; lane 3, bound proteins eluted with 0.1 M glycine, pH 2.0.

According to the experiences with plant and animal legumain isolation, a similar experiment was performed with a macrocypin CIM epoxy disk. The protein sample of germinated bean seeds was loaded onto a CIM epoxy disk and bound proteins were eluted by lowering the pH. Separation of legumain was confirmed by SDS-PAGE analysis, where a band at 48 kDa is visible (Fig. 12, lane 3). Isolation of proteolytically active legumain (determined by hydrolysis of the legumain specific substrate Z-Ala-Ala-Asn-AMC) using macrocypin immobilized to the monolith support confirms the latter's applicability for separation of cognate proteases from complex protein mixtures.

#### 4. Conclusion

Protein protease inhibitors from mushrooms bound to solid matrices proved to be a useful tool for isolation of proteases from various natural sources. Protease inhibitors from higher fungi offer inhibitory patterns different from those from other sources, together with superior characteristics as affinity chromatography ligands, in terms of pH and temperature stability. They withstand the harsh conditions during immobilization procedures and retain their inhibitory activity through several elution cycles of extreme pH changes.

In most cases, the ligands of interest are immobilized onto conventional particle based chromatographic supports. This represents a widely used and well established technique used for selective isolation and purification of proteases. The Sepharose immobilized inhibitors were effective in isolating several different target proteases from various sources, however the method is applicable only on the analytical or laboratory scale. The well-known drawbacks of this type of matrix are their low intrinsic velocity of operation and mass transfer limitations. These can be effectively overcome by using monolith supports. Monolith supports are characterized by an open pore structure where the mass transfer between the mobile and the stationary phases is greatly enhanced by the convective flow. In this work, the monolith support with immobilized macrocypin provided a convenient approach for isolation of various target proteases. Selectivity of the columns was tested by SDS-PAGE and the best results were obtained with a glutaraldehyde spacer arm, indicating that steric hindrance was one of the reasons for low efficacy of the other systems tested. Thus the steric interference of analyte binding to the immobilized ligand is important to consider when designing an affinity column or disk. In conclusion, the CIM disk with macrocypin immobilized through the glutaraldehyde spacer arm could be used for rapid and effective purification of cysteine proteases from various sources.

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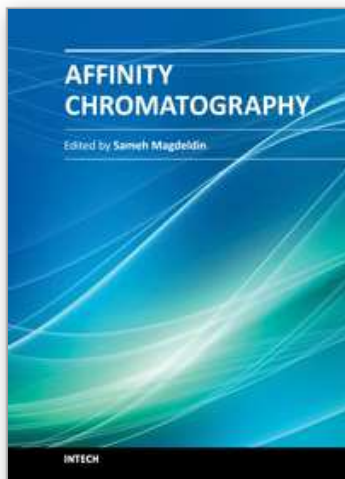
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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific 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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