Trends in Chromatography
Vol. 6, 2010

Mini-Review

Protein purification by affinity chromatography with peptide ligands selected from the screening of combinatorial libraries

Silvia A. Camperi[1], Mariela M. Marani[1], María C. Martínez-Ceron[1], Fernando Albericio[2,3,4], and Osvaldo Cascone[1,*]

[1]Cátedra de Microbiología Industrial y Biotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Ciudad Autónoma de Buenos Aires, Argentina. [2]Institute for Research in Biomedicine, [3]CIBER-BBN, Networking Centre on Bioengineering Biomaterials and Nanomedicine, Barcelona Science Park, University of Barcelona, Baldiri Reixac 10, 08028-Barcelona, [4]Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1, 08028-Barcelona, Spain

ABSTRACT

Affinity chromatography is likely to play an ever-increasing important role in protein purification as it is the most effective method for the direct isolation and purification of biomolecules from complex mixtures. Successful separation by affinity chromatography requires the availability of a selective ligand. Short peptides are excellent ligands for affinity separations as they have higher selectivity than dyes and metals, they are more stable than antibodies to elution and cleaning conditions, and they are not likely to cause an immune response in case of leakage into the product. Furthermore, the combinatorial synthesis of peptide libraries allows obtaining millions of peptides, thus greatly facilitating the discovery of suitable affinity ligands for any given protein of interest. After screening of the library the peptides with affinity for the target protein can be identified, typically by Edman microsequencing or mass spectrometry in the case of synthetic libraries, or by DNA sequencing in the case of biological libraries. Numerous proteins have been purified in only one step with chromatographic matrices made of peptide-ligands selected from the screening of combinatorial libraries, attached to different supports.

KEYWORDS: phage display, one-bead-one-compound, split–mix–split, divide-couple-recombine

ABBREVIATIONS

Ab, antibody; AC, affinity chromatography; anti-GMCSF, anti-granulocyte macrophage-colony stimulating factor; ELISA, enzyme-linked immunosorbent-assay; FDA, Food and Drug Administration; GMP, good manufacturing practices; HMBA 4-hydroxymethylbenzoic acid; Ig, immunoglobulin; mAb, monoclonal antibody; MALDI-TOF-MS, matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry; OBOC, one-bead-one-compound; pAbs, polyclonal antibodies; SEB, staphylococcal enterotoxin B; uPAR, plasminogen activator receptor; vWF, von Willebrand Factor

INTRODUCTION

This mini-review is mainly focused on the library preparation and screening, ligand peptide identification and application to protein purification by affinity chromatography (AC). The process from the construction of a library to the purification of a protein by AC with a peptide ligand is reviewed.

*Corresponding author
ocasco@ffyb.uba.ar

Affinity chromatography in protein purification

The use of affinity for purification of proteins through chromatography was first described in the late 60s [1]. AC relies on the use of an affinity ligand coupled to a support to allow specific capture of the product from a complex mixture. Due to the very selective interaction between the immobilised ligand and the target protein, an essentially pure product is usually obtained in a single operation. So, AC eliminates steps, increases yield and eases the experimental procedures, hence it is ideally suited for purification of biomolecules, especially therapeutic proteins [2].

Many of the current affinity adsorbents are based on natural biological ligands and the most widely utilised affinity columns are prepared by exploiting the antibody-antigen interaction (immunoaffinity chromatography) [3]. Antibodies (Abs) are immobilised on a solid support and their great affinity for a specific molecule allows them to retain the analyte. Although offering defined selectivity, this kind of affinity matrices is rather fragile, expensive and not readily amenable to be scaled up and endure the harsh manufacturing of the pharmaceutical industry. Production by hybridoma cell cultures and subsequent purification of the monoclonal Abs (mAbs) is a lengthy and costly process, and the purity and activity of the Abs may vary. Moreover, Abs usually have very high binding affinity, which may require harsh elution conditions to disrupt the complexes formed with their respective ligands, this leading to the leakage of the Abs from a column, shortening column lifetime and resulting in serious product contamination due to the immunogenicity of those proteins. Protein ligands, such as Abs, make the procedures for obtaining regulatory approval of an industrial scale purification of therapeutic proteins lengthy [4, 5].

Triazinic dyes [6] and immobilised transition metal ions [7] have been extensively utilised to obtain pseudo-biospecific ligand AC matrices due to their low cost, availability, simple immobilisation reaction, and biological and chemical degradation resistance. However, their selectivity is not always as high as desired.

Peptides as affinity ligands

Small peptides consisting of a few amino acids represent promising affinity ligand candidates for industrial separations [8]. Peptide ligands are much more physically and chemically stable than Abs ligands and very resistant against proteolytic cleavage. They can be readily synthesised in bulk quantities at a lower cost under good manufacturing practices (GMP) by standard chemistry with the advantage of more than 30 years experience in peptide synthesis. Also, peptides may be easily modified by existing chemical methods to facilitate product elution under mild conditions. Furthermore, a site-directed immobilisation is possible, a high ligand density can be achieved and the matrices are more robust during elution and regeneration as compared to protein-based affinity matrices such as mAbs. Moreover, peptides hardly cause poisoning and immune responses compared with, for instance, proteins, dyes or transition metal ion ligands in case of leakage into a product [9]. Even if any leakage occurs, small peptide molecules can be easily removed from a macromolecular product.

Combinatorial peptide libraries

The synthesis of a peptide library from which we can select a ligand with the best recognition properties is the method of choice for the development of a peptide with high affinity and specificity for a target protein. The advantage offered by the combinatorial approach is the possibility of screening a great number of variables and combinations, increasing the probability of obtaining a suitable peptide ligand. Binding molecules from first generation libraries can be used as parental scaffolds to produce additional development.

There are different ways to generate peptide libraries: the genetic and the synthetic approach. The synthetic approach offers the additional option to include structural elements other than the natural L-amino acids.

Genetic approach

Genetic engineering allows modification of bacterial and bacteriophage genes, which code for surface proteins, enabling display of random peptides on the surface of these microbial vectors. Biologic peptide libraries thus formed are used for high-throughput screening of clones bearing peptides with high affinity for target proteins. There are many reports of successful affinity

selections performed with phage display libraries and substantially fewer cases describing the use of bacterial display systems.

Phage display libraries

The phage display method was introduced in 1988 by Parmley and Smith [10]. They developed a library of peptides by inserting foreign DNA into a mixture of phage clones, each exposing a peptide sequence on the virion surface. This mixture was faced with minute amounts of an antibody of interest to select the phages that expose peptides determinant that have affinity from a 10^8-fold excess of phage not bearing the determinant. These methods enable one to easily and rapidly generate and screen libraries with millions to billions of random peptides. A lot of works described the use of these libraries to select peptides binding to proteins [11, 12], mAbs [13-15] and polyclonal Abs (pAbs) and to cell surfaces [16].

Epitope library construction consist of transformed fusion phages in *Escherichia coli* through oligodesoxi-nucleotides $(KKN)^n$ of different length-were n corresponds to the number of triplets, K to a mixture of all four desoxinucleotides and N to a mixture of guanidine and thymidine. Every triplet is a mixture of 32 triplets that include codons for all of the 20 natural amino acids but only for one stop codon. That means 32^n different molecular species collectively encoding all 20^n possible n-residue peptides. Fusion phages m13 [11, 17], f1 [18] and fd [14] are mostly used. The multiple protein production takes place mainly in one of the minor coat protein called pIII [11, 14] or in the major coated pVIII protein [19]. Those proteins are coded by the phage and they are expressed at the tip (pIII) or in several thousand copies at the surface (pVIII) of the virion with little effect on phage function [10, 20]. In general, foreign peptides are fused to regions of pVIII and pIII that are known to be exposed to the exterior: the N-terminus of pVIII (type 8 vector) and the N-terminus and middle of pIII (type 3 vector) [10, 21, 22]. However, when a relatively large foreign peptide is displayed, mosaic particles can be made where two types of pIII (type 33 and 3+3 vector) or pVIII (type 88 and 8+8 vector) particles are exposed in the phage surface [19, 23, 24], one with the foreign peptide and the other one in the wild-type form. That is made to maintain the infective properties of the virion.

Although the most common type of phage-display constructs are "random" peptide libraries there are constructs that display all or part of natural peptide or protein domains. In the first case, the DNA inserts are derived from "degenerate" oligonucleotides, which are synthesised chemically by adding mixtures of nucleotides to a growing nucleotide chain. In the second case different types of libraries exist: "genomic libraries", were the inserts are fragments of total chromosomal DNA (genome) [25, 26]; "cDNA libraries", were the insert are DNA copies of messenger RNAs [27, 28] or libraries were the phage display all or part of a specific peptide or protein domain [10, 20, 29-36]. In those kinds of libraries while some positions are fixed others can be "randomised" in order to create libraries of sequence variants, to select new clones with enhanced function or clones in which the displayed domain has acquired a new function [37-39].

The biological library method provides a large number of peptide entities to be screened, and longer peptides can be constructed easier than with synthetic methods. Usually, a random peptide library has about 10^8-10^9 phage clones and there are also libraries up to 10^{10} clones [37, 40]. In different works libraries of 4-mer up to 40-mer peptides were constructed [41]. For an exhaustive description of different biological libraries see the review by Smith and Petrenko [40]. Furthermore, the phage display method can take advantage of known protein folds (e.g., alpha helix fold, beta-sheet fold, immunoglobulin fold, zinc-finger fold, or conotoxin fold) by grafting random oligopeptides on such tertiary folds [42-44]. The big disadvantage is the limitation on the chemical nature of the libraries that only includes natural compounds and incorporation of unnatural amino acids or other organic building blocks into these libraries is not feasible. Therefore, this kind of combinatorial libraries has less diversity than synthetic libraries.

The screening consists in the selection, from a very large initial library, of a tiny fraction of phage displayed peptides with affinity for the target studied. Usually, it can be performed in two different ways. The target is immobilised and incubated with the library or it is pre-incubated

with the library prior to capture on a solid support. In the first case surface supports polystyrene dishes [22], impermeable plastic beads [45], nitrocellulose membranes [46], paramagnetic beads [21] or permeable beaded agarose gels [47] can be used as solid supports to which target are immobilised, this is called AC selection. Targets can be attached directly to the solid support by chemical coupling [45, 47] or by non-covalent adsorption to a hydrophobic plastic surface [22]. When the target is pre-incubated with the library, phage expressing peptides with affinity for the target could be isolated by a method known as panning, described by Parmley and Smith [10]. The target molecules can be biotinylated prior to incubate them with the library, and then allowed to bind to a surface that has already been coated with avidin or streptavidin, allowing the super strong biotin-avidin or biotin-streptavidin bond [10, 37]. If the target cannot be biotinylated, alternatively, the target with the phage displayed affinity peptides could be selected - after interacting with the library - by an Ab biotinylated (or a second Ab biotinylated in the case of mAbs or pAbs used as targets). After the screening, non interacting phage displayed peptides are washed away and then the interacting phages are eluted specifically or non-specifically.

Another form to screen was proposed by Matthews and Wells [48] to select protease substrates. In the library, a randomised amino acid sequence is fused to a peptide or protein domain with high affinity for a convenient receptor and fused in the pIII coat protein. The phage library is bound to a solid support coated with the receptor and treated with the target protease. This can be made also with a "tether" sequence fused to the randomized sequence, in other to use an antibody to bind the phage library to a solid support prior to the treatment with the corresponding target as proposed by Smith *et al.* [49].

Those phages with good target substrate are released from the solid support. The selected phages are propagated by infecting fresh bacterial host cells to increase their copy number up to millions of copies in the amplified stock. The amplified population can then be subjected to further rounds of selection to obtain an ever-fitter subset of the starting peptides. This screening/amplification process can be repeated as necessary to obtain higher-affinity phage display peptides.

Once obtaining the higher-affinity phage display peptides by the screening/amplification process, identification are individually determined by sequencing the coding region of the isolated phage DNA [50].

The amino acid sequences of the ligands, likely to be important to binding are investigated and the ligand(s) evaluated in binding experiments, before the 'winner' ligand is finally chosen.

Bacterial surface display libraries

Another genetic approach is the bacterial surface peptide display library, which consists of cloning and bacterial expression of random DNA fragments as fusion-protein utilising flagella to display random peptides. *Escherichia coli* cells harbour a plasmid engineered to express a fusion protein containing random peptides that are inserted into the active loop of thioredoxin, which itself is inserted into the dispensable region of the flagellin gene, the major constituent of flagellar filaments. When the fusion protein becomes an integral part of the flagellar filaments on the bacterial cell surface, the peptides become available to interact with target proteins. Specifically bound clones can be eluted from the immobilised target by mechanical shearing of the flagella. The screening and sequence analysis of positive clones is developed by a binding assay similar to those used in phage display libraries [51].

Synthetic approach

The first methods for the synthesis of equimolar large peptide libraries consisted of parallel synthesis such as the multi-pin technology [52] and the spot synthesis [53].

Parallel synthesis

The multi-pin technology consists of the simultaneous parallel peptide synthesis on polyethylene pins arranged in a microtiter plate format. Polyethylene rods (diameter, 4 mm; length, 40 mm) are activated and assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tip of the rods are carried out utilising conventional solid-phase peptide chemistry. The peptides, still attached to the support used for their synthesis, are tested using the enzyme-linked immunosorbent-assay (ELISA) [54]. On the other

hand, the spot technology, developed by Frank [53], consists of simultaneous parallel peptide synthesis at different positions on a membrane support (i.e. cellulose paper sheets). The paper sheet needs to be chemically modified to introduce suitable anchor functions for peptide synthesis at the spot positions. These anchors also serve as spacer arms to improve the accessibility of the immobilised peptides. Peptides are assembled by manual or automated spotting of small aliquots of solutions containing the activated amino acid derivatives onto marked positions on the sheets utilising conventional Fmoc/tBu chemistry. Target protein binding to the peptides can be identified by ELISA, although other labelling techniques employing fluorescent dyes are also adequate.

Split–mix–split or divide-couple-recombine technique

The 'split–mix–split' combinatorial technique, also called 'divide-couple-recombine' [55-57], allows preparing new compounds in practically unlimited number. That procedure is based on the solid phase method and entails: (i) dividing the solid support into equal portions, (ii) coupling each portion individually with a different building block and, (iii) mixing the portions (Figure 1). This assures a theoretically even representation of the library members and results in the display of many copies of the same compound on one single bead known as One-Bead-One-Compound (OBOC) distribution. The number of divide-couple-recombine or split–mix–split cycles should equal the number of monomers (building blocks) in the final oligomeric ligand. The theoretical number of ligands present in the combinatorial library is calculated from the following formula:

$$N_{ligand} = (N_{monomers})^{\eta}$$

where N_{ligand} is the number of ligands in the library, $N_{monomers}$ is the number of building blocks used or the number of reaction vessels, and η is the number of times each vessel is used or number of repeated cycles or number of monomers in the ligand.

For the synthesis of equimolar large peptide libraries the split-mix-split synthesis is a useful and easier method than the previously reported

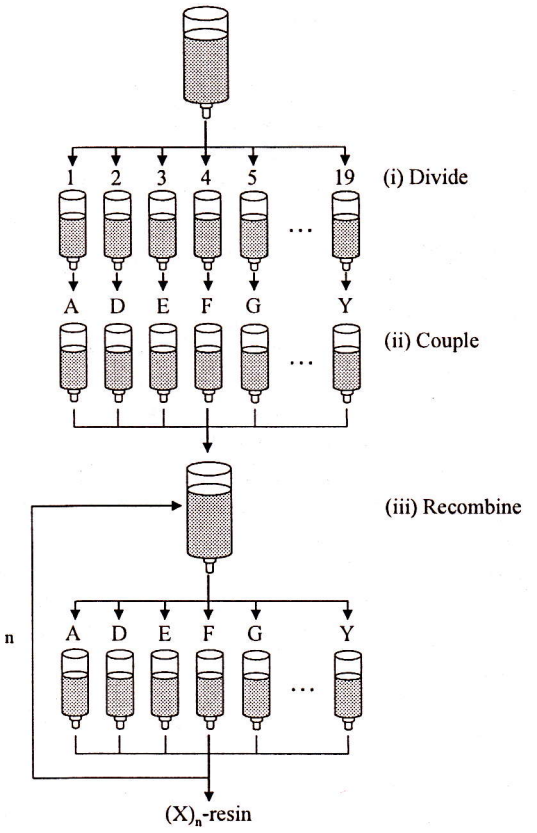

Figure 1. Divide-couple-recombine technique.

methods of parallel synthesis such as the multi-pin technology [52] and the spot technology [53].

To screen the OBOC combinatorial libraries, tens of thousands to millions of compound beads are first mixed with a small quantity of the target protein. The beads that interact with the target molecule will be identified and then isolated for structure determination. For target molecules that cannot be visualized directly through a microscope, a reporter group such as an enzyme [57], fluorescent probe [58], or radionuclide [59] is conjugated to the target molecule. If an antibody to the target molecule is readily available, an alternative method uses this antibody with a reporter group or a second antibody with a reporter group. Otherwise, the target molecule can be biotinylated and probed with streptavidin with the reporter group [60].

Unlike parallel synthesis that yields known products, the identified beads that interact with

the target protein must be isolated for compound structure determination.

OBOC peptide library screening often generates many positive beads that need to be individually characterised. Sequencing of resin-bound peptides by Edman degradation, a common used technique [57, 61] is expensive and time-consuming. On the other hand, the high sensitivity and mass accuracy of mass spectrometry together with the speed of the analyses and the large amount of information generated in each experiment make it a technique of choice for peptide and protein sequencing [62, 63]. Different strategy for sequencing hits from peptide combinatorial libraries have been used [64-66]. In our laboratory, we developed a rapid and non-expensive strategy for the identification of peptides contained on positive beads by using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) based on 4-hydroxymethylbenzoic acid (HMBA) linker and ChemMatrix resin. Optimisation of beads analysis by MALDI-TOF-MS after the screening of one-bead-one-peptide combinatorial libraries was achieved, involving the fine tuning of the whole process [67-70].

Examples of protein purification by affinity chromatography with peptide ligands selected from the screening of combinatorial libraries

Peptides selected from combinatorial libraries - immobilised on different supports - have been used for affinity purification of therapeutic proteins and antibodies. The efficient recovery and purification of these proteins are difficult tasks because the target molecule is often present at low concentrations in complex streams that contain many proteins with similar characteristics. The manufacturing process of biotherapeutics must follow GMP guidelines, the final product should be a "well characterised biologic" complying with the exigencies from regulatory bodies, such as the Food and Drug Administration (FDA) [71].

Monoclonal antibodies

MAbs are presently the most rapidly expanding category of biopharmaceuticals and are being applied across a wide range of previously untreatable diseases representing an important and growing class of biotherapeutics, with a multibillion dollar market [72, 73].

Engineering the downstream processing of antibodies has been a main task in research and industry, by exploring different types of interactions and separation techniques. AC is undoubtedly the most widespread technique in use for the purification of antibodies. Biospecific affinity ligands, mainly immunoglobulin (Ig)-binding proteins isolated from the bacterial surface (proteins A and G), have been the most popular ligands for antibody purification. Despite their lack in specificity, the "traditional" pseudobiospecific affinity matrices including thiophilic, hydrophobic, and mixed-mode adsorbents, are also well liked for Ab purification purposes [74]. The search for synthetic ligands able to bind to Igs has been undertaken mainly through the synthesis and screening of combinatorial peptide libraries.

By synthesising and screening a multimeric combinatorial OBOC library composed of randomised synthetic tripeptide tetramers Fassina *et al.* [75] identified a new synthetic peptide, denoted PAM (protein A mimetic, TG19318), able to bind specifically and selectively to the constant portion (Fc) of Igs. This ligand is a tetrameric tripeptide of the formula $(R\text{-}T\text{-}Y)_4\text{-}K_2\text{-}K\text{-}G$. Ligand specificity was broader than protein A, since IgG deriving from human, cow, horse, pig, mouse, rat, rabbit, goat and sheep sera, IgY obtained from egg yolk, and IgM, IgA and IgE were efficiently purified on TG19318 affinity columns. TG19318 affinity columns proved useful for a very convenient one-step purification of mAb directly from crude sources such as ascitic fluid and cell culture supernatants [76-80]. D-PAM, peptide, which is obtained from the PAM peptide by replacing the natural amino acids with the corresponding D isomers, maintains the recognition properties for Igs and is not degraded by treatment with proteolytic enzymes, such as trypsin and chymotrypsin, and after prolonged incubation with mouse serum [81].

In other approach, peptides interacting with the Fc portion of human IgG (IgG Fc) were selected from a phage display decapeptide library [82].

Two different methods were used to select interacting phage peptide clones; binding phage peptides were eluted either with protein A or at low pH. Both methods produced peptide phage clones that interacted with IgG Fc and differed in amino acid content depending on which eluent was used. The predominating amino acid sequences found were FGRLVSSIRY, eluted with protein A, and TWKTSRISIF, eluted at low pH.

Also, Erlich and Bailon [83] used phage-display technology to identify peptides or peptidomimetics for use as a Protein A alternative in the affinity purification of mAbs. The best binding immobilised peptide was EPIHRSTLTALL.

Besides, other groups identify peptide ligands with unique specificity for a mAb. Phage display technology was used to identify peptide ligands with unique specificity for a model mAb, MK16, that recognises the human multiple sclerosis associated MHC class II molecule DR2 in complex with a myelin basic protein (MBP)-derived peptide corresponding to residues 85-99. Several peptide epitopes were identified and all of them recognised specifically MK16. One peptide, CNYSVAHLC, was selected and linked to beaded agarose, demonstrating an excellent performance as an AC matrix. This epitope matrix was efficient in the purification of MK16 Fab fragments and had no affinity for other antibodies. Using this peptide matrix MK16 IgG could be purified from cell culture supernatants thereby separating MK16 IgG from bovine IgG normally present in the enriched growth media used for such cells [84].

In our laboratory peptide ligand, Ala-Pro-Ala-Arg (APAR), was selected from the screening of a OBOC combinatorial solid phase tetrapeptide library as the ligand for affinity purification of an anti-Granulocyte Macrophage-Colony Stimulating Factor (anti-GMCSF) mAb developed in mouse ascitis. Pure anti-GMCSF could be obtained in a single step [85].

Plasma proteins

Many plasma proteins with therapeutic use were purified using peptides obtained from the screening of combinatorial libraries.

Phage peptide library screening yielded a lead peptide (RLRSFY) that interacts with von Willebrand Factor (vWF). Conservative substitutions of terminal residues of the lead peptide led to a second peptide, RVRSFY, which was more efficient in the affinity chromatographic purification of vWF from protein mixtures [86].

Several peptides were developed for the recombinant human blood coagulation factor VIII purification. Spot technology using cellulose sheets has been applied for this purpose. Among the different peptides selected for affinity purification, the peptide with the sequence EYKSWEYC showed the better performance as a ligand for AC of factor VIII [87, 88]. The same laboratory used the region of vWF, which is involved in the complex formation with factor VIII, to generate a panel of octapeptides. A peptide ladder was generated from the vWF region aa40 to aa100 and was synthesized on cellulose membranes by spot technology. Four peptides with affinity for factor VIII were identified by incubation with plasma-derived factor VIII and recombinant factor VIII. The peptides LCPPGMVRHE, RCPCFHQGK, CFHQGKEYA and RDRKWNCTDHVC were further characterised by small scale AC. These experiments showed that the peptides directed against the light chain of factor VIII were ligands suitable for AC [89].

An affinity resin containing the peptide ligand FLLVPL has been developed for the purification of fibrinogen by Kaufman y col. *et al.* [90]. The ligand was identified by screening a OBOC combinatorial library. The ligand immobilised and the corresponding adsorbent was used to purify human fibrinogen. Acetic acid at pH 3.0 was used successfully to elute the adsorbed fibrinogen from the column with high purity, and minimal loss of its biological activity.

Jacobsen *et al.* [91], developed a new method for the affinity purification of recombinant urokinase-type plasminogen activator receptor (uPAR) from crude *Drosophila melanogaster Schneider 2* cell culture medium based on a high-affinity synthetic peptide antagonist, denoted AE152. The AE152 (Figure 2) was originally selected from a phage-display library

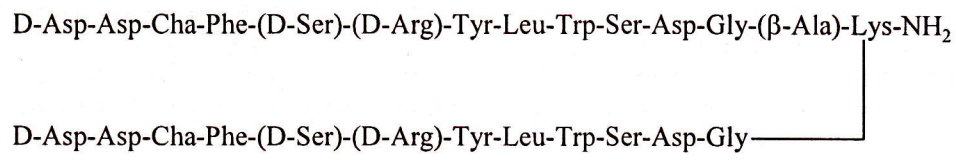

Figure 2. Primary structure of peptide AE 152.

and subsequently affinity-matured by combinatorial chemistry. It is a pseudosymmetrical dimer with respect to the α-carbon atom of lysine and has four aspartic acid extra residues introduced to increase its solubility at physiological pH. In addition, Yu *et al.* [92] purified insulin in one step by affinity chromatography with the heptapeptide HWWWPAS with high affinity for insulin. The peptide was found by screening a heptapeptide phage display library.

Milk proteins

Only two proteins from milk, α-lactalbumin and lactoferrin, were purified using peptide affinity ligands derived from peptide libraries.

α-Lactalbumin is a whey protein with high digestibility and low potential for causing allergic problems in infants, making it a strong candidate for use in infant formulas. To allow its use on a large scale, the development of an efficient and scalable process for isolation of α-lactalbumin is necessary. Gurgel *et al.* [93] identify the hexapeptide WHWRKR from the screening of a combinatorial library, which shows affinity for α-lactalbumin. A hexamer peptide library was used and a process for whey protein fractionation was developed [94]. The library was generated by the divide-couple-recombine technique.

Noppe *et al.* [95, 96] developed an affinity purification procedure for the direct purification of lactoferrin from defatted milk. The procedure is based on the use of selected phage clones expressing APRQPP, DQDQDT, EGKQRR or HQHRQR peptides with high binding affinity for lactoferrin which were covalently coupled to a macroporous poly (dimethylacrylamide) monolithic column.

Toxins

Some toxins were purified using peptides as well. For instance, combinatorial phage display was used to discover peptides that selectively bind to the α-cobratoxin (neurotoxin) component of the multi-component venom of the Thai cobra *Naja kaouthia*. Peptide sequences determined in this way were synthesised chemically and were covalently attached to agarose through the amino terminus. Such AC matrices bound selectively the α-cobratoxin component from crude venom; the passage of the crude venom through a matrix column selectively depleted the venom of this component [97]. Some deduced peptide sequences were: SWWRHAAVYEWD, YSGSWWPPTYNNE VPL, HTWWYNPPSYMGLEAS, TLWGLFPPVY EDSFGL and PWTSWWPPVYEGSTTN.

In addition, a short peptide ligand that selectively binds to staphylococcal enterotoxin B (SEB), YYWLHH, has been identified from a solid-phase combinatorial peptide library by using 'split synthesis' [98]. YYWLHH binds with high affinity and selectivity to SEB, but only weakly to other SEs that share sequence and structural homology with SEB. Using column AC with an immobilised YYWLHH stationary phase, it was possible to separate quantitatively SEB from *Staphylococcus aureus* fermentation broth, a complex mixture of proteins, carbohydrates and other biomolecules. The immobilised peptide was also used to purify native SEB from a mixture containing denatured and hydrolysed SEB, showing little cross-reactivity with other SEs. The influence of mass transfer and adsorption–desorption kinetics on the binding of SEB to an affinity resin with this peptide ligand have been studied [99].

Table 1 summarises the examples of proteins purified by AC with peptide ligands obtained from the screening of libraries.

Table 1. Examples of proteins purified by affinity chromatography with peptides obtained from libraries.

Target	Library	Peptide selected	Reference
Antibodies			
Antibodies (IgG, IgA, IgM, IgE, IgY)	Split–mix–split	$(RTY)_4K_2KG$ TG19318 (PAM)	[75-81]
Antibodies (IgG)	Phage display	FGRLVSSIRY TWKTSRISIF	[82]
Antibodies (IgG)	Phage display	EPIHRSTLTALL	[83]
mAb MK16	Phage display	CNYSVAHLC	[84]
mAb anti-GM-CSF	Split–mix–split	APAR	[85]
Plasma proteins			
vWF	Phage display	RLRSFY RVRSFY	[86]
Factor VIII	Spot technology	EYKSWEYC	[87, 88]
Factor VIII	Spot technology	LCPPGMVRH RCPCFHQGK CFHQGKEYA RDRKWNCTDHVC	[89]
Fibrinogen	Split–mix–split	FLLVPL	[90]
uPAR	Phage display*	AE152[#]	[91]
Insulin	Phage display	HWWWPAS	[92]
Milk proteins			
α-lactalbumin	Split–mix–split	WHWRKR	[93, 94]
Lactoferrin	Phage display	APRQPP DQDQDT EGKQRR HQHRQR	[95, 96]
Toxins			
α-cobratoxin	Phage display	SWWRHAAVYEWD YSGSWWPPTYNNEVPL HTWWYNPPSYMGLEAS GTWTWWPPTYAGMDHL TLWGLFPPVYEDSFGL PWTSWWPPVYEGSTTN	[97]
SEB	Split–mix–split	YYWLHH	[98, 99]

*Phage display and subsequently subjected to affinity maturation by combinatorial chemistry.
[#]AE152: See Figure 2.

CONCLUSION

Peptide ligands attached to hydrophilic supports are very useful tools for protein purification by affinity chromatography, as evidencing by the numerous proteins successfully purified with this approach.

REFERENCES

1. Cuatrecasas, P., and Wilchek, M. 1968, Biochem. Biophys. Res. Commun., 33, 235.
2. Roque, A. C., and Lowe, C. R. 2008, Meth. Mol. Biol., 421, 1.
3. Van Emon, J. M., Gerlach, C. L., and Bowman, K. 1998, J. Chromatogr. B, 715, 211.
4. Tozzi, C., and Giraudi, G. 2006, Curr. Pharm. Des., 12, 191.
5. Huang, P. G., and Carbonell, R. Y. 1995, Biotechnol. Bioeng., 47, 288.
6. Scopes, R. K. 1986, J. Chromatogr., 376, 131.
7. Porath, J. 1992, Protein Expr. Purif., 3, 263.
8. Baumbach, G. A., and Hammond, D. J. 1992, BioPharm., 5, 24.
9. Boyer, P. M., and Hsu, J. T. 1992, Chem. Eng. Sci., 47, 241.
10. Parmley, S. F., and Smith, G. P. 1988, Gene, 73, 305.
11. Devlin, J. J., Panganiban, L. C., and Devlin P. E. 1990, Science, 249, 404.
12. Kelley, B. D., Booth, J., Tannatt, M., Wub, Q. L., Ladner, R., and Yuc, J. 2004, J. Chromatogr. A, 1038, 121.
13. Scott, J. K., and Smith, G. P. 1990, Science, 249, 386.
14. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. 1990, Proc. Natl. Acad. Sci. USA, 87, 6378.
15. Ehrlich, G. K., and Bailon, P. 2001, J. Biochem. Biophys. Methods, 49, 443.
16. Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., and Cortese, I. 1996, Curr. Opin. Biotechnol., 7, 616.
17. Sidhu, S. S. 2001, Biomol. Eng., 18, 57.
18. DeGraaf, M. E., Miceli, R. M., Mott, J. E., and Fischer, H. D. 1993, Gene, 128, 13.
19. Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., and Cesareni, G. 1991, J. Mol. Biol., 222, 301.
20. Markland, W., Roberts, B. L., Saxena, M. J., Guterman, S. K., and Ladner, R. C. 1991, Gene, 109, 13.
21. Fowlkes, D. M., Adams, M. D., Fowler, V. A., and Kay, B. K. 1992, Biotechniques, 13, 422.
22. Smith, G. P. 1985, Science, 228, 1315.
23. Iannolo, G., Minenkova, O., Petruzzelli, R., and Cesareni, G. 1995, J. Mol. Biol., 248, 835.
24. Greenwood, J., Willis, A. E., and Perham, R. N. 1991, J. Mol. Biol., 220, 821.
25. Jacobsson, K., and Frykberg, L. 1995, Biotechniques, 18, 878.
26. Jacobsson, K., and Frykberg, L. 1996, Biotechniques, 20, 1070.
27. Jespers, L. S., Messens, J. H., De, K. A., Eeckhout, D., Van, D. B. I., and Gansemans, Y. G. 1995, Biotechnology, 13, 378.
28. Crameri, R., and Blaser, K. 1996, Int. Arch. Allergy Immunol., 110, 41.
29. Wang, L. F., Du Plessis, D. H., White, J. R., Hyatt, A. D., and Eaton, B. T. 1995, J. Immunol. Methods, 178, 1.
30. Van Zonneveld, A. J., van den Berg, B. M. M., van Meijer, M., and Pannekoek, H., 1995, Gene, 167, 49.
31. Petersen, G., Song, D., Hugle-Dorr, B., Oldenburg, I. and Bautz, E. K., 1995, Mol. Gen. Genet., 249, 425.
32. Wang, C. I., Yang, Q. and Craik, C. S., 1996, Methods Enzymol., 267, 52.
33. Soumillion, P., Jespers, L., Bouchet, M., Marchand-Brynaert, J., Winter, G., and Fastrez, J. 1994, J. Mol. Biol., 237, 415.
34. Lowman, H. B., Bass, S. H., Simpson, N., and Wells, J. A. 1991, Biochemistry, 30, 10832.
35. McCafferty, J., Jackson, R. H., and Chiswell, D. J. 1991, Protein Eng., 4, 955.
36. Djojonegoro, B. M., Benedik, M. J., and Willson, R. C. 1994, Biotechnology, 12, 169.
37. Bonnycastle, L. L., Mehroke, J. S., Rashed, M., Gong, X., and Scott, J. K. 1996, J. Mol. Biol., 258, 747.
38. O'Boyle, D. R., Pokornowski, K. A., McCann, P. J., and Weinheimer, S. P. 1997, Virology, 236, 338.
39. Nord, K., Gunneriusson, E., Ringdahl, J., Stahl, S., Uhlen, M., and Nygren, P. A. 1997, Nat. Biotechnol., 15, 772.

40. Smith, G. P., and Petrenko, V. A. 1997, Chem. Rev., 97, 391.
41. Kay, B. K., Adey, N. B., He, Y. S., Manfredi, J. P., Mataragnon, A. H., and Fowlkes, D. M. 1993, Gene, 128, 59.
42. Liu, R., Enstrom, A. M., and Lam, K. S. 2003, Exp. Hematol., 31, 11.
43. Bianchi, E., Folgori, A., Wallace, A., Nicotra, M., Acali, S., and Phalipon, A. 1995, J. Mol. Biol., 247, 154.
44. McConnell, S. J., and Hoess, R. H. 1995, J. Mol. Biol., 250, 460.
45. Bass, S., Greene, R., and Wells, J. A. 1990, Proteins, 8, 309.
46. Dyson, M. R. and Murray, K. 1995, Proc. Natl. Acad. Sci. USA, 92, 2194.
47. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. 1990, Nature, 348, 552.
48. Matthews, D. J., and Wells, J. A. 1993, Science, 260, 1113.
49. Smith, M. M., Shi, L., and Navre, M. 1995, J. Biol. Chem., 270, 6440.
50. Sanger, F., Nicklen, S., and Coulson, A. R. 1977, Proc. Natl. Acad. Sci. USA, 74, 5463.
51. Lu, Z., Murray, K. S., Van Cleave, V., LaVallie, E. R., Stahl, M. L., and McCoy, J. M. 1995, Nature Biotechnol., 13, 366.
52. Geysen, H. M., Meloen, R. H., and Barteling, S. J. 1984, Proc. Natl. Acad. Sci. USA, 81, 3998.
53. Frank, R. 1992, Tetrahedron, 48, 9217.
54. Bray, A. M. 1997, Multiple peptide synthesis with commercially available Multipin kits. Immunology Methods Manual 2, 809.
55. Furka, A., Sebestyen, F., Asgedon, M., and Dibo, G. 1991, Int. J. Peptide Protein Res., 37, 487.
56. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., and Cuervo, J. H. 1991, Nature, 354, 84.
57. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., and Knapp, R. J. 1991, Nature, 354, 82.
58. Needles, M. C., Jones, D. G., Tate, E. H., Heinkel, G. L., Kochersperger, L. M., Dower, W. J., Barrett, R. W., and Gallop, M. A. 1993, Proc. Natl. Acad. Sci. USA, 90, 10700.
59. Kassarjian, A., Schellenberger, V., and Turck, C. W. 1993, Pept. Res., 6, 129.
60. Lam, K. S., Lehman, A. L., Song, A., Doan, N., Enstrom, A. M., Maxwell, J., and Liu, R. 2003, Methods Enzymol., 369, 298.
61. Lebl, M., Krchnak, V., Sepetov, N. F., Seligmann, B., Strop, P., Felder, S., and Lam, K. S. 1995, Biopolymers, 37, 177
62. Karas, M., and Hillenkamp, F. 1988, Anal. Chem., 60, 2299.
63. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. 1989, Science, 246, 64.
64. Youngquuist, R. S, Fuentes, G. R., Lacey, M. P., and Keough, T. 1995, J. Am. Chem. Soc., 117, 3900.
65. Liu, R., Marik, J., and Lam K. S. 2002, J. Am. Chem. Soc., 124, 7678.
66. Vágner, J., Barany, G., Lam, K. S., Krchnák, V., Sepetov, N. F., Ostrem, J. A., Strop, P., and Lebl, M. 1996, Proc. Natl. Acad. Sci. USA, 93, 8194.
67. Camperi, S. A., Marani, M. M, Iannucci, N. B., Côte, S., Albericio, F., and Cascone, O. 2005, Tetrahedron Lett., 46, 1561.
68. Marani, M. M., Oliveira, E., Côte, S., Camperi S. A., Cascone O., and Albericio, F. 2007, Anal. Biochem., 370, 215.
69. Marani, M. M., Martínez Ceron, M. C., Giudicessi, S. L., Oliveira, E., Côte, S, Erra-Balsells, R., Albericio, F., Cascone, O., and Camperi, S. A. 2009, J. Comb. Chem., 11, 146.
70. Martínez-Ceron, M. C., Giudicessi, S. L., Marani, M. M., Albericio, F., Cascone, O., Erra-Balsells, R., and Camperi, S. A. 2010, Anal. Biochem., 400, 295.
71. Lowe, C. R., Lowe, A. R., and Gupta, G. 2001, J. Biochem. Biophys. Methods, 49, 561.
72. Stockwin, L. H., and Holmes, S. 2003, Biochem. Soc. Trans., 31, 433.
73. Walsh, G. 2004, Eur. J. Pharm. Biopharm., 58, 185.
74. Huse, K., Bohme, H. J., and Scholz, G. H. 2002, J. Biochem. Biophys. Methods, 51, 217.
75. Fassina, G., Verdoliva, A., Odierna, M., Ruvo, M., and Cassani, G. 1996, J. Mol. Recognit., 9, 564.

76. Fassina, G., Verdoliva, A., Palombo, G., Ruvo, M., and Cassani, G. 1998, J. Mol. Recognit., 11, 128.
77. Palombo, G., Verdoliva, A., and Fassina, G. 1998, J. Chromatogr. B, 715, 137.
78. Palombo, G., Rossi, M., Cassani, G., and Fassina, G. 1998, J. Mol. Recognit., 11, 247.
79. Verdoliva, A., Basile, G., and Fassina, G. 2000, J. Chromatogr. B, 749, 233.
80. Fassina, G., Ruvo, M., Palombo, G., Verdoliva, A., and Marino, M. 2001 J. Biochem. Biophys. Methods, 49, 481.
81. Verdoliva, A., Pannone, F., Rossi, M., Catello, S., and Manfredi, V. 2002, J. Immunological Methods, 271, 77.
82. Krook, M., Mosbach, K., and Ramström, O. 1998, J. Immunol. Methods, 221, 151.
83. Ehrlich, G. K., and Bailon, P. 2001, J. Biochem. Biophys. Methods, 49, 443.
84. Jensen, L. B., Riise, E., Nielsen, L. K., Dziegiel, M., Fuggerc, L., and Engberg, J. 2004, J. Immunol. Methods, 284, 45.
85. Camperi, S. A., Iannucci, N. B., Albanesi, G. J., Oggero Eberhardt, M., Etcheverrigaray, M., Messeguer, A., Albericio, F., and Cascone, O. 2003, Biotechnol. Lett., 25, 1545.
86. Huang, P. Y., Baumbach, G. A., Dadd, C. A., Buettner, J. A., Masecar, B. L., Hentsch, M., Hammond D. J., and Carbonell, R. G. 1996, Bioorg. Medicinal Chem., 4, 699.
87. Amatschek, K., Necina, R., Hahn, R., Schallaun, E., Schwinn, H., Josic, D., and Jungbauer, A. 2000, J. High Resol. Chromatogr., 23, 47.
88. Pflegerl, K., Hahn, R., Berger, E., and Jungbauer, A. 2002, J. Pept. Res., 59, 174.
89. Necina, R., Amatschek, K., Schallaun, E., Schwinn, H., Josic, D., and Jungbauer, A. 1998, J. Chromatogr. B, 715, 191.
90. Kaufman, D. B., Hentsch, M. E., Baumbach, G. A., Buettner, J. A., Dadd, C. A., Huang, P. Y., Hammond, D. J., and Carbonell, R. G. 2002, Biotechnol. Bioeng., 77, 278.
91. Jacobsen, B., Gardsvoll, H., Funch, G. J., Østergaard, S., Barkholt, V., and Ploug, M. 2007, Protein Expr. Purif., 52, 286.
92. Yu, H. Q., Dong, X. Y., and Sun, Y. 2004, Chromatographia, 60, 379.
93. Gurgel, P. V., Carbonell, R. G., and Swaisgood, H. E. 2001, Sep. Sci. Technol., 36, 2411.
94. Gurgel, P. V., Carbonell, R. G., and Swaisgood, H. E. 2000, Bioseparation, 9, 385.
95. Noppe, W., Plieva, F. M., Galaev, I. Y., Vanhoorelbeke, K., Mattiasson, B., and Deckmyn, H. 2006, J. Chromatogr. A., 1101, 79.
96. Noppe, W., Vanhoorelbeke, K., Galaev, I. Y., Mattiasson, B., and Deckmyn, H. J. 2004, J. Dairy Sci., 87, 3247.
97. Byeona, W. H., and Weisblum, B. 2004, J. Chromatogr. B, 805, 361.
98. Wang, G., De, J., Schoeniger, J. S., Roe, D. C., and Carbonell, R. G. 2004, J. Peptide Res., 64, 51.
99. Wang, G., and Carbonell, R. G. 2005, J. Chromatogr., 1078, 98.