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Research Paper

Covalent capture: a new tool for the purification of synthetic and recombinant polypeptides

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Abstract

Background: Purification of polypeptides and proteins derived from recombinant DNA techniques and of long synthetic polypeptides often represents a challenge. Affinity methods exist, but generally require addition of a large recognition unit to the target protein and use of expensive purification media. Use of large units is dictated by the characteristics of non-covalent complexes, where the energy necessary to form the complex derives from the sum of multiple weak energy interactions. Covalent interactions in contrast are of high energy, even when only a few bonds are formed. We decided to explore the use of the reversible covalent bond formed between N-terminal cysteine and threonine residues with an aldehyde as a method of protein purification.

Results: A series of test peptides with N-terminal cysteine and threonine were captured by a polyethyleneglycol–polyacrylamide resin to which an aldehyde function had been grafted. Peptides with other amino acids at the N-terminus did not interact with the resin. A recombinant polypeptide with N-terminal cysteine was purified to 90% purity in one step. Polypeptides were eluted from the resin simply by adding a hydroxylamine derivative, which reacts with aldehyde functions to form an oxime.

Conclusions: Polypeptides possessing N-terminal cysteine or threonine can be easily purified using this 'covalent capture' approach.

Keywords: Covalent capture; Polypeptide purification; Recombinant protein purification

1. Introduction

Many methods exist for the affinity purification of proteins, but none is ideal [1]. For proteins produced by recombinant DNA techniques, some methods rely on the use of relatively inexpensive purification media, but in contrast require fusion of the target protein with binding domains (maltose binding protein, chitin binding domain, thioredoxin, cellulose binding domain, glutathione *S*-transferase or polyhistidine tag), which are large enough, or peculiar enough, to possibly affect function. While these domains may sometimes be removed by enzymatic techniques, yields are often low due to low accessibility of the cleavage site, and such cleavage can be expensive to perform on a large scale. Other approaches rely on fusion with a small peptide tag, such as S-peptide and the Flag peptide [1], but require expensive antibody-based purification media. For polypeptides produced by chemical techniques (solid phase peptide synthesis), N-terminal tags in combination with capping at every coupling step have been explored with good results either for affinity-type purification or to modify the chromatographic properties of the correct peptide [2–5]. Unfortunately the harsh conditions required for tag removal (cyanogen bromide, 20% piperidine and pH 12) limited their use.

We decided to exploit as a purification strategy a well-known organic chemistry reaction, the formation of a five-membered heterocyclic ring arising from the condensation of a carbonyl group with a molecule carrying two vicinal nucleophilic groups (–NH_2, –OH, –SH) [6]. Two natural amino acid residues, cysteine and threonine, when in the N-terminal position, can undergo this reaction in an aqueous or semi-organic environment [7–9] while the same amino acids elsewhere in a peptide sequence are completely

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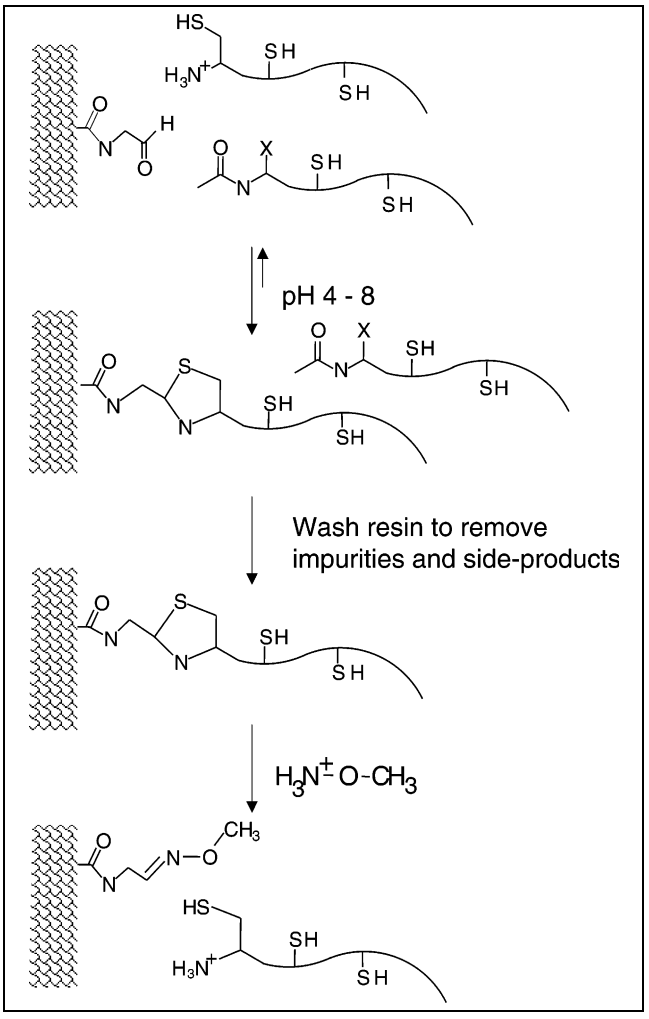

Fig. 1. Covalent capture concept. A peptide with an N-terminal cysteine (or threonine, not shown) reacts with a resin possessing an aldehyde function, forming a five-membered heterocyclic ring. Peptides lacking this amino acid in the N-terminal position, or with an acetylated N-terminus, do not react with the resin. Cysteines at intermediate positions are not reactive. Once the correct sequence is covalently linked to the resin, impurities and by-products are removed by filtration and the resin extensively washed with the same buffer. The purified peptide is eluted with a solution of *O*-methylhydroxylamine, and recovered by filtration.

unreactive due to the low nucleophilicity of the amide nitrogen (Fig. 1). Curiously, N-terminal serine reacts poorly with aldehyde groups, even in the presence of organic solvents. Our approach was to use a solid support (resin) to which aldehyde groups were attached. A peptide possessing an N-terminal cysteine (or threonine) reacts with the resin while impurities and by-products such as acetylated truncated peptides do not react. The ring formation reaction proceeds to an equilibrium, which is strongly in favor of product formation in the range pH 4.5–8, and permits extensive washes. This equilibrium can be perturbed by introducing a competitor for the aldehyde group, such as an aminoxy group, that rapidly reacts to form an oxime, conveniently releasing the captured peptide or protein (Fig. 1).

To demonstrate the applicability of the covalent capture approach to the purification of synthetic and recombinant DNA-derived polypeptides, we used sequences from the chemokine Rantes. Certain derivatives of Rantes, modified at the N-terminus, are useful in inhibiting entry of HIV-I into macrophages, but require the chemical synthesis of these modified proteins. Production by recombinant techniques, and purification by covalent capture of a large fragment of Rantes compatible with native chemical ligation, could simplify the production of these anti-HIV drugs [10,11].

2. Results

2.1. Capture of short peptides with N-terminal cysteine and threonine

We began with a series of synthetic peptides with at the N-terminal position: cysteine (CYAKYAKL, referred to as Cyak); threonine (TYAKYAKL, referred to as Tyak); serine (SYAKYAKL, referred to as Syak), and as a negative control tyrosine (YAKYAKL, referred to as yak). Since peptides after synthesis are often solubilized in a mixture of water/acetonitrile, we chose a solid support (resin) compatible with these solvents in the form of the commercially available amino PEGA resin from Novabiochem. This resin, prepared from polymerization of bisacrylamido polyethyleneglycol, monoacrylamido polyethyleneglycol and *N*,*N*-dimethyl acrylamide, presents good swelling properties in both aqueous and organic media [12]. The amino groups on the resin were derivatized with an aldehyde group. This aldehyde resin captured Cyak quantitatively in a mixture of water and acetonitrile either at pH 6.4 (Fig. 2) or at pH 4.5, simply upon incubation of the peptide solution with the resin under gentle agitation. At pH 6.4 capture was complete within 4 h using two equivalents of aldehyde per equivalent of peptide. Capture rate was a function of the resin quantity and the peptide concentration, as expected for a bi-molecular reaction, and working with a larger excess of resin (five equivalents) the reaction was completed in 1 h. Tyak was less reactive, and 80% of the peptide was captured in 12 h at pH 6.4 (Fig. 2), while only 5% was captured in the same time at pH 4.5. The peptide Syak failed to react with the resin and so did the control peptide yak, which lacks an appropriate reactive amino acid at the N-terminus. In the case of Cyak, the presence of a reducing or chelating agent (dithiothreitol (DTT), Tris(2-carboxyethyl) phosphine (TCEP) or EDTA, 10 mM) in the capture buffer increased the yield of capture since it prevented the formation of disulfide dimer, which cannot react with aldehyde resin. Care should be taken when employing DTT since an excess of this reagent might block the aldehyde binding sites, a problem that we did not encounter using TCEP. Once trapped on the resin the Cyak peptide was not released by

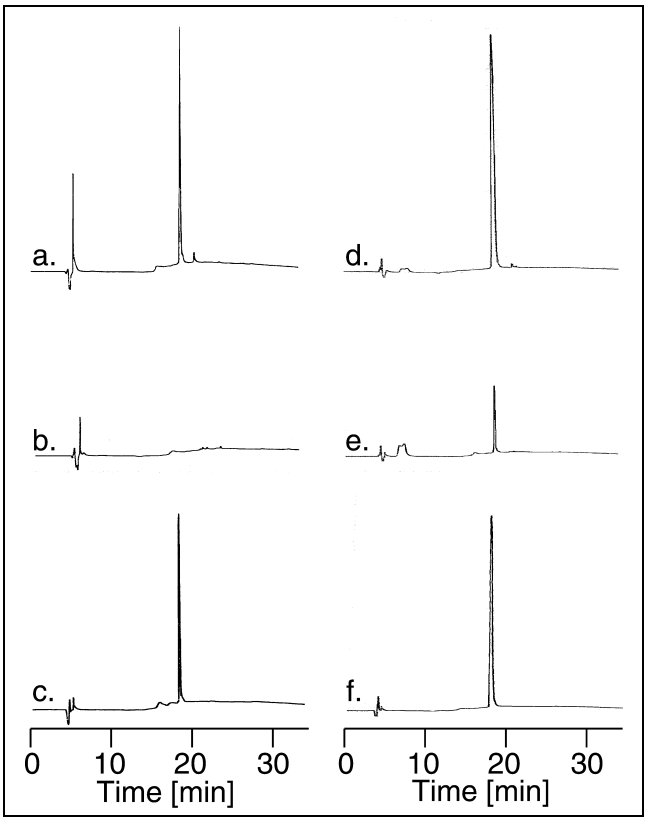

Fig. 2. Analytical RP-HPLC of the supernatant at different stages of Cyak and Tyak capture, using a gradient of 3%/min of solvent B. Volumes injected were kept constant. (a) Initial conditions prior to capture of Cyak; (b) 4 h capture of Cyak; (c) 6 h elution with *O*-methylhydroxylamine hydrochloride; (d) initial conditions prior to capture of Tyak; (e) 12 h capture of Tyak; (f) 6 h elution with *O*-methylhydroxylamine hydrochloride. All the materials were analyzed by mass spectroscopy, and the eluted material did not show any sign of modification.

repetitive washes with the capture buffer. Tyak binding was less stable, but could still sustain repetitive washes without great loss. Bound Cyak and Tyak were recovered quantitatively from the resin by treating the resin with a solution of *O*-methylhydroxylamine at pH 3 (Fig. 2) for 6 h. This is the optimal pH for release, but full release could be obtained at higher pH (4.5) by extending the elution time.

2.2. Selectivity of the aldehyde resin

To investigate the influence of peptide size on the capture process, and to check the selectivity of the resin for N-terminal cysteine peptides, we used a synthetic 32-mer peptide possessing N-terminal cysteine (pep1). The same sequence was extended in the N-terminal direction with an alanine residue (pep2) to check the reactivity of cysteine when not in the N-terminal position. Using the same conditions as for Cyak (pH 6.4), pep1 and pep2 were mixed (Fig. 3a) and incubated with the aldehyde resin. Pep1 was completely captured in 4 h, while pep2 failed to react with the resin (Fig. 3b). After the washing steps, purified pep1 was eluted from the resin (Fig. 3c). The degree of purification achieved by this simple procedure is considerable.

2.3. Synthesis and purification of Rantes 34–68 from truncated sequences

To investigate the applicability of covalent capture to the case of a more difficult synthetic peptide purification, we synthesized by solid phase peptide synthesis (SPPS) a 35-mer peptide, Rantes 34–68 (fragment containing the C-terminal 35 residues of the human chemokine Rantes), using capping at every step before Boc removal. This peptide possesses an N-terminal cysteine as well as cysteine and threonine residues in internal positions. In order to evaluate further the specificity of our covalent capture purification method, at different steps of the synthesis a portion of the resin was manually capped with acetic anhydride or with pyroglutamic acid (a common side reaction of glutamine residues). These *N*-acylated fragments were mixed with Rantes 34–68 and mixed with the aldehyde resin (Fig. 4a). Only Rantes 34–68 was captured, while the different truncated peptides were not retained (Fig. 4b). The eluted material contained mainly Rantes 34–68 (Fig. 4c). The small impurities present were identified by mass spectrometry as the Met_{68} oxidized product and as *p*-cresol adducts (Fig. 4c). The degree of purification achieved (Fig. 4c vs. a) is considerable.

Further experiments were performed with synthetic pep-

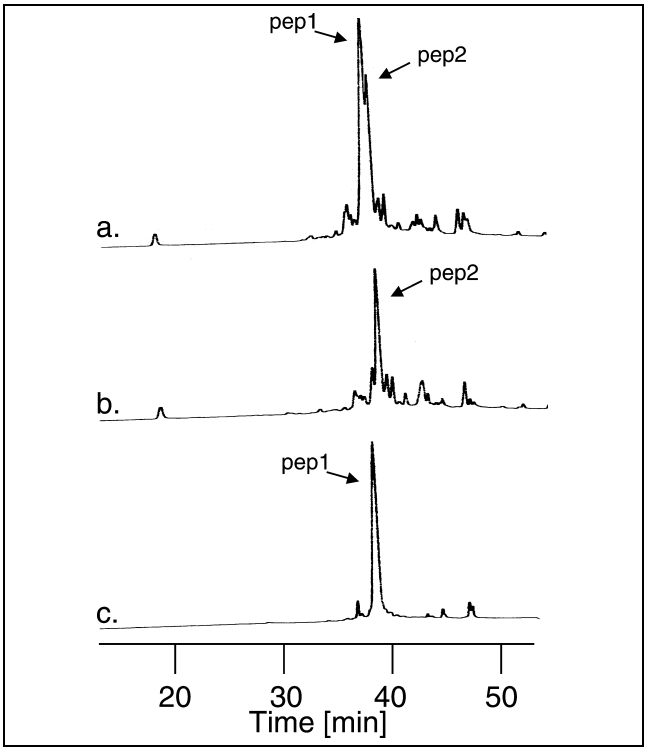

Fig. 3. Analytical RP-HPLC of the supernatant of mixed pep1 and pep2, using a gradient of 0.8%/min of solvent B. Volumes injected were kept constant. (a) Initial conditions prior to capture; (b) 4 h capture; (c) 6 h elution with *O*-methylhydroxylamine hydrochloride.

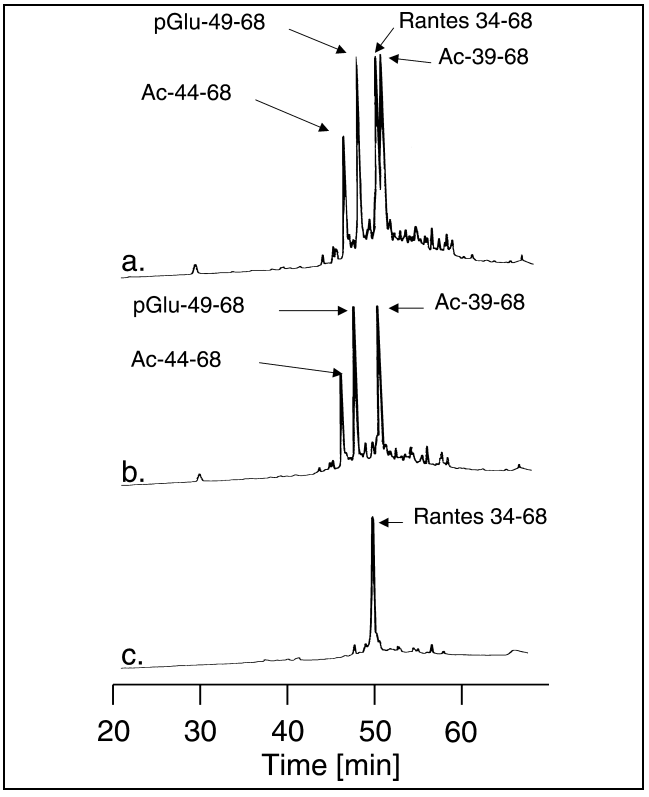

Fig. 4. Analytical RP-HPLC of the supernatant of mixed acylated fragments of Rantes 34–68, and correct Rantes 34–68 capture, using a gradient of 0.8%/min of solvent B. Volumes injected were kept constant. (a) Initial condition prior to capture; (b) 4 h capture; (c) 8 h elution with *O*-methylhydroxylamine hydrochloride.

tides possessing N-terminal Cys (Rantes 50–68 and Rantes 10–68), and Thr (Rantes 30–68). These experiments demonstrated that all N-terminal cysteine fragments were quantitatively captured by the resin. With increasing length, peptide capture became slower, but in all cases it was complete within 4 h at pH 6.4. The N-terminal threonine peptide was captured in approximately 80% yield in 12 h. Interaction with the resin was not affected by repetitive washes, and no peptide was detected in the buffer used for washing steps. Elution with *O*-methylhydroxylamine was quantitative and was achieved within 8 h. All the eluted fragments possessed the expected mass spectrum (data not shown).

2.4. Development of a capture resin for recombinant polypeptides

To investigate the applicability of the covalent capture approach to a polypeptide obtained through recombinant DNA technology we developed a different type of aldehyde resin. This resin is based on a cross-linked dextran support, compatible with solutions containing 6 M guanidinium chloride used when handling inclusion bodies produced by recombinant techniques. Rantes 10–68, a fragment of wild-type Rantes possessing cysteine at the N-terminus, was expressed in *Escherichia coli* using standard recombinant techniques with an initiating methionine residue. We expected that the translation machinery of the cell would remove the initiator methionine to liberate the N-terminal cysteine. The inclusion bodies were collected by centrifugation after cell lysis, solubilized directly in a buffer containing 6 M guanidinium chloride and loaded onto the dextran-based aldehyde resin with gentle agitation overnight at 4°C. Reverse-phase high performance liquid chromatography (RP-HPLC) and mass spectroscopy analysis of the crude material indicated the presence of: the desired Rantes 10–68 (about 30% of the total material); a protein corresponding to Met-Rantes 10–68 (+132 amu, expected +131); oxidized Met-Rantes 10–68 (+147 amu, expected +147), and doubly oxidized Met-Rantes 10–68 material (+163 amu, expected +163) (Fig. 5a). After capture, the resin was washed extensively and the captured material eluted with *O*-methylhydroxylamine. Neither Met-Rantes 10–68 nor the oxidized materials were retained by the resin. From 1 liter of induction medium of Rantes 10–68, 0.5 mg of material with a purity of over 90% (Fig. 5c) was recovered, and this was ready for further use after a desalting step.

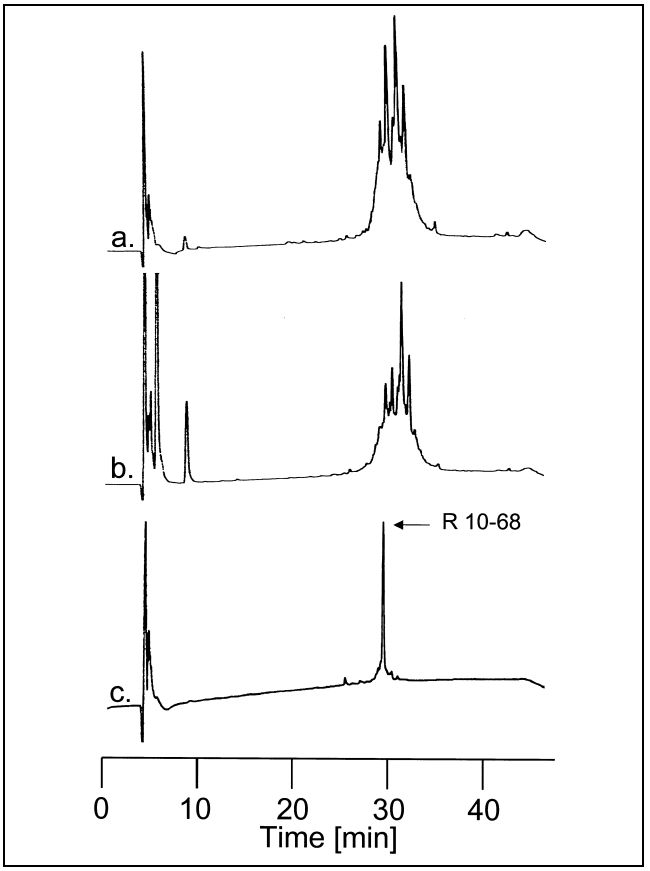

Fig. 5. Analytical RP-HPLC of the supernatant of crude recombinant Rantes 10–68, using a gradient of 1%/min of solvent B. Volumes injected were kept constant. (a) Initial condition prior to capture; (b) 4 h capture; (c) 8 h elution with *O*-methylhydroxylamine hydrochloride.

3. Discussion

The results reported here clearly demonstrate that it is possible to use the reversible covalent reaction of thiazolidine or oxazolidine formation as a basis for polypeptide purification. The advantages of such a purification approach are multiple. First is the use of inexpensive purification media, available with high capacity (50 mM or greater), stable for many months and compatible with the conditions employed after peptide synthesis and after protein production by recombinant techniques. These media can be simply added to the solution containing the protein or polypeptide, and the capture reaction performed in batch mode, without a chromatographic step. Washes can be performed similarly, since the stability of a covalent interaction eliminates diffusion problems. Elution is achieved with a compound that reacts specifically with the resin, again in batch mode. Since no chromatographic step is involved, the protein can be eluted in a minimal volume, and isolated from the resin by simple filtration.

Second, use of a natural amino acid as the capture tag allows this technique to be adopted in the case of recombinant proteins. If the target protein happens to possess a cysteine or threonine at the N-terminus, no further elaboration of the sequence is necessary. If not, different options are possible. Extension or substitution of the natural sequence with a threonine or a cysteine capture tag would probably not affect the biological activity of the target protein and is the simplest solution. Threonine seems the better option, since it avoids the problem of incorrect disulfide bridge formation. Alternatively, an enzymatically cleavable sequence could follow the terminal cysteine or threonine, and be removed after the purification, but adds expense and complexity. The results demonstrated are encouraging but of course the size limit of recombinant-derived polypeptides and proteins which may be purified by covalent capture remains to be determined, and larger proteins may require a Sepharose-based matrix rather than the PEGA and Sephadex matrices used here.

Third, if a cysteine or threonine capture tag is employed in SPPS in conjunction with a capping step after every amino acid coupling, it will allow the selective recovery of the correct sequence from among the multiple incomplete sequences produced, creating a simpler semi-purified product for final HPLC purification. A fourth important advantage in using cysteine as a capture tag lies in the increasing use of native chemical ligation (NCL) [13,14]. Since the introduction of NCL and expressed protein ligation [15], the number of applications of peptides and proteins with N-terminal cysteine has increased exponentially. NCL is used to join two or more protein or peptide fragments by transthioesterifying an N-terminal cysteine thiol in one fragment with a C-terminal thioester in the other fragment. The unstable thioester bond first formed then rearranges by an S→N acyl shift and yields a natural amide bond. For NCL, the process requires use of fairly pure peptides, limiting the length of precursor fragments employed in each ligation step to 30–35 residues. With our purification technique we believe it will be possible to extend the length of a peptide precursor beyond the 59-residue recombinant example shown here, expanding enormously the number of proteins accessible to NCL.

Finally, an N-terminal cysteine represents an optimal selective reactive group for the introduction of any type of reporter tag carrying an aldehydic or ketonic function [7]. This would be of enormous advantage in protein expression, since such a polypeptide product contains both the purification tag and the derivatization tag, thus simplifying biochemical applications. By inserting a linker which is removable (chemically or enzymatically) under mild conditions, between a cysteine or threonine capture unit and the target protein the covalent capture approach becomes generally applicable.

4. Significance

After a protein or polypeptide has been produced, by chemical or recombinant DNA means, it is necessary to purify it. Quite often, the crude sample contains some protein or polypeptide species which are quite close in structure and properties to the wanted product and purification can be a lengthy and expensive process with sometimes low yields. We describe a rapid, simple and high-yield one-step purification process which is based on chemical rather than physical properties. The technique is particularly suited for the purification of polypeptides used in fragment condensations such as NCL or expressed chemical ligation. By eliminating bulk impurities in a simple step, covalent capture facilitates final purification at a smaller scale by complementary techniques.

5. Materials and methods

5.1. Peptide synthesis

All peptides were prepared by SPPS using machine-assisted protocols on a custom-modified Applied Biosystems model 430A peptide synthesizer using the in situ neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation procedure for Boc chemistry as described [16]. Peptide identity was confirmed by MALDI-TOF spectroscopy on a Voyager Elite or STR DE machine (PE Biosystems, Foster City, CA, USA).

The short peptide sequences used were: Cyak, CYAKYAKL; Tyak, TYAKYAKL; Syak, SYAKYAKL; and yak, YAKYAKL. These peptides were purified by preparative RP-HPLC. The longer peptides were used directly as crude material: Pep1 was CAVVFVTRKNRQVSANPEKKAVREYINSLELA and Pep2 ACAVVFVTRKNRQVSANPEKKAVREYINSLELA. Rantes 34–68 was CSNPAVVFVTRKNRQVCANPEKKWV-

REYINSLEMS, while the truncated fragments of Rantes 34–68 were Ac-39–68, Ac-VVFVTRKNRQVCANPEKKWVREYINSLEMS; Ac-44–68, Ac-RKNRQVCANPEKKWVREYINSLEMS; and pGlu-49–68, pGlu-VCANPEKKWVREYINSLEMS.

5.2. Capture resin preparation

The polyethyleneglycol–polystyrene (PEG)-based aldehyde resin was prepared starting from Amino PEGA resin (Novabiochem, Laufelfingen, Switzerland) with a nominal free amine substitution of 0.05 mmol/g of wet resin. The resin was rinsed with dimethylformamide (DMF), then treated with succinic anhydride/*N*-hydroxybenzotriazole/diisopropylethylamine (10/10/10 equivalents calculated over the amine substitution) in DMF overnight. Excess reagents were removed by filtration and the resin washed with DMF. The same washing procedure was applied to all the following steps. The carboxylic resin thus obtained was activated with 40 eq. of 1,1′-carbonyl-diimidazole in DMF for 1 h, washed, and functionalized with 30 eq. of 2-amino-acetaldehyde diethyl acetal in the presence of 20 eq. of *N*-hydroxybenzotriazole. The aldehyde function was liberated from the acetal by treating the resin with trifluoroacetic acid (TFA)/H_2O (1:1) for 1 h and rinsing with H_2O/acetonitrile (1:1). Aldehyde resin substitution was evaluated using a fluorescence method based on the dye dansylhydrazine [17]. The evaluation was conducted on the resin washed with dichloromethane and dried under vacuum overnight. The total amount of dansylhydrazine found bound to the resin was corrected by subtracting the amount of dye molecules associated non-covalently with the same amount of amino PEGA resin capped with acetic anhydride. We calculated an aldehyde substitution of 0.4 mmol/g of dry resin. This value is consistent with the expected value (0.4 mmol/g of dry resin) considering the orginal resin substitution reported by the producer (0.05 mmol/g of wet resin). We measured a loss of weight after the drying procedure of 7 parts in 8, so the wet resin used possessed a nominal aldehyde substitution of 0.05 mmol/g. The resin was stored at 4°C as a slurry in H_2O/acetonitrile for up to 5 months.

The dextran support was prepared starting from Sephadex CM C50 (Pharmacia, Uppsala, Sweden), with a nominal carboxylic acid substitution of 4.5 mmol/g of dry resin. The resin was swollen according to the manufacturer's instructions, and equilibrated with 200 mM sodium phosphate buffer, pH 6.5. The carboxylic function was activated with a water solution of *N*-hydroxysuccinimide and 1′-ethyl-(3′-dimethylaminopropyl)-carbodiimide·HCl (2 eq. and 4 eq. respectively over the carboxylic groups) for 8 min. The resin was rapidly rinsed with H_2O and incubated for 1 h with a water solution of 2-amino-acetaldehyde diethyl acetal (1 M) and 200 mM 2-morpholino-ethanesulfonic acid (MES) adjusted to pH 6.4 with HCl. After 1 h the aldehyde precursor was eliminated, any unreacted sites were blocked by incubation with a solution of ammonium acetate 1 M pH 7.5 for 10 min, and the resin rinsed thoroughly with 10 mM HCl to liberate the aldehyde function. The resulting aldehyde resin was equilibrated with 0.1 M acetic acid prior to storage at 4°C. It was active for up to 5 months.

5.3. Cloning and expression of Rantes 10–68

Met-Rantes 10–68, MCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS, was made from single-strand phage DNA encoding the full-length native molecule. The truncated gene was created using classical PCR techniques and inserted into the expression vector pET-22b. Plasmid production was obtained using *E. coli* XL1-blu strain. The purified plasmid was introduced into *E. coli* BL21 strain. Cells were grown on 5 l of LB medium [18] and induced for 4 h at 37°C with 1 mM IPTG. After centrifugation, cells were processed with lysis buffer, sonicated and washed with 2 M urea/5 mM DTT. The inclusion body fraction was collected by high speed centrifugation.

5.4. Covalent capture protocol

In a typical capture experiment on the PEG resin, peptides were dissolved in a solution of H_2O/acetonitrile 1:1 containing either 0.1 M MES at pH 6.4 or 0.1 M sodium acetate at pH 4.5. For peptides containing cysteine, 10 mM TCEP was used to limit disulfide bond formation. Peptides were used at a concentration of 1 mM or 5 mM. Peptide solutions were incubated with the resin previously conditioned with the same buffer, and kept under gentle agitation. The volume of settled resin corresponded to a quarter of that of the peptide solution. Experiments were conducted using either equimolar amounts of resin-bound aldehyde and peptide, or an excess of two equivalents of aldehyde over peptide, or an excess of 10 equivalents of aldehyde over peptide. In different experiments, at different time points (from 1 to 24 h) the supernatant was eliminated by filtration, and the resin was incubated with a washing buffer with the same composition and volume as the loading buffer, and washed for 10 min three times. Elution was accomplished using a solution of 200 mM *O*-methylhydroxylamine hydrochloride in H_2O/acetonitrile (1:1) unbuffered (pH 2.9). The eluted material was collected by filtration. Extent of capture was evaluated by analytical RP-HPLC, performed on a Brownlee Lab HPLC system with 214-nm UV detection, using a Macherey-Nagel C18 analytical column 250×2 mm i.d. at a flow rate of 0.15 ml/min and linear gradients of solvent B in solvent A as indicated in the figure legends. Solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in 90% acetonitrile and 9.9% water.

For recombinant Rantes 10–68, the inclusion body fraction recovered from a 1 liter induction was suspended in 10 ml of 6 M guanidinium chloride, 0.1 M MES, 10 mM TCEP. The fraction not solubilized was discarded, and the solution added to 1 ml of dextran resin slurry, previously conditioned with the same buffer. After 12 h capture the resin was washed with 10 ml buffer three times for 10 min, and the peptide eluted with 2 ml of a water solution of 400 mM *O*-methylhydroxylamine hydrochloride and 10 mM TCEP. The recovered quantity was evaluated by analytical RP-HPLC using as standard Rantes and correcting for the change in extinction coefficient.

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References

[1] D.E. Meyer, A. Chilkoti, Purification of recombinant proteins by fusion with thermally-responsive polypeptides, Nature Biotechnol. 17 (1999) 1112–1115.
[2] M.A. Roggero, C. Servis, G. Corradin, A simple and rapid procedure for the purification of synthetic polypeptides by a combination of affinity chromatography and methionine chemistry, FEBS Lett. 26 (1997) 285–288.
[3] A.R. Brown, M. Covington, R.C. Newton, R. Ramage, P. Welch, The total chemical synthesis of monocyte chemotactic protein-1 (MCP-1), J. Peptide Sci. 2 (1996) 40–46.
[4] S. Funakoshi, H. Fukuda, N. Fujii, Chemoselective one-step purification method for peptides synthetized by the solid-phase technique, Proc. Natl. Acad. Sci. USA 88 (1991) 6981–6985.
[5] L.E. Canne, R.L. Winston, S.B.H. Kent, Synthesis of a versatile purification handle for use with Boc chemistry solid phase peptide synthesis, Tetrahedron Lett. 38 (1997) 3361–3364.
[6] J.C. Spetzler, J.P. Tam, Unprotected peptides as building blocks for branched peptides and peptide dendrimers, Int. J. Peptide Protein Res. 45 (1995) 78–85.
[7] L. Zhang, J.P. Tam, Thiazolidine formation as a general and site-specific conjugation method for synthetic peptides and proteins, Anal. Biochem. 1 (1996) 87–93.
[8] T. Wöhr, B. Rohwedder, F. Wahl, M. Mutter, Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis, J. Am. Chem. Soc. 118 (1994) 9218–9227.
[9] J.P. Tam, C. Rao, C. Liu, J. Shao, Specificity and formation of unusual amino acids of an amide ligation strategy for unprotected peptides, Int. J. Peptide Protein Res. 45 (1994) 209–216.
[10] T. Kawamura, S.S. Cohen, D.L. Borris, E.A. Aquilino, S. Glushakova, L.B. Margolis, J.M. Orenstein, R.E. Offord, A.R. Neurath, A. Blauvelt, Candidate microbicides block HIV-1 infection of human immature langerhans cells within epithelial tissue explants, J. Exp. Med. 20 (2000) 1491–1500.
[11] G. Simmons, P.R. Clapham, L. Picard, R.E. Offord, M.M. Rosenkilde, T.W. Schwartz, R. Buser, T.N. Wells, A.E. Proudfoot, Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist, Science 11 (1997) 276–279.
[12] M. Meldal, Pega: a flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis, Tetrahedron Lett. 33 (1992) 3077–3080.
[13] P.E. Dawson, T.W. Muir, I. Clark-Lewis, S.B.H. Kent, Synthesis of proteins by native chemical ligation, Science 266 (1994) 776–779.
[14] J.P. Tam, Q. Yu, Z. Miao, Orthogonal ligation strategies for peptide and protein, Biopolymers 51 (1999) 311–332.
[15] T.W. Muir, D. Sondhi, P.A. Cole, Expressed protein ligation: a general method for protein engineering, Proc. Natl. Acad. Sci. USA 95 (1998) 6705–6710.
[16] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S.B.H. Kent, In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences, Int. J. Peptide Protein Res. 40 (1992) 180–193.
[17] B. Yan, W. Li, Rapid fluorescence determination of the absolute amounts of aldehyde and ketone groups on resin supports, J. Org. Chem. 62 (1997) 9354–9357.
[18] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.