A simple and rapid procedure for the purification of synthetic polypeptides by a combination of affinity chromatography and methionine chemistry

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Abstract Chemical synthesis of bioactive peptides has become a widespread and rapidly growing technique due to automated and efficient protocols for chain assembly. For most applications, the crude synthetic product must be purified to remove residual reactants, failure sequences and chemically modified peptide species. We propose here a method of universal applicability based on immobilized metal ion affinity chromatography, CNBr cleavage and use of reversible Met-sulfoxide protection. With this method we were able to purify to homogeneity in high yield the PbCS 242–310 polypeptide corresponding to the C-terminal region of *Plasmodium berghei* CS protein.

Key words: Peptide synthesis; Peptide purification; Affinity chromatography

1. Introduction

During the last decade solid phase peptide synthesis, using either t-Boc or F-moc strategies, has been largely improved. Sophisticated protocols of synthesis have allowed the preparation of polypeptides of 70 or more residues [1–5]. Nevertheless, incomplete coupling and chain termination that may occur during any cycle of peptide assembly lead to the formation of deletion and truncated sequences. Such problems together with the possible occurrence of side reactions observed mainly during final cleavage from the resin hamper straightforward separation of the desired peptide from other impurities. The purification of long synthetic polypeptides is a major problem in the production of products useful for biological studies or for human and animal use where a high level of purity is mandatory. In particular, when sequences containing 30 or more residues are synthesized, differences in physical properties such as size, charge and hydrophobicity between the desired product and deleted, truncated or modified peptide impurities may be too small to allow adequate purification. In addition, modern powerful separation techniques, such as reverse phase HPLC, are often limited by low yields and small sample 'loadability' which is time consuming and expensive. Different approaches to circumvent these limitations have been tested. Biotinylation of a 153 residue IL-1 synthetic protein [6] and a 99 residue SIV protease synthetic protein [7] was performed and the biotinylated chains were isolated on avidin-agarose columns. Ball et al. [8] recently proposed a purification procedure based on the addition of a reversible protecting group bearing a lipophilic, acidic or basic function to the last residue of the peptide chain. More specific chromatographic methods have been optimized exploiting the presence of particular residues in the synthesized sequences. For example, cysteine-containing peptides have been purified by reaction with immobilized mercury derivatives [9] or activated thiols [10]. In addition, immobilized metal ion affinity chromatography (IMAC) [11] has been successfully applied for the purification of peptides containing histidine or tryptophan [12] or recombinant proteins to which a histidine tail was purposely added. Taking advantage of this latter approach we developed a purification procedure of general applicability based on the combination of: (1) a simple capping protocol and replacement of methionine residue(s), if present, with methionine sulfoxide during assembly of the native sequence; (2) N-terminal elongation of the desired peptide with 1 methionine, 2 glycine residues and a terminal stretch of 6 histidines; (3) after acid cleavage, purification of the peptide by immobilized Ni affinity chromatography followed by cyanogen bromide cleavage of the histidine tail and, if necessary, final reduction of methionine sulfoxide to methionine. This simple strategy allowed rapid purification to homogeneity of the 69 residue polypeptide PbCS 242–310, covering the C-terminal region of the *P. berghei* CS protein [13], in high yield and using only inexpensive chromatographic procedures.

2. Materials and methods

2.1. Reagents and solvents

Chemicals and solvents used for the peptide synthesis were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland) and Fluka (Buchs, Switzerland).

2.2. Peptide synthesis

The polypeptide PbCS 242–310 covering the C-terminal region of *P. berghei* CS protein [13] was chemically synthesized using solid phase F-moc chemistry in an Applied Biosystems 431A Peptide Synthesizer. The polypeptide was prepared on a Fmoc-Ser(t-butyl)-p-alkoxybenzylalkohol resin (Wang resin) with a degree of substitution of 0.43 mmol/g at the 0.1 mmol scale. The synthesis was performed using a five-fold excess of F-moc amino acid derivatives, DCCI and HOBt as activating agents, a 60 min coupling time for the first 34 amino acids and a double coupling for the following residues. Capping with acetic anhydride was performed at the end of each cycle. Side chain protecting groups included: t-butylthio group for Cys; pentamethylchroman-sulfonyl group for Arg; triphenylmethyl group for Asn, Gln and His; t-butoxycarbonyl group for Lys and Trp; t-butyl group for Asp, Glu, Ser, Thr and Tyr. Met 306 was inserted as Fmoc-Met-sulfoxide. The peptide was then elongated N-terminally with the sequence His-His-His-His-His-His-Gly-Gly-Met using the same conditions described above but capping was omitted after coupling of the second Gly. Crude peptide was obtained by treating the peptide resin with 2.5% H_2O, 5% triethylsilan in TFA for 2 h at room temperature.

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Abbreviations: IMAC, immobilized metal ion affinity chromatography; P. berghei, *Plasmodium berghei*; TFA, trifluoroacetic acid; DIPC, diisopropylcarbodiimide; HOBT, hydroxybenzotriazole

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Synthetic peptide was desalted and partially purified from low MW fragments by size exclusion liquid chromatography (Sephadex G50 column 70×2.5 cm using 50% acetic acid/H_2O as mobile phase).

2.3. IMAC and CNBr cleavage

A Ni-column was prepared with 25 ml Ni-NTA agarose resin (Qiagen Inc., Chatsworth, USA) and equilibrated with buffer A (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris, pH adjusted to 8.0 with H_3PO_4). Size exclusion purified His tag PbCS 242–310 polypeptide was dissolved in buffer A and loaded on the column with a flow rate of 15 ml/h. The column was washed with buffer A (flow rate 15 ml/h) and buffer B (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris, pH adjusted to 6.3 with H_3PO_4) containing 50 mM imidazole at a flow rate of 30 ml/h. The His tag PbCS 242–310 polypeptide was then eluted (flow rate 30 ml/h) with buffer B containing 250 mM imidazole. The eluted material was desalted by a Sephadex G25 column (50×2.5 cm using 50% acetic

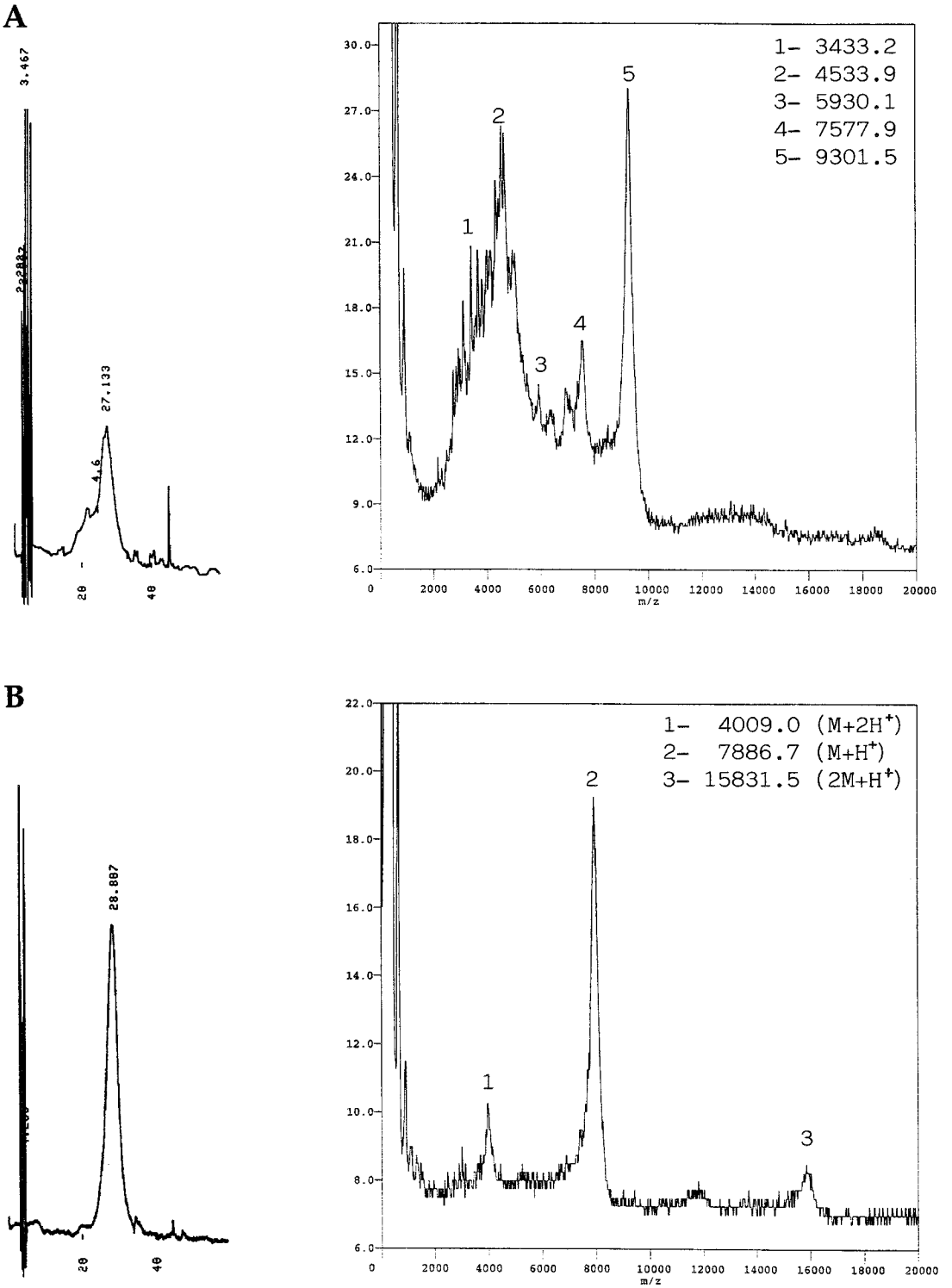

Fig. 1. Analytical HPLC profiles and mass spectra of A: crude, and B: purified peptide. The HPLC chromatograms were obtained injecting 100 μg of each sample and monitoring at 280 nm with an a.u.f.s. of 0.2.

acid/H_2O as mobile phase), lyophilized and treated for 8 h at RT at a concentration of 20 mg/ml in 70% TFA using a 100-fold molar excess of CNBr. The digested material was lyophilized, solubilized in buffer A and loaded again on the Ni-NTA agarose column. The flow through of the column was desalted by a Sephadex G25 column (50×2.5 cm using 50% acetic acid/H_2O as mobile phase) and lyophilized.

2.4. Cys deprotection and Met-sulfoxide reduction

The CNBr treated and IMAC purified material was treated with 10% β-mercaptoethanol [14] in buffer A and further purified by gel filtration (Sephadex G25 column 250×4.4 mm).

2.5. Peptide analysis

The purity of peptide was analysed by RP-HPLC using a C4 W-Porex 250×4.6 mm column and a 10–90% CH3CN gradient in 0.1% TFA/H_2O in 60 min with a flow rate of 1.0 ml/min. The amino acid composition was determined according to Knecht and Chang [15].

2.6. Mass spectrometry

Mass spectrometry analysis was performed using a time of flight mass spectrometer LDI 1700 Mass Monitor (Linear Scientific Inc., Reno, NV, USA). Five μl of 1 mg/ml of polypeptide were mixed with 5 μl of trans-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) 20 mg/ml in acetonitrile (Linear Scientific Inc., Reno, NV, USA) and 1.0 μl of this solution was placed on the mass spectrometer probe tip and dried with a gently vacuum. The sample was irradiated with 3 ns laser pulses (wavelength 337 nm) from a N_2 laser. Time of flight was measured with a digital oscilloscope (series 9304; Le Croy Research Systems, Corp., Spring Valley, NY, USA) which was converted into mass spectrum using the Peptide MALDI-TOF MS Calibration Standard (Linear Scientific Inc., Reno, NV, USA).

3. Results

The 69 residue polypeptide PbCS 242–310 corresponds to the C-terminal region of *P. berghei* CS protein [13]. Synthesis of His tag PbCS 242–310 was performed using an automated protocol in which a capping step was included after each coupling as described in Section 2. More than 150 mg of crude polypeptide were obtained by treating 600 mg of the corresponding peptide resin. Mass spectral analysis indicated the presence of the species of interest (9301.5, expected MW = 9305) together with other low MW components (Fig. 1A). The peptide mixture was partially purified by size exclusion liquid chromatograph. Ninety mg of size exclusion purified His tag PbCS 242–310 polypeptide were purified by IMAC on a 25 ml Ni-column (Fig. 2). The purified material was CNBr digested and loaded again on the Ni-NTA agarose column to eliminate the histidine tail and undigested peptide. The purified PbCS 242–310 polypeptide was then treated with β-mercaptoethanol to deprotect the blocked cysteines and to reduce Met-sulfoxide 306 to methionine. After gel filtration, 19 mg of pure polypeptide were finally recovered, corresponding to approximately 20% of the initial crude material. In Fig. 1, the analytical chromatographic profiles and mass spectra of crude (A) and final purified peptide (B) are compared. The difference of retention times between the two runs is due to the absence of the highly charged His tail in the purified material. Purified PbCS 242–310 was found to be approximately 95% pure based on the integration of peak areas when analysed at 214 nm. The amino acid composition of the CS polypeptide was consistent with that expected for this peptide (data not shown). Mass spectral analysis gave a MW of 7886.7 instead of the expected 7869 MW. This difference is probably due to the presence of a sodium ion.

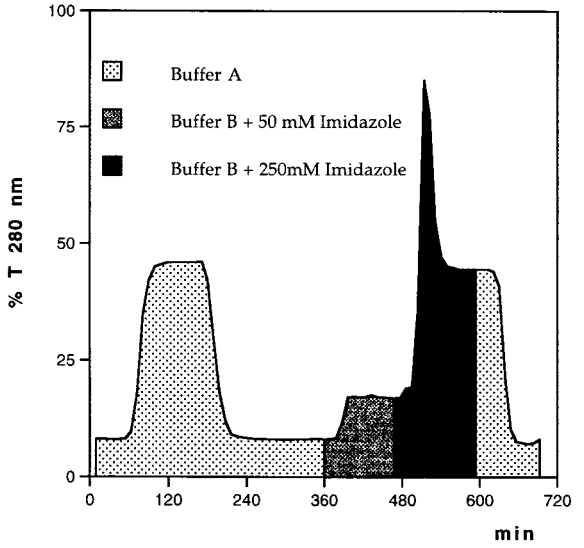

Fig. 2. Chromatographic profile of IMAC purification.

4. Discussion

The purification methods described so far for long peptides are not completely satisfactory since they are still time consuming and/or leave covalently derivatized peptides in the final purified products which may interfere or alter their biological and physico-chemical properties and their eventual utilization in animal and human experiments. In contrast, the procedure described here appears to meet the requirements of universality, high yield of recovery and easiness of the purification steps involved. This was achieved by insertion of a methionine residue before the affinity tag (stretch of 6 histidine residues or other purification facilitating compounds) followed, after appropriate purification steps, by elimination of the histidine tail by CNBr digestion, an inexpensive and very efficient process. The problem of the presence of Met residues in the desired sequence, as in our case, is easily circumvented using Met-sulfoxide residues which are resistant to CNBr treatment and quantitatively reducible with β-mercaptoethanol [14]. We have successfully applied this protocol to the purification of the polypeptide PbCS 242–310, a 69 residue chain corresponding to the C-terminal region of *P. berghei* CS protein [13]. Although many peptide impurities were present in the crude material after cleavage from the resin as shown by mass spectral analysis reported in Fig. 1, we were able to purify the target peptide to homogeneity in high yield and in relatively short time. The complete protocol of purification yielded 19 mg of purified PbCS 242–310, corresponding to approximately 20% of the crude material.

In conclusion, we have demonstrated here that purification using an His affinity tag, the specificity of the CNBr cleavage, and use of a reversible Met-sulfoxide protection in native sequence assembly can represent a novel general protocol for the efficient purification of solid phase synthesized polypeptides.

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