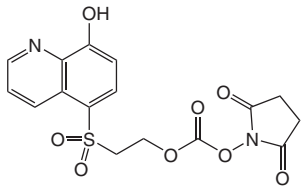
innovations 3 Vol. 12

Novabiochem®
NEW • Orthogonal purification strategy for long peptides

- IMAC purification for synthetic peptides
- Higher recoveries than RP-HPLC
- Effective for removal of closely eluting impurities

NEW • IMAC-based chemoselective tag for affinity purification of long peptides

IMAC Tag



In spite of the numerous methodological advances made over the last two decades, the preparation of large peptides and proteins by step-wise solid phase synthesis still remains problematic. This is principally due to difficulties in separating the target molecule from the mixture of closely related truncated and deletion products which arise during the synthetic process. In addition, the purified products, despite giving the appearance of being homogeneous by HPLC analysis, are often heterogeneous, being contaminated with numerous co-eluting sequences which, because they are individually only present in small amounts, escape detection by mass spectrometry.

One solution is to use a method of purification which has a selectivity orthogonal to RP-HPLC. The new chemoselective IMAC tag meets these requirements. It is extremely easy-to-use, gives higher recoveries than RP-HPLC, and is more effective at removing closely eluting impurites. Furthermore, as the purification is an on-off process, it can be readily automated using standard HPLC or FPLC instrumentation.

The mechanism of purification is analogous to HisTag affinity purification, the traditional method in use for isolation and purification of recombinant proteins. The process involves reacting the full length peptide sequence on resin with a cleavable tag which has an affinity for a IMAC column. During peptide synthesis, any failed couplings are capped forming truncated sequences, and so the only amino functionality available to react with the tag is the *N*-terminus of the full length peptide. Following IMAC purification, the tag is removed to give the desired sequence without relying on HPLC alone to obtain the required purity.

IMAC Tag

Features

- Reversible TAG for purification of peptides using immobilised metal affinity chromatography (IMAC) which is an orthogonal purification method to HPLC.
- Involves the temporary labelling of a peptide with a tag which binds to an IMAC column.
- Due to the capping step commonly employed in peptide synthesis, the tag only attaches to the desired, full length peptide and not to truncated sequences.
- Improved yields and purities compared with HPLC purification.
- Separation of large polypeptides from truncated sequences that co-elute during HPLC purification.
- Tag can be completely removed after the IMAC purification process.

Principles of use

The peptide is synthesized using standard methods. After each coupling unreacted amino groups must be capped using Ac_2O or Z(Cl)-OSu. Once the final Fmoc group is removed, the IMAC tag is attached to the peptide *via* an OSu carbonate.

Peptides are then cleaved from the resin under standard conditions and purified using a column functionalised with iminodiacetic acid (IDA) loaded with Cu^{2+} ions. Tagged peptide is bound to the column at pH 6.5 – 8.0 and truncated sequences are washed away before the tagged peptide is eluted from the column by adjusting the pH to 3.5. Buffers incorporating sodium phosphate, sodium chloride and urea are used to ensure maximum solubility of substrates.

Once the purified tagged peptide is eluted from the column, the TAG can be removed by simply raising the pH to 11.0 using 2 M NaOH for a short period. The liberated TAG and purified peptide can then be separated using the same IMAC column or an RP-HPLC column for combined isolation and de-salting. As reducing agents are not compatible with IDA, any cysteines present in peptides must be protected during the IMAC step. This can be done simply and reversibly using for example StBu protection.

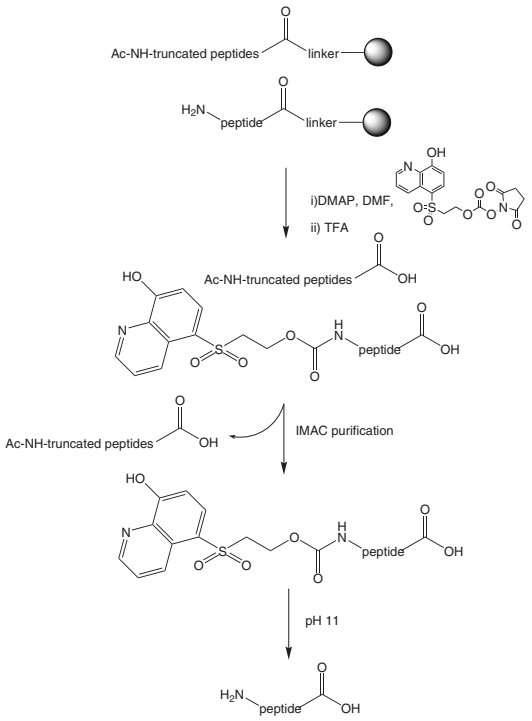


Fig. 1: IMAC purification of peptides.

Protocols

The procedure of attachment of the IMAC and cleavage of tagged peptide from the resin are shown in Method 1.

Method 1: Formation of IMAC-tagged peptide

Attachment of IMAC tag

1. Dissolve Tag-OSu in the minimum volume of DMF and add to pre-swollen resin. Coupling has been shown to be effective using as little as 1 eq Tag-OSu with respect to the initial resin loading.
2. Agitate the mixture for one hour, add a catalytic amount of DMAP and agitate for a further hour.
3. Wash the resin with DMF, DCM and diethyl ether before drying under vacuum.

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Cleavage of tagged peptide from the resin

1. Cleave non-cysteine containing peptides from the solid phase with concomitant side-chain deprotection by treatment with 90%TFA v/v, 5% H_2O v/v, 2.5% TIS v/v, 2.5% EDT v/v for 4.5 hours.
2. Cleave peptide-resins containing Cys(StBu) by treatment with 90% TFA v/v, 5% H_2O v/v, 5% TIS v/v for 4.5 hours.

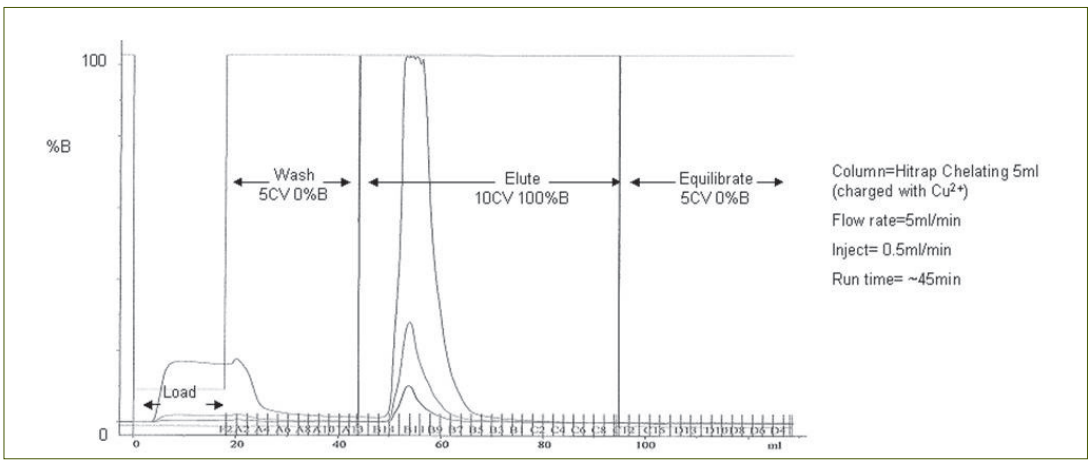

Fig. 2: A typical IMAC purification trace obtained using a GE Akta Explorer purification system. Untagged truncates elute during the load and wash step and tagged full length product remains bound to the column until the elute step.

The IMAC purification using a copper (II) loaded IDA column is given in method 2. Columns such as HiTrap Chelating HP worked well for this application.

Method 2: Purification of tagged peptide

Buffer preparation

1. Binding buffer for the IMAC chromatography may consist of 20 mM sodium phosphate, 2-8 M urea (depending on peptide solubility) and 0.5 M NaCl. A pH range of 6.5-8.5 can be used.
2) Elution Buffer should have a pH of 3.5. A suggested buffer within this range is citric acid-sodium phosphate system. Elution buffer should also contain Urea (2-8 M) and NaCl (0.5 M).

IMAC purification

1. Charge column with 0.5 column volumes (CV) of 0.1 M CuSO4 in H2O.
2. Wash column with 2 CV H_2O, 7.5 CV elution buffer, 10 CV binding buffer.
3. Solubilise crude tagged peptide in binding buffer.
4. Load sample onto IMAC column.
5. Wash any unbound material from the column with 10 CV binding buffer.
6. Elute tagged peptide from column using 20 CV elution buffer.

Method 3: Removal of tag

1. Combine fractions containing purified tagged product. Adjust to pH 11 with 2 M NaOH. If product contains Cys(StBu) add TCEP up to a concentration of 5 mM to remove S-tBu protecting groups simultaneously.
2. Agitate reaction for 1-2 hours at room temperature and then adjust pH to ~ 4 with 2 M HCl.
3. Separate liberated tag and purified peptide using a second IMAC step or carry out RP-HPLC to combine isolation with desalting.

Example purifications

Application 1: Purification of YLLPRRGPRLGV

The HCV 12mer sequence was tagged and purified using IMAC methodology. The crude peptide was shown to contain a major impurity identified as a capped truncate formed during SPPS (Figure 3). A recovery of 38% pure peptide (99% purity) was obtained after IMAC purification and tag cleavage. For comparison, purification using RP-HPLC gave pure peptide (95%) with a lower recovery of 21%.

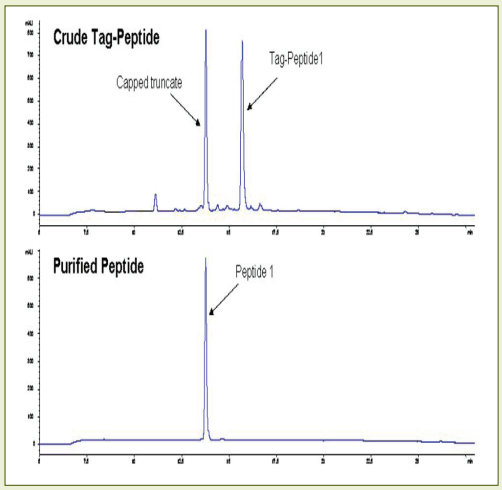

Fig. 3: HPLC profiles of YLLPRRGPRLGV before and after IMAC purification.

Application 2: Purification of Gly-GLP (2-36)

Gly-GLP (2-36) was also tagged and purified using IMAC methodology. The crude peptide was shown to contain several impurities identified as capped truncates formed during SPPS (Figure 4). A recovery of 22% pure peptide (89% purity) was obtained after IMAC purification and TAG cleavage. For comparison, purification using RP-HPLC gave pure peptide (81%) with a lower recovery of 5%.

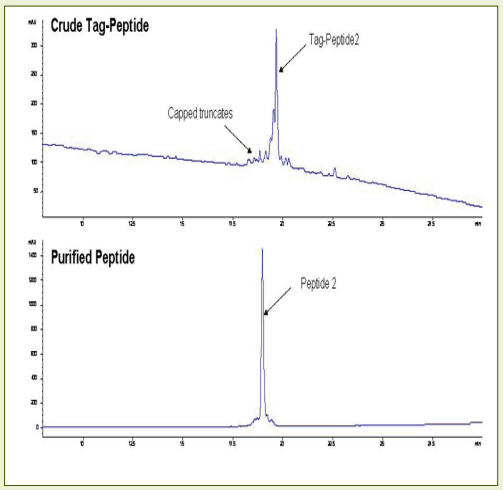

Fig. 4: HPLC profiles of Gly-GLP 2-36 before and after IMAC purification.

Application 3: Purification of Ubiquitin

Ubiquitin was also tagged and purified using IMAC methodology. The crude peptide was shown to contain several impurities identified as capped truncates formed during SPPS (Scheme 4). A recovery of 62% pure peptide (89% purity) was obtained after IMAC purification and TAG cleavage. For comparison, purification using RP-HPLC gave pure peptide (82%) with a lower recovery of 21%..

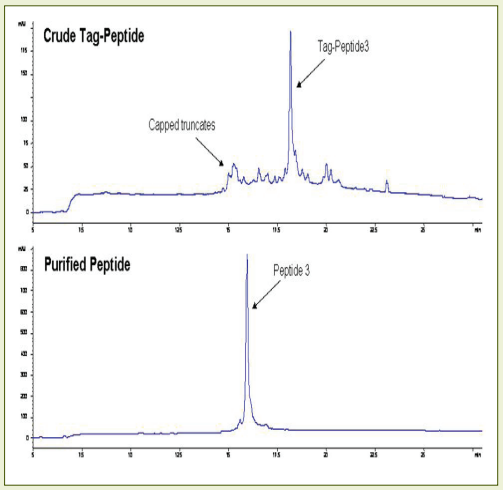

Fig. 5: HPLC profiles of Ubiquitin before and after IMAC purification.

Ordering Information

Cat.No.	Product	Contents	Price EUR
851208	IMAC tag	250 mg	85.00
NEW	Sold for research use only.	1 g	325.00
	Other tags		
851092	4-Nitrophenyl 2-(octadecylsulfonyl)ethyl carbonate	250 mg	52.00
		1 g	180.00

References

1. PCT Application No.: PCT/GB2011/000363

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