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Intein-mediated purification of a recombinantly expressed peptide

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A 26 amino acid peptide has successfully been purified *via* recombinant expression as an intein fusion protein accompanied by cleavage without the need for any exogenous proteases or cofactors, thus offering a practical, inexpensive approach to produce isotopically labelled peptides.

Peptides are involved in the physiology of normal biological pathways such as Ca^{2+} and insulin signalling as well as pathological processes such as Alzheimer's disease and type 2 diabetes. Bioactive peptides such as hormones, antibiotics, growth factors and toxins can be used as tools to probe physiological mechanisms. Peptides that adopt discrete folds, or mini-proteins, are useful prototypes for the study of full-length proteins due to their small size and natural amino acid construction.[1] For example, mini-proteins produced *de novo* have been used as models to study protein structure, protein folding, protein–protein interactions, DNA binding and the incorporation of proteins into membranes.[2–5] Peptides have also been used to probe protein activity such as phosphorylation and ion channelling and they have been called "natural pharmaceuticals" for their potential as drugs.[6–8]

The inherent conformational flexibility of peptides causes difficulties in production. Besides the well characterized chemical synthesis, other techniques of purification have been attempted such as recombinant expression as a fusion protein with protease cleavage[9] and recombinant expression with chemical cleavage.[10,11] Purification of peptides through traditional recombinant techniques is often a desired alternative to the well characterized method of solid-state peptide synthesis (SSPS). Peptide yields from SSPS are relatively low and more importantly, the high price of isotopically labelled peptides generated in this way make it an impractical technique to produce the yields needed for NMR. The advantage of recombinant expression is the peptide is translated by the normal *E. coli* ribosomal machinery. This enables the uniform incorporation of isotope tags such as ^{2}H, ^{13}C and ^{15}N when expressed in the appropriate media. Such peptides can be useful tools for segmental labelling of proteins[12,13] or complete labelling of peptides for NMR structure determination.[14]

Until recently, recombinant expression of peptides has been either unachievable or impractical due to difficulties in the purification process. It is likely that this is due to their small size and lack of defined structure. Small peptides are likely to interfere with the normal cellular processes and make good targets for protease degradation. To circumvent these problems, expression as a fusion protein followed by site-specific cleavage by either a protease or a chemical reagent has been the most successful strategy for purification; however, these techniques are limited. Cleavage by proteases such as thrombin or factor Xa often results in non-specific degradation of the unstructured peptide.[9] Chemical cleavage is limited because of the specific amino acid sequences required. For example, hydroxylamine cleavage requires an Asn–Gly peptide bond at the site of cleavage, resulting in an unavoidable glycine at the N-terminus of the peptide. Additionally, cyanogen bromide cleavage disallows the presence of methionine in the peptide.[10,11]

In this communication, a 26 amino acid peptide is recombinantly expressed and purified without the requirement for any exogenous factor and therefore no specific amino acid constraint. Peptide yields are comparable to those of other recombinant techniques. The approach employs the natural fold of inteins to promote peptide bond cleavage at the C-terminal end of the intein *via* succinimide formation by cyclization of asparagine,[15] thus allowing directed cleavage upon a change in pH and temperature[16] (Fig. 1). The benefit of this technique is that there is no protease treatment and no specific amino acid requirement in the peptide. The cysteine at the N-terminus of the peptide is not involved in the purification, however it is necessary for the subsequent peptide ligation in the expressed protein ligation (EPL)[17,18] described below.

The 26 amino acid peptide generated in this study corresponds to the 26 amino acids at the C-terminal end of rabbit fructose 1,6-bisphosphate aldolase (EC 4.1.2.13). DNA encoding the aldolase C-terminal peptide was fused in frame to the C-terminal end of the *Ssp* DnaB intein supplied by the vector pTWIN1 (New England Biolabs, Beverly, MA). The open reading frame also encodes a chitin-binding domain at its N-terminal end (Fig. 1). Expression was carried out as per the manufacturers' instructions (NEB). Briefly, cells containing the pTWIN/peptide fusion plasmid were induced with 0.4 mM IPTG and grown for an additional 16–20 h at 18 °C then washed with buffer (20 mM Na-HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.5) and lysed using a French-press at 10 000–15 000 psi in buffer supplemented with 2.5 mM PMSF. Clarified cell extract was run over a chitin column containing approximately 15 ml of sedimented chitin pre-equilibrated with the same buffer. The column was washed with >20 column volumes of buffer. Cleavage of the fusion protein was induced by running about 2–5 ml of buffer at pH 6.0 over the column at 26 °C, at which time the column flow was stopped and the column incubated for 17 h. Flow was restarted and the first 15 ml of eluant was collected and desalted by running over a 35 cc, C18 Sep-Pak column (Waters, Milford, MA) which had been prewashed with 0.1% trifluoroacetic acid (TFA). The C18 column was washed with >4 column volumes 0.1% TFA followed by 15 ml of 75% acetonitrile, 0.1% TFA. Elution off the C18 column was achieved with 15 ml of 85% acetonitrile, 0.1% TFA. The sample was then frozen in a dry ice

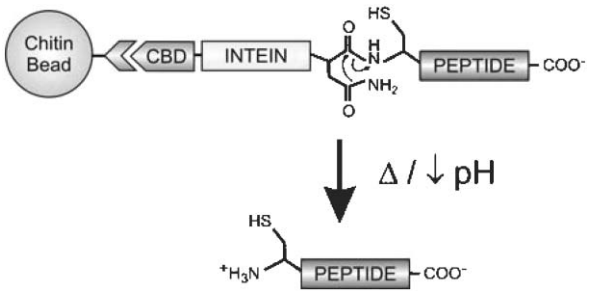

Fig. 1 A diagrammatic representation of the recombinant peptide purification scheme. The peptide, corresponding to the 26 amino acids at the C-terminus of rabbit aldolase A (aa 338–363), is fused in-frame to the C-terminal end of the *Ssp* DnaB intein (NEB). After the fusion protein is purified *via* a chitin column, the unique fold of the intein induces succinimide formation *via* asparagine cyclization, with cleavage upon an increase in temperature and a decrease in pH. This results in the purification of a peptide without the addition of any exogenous proteases or cofactors. Abbreviation: CBD: chitin-binding domain.

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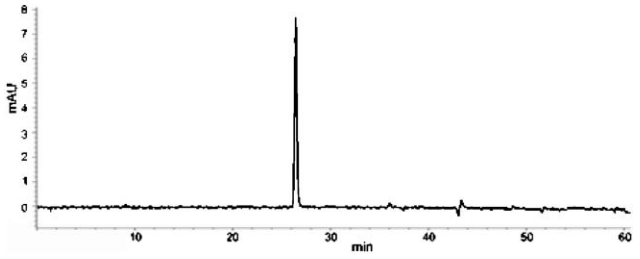

Fig. 2 HPLC analysis of the purified peptide. The lyophilized peptide was dissolved in 2% acetonitrile, 0.1% TFA to a final concentration of 0.5 mM. 20 μl was injected onto a Symmetry® C18 4.6 × 150 mm HPLC column (Waters) and run at 1 ml min^{-1} with increasing concentration of acetonitrile from 2–60% at 1% min^{-1}. Absorbance was monitored at A_{254} and the background chromatogram was subtracted.

bath and lyophilized. The average weight of precipitant was 1.4 mg per 1 l of culture. The lyophilized product was run on a C18 HPLC column (Fig. 2). The presence of a single peak confirms the purity of the peptide and verifies a final yield of 1.4 mg of pure peptide per l of culture. Ion-spray mass spectrometry revealed the presence of a 2659 MW polypeptide which is the expected size of the encoded 26 amino acid peptide (data not shown). ^{15}N labelled peptide was purified using the same protocol after expression in M9 minimal media supplemented with 0.1% [^{15}N]-NH_4Cl (Cambridge Isotope Laboratories, Inc., Andover, MA) with a final yield of 1.6 mg of labelled peptide per l of culture.

One of the more recent uses of peptides has been as molecular building blocks which ligate to proteins using the technique of EPL.[17,18] These experiments were performed with chemically synthesized peptides. Concomittantly, EPL has been used to isotopically label only partial segments of full-length proteins.[12,13] One of the advantages of the purification described herein is the ability to easily and inexpensively generate isotopically labelled peptides. Thus, EPL is a good technique with which to test the efficacy of this purification method by attempting to ligate the purified peptide to a larger protein which was done by testing the ability of the aldolase peptide to ligate to the rest of the aldolase protein. A truncated form of rabbit aldolase A, Ald-337, consisting of the first 337 amino acids (out of 363) was purified as a fusion protein to the *Sce* VMA intein using the pTYB1 expression vector supplied in the IMPACT™-CN system (NEB). Expression and purification took place as per the manufacturers' instructions and included elution from a chitin column with the addition of the thiol reagent, 2-mercaptoethanesulfonic acid (MESNA). The activity of pure Ald-337 was tested by measuring the cleavage of fructose 1,6-bisphosphate as indicated by a decrease in A_{340} in an assay coupled to NADH oxidation by glycerol 3-phosphate dehydrogenase.[19] The specific activity of Ald-337 was only 4% that of wild-type enzyme (Table 1). This result is consistent with the 20-fold reduction of activity seen upon removal of amino acids at the C-terminus after carboxypeptidase treatment of wild-type aldolase A.[20]

After purifying Ald-337, ligation of the C-terminal peptide was tested utilizing the naturally occurring cysteine at position 338, which in this system, is located on the N-terminus of the peptide. Ligation was accomplished by dissolving the lyophilized peptide, to a final concentration of 500 μM, in a 100 μM solution of Ald-337 in 20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0. The ligation reaction proceeded to a maximum of 35% completion after 22 h at 37 °C (Table 1). At 22, 37 and 45 °C, the % ligation was essentially the same, yet specific activity decreased at 45 °C. At 4 °C, both the % ligation and specific activity were slightly less than at 22 and 37 °C (Table 1). Ligation efficiency was measured by following the shift in size of aldolase from 37 kDa to 40 kDa on a 15% SDS-PAGE gel. In order to determine if ligation of the peptide to Ald-337 disrupted aldolase structure and/or function, recovery of aldolase activity after ligation was measured. The specific activity of

Table 1 Recovery of aldolase activity upon the ligation of the C-terminal peptide (22 h incubation)

Enzyme	C-terminal peptide added	Ligation temp/°C	% Ligation	Specific activity/ units mg^{-1}
Aldolase A	N	45	n/a	1.3
Ald-337	N	45	n/a	0.05
Ald-337	Y	4	25	1.4
Ald-337	Y	22	33	2.4
Ald-337	Y	37	35	2.2
Ald-337	Y	45	31	1.1

ligated protein was indistinguishable from that of wild-type after incubating at the same temperature, 45 °C (Table 1). This result demonstrates that a peptide purified in this manner is able to ligate to a protein *via* EPL and restore enzymatic activity. This occurs without the need to denature and renature the protein.

These experiments describe a novel approach to peptide expression and purification. The protocol as a whole is a series of familiar molecular biological and biochemical techniques that are well characterized. Although these studies tested the applicability of a single peptide, there are seemingly no limitations on the composition of the peptide. Possibly even more significant is the demonstrated utilization of this technique in protein engineering, specifically for use in the construction of segmentally labelled proteins for use in NMR.

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