Intein-mediated Rapid Purification of Recombinant Human Pituitary Adenylate Cyclase Activating Polypeptide

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Abstract In order to obtain the recombinant human PACAP efficiently by intein-mediated single column purification, a gene encoding human PACAP was synthesized and cloned into Escherichia coli expression vector pKYB. The recombinant vector pKY-PAC was transferred into E. coli ER2566 cells and the target protein was over-expressed as a fusion to the N-terminus of a self-cleavable affinity tag. After the PACAP-intein-CBD fusion protein was purified by chitin-affinity chromatography, the self-cleavage activity of the intein was induced by DTT and the rhPACAP was released from the chitin-bound intein tag. The activity of the rhPACAP to stimulate cyclic AMP accumulation was detected using the human pancreas carcinoma cells SW1990. Twenty-two milligrams of rhPACAP with the purity over 98% was obtained by single column purification from 1 liter of induced culture. The preliminary biological assay indicated that the rhPACAP, which has an extra Met at its N-terminus compared with the native human PACAP, had the similar activity of stimulating cAMP accumulation with the standard PACAP38 in the SW1990 cells. A new efficient production procedure of the active recombinant human PACAP was established.

Key words intein; recombinant human pituitary adenylate cyclase activating peptide (rhPACAP); purification

Pituitary adenylate cyclase activating polypeptide (PACAP), a neuropeptide released by the hypothalamus, was originally identified by its property of dramatically stimulating adenylate cyclase activity in anterior pituitary cells [1]. PACAP belongs to the superfamily of metabolic, neuroendocrine and neurotransmitter peptide hormones that include vasoactive intestinal polypeptide (VIP), secretin, glucagon, growth hormone releasing factor (GRF) and corticotropin-releasing factor (CRF) [2]. The biologically active peptides released from the biosynthetic precursor exist in two molecular forms: a 38-amino acid peptide (PACAP-38) and a 27-amino acid peptide (PACAP-27) with an amidated carboxyl terminus [2]. The highest concentrations of both forms are found in the central and peripheral nervous system [3]. They are also widely distributed in peripheral tissues, such as adrenal gland, stomach, pancreas, lung, intestine, testis and central and peripheral lymphoid organs [4]. The effects of PACAP are mediated by PACAP-prefering type 1 (PAC1) and two VIP-shared type 2 receptors (VPAC1 and VPAC2), which are G-protein-coupled receptors and exert their action through the cAMP-mediated and other Ca^{2+}-mediated signal transduction pathways [5]. PACAP has comparable activities toward all three receptors. In addition to functioning as a hypophysiotropic hormone, neurotransmitter and neuromodulator [1,2], PACAP displays diverse biological effects in neurogenesis [6], reproduction [7,8] stimulating insulin secretion [9], inducing selective neuronal differentiation of embryonic stem cells [10], modulating the immune system [11] and protecting cardiovascular system [12]. Due to the important role of PACAP in physiology, the therapeutic uses of PACAP have been widely explored, such as the therapy for type 2 diabetes [13], erectile dysfunction [14] and nerve injury [15], or the prospective therapeutic tools as the immuno-
PACAP is usually produced by chemical synthesis that is time-consuming and costly. To achieve rapid and efficient purification of the recombinant PACAP, we fused PACAP as a target protein to the N-terminus (Cys1) of a modified intein from *Saccharomyces cerevisiae* (*Sce* VMA intein, 454 amino acids), which in turn links to the chitin-binding domain (CBD) [17]. The CBD allowed the binding of the fusion protein to a chitin column and the intein was capable of undergoing peptide bond cleavage at its terminus. The C-terminal residue (Asn454) of the intein has been mutated to an alanine. This blocks the splicing reaction but still allows an N-S acyl rearrangement to occur at the intein N-terminus (Cys1) resulting in the formation of a thioester linkage between the target protein and the intein. Cleavage of the thioester bond can be induced by thiol reagents, such as 1,4-dithiothreitol (DTT), β-mercaptoethanol or cysteine. Use of DTT or β-mercaptoethanol results in the formation of a thioester bond between the thiol compound and the C-terminal residue of the target protein. This thioester is not stable and hydrolyzes to yield a free C-terminus. As a result, the rhPACAP with an extra N-terminal methionine was released from its affinity tag without the use of a protease and separated from its fusion partner on the same column.

Due to the specificity of the cleavage reaction and very mild cleavage/elution conditions, the rhPACAP with high purity was obtained. It was found that the purified rhPACAP has similar specific activity of promoting the cAMP in the cell line SW1990 of human pancreas carcinoma as the standard PACAP obtained by chemical synthesis.

**Materials and Methods**

**Strains, plasmids and cell lines**

Plasmid pKYB was purchased from New England Biolabs (NEB). *E. coli* strain ER2566 ([*fhuA2 (lon) ompT lacZ::T7 gene1 galsulA1 D(mcrC-mrr)114::IS10 R(mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10-TetS) endA1 (dcm)]), which allowed IPTG-regulated expression of T7 RNA polymerase, was presented by NEB. The human pancreas carcinoma cell line SW1990 was purchased from Zhongshan University.

**Enzymes, reagents and kits**

Restriction enzyme *SapI, NdeI* and chitin beads were purchased from NEB. T4 ligase and *Taq* polymerase were purchased from TaKaRa. DNA purification kit of QIAquick column was purchased from Qiagen. The enzyme immunoassay kit for cyclic AMP was purchased from Cayman Chemical. The standard PACAP-38 was from ANASPEC, and the goat polyclonal antibody raised against human PACAP was purchased from Santa Cruz. Low range protein marker was purchased from Amresco.

**Synthesis of PACAP gene**

The gene encoding PACAP was designed according to the codon bias of *E. coli* to ensure the high expression. The PACAP gene was synthesized and amplified in two steps using three Oligonucleotides primers (Fig. 1). First, the PACAP gene chain extension reaction was carried out as follows: the reaction mixtures (100 µl) containing TaKaRa LA Buffer (containing 4 mM MgCl2), 40 pmol of each primer F1 and F2, 300 nM each dNTP were denatured at 94 °C for 10 min and then annealed at 55 °C, 1 µl LA *Taq* DNA polymerase was added, and extension was undertaken at 72 °C for 5 min. Second, the PACAP gene was amplified using the chain extension product as the template. 100 µl PCR mixture were composed of LA Buffer (TaKaRa), 300 nM each dNTP, 40 pmol primer F1.
and F3, 10 µl of chain extension product and 2.0 u LA Taq DNA polymerase (TaKaRa). Amplification was carried out using a Biolab thermal cycler at 94 ºC for 45 s, 55 ºC for 45 s, and 72 ºC for 1.5 min, for 35 cycles.

F1: 5’-GGTGGTGTCAATGCACTCTGACGGCATCTCCTACAGACTTTCCGCTTACCGAAAACAAATGGCTGTCA-3’ (Ndel site underlined)
F2: 5’-TCCCCTACCGAAAAACAAATGCTGTCAAGAAATACCTGGGCGCGTGCTAGGGAAAAAGG-3’
F3: 5’-GGTGGTTGCTCTTCCCGCATTTTTTAA-CCCTCTGTATTACCTTTCCTAGCACGCAGCCGC-3’ (SapI site underlined)

**Construction of recombinant vector**

The PCR product containing the synthesized gene was purified on the QIAquick column. After double digestion with Ndel and SapI, the purified DNA fragment was directly ligated to Ndel/SapI double digested pKYB vector to yield pKY-PAC (Fig. 1). Insertion of the PACAP gene was verified by restriction digestion and DNA sequencing.

**Fusion protein expression**

The pKY-PAC plasmid was transformed to E. coli strain ER2566. Cells were grown in an air shaker (250 rpm) at 37 ºC in LB medium containing 50 mg/ml of kanamycin. Fusion protein expression was verified by restriction digestion and DNA sequencing.

**Activity assay for purified recombinant PACAP**

The cell line SW1990 of human pancreas carcinoma was used in the activity assay of the rhPACAP to stimulate the cAMP production. The SW1990 cells cultured in DMEM nutrient at 37 ºC were scraped off the surface with the scraper or rubber policeman, washed with PBS for two times and the density of the cells was adjusted to 2×10^6 cells/ml. Equal amount of the purified rhPACAP and standard PACAP-38 was added to 500 µl cell suspension respectively, and the working concentrations of the peptides were changed from 10^{-12} M to 10^{-6} M. The reactions were incubated at 37 ºC for 5 min, and then vortexed after 2 ml ice-cold ethanol was added. The precipitate was removed by centrifugation at 1500 g for 10 min, and the supernatant was collected to the clean test tube and dried by vacuum centrifugation. The cAMP quantities in the samples were measured using the enzyme immunoassay kit for cyclic AMP according to the operating instructions.

**Tricine-SDS-PAGE**

Tricine-SDS-PAGE was carried out according to Yang et al. [18], which was suitable for the analysis of protein with molecular weight less than 20 kDa. The proteins in the gels were stained with 0.1% Coomassie brilliant blue G-250. The molecular weight of the enzyme was estimated using low molecular weight marker.

**Protein analysis**

The concentration of proteins in the clear lysate was determined by the BCA method [19] using bovine serum albumin as a standard. The percentage or the purity of target proteins were estimated by analyzing the Tricine-SDS-PAGE gel stained with Coomassie blue using Chemilnager V5.5 software for image collecting and analyzing. The cleavage efficiency was represented by the percentage of the intein-CBD to the total proteins (including the uncleaved fusion precursor and the intein-CBD tag).
eluted by strip buffer (1% SDS).

The binding efficiency \( (E) \) of the fusion precursor to the chitin resin was calculated just when the chitin column will not be saturated by the precursor according to the following formula:

\[
E = \frac{P1 - P2}{P1}
\]

\( P1 \) represents the percentage of fusion precursor to the total proteins of the clarified cell extract before loading onto the chitin column, and \( P2 \) represents the percentage of fusion precursor to the total proteins of the flow through.

Results

Construction the recombinant plasmid pKY-PAC

The synthesized human PACAP gene was inserted into the plasmid vector pKYB by \( NdeI \) and \( SapI \), and the site recognized by \( SapI \) disappeared in the recombinant plasmid pKY-PAC. The recombinant clone identified by PCR and DNA sequencing (Fig. 2) was transformed to \( E. coli \) strain ER2566, and the engineering strain pKY-PAC-ER2566 was constructed.

Affinity purification and cleavage of fusion protein

After IPTG induction, the PACAP-intein-CBD fusion precursor accumulated as a soluble product observed in the clarified lysate and reached 28% of the total soluble proteins (Fig. 3, lane 4). After passing an excessive supernatant over the chitin column, more than 98% of the fusion precursor was bound to the resin as determined according to the method mentioned (Fig. 3). This was due to extremely high affinity of the CBD for chitin. In addition, since the CBD cannot be eluted from the chitin resin under nondenaturing conditions, extensive washing was performed to remove most nonspecifically bound contaminating proteins (Fig. 4, lane 6).

Flushing the column with DTT triggered the intein-
mediated cleavage reaction, which released the rhPACAP whereas the intein-CBD fusion partner remained bound to the resin. By additional column buffer through the resin the rhPACAP was eluted (Fig. 4, lanes 7,8). The purity of the peptide was over 98% using the stringent washing conditions.

The uncleaved fusion precursor and the intein-CBD were stripped from the resin by 1% SDS and examined by Tricine-SDS-PAGE, and the concentration of the proteins were determined using image analysis software. The cleavage efficiency was represented by the percentage of the intein-CBD to the total proteins eluted by the strip buffer.

It was shown that incubation of the column at 16 °C for 16 h allowed the nearly complete cleavage reaction (Fig. 4, lane 10) and lower temperature (4 °C) for the cleavage reaction resulted in 80% cleavage efficiency (Fig. 4, lane 9).

DTT was removed from the elution by dialysis. The tricine-SDS-PAGE showed that the peptide was stable after dialysis in deionized water overnight at room temperature. The purified peptide was further demonstrated by Western blot analysis with human PACAP antibody (Fig. 5). The Laser flying mass spectrometry showed the precise

**Fig. 3** Identification of the binding efficiency of the fusion precursor to the chitin column

1, protein marker; 2, crude extract from uninduced cells; 3, crude extract from cells, induced at 30 °C for 16 h; 4, clarified crude extract from induced cells; 5, chitin column flow through.

**Fig. 4** Identification of inducing expression and purification of the recombinant PACAP on tricine-SDS-PAGE

1, protein marker; 2, crude extract from uninduced cells; 3, crude extract from cells, induced at 30 °C for 16 h; 4, clarified crude extract from induced cells; 5, chitin column flow through; 6, chitin column wash; 7 and 8, fractions of eluted rhPACAP after self-cleavage reaction; 9, SDS stripping of remaining proteins bound to chitin column (incubation at 4 °C for 16 h); 10, SDS stripping of remaining proteins bound to chitin column (incubation at 16 °C for 16 h).

**Fig. 5** Tricine-SDS-PAGE and Western blot of purified rhPACAP

(A) Tricine-SDS-PAGE analysis of purified rhPACAP. (B) Western blot analysis of purified rhPACAP. 1, low range protein marker; 2, purified rhPACAP after dialysis.
molecular weight of rhPACAP was 4536.8 u (Fig. 6), which was consistent with the theoretical molecular weight.

**Activity assay of purified recombinant PACAP in vitro**

The effects of the rhPACAP and the standard PACAP-38 on cyclic AMP accumulation in the cell line SW1990 of human pancreas carcinoma were shown in Fig. 7. Both the rhPACAP and the standard PACAP38 significantly stimulated cyclic AMP accumulation in a dose-dependent when the working concentrations varied from $10^{-12}$ M to $10^{-8}$ M. When the working concentration was higher than $10^{-8}$ M, the effects of the peptides to stimulate the accumulation of cyclic AMP reduced. The preliminary in vitro assay indicated the rhPACAP had almost the identical activity of promoting the cAMP production in SW1990 cells to the standard PACAP-38.

**Discussion**

PACAP is usually produced by chemical synthesis that is time-consuming and costly. A recombinant expression system was recently reported in which the PACAP derived mutant peptide was purified as a fusion to the glutathione S-transferase (GST) using an affinity column. The fusion protein was then treated with protease to separate the target peptide from its fusion tag, GST, followed by an extra chromatographic step to purify the PACAP mutant peptide from the protease and GST [20]. To achieve rapid purification of the recombinant PACAP using affinity chromatography and at the same time avoiding protease treatment and extra steps, we expressed PACAP as a fusion protein with the N-terminal fused to a modified protein-splicing element (called intein). Protein splicing is a posttranslational processing event in which an internal protein segment, the intein, can catalyze its own excision from a precursor protein and concomitantly ligate the flanking regions, the exteins, to form a mature protein [21, 22]. Intein fusion systems have been used to purify target proteins from their recombinant precursors without the addition of exogenous proteases [23–26]. In this report, the IMPACT system (intein-mediated purification with an affinity chitin-binding tag) from New England Biolabs was used to achieve the one-step purification of recombinant PACAP.

A target protein can be fused to either the N-terminus (upstream) or the C-terminus (downstream) of the intein [27]. Since the N-terminal histidine of the natural PACAP was less suitable for the intein’s C-terminal cleavage (the cleavage efficiency being lower than 50% at 16 °C), and the lysine at the C-terminal of PACAP was optimal for its N-terminal cleavage (the cleavage efficiency being 75%–100% at 16 °C) [28], we constructed a plasmid encoding recombinant PACAP with N-terminal fused to the modified See VMA1 intein that allowed thiol inducible cleavage. Furthermore the codons of the synthesized gene encoding the hPACAP had been chosen to ensure the high expression of the artificial gene in *E. coli*. In this paper the expression of the fusion protein reached 32% of total bacterial protein and 28% of the total soluble proteins (Table 1). A small fraction of the fusion protein formed insoluble inclusion bodies resulting the low recovery after sonication and centrifugation (data not shown).

The final yield of the purified rhPACAP was mainly
Further experiments were in plan to identify the selectivity of at least one PACAP receptor. The expression of the rhPACAP on the SW1990 cells, though the identification that which receptor was involved in the functional increased its selectivity and potency [33]. It had not been observed with a VPAC2 receptor preferring ligand markedly decreased the biological effect [32], and the N-terminal hexanoylation of GST to PACAP did not influence the PAC1-mediated pathway [31]. In the other hand, the N-terminal fusion by both adenosine cyclase and calcium/calmodulin receptor single transduction of PACAP may be mediated in pancreatic acini [30]. PACAP-38 may play an important role in the proliferation of human pancreatic cancer cells such as JF303, HS766T and ASPC1; and the post-receptor single transduction of PACAP may be mediated by both adenosine cyclase and calcium/calmodulin pathways [31]. In the other hand, the N-terminal fusion of GST to PACAP did not influence the PAC1-mediated biological effect [32], and the N-terminal hexanoylation with a VPAC2 receptor preferring ligand markedly increased its selectivity and potency [33]. It had not been identified that which receptor was involved in the function of the rhPACAP on the SW1990 cells, though the expression of at least one PACAP receptor was sure. Further experiments were in plan to identify the selectivity and potency of rhPACAP to each type of the receptors using the CHO cells that expressed the three receptors respectively.

### References


### Table 1  The purification of rhPACAP from 1 L culture

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (g) a</th>
<th>Target protein purity (%) b</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
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<tr>
<td>Total bacterial production</td>
<td>1.550</td>
<td>32</td>
<td>ND</td>
<td>100</td>
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<td>Cleared lysate</td>
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<td>1.0</td>
<td>72</td>
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<td>Chitin column and intein-mediated cleavage</td>
<td>0.022</td>
<td>98</td>
<td>3.5</td>
<td>65</td>
</tr>
</tbody>
</table>

ND, not determined; a concentration of the proteins in the cleared lysate was determined by the BCA assay using BSA as a standard; b purity of target proteins estimated by analyzing images of the trice-SDS-PAGE gel stained with Coomassie blue using Chemilnager V5.5 software.


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