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High yield production of recombinant native and modified peptides exemplified by ligands for G-protein coupled receptors

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Abstract

G-protein coupled receptors (GPCRs) comprise a large family of membrane proteins and attract pharmaceutical interest as therapeutic targets. Two examples of class B GPCRs that are involved in metabolic diseases are the Parathyroid hormone receptor 1 (PTHR1) and the Glucagon-like-peptide-1 receptor (GLP-1R) which play central roles in osteoporosis and diabetes mellitus type II, respectively. Class B GPCRs are characterised by a large extracellular N-terminal domain with a typical disulfide bridge pattern. This domain is responsible for the binding of peptide hormone ligands. Here we report the recombinant expression of these ligands in natural and several modified forms for their use in functional assays, NMR analyses or affinity purification of receptor/ligand complexes for crystallisation. Applying the SUMO system, low cost expression of soluble fusion-proteins is achieved. Moreover, via the SUMO cleavage site, the authentic N-terminal sequence which is essential for ligand–receptor interactions can be obtained. Purification of the peptide by RP-HPLC results in >98% pure preparations. The strategy can also be adopted for many other purposes, especially if small peptides are needed at either large amounts or with specific features like isotope, affinity or fluorescent labels. Furthermore, for the growing demand for therapeutic peptides, this method could represent a straightforward production process.

Keywords: Peptide expression; Isotopic labelling; SUMO fusion protein; SUMO protease; G-protein coupled receptor; GLP-1; Glucagon like peptide-1; GLP-1 receptor; PTH; Parathyroid hormone; PTH receptor 1

Membrane proteins occupy key functions in the communication between the extracellular and intracellular space. In contrast to their importance and the plethora of different types of membrane proteins, only little information is available about their molecular structure. Currently, a lot of interest and work is focused on structural investigations of membrane proteins. The inherent problems are reflected by the small number of solved structures for membrane proteins which are mostly from non-mammalian sources [1].

G-protein coupled receptors (GPCRs)[1] comprise a large gene family of membrane proteins with more than 1000 members in human [2,3]. The relevance of these proteins is mirrored by the fact that more than 30% of the drugs on the market affect signalling via GPCRs [4]. The lack

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[1] *Abbreviations used:* SUMO, small ubiquitin related modifier; GPCR, G-protein coupled receptor; GLP-1, glucagon like peptide-1; GLP-1R, GLP-1 receptor; nGLP-1R, N terminal domain of the GLP-1R; ECD, extracellular ligand binding domain; PTH, parathyroid hormone; PTHR1, PTH receptor 1.

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of detailed structural information limits the development of highly effective, specific and save pharmaceuticals. Currently the only example of a high resolution structure of a GPCR is that of bovine rhodopsin [5]. A major problem is that most of the GPCRs are expressed at low levels in their respective tissues. To produce sufficient amounts of the receptors for biophysical characterisation we use recombinant expression in *Escherichia coli* inclusion bodies. The solubilised and renatured protein is subsequently tested for functionality and ligand binding is a critical parameter [6,7]. Therefore the aim of the present study was to express variants of the corresponding peptide ligands for the Parathyroid hormone receptor (PTHR1) and the Glucagon-like-peptide-1 receptor (GLP-1R) for analytical purposes in *E. coli*.

In the past 20 years peptides have gained prominence as pharmaceuticals. Examples are cytokines, growth factors and hormones. Also peptide agonists of PTHR1 and GLP-1R are applied as therapeutics. PTH(1–34) (teriparatide, Forteo™) exerts influence on the calcium homeostasis of osteogenic and renal cells thereby promoting bone formation in osteoporosis [8]. A GLP-1 related peptide from a lizard, exendin-4 (exenatide, Byetta®) enhances insulin while suppressing glucagon secretion and represents an approved drug for therapy of diabetes type II [9]. A lot of effort is made to find compounds with improved therapeutic features.

Therefore the availability of peptides is crucial in two ways: first for the characterisation of the respective receptors and understanding of the receptor ligand interaction and second for the development of drugs by modification of the natural species. The use of solid-phase chemical synthesis is limited if isotope labelled peptides, long peptides or fusion constructs have to be generated. On the other hand, recombinant expression of peptides shorter than 50 amino acids in *E. coli* is usually poor due to proteolytic degradation [10]. One strategy to obtain stable, structured products is to connect the peptide sequence to well characterised fusion partners like maltose binding protein [11], glutathione *S*-transferase [12] or cellulose binding domain [13]. The critical point of this approach is the subsequent elimination of the fusion partner which can be accomplished by insertion of a consensus sequence for a protease into the expression construct. Most of the proteases used for this purpose, however, exhibit also disadvantages like additional cleavage within the product, impurities in the protease preparation and amino acid residues from the consensus sequence remaining at the N-terminus of the peptide. Since in the case of PTH and GLP-1, a free and authentic amino terminus is essential for high affinity receptor binding [7,14] we developed a strategy based on the SUMO protein (small ubiquitin-related modifier, Smt3 protein from *Saccharomyces cerevisiae*) as an expression partner for our constructs. The system combines several desirable features: high expression levels of soluble protein, an N-terminal His-tag for purification and a unique cleavage site for the highly specific SUMO protease not depending on a consensus sequence and therefore not leaving additional residues at the N-terminus of the product [15]. With this system we could produce PTH, GLP-1 and exendin wild type peptides and variants in isotope labelled and tagged form, furthermore as fusions with fluorescent proteins. The recombinant peptides were highly pure (>98%), functional and were obtained at high yields.

Materials and methods

SUMO expression plasmids

The SUMO system from Invitrogen was used for some of the constructs according to the instructions of the supplier.

The expression plasmid pET SUMOadapt was derived by inserting a short DNA stretch constructed by two synthetic oligonucleotides (MWG) as a multiple cloning site into the pET SUMO vector via A/T cloning (Fig. 1A). The 5′-phosphorylated oligonucleotides SUMOadaptf (5′-AGAGACCTCAGGATCCAAGCTTGCGGCCGCC TCGAGA-3′) und SUMOadaptr (5′- CTCGAGGCGG CCGCAAGCTTGGATCCTGAGGTCTCTA-3′) were annealed and cloned into the linear form of pET SUMO with T-overhangs. The proper insertion into the vector

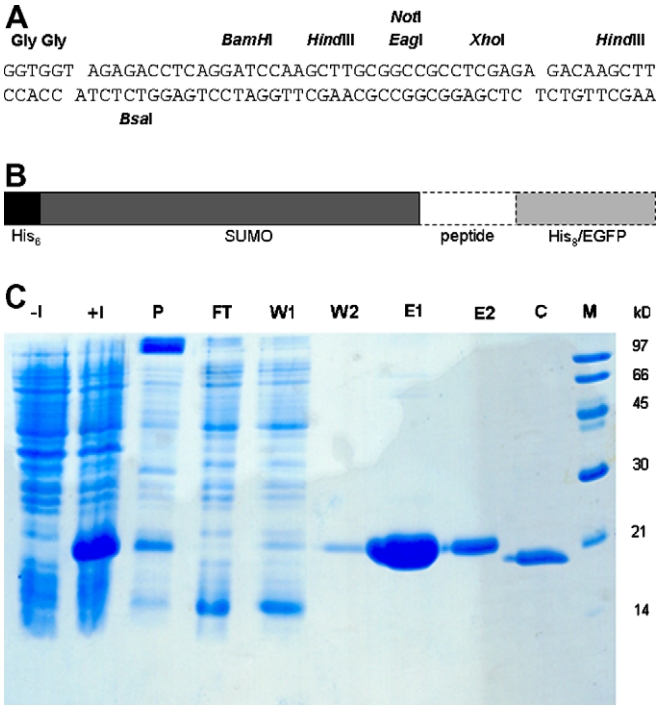

Fig. 1. Expression and purification of SUMO peptide fusion proteins. (A) Oligonucleotide for insertion of a multiple cloning site into pET SUMO via A/T cloning (flanking vector sequences shown). (B) Schematic structure of the expression cassette under control of the T7 promoter. The C-terminal His-tag or EGFP is optional, *Hind*III is not unique. (C) SDS–PAGE of the expression, purification and cleavage of SUMOPTH(1–34).Lane 1 and 2 represent *E. coli* lysates before (−I) and after (+I) induction, P corresponds to the insoluble fraction, FT, W1 and W2 are flow through and washing steps from the purification, E1 and E2 are elution fractions and C is the cleaved off SUMO protein.