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Expression and Purification of Delta Sleep-Inducing Peptide in *Escherichia coli*

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Abstract The delta sleep-inducing peptides (DSIP, Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) is an important regulatory hormone, controlling hypothalamus and pituitary functions. In the current study, an expression system was designed for the rapid and economic expression of recombinant DSIP for biophysical studies. Artificially synthesized oligonucleotides encoding DSIP were cloned into a pGEX-KG vector and expressed in *E. coli* (BL21). The recombinant GST-DSIP was then readily purified using a GST affinity column. To obtain intact DSIP from the GST-DSIP, thrombin cleavage and a CNBr reaction were successively carried out. The DSIP in the CNBr reaction mixture was subjected to RP-HPLC purification to yield 1.2 mg DSIP from a 1 liter culture of *E. coli*. Identification of the DSIP was performed using MALDI-MS and an amino acid composition analysis.

Key words: DSIP, expression, peptide production, labeling of small peptide

The delta sleep-inducing peptide (DSIP, WAGGDASGE) was originally isolated from cerebral venous blood of rabbits following electrical stimulation of the thalamus, which induces delta sleep in rabbits [24, 17]. Aside from its sleep-inducing function, DSIP has many additional functions; resistance against emotional stress [26], circadian hormone regulation from hypothalamus and pituitary [5–7, 9, 10, 23], activation of the GABA-ergic system [20], induction of cerebral monoamine oxidase activity [12], reduction of opioid and alcohol withdrawal symptoms [2], and alleviation of depression [27]. DSIP can also permeate the brain blood barrier *in vivo* and *in vitro* [3, 22]. Circular dichroism (CD) and molecular dynamic analyses of DSIP suggest hydrophobicity-driven diffusion across a bilayer [21]. Furthermore, DSIP is readily degraded by proteases in the apical side of Caco-2 cells *in vitro* [1, 4]. Although this fragile property of DSIP is a major obstacle for oral administration, DSIP has still received great interest, because of its functions related to depression, stress, and the withdrawal symptoms of alcohol and opiates [2, 26, 27]. Although DSIP was originally discovered and characterized in the late 1970s, further investigations of DSIP have been difficult due to its labile features [20]. Therefore, instead of DSIP *per se*, DSIP-like immunoreactivity (DSIP-LI) has been preferred in experiments, where about 70 amino acids with no matching sequence to DSIP, exhibit the same immunoreactivity to antiserum as DSIP [25]. As such, it is assumed that DSIP-LI is a precursor form or aggregate of DSIP [11]. To date, no crystallographic data have been published for DSIP as either a free ligand or bound to its receptor. Thus, in the absence of any direct structural information, the goal of the current study was to produce sufficient quantities of the peptide labeled with NMR-sensitive isotopes to facilitate structural multinuclear NMR studies of DSIP in both a solid and solution state.

Therefore, to obtain a relatively larger quantity of recombinant DSIP, a glutathione *S*-transferase (GST) fusion protein system (pGEX-KG) was used to express DSIP as a GST-DSIP fusion protein in *E. coli*. Since small peptides are not expressed stably and are easily degraded, the strategy of a fusion protein system (pGEX-KG) is to overexpress them and thereby protect them from degradation [13–16, 19].

For the synthesis and cloning of the oligonucleotides, two oligonucleotides were artificially synthesized: GATC-CATGTGGGCGGGTGGCGATGCCAGCGGCGAATAAA and AGCTTTTATTCGCCGCTGGCATCGCCACCC-GCCCACATG.

The oligonucleotides were then phosphorylated individually by T4 polynucleotide kinase in a kinase buffer containing 0.5 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, and 50 mM dithiothreitol. After incubation at 37°C for 2 h, the oligonucleotides were mixed in an annealing buffer

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containing 50 mM NaCl, 1 mM EDTA (pH 8.0), and 10 mM Tris-HCl. The mixture was then boiled for 3 min and cooled slowly to room temperature. The annealed DNA was cloned into the expression vector pGEX-KG, derived from pGEX-2T, to facilitate thrombin cleavage for improved purification [8].

The precultured cells (1/50 vol) were inoculated into an LB broth containing ampicillin and 0.4% glucose. The culture was incubated at 37°C until the $O.D._{600}$ reached 0.5-0.6, induced by 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) for 4 h ($O.D._{600}$=1.7), and then centrifuged at 4,000 rpm at 4°C. The harvested cells were resuspended in 1/25 vol of 100 mM ammonium bicarbonate and sonicated 4 times each for 30 sec. The cell lysate was mixed with 1% Triton X-100 (v/v), agitated for 30 min at 4°C, and then centrifuged at 13,000 rpm for 20 min. Next, the supernatant was applied to a GST affinity column, and the column was washed with 10 column volumes of 100 mM ammonium bicarbonate. The recombinant DSIP was obtained by thrombin cleavage for 20 h at room temperature, while the GST was still bound to the resin. The flow-through was filtrated to separate the DSIP and the thrombin, then the DSIP that passed through the filter was lyophilized. To remove any N-terminal remnant in the recombinant DSIP, i.e., an Met in the sequence, the recombinant DSIP was subjected to CNBr reaction [13]. Briefly, CNBr dissolved in acetonitrile was added to the DSIP solution, then formic acid was added to the mixture at a final concentration of 70%. The mixture was sealed and reacted for 20 h in the dark. The gel-like pellet formed after the reaction was resuspended in 40% acetonitrile and 0.1% trifluoroacetic acid (TFA), then stirred for 15 h at room temperature. The resulting solution was centrifuged at 15,000 rpm and 4°C for 20 min, and the supernatant was analyzed by RP-HPLC. The HPLC was performed on an Agilent 1100 Series from Agilent (Foster, CA, U.S.A.). The DSIP in the CNBr mixture was purified by RP-HPLC with a mobile phase of 5% acetonitrile with 0.1% TFA (A) and 80% acetonitrile (B). The column used was a μBondapak C_{18} column (3.9 i.d.x300 mm) from Waters (Milford, MA, U.S.A.) with a flow-rate of 0.5 ml/min and UV detector at 280 nm. Buffer B was increased from 0% (0 min) to 30% (30 min), rapidly increased up to 95% (33 min), isocratic elution was maintained for 2 min, buffer B was decreased to 0% (39 min), and then the column was washed for 20 min with buffer A. The molecular mass and amino acid composition of the recombinant DSIP were determined using a Voyager-DE STR from Applied Biosystems (Foster, CA, U.S.A.) and Biochrom 20 from Pharmacia Biotech (UK), respectively.

Artificial oligonucleotides encoding DSIP were synthesized, as the DSIP gene has not yet been found. The codons used in the gene were optimized depending on the codon usage of *E. coli*, as presented by the Web site http://www.kazusa.or.jp/codon [18]. The synthesized oligonucleotides did not have a phosphate group at the 5' end, therefore, they were

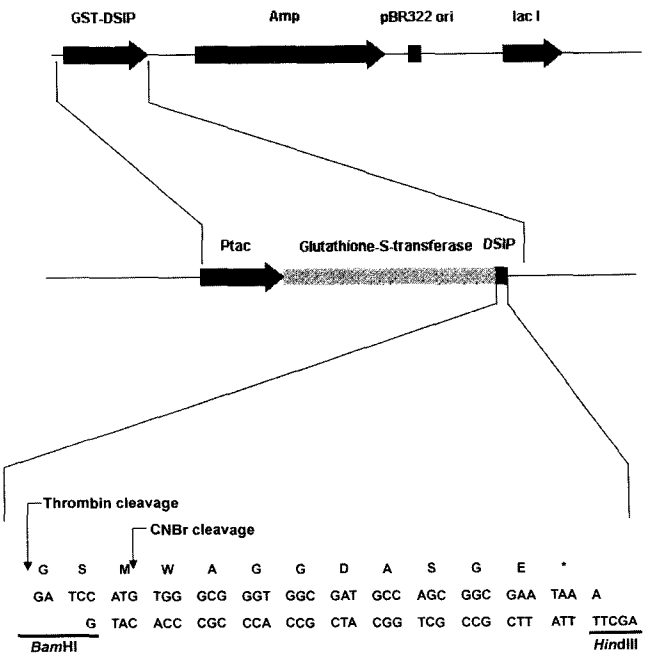

Fig. 1. Structure of synthetic gene for expression of GST-DSIP, showing synthesized oligonucleotides, and relevant protease/chemical cleavage sites.

The synthesized oligonucleotides had two restriction sites, *Bam*HI and *Hind*III, to facilitate insertion into a pGEX-KG vector. At the end of DSIP, a termination codon was used to stop the expression, thereby avoiding the attachment of any residue to the C-terminus.

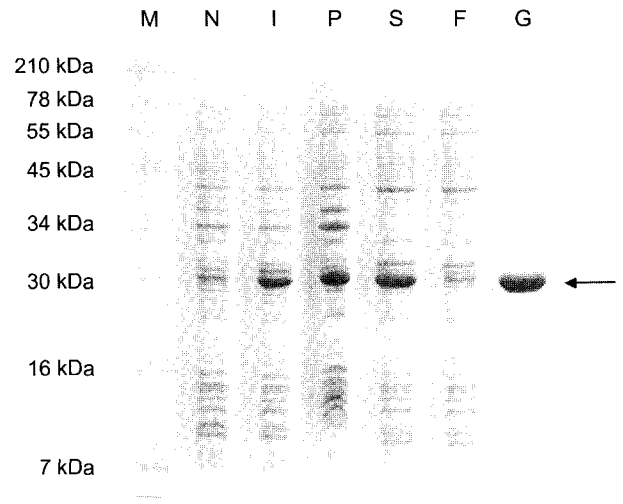

Fig. 2. Analysis of expressed and purified GST-DSIP. A dense band of 27 kDa corresponding to GST-DSIP is indicated by an arrow.

Lane M, protein standard; lane N, noninduced cells; lane I, 0.4 mM IPTG-induced cells; lane P, postsonicated pellet; lane S, postsonicated supernatant; lane F, flow-through; lane G, eluate of GST-cleaved by thrombin.

phosphorylated individually to ligate into a pGEX-KG vector harboring GST as the fusion protein. GST was chosen to solubilize and overexpress DSIP, thereby facilitating purification. Figure 1 shows a diagram of the constructed vector including the DSIP gene.

Figure 2 shows an analysis of the expressed and purified GST-DSIP in 15% SDS-PAGE. The GST-DSIP was detected as a molecular mass of 27 kDa (indicated by arrow). Lane I shows that the GST-DSIP expressed by IPTG induction was stable. When measuring the band intensity using densitometry at 595 nm, the expressed GST-DSIP was approximately 20% of that of the postsonicated supernatant (lane S in Fig. 2). During GST affinity chromatography, the DSIP was cleaved from the GST by thrombin, and the thrombin and DSIP were separated from the eluted solution by filtration. The filtered DSIP was then lyophilized. Since the purified DSIP was small in size, it was not detectable by PAGE, therefore, the GST cleaved by thrombin was electrophoresed (lane G in Fig. 2). The recombinant DSIP obtained by GST affinity chromatography exhibited a peak at 1125.4221 m/z, $[M+H]^+$ in MALDI-MS, reflecting the presence of an N-terminal fragment (data not shown). The N-terminal fragment

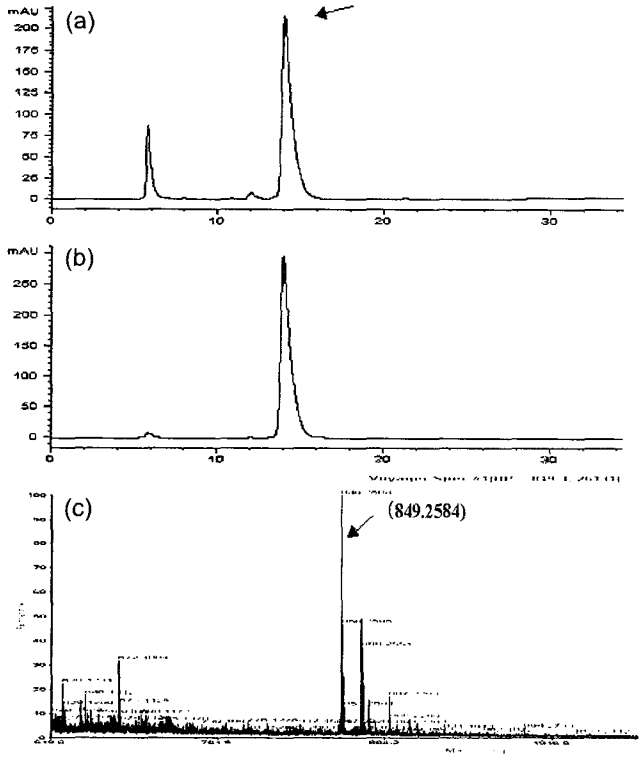

Fig. 3. Reverse-phase HPLC chromatogram and MALDI-MS of purified DSIP.
Profile of CNBr reaction mixture (panel A). The peak at the retention time of 13.9 min was collected and reinjected for verification (panel B), and analyzed by MALDI-MS (panel C). Intact DSIP was detected at 849.2584 m/z (arrow in panel C).

Table 1. Results of MALDI-TOF and amino acid composition analysis.

MALDI-TOF	Amino acid composition		
	Amino acid	Expected (%)	Real (%)
849.2584 Da	Asp	11.1	8.26
	Glu	11.1	11.25
	Ser	11.1	13.77
	Gly	33.3	38.10
	Ala	22.2	20.57
	Trp	11.1	8.05

in the recombinant DSIP was successively removed by CNBr reaction, and the mixture was analyzed by RP-HPLC (Fig. 3A). The peak at the retention time of 13.9 min (Fig. 3B) was subjected to MALDI-MS and an amino acid composition analysis, as shown in Table 1 and Fig. 3C. The peak for DSIP, at 849.2584 m/z $[M]^+$, matched the calculated molecular mass for DSIP. The amount of DSIP at each step of purification was measured and calculated by UV-spectroscopy with an A_{280} of 1 mg/ml DSIP (6.704) (Table 2). For the final purification by RP-HPLC, about 1.2 mg of DSIP was obtained from a 1 liter culture of *E. coli*.

Many recombinant peptides produced in *E. coli* are expressed in a tagged form. Generally, this tag facilitates the purification of the expressed protein from the extract using affinity chromatography, yet cleavage of the tag is usually required prior to measuring biological activity. Furthermore, unwanted amino acid residues often remain in the purified protein. When producing small peptides such as DSIP, such additional amino acid residues, which can result in a large change in the tertiary structure, must be removed if possible. However, the methods employed in the current study resulted in a high-level expression of the DSIP peptide that appeared to be identical to the native peptide. Accordingly, this method is proposed as an efficient and economic method for the uniform labeling of

Table 2. Purification of DSIP from *E. coli*.

Purification step	Total protein (mg)	Peptide of interest (mg)
Crude extract[a]	579.3[b]	115.8[c]
Glutathione chromatography	87.7	3.8[c]
Filtration	3.4[d]	3.0[e]
HPLC (after CNBr reaction)	1.2	1.2[e]

[a]The starting material was crude extract from 1 liter of an induced *E. coli* culture.
[b]Determined by Bradford assay using bovine serum albumin as the standard.
[c]Calculated by densitometry of gel at 595 nm.
[d]Calculated from HPLC profile. Each peak area was measured and compared.
[e]Measured by UV-spectroscopy at 280 nm. The absorbance of 1 mg/ml DSIP was 6.704, while that of 1 mg/ml Met-uncleaved DSIP was 5.062.

small peptides with an isotope easily identifiable in NMR and other biophysical studies.

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