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Synthesis of the *C*-terminal region of opioid receptor like 1 in an SDS micelle by the native chemical ligation: effect of thiol additive and SDS concentration on ligation efficiency

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Abstract: In the process of developing a method for the synthesis of membrane proteins, the conditions for native chemical ligation, namely, detergent concentration and the chemical characteristics of the thiol additive were investigated in detail. The *C*-terminal region of the opioid receptor like 1, ORL1(288–370), which contains *C*-terminal intracellular and transmembrane domains, was chosen as a model. The building blocks, ORL1(329–370) and ORL1(288–328)-SR-Gly-Arg_5-Leu (-SR- : -SCH_2CH_2CO-) were most effectively condensed slightly below the critical micelle concentration of SDS and in the presence of mercaptoethanesulfonic acid as a thiol additive. The results showed that the concentration of SDS and the charge on the thiol additive are crucial factors for the effective synthesis of a membrane protein by native chemical ligation.

Keywords: membrane protein; native chemical ligation; SDS micelle; GPCR; 2-mercaptoethanesulfonic acid

INTRODUCTION

Ligation chemistry is now a well established technique, since the introduction of a peptide thioester as a building block for the chemical synthesis of proteins [1–3]. Two ligation chemistries, the thioester method [1] and native chemical ligation [2,3] have been utilized successfully in the preparation of a variety of peptides [4–6]. Other techniques such as auxiliaries for ligation reactions [7–10] or expression, for use in preparing building blocks [11–14] represent new methodologies for the synthesis of proteins. These ligation methodologies are most effective in the preparation of a polypeptide that is difficult to obtain by a biological route. Membrane proteins are representative of such proteins. It is well known that the slow progress in the field of membrane protein research is due to difficulties in sample preparation. Therefore chemical synthesis based on ligation chemistry can be a powerful technique for the preparation of membrane proteins.

Our previous report [15] on the chemical synthesis of a protein with two transmembrane regions, F_1F_0 ATP synthase subunit c, via the thioester method, was the first trial to examine whether a membrane protein with multiple transmembrane domains could be synthesized by chemical procedures. During the study, effective conditions were found for ligation by the thioester method for the synthesis of a protein with two transmembrane domains. In addition, optimum conditions were determined for the RP-HPLC purification of a peptide containing transmembrane domain(s). Native chemical ligation has also been applied successfully to the synthesis of membrane proteins such as influenza A virus M2 [16] and a potassium channel KcsA [17]. These successes clearly show that ligation strategies, particularly the thioester method and native chemical ligation, can be used effectively for the synthesis of membrane proteins.

To expand the usefulness of ligation methodologies for the preparation of membrane protein, more general conditions need to be developed. In order to establish a strategy for membrane protein synthesis based on ligation chemistry, more challenges must be met for the efficient preparation of transmembrane peptide thioesters and for the optimization of conditions for the ligation reaction. One of the problems that can be easily imagined during the synthesis is the difficulty associated with solubilizing an extremely hydrophobic building block under conditions that are suitable for ligation, especially, in the case of the native chemical ligation, in which the ligation is performed in an aqueous buffer. A search for a solubilizing reagent such as a detergent is a general way to solve this problem. Indeed, for the synthesis of potassium channel KcsA [17], Valiyaveetil *et al.* examined additives such as TFE, guanidium chloride, urea and SDS for their abilities to dissolve hydrophobic building blocks. As a result, they synthesized the potassium channel protein via native chemical ligation using an aqueous solution of SDS (1%). However, a problem associated with the use of a detergent is that there is no best detergent for all cases. Therefore, the protocols for membrane protein synthesis by native chemical ligation must be investigated in detail. For

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this purpose, the synthesis of opioid receptor like 1 (ORL1) was initiated as a model compound. ORL1, of human origin, was first cloned as an orphan opioid receptor-like receptor [18] and is considered to be a G protein coupled receptor. The protein comprises seven transmembrane domains and plays a crucial role in signal transduction in cells. This report details an investigation of the application of native chemical ligation to the preparation of a membrane protein through the synthesis of ORL1(288–370) comprising the seventh transmembrane domain and the *C*-terminal intracellular domain of ORL1. During the synthesis, a technique was developed for enhancing the solubility of the building block containing a transmembrane domain. The results may also provide the basis for a procedure for the synthesis of membrane proteins.

MATERIALS AND METHODS

Boc-Leu-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives and the CLEAR acid resin were purchased from the Peptide Institute Inc. (Osaka, Japan). Ether, used for the washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). The amino acid compositions of peptides, except for peptide **3**, were analysed using an L-8500 amino acid analyser (Hitachi Ltd, Tokyo, Japan) after hydrolysis with a mixture of concentrated hydrochloric acid and trifluoroacetic acid (2 : 1) at 166 °C for 50 min in an evacuated sealed tube. Hydrolysis of peptide **3** was carried out with constant boiling point hydrochloric acid for 24 h at 110 °C. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA). Yields in the preparation of peptide or peptide thioesters were based on the amount of amino acid on the starting resin.

Synthesis of Boc-Gly-(Arg(Tos))$_5$-Leu-OCH_2-Pam Resin

Starting with the Boc-Leu-OCH_2-Pam resin (0.45 g, 0.74 mmol/g), the synthesis of Boc-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin, was performed manually in a stepwise fashion by the solid-phase method using the *in situ* neutralization protocol of Boc chemistry [19]. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1- to 2-min flow wash with DMF, a 10- to 20-min coupling time with preactivated Boc-amino acid in the presence of excess DIEA, and a second DMF wash. N^{α}-Boc-amino acids (1.0 mmol) were preactivated for 2 min with 1.0 mmol of HBTU in the presence of DIEA (1.4 mmol). After each coupling step, the yields were determined by measuring the residual free amine content on the peptide-resin using a quantitative ninhydrin assay.

Synthesis of Boc-Ala-SCH_2CH_2CO-Gly-(Arg(Tos))$_5$-Leu-OCH_2-Pam Resin

Boc-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin was treated with a 50% TFA/CH_2Cl_2 solution (1 × 5 min and 1 × 20 min), washed with CH_2Cl_2 (3 × 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 × 1 min), and then washed with NMP (3 × 1 min). Trt-$SCH_2CH_2CO_2H$ (0.35 g, 1.0 mmol), HOBt monohydrate (0.15 g, 1.0 mmol) and DCC (0.20 g, 1.0 mmol) in NMP (10 ml) was mixed for 30 min and the resulting solution was added to the resin. The suspension was shaken for 1 h, followed by a ninhydrin assay to measure the residual free amine on the resin, and washed with NMP (3 × 1 min). The resin was treated with an NMP solution containing 10% acetic anhydride and 5% DIEA (1 × 10 min) and then washed with NMP (5 × 1 min). The resulting resin was treated with TFA containing 5% ethanedithiol (1 × 5 min, 1 × 10 min, 1 × 20 min and 1 × 30 min) and then washed with CH_2Cl_2 (3 × 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 × 1 min), and then washed with NMP (3 × 1 min). A solution of Boc-Ala (0.19 g, 1.0 mmol), HOBt monohydrate (0.15 g, 1.0 mmol) and DCC (0.20 g, 1.0 mmol) in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and the suspension mixed for 14 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 × 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 × 10 min), washed with NMP (5 × 1 min) to give the Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin (0.98 g, Ala content in the resin found to be 0.15 mmol/g).

Fmoc-ORL1(288–328)-SCH_2CH_2CO-Gly-Arg$_5$-Leu-OH (1)

Starting with Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin (0.98 g; 0.15 mmol/g), the synthesis of Fmoc-Leu-Gly-Val-Gln-Pro-Ser(Bzl)-Ser(Bzl)-Glu(OBzl)-Thr(Bzl)-Ala-Val-Ala-Ile-Leu-Arg(Tos)-Phe-Cys-Thr(Bzl)-Ala-Leu-Gly-Tyr (Br-Z)-Val-Asn-Ser(Bzl)-Cys-Leu-Asn-Pro-Ile-Leu-Tyr(Br-Z)-Ala-Phe-Leu-Asp(OcHex)-Glu(OBzl)-Asn-Phe-Lys(Cl-Z)-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin, was performed manually in a stepwise fashion by the solid-phase method using the *in situ* neutralization protocol of Boc chemistry. Each synthetic cycle consisted of N^{α}-Boc removal by means of a 1- to 2-min treatment with neat TFA, a 1–2 min flow wash by DMF, a 10–20 min coupling time with preactivated Boc-amino acid in the presence of excess DIEA, and a second NMP wash. N^{α}-Boc-amino acids (1.0 mmol), except for N^{α}-Boc-Asn, which were preactivated for 2 min with 1.0 mmol of HBTU (0.5 M in DMF) in the presence of DIEA (1.4 mmol). Preactivation of N^{α}-Boc-Asn was performed with HOBt (1.0 mmol) and DCC (1.0 mmol) for 30 min followed by the addition of DIEA (1.4 mmol) before coupling. After each coupling step, the yields were determined by measuring the residual free amine content on the peptide-resin with a quantitative ninhydrin assay. After coupling of the Gln residues, a DCM flow wash was used before and after deprotection using TFA, to prevent possible high temperature (TFA/DMF)-catalysed pyrrolidone formation. The amount of fully protected peptide resin obtained was 1.31 g.

This fully protected peptide resin (300 mg) was deprotected and cleaved from the resin by treatment with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml) and 1,4-butanedithiol (0.75 ml) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, the resulting precipitate was washed three times with

ether and dissolved in TFA. This solution was passed through a glass filter and the product precipitated by the addition of cold ether. After washing the precipitate with ether, it was mixed with 50% aqueous acetonitrile and freeze-dried to give a crude powder (130 mg). Purification was performed by RP-HPLC to give the purified Fmoc-ORL1(288–328)-SCH_2CH_2CO-Gly-Arg_5-Leu-OH (4.5 mg) in the yield of 1.3%. MALDI TOF: found for *m/z*: 5710.8 (calcd for $[M + H]^+$: 5711.6: amino acid analysis: $Asp_{4.0}Thr_{1.6}Ser_{1.9}Glu_{3.3}Pro_{nd}Gly_3Ala_{5.1}Cys_{nd}Val_{3.0}Ile_{1.8}Leu_{7.0}Tyr_{1.3}Phe_{3.5}Lys_{0.9}Arg_{5.6}$.

Synthesis of ORL1(329–370) (3)

Peptide chain elongation was carried out with a peptide synthesizer 433 A (Applied Biosystems, Inc.) using the *FastMoc 0.25 mmol MonPrevPk* protocol with end capping by acetic anhydride. The first 25 amino acids were coupled in a single coupling protocol and the remainder in a double coupling protocol.

Starting with Fmoc-Ala-CLEAR acid resin (0.33 mmol/g, 0.80 g), a protected peptide resin corresponding to the sequence of peptide **3**, Cys(Trt)-Phe-Arg(Pmc)-Lys(Boc)-Phe-Cys(Trt)-Cys(Trt)-Ala-Ser(^{t}Bu)-Ala-Leu-Arg(Pmc)-Arg(Pmc)-Asp(O^{t}Bu)-Val-Gln(Trt)-Val-Ser(^{t}Bu)-Asp(O^{t}Bu)-Arg(Pmc)-Val-Arg(Pmc)-Ser(^{t}Bu)-Ile-Ala-Lys(Boc)-Asp(O^{t}Bu)-Val-Ala-Leu-Ala-Cys(Trt)-Lys(Boc)-Thr(^{t}Bu)-Ser(^{t}Bu)-Glu(O^{t}Bu)-Thr(^{t}Bu)-Val-Pro-Arg(Pmc)-Pro-Ala-CLEAR acid resin was obtained (1.3 g). This peptide resin (100 mg) was treated with a mixture of TFA, phenol, water, thioanisole and ethanedithiol (82.5 : 5 : 5 : 5 : 2.5 (v/v), reagent K [20]) with stirring for 150 min. Ether was added to the reaction mixture followed by stirring for 20 min. The resulting precipitate was washed with ether three times and then dissolved in a mixture of water and acetonitrile. The solution was passed through the disposable ODS column and then freeze-dried to give a crude powder (21 mg). Purification of peptide **3** was performed on a Cosmosil 5C18ARII column (10 × 250 mm, Nacalai Tesque), and a linear gradient of 0.1% TFA containing acetonitrile over 0.1% aqueous TFA at a flow rate of 2.5 ml/min was employed. The crude powder (27 mg) was applied to the column to give the purified peptide (4.6 mg) in a yield of 10% [mass (MALDI TOF) found: *m/z* 4656.8. Calcd for $[M + H]^+$ 4656.5 (average). Amino acid analysis: $Asp_{3.0}Thr_{2.3}Ser_{3.5}Glu_{2.2}Pro_{1.5}Ala_6Cys_{nd}Val_{4.6}Ile_{1.0}Leu_{2.2}Phe_{1.9}Lys_{2.8}Arg_{5.6}$].

Evaluation of the Synthesis of Fmoc-ORL1(288–370) via Native Chemical Ligation

Peptide thioester **1** (0.3 mg, 53 nmol) and peptide **3** (0.3 mg 64 nmol) were dissolved in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, 3.4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), thiol and SDS. As a thiol additive, 2-mercaptoethanesulfonic acid or thiophenol was used. For a detergent, SDS (35 mM or 7 mM) or hexadecyltrimethylammonium bromide (0.9 mM) was used. The reaction was monitored by RP-HPLC [Cosmosil 5C4 AR-300 column (4.6 × 150 mm), using a linear gradient of formic acid and 1-propanol (4 : 1) over formic acid and water (2 : 3) at the flow rate of 0.65 ml/min].

Synthesis of Fmoc-ORL1(288–370) (6)

Fmoc–ORL1(288–328)-SCH_2CH_2CO-Gly-Arg_5-Leu (1.2 mg, 46 nmol) and ORL1(329–370) (1.2 mg, 70 nmol) were dissolved in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, 3.4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 60 mM 2-mercaptoethanesulfonic acid sodium salt and 7 mM SDS. The mixture was stirred for 24 h at room temperature. The mixture was treated with DTT, and then loaded on the column for purification. The purification was performed by RP-HPLC (column: Cosmosil 5C4 AR 300 (4.6 × 150 mm); a linear gradient from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1); flow rate: 0.65 ml/min) to give the peptide component **6** in a yield of 25% (12 nmol; amino acid analysis: $Asp_{6.8}Thr_{3.1}Ser_{4.0}Glu_{4.8}Pro_{3.3}Gly_2Ala_{11.2}Val_{7.1}Ile_{2.8}Leu_{8.2}Tyr_{1.7}Phe_{5.1}Lys_{3.4}Arg_{6.7}$).

RESULTS AND DISCUSSION

Segment Preparation, Introduction of a Sequence of Arginines to the Thioester Moiety

The amino acid sequence of the model compound, ORL1(288–370), together with the site of coupling is shown in Figure 1. The scheme for the synthesis of Fmoc-ORL1(288–370) is outlined in Scheme 1. The *N*-terminal Fmoc group is introduced for further synthesis towards ORL1(1–370) by successive condensation of peptide thioesters via the combination of the thioester method and native chemical ligation.

A general problem in dealing with a transmembrane peptide is its low solubility. To achieve sufficient solubility and to permit efficient purification and a ligation reaction, a peptide thioester, Fmoc-ORL1(288–328)-SCH_2CH_2CO-Gly-Arg_5-Leu **(1)**, was prepared. An Arg_5 sequence was introduced into the thioester moiety. The peptide thioester **1** was synthesized by Boc chemistry using the *in situ* neutralization protocol [19] with 2-(1H-benzo-triazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) activation. For the purification, a mixture of formic acid, 1-propanol and water was used as the eluent [15]. The RP-HPLC elution profile of the crude peptide thioester **1** is shown in Figure 2. Peptide thioester **1** was obtained from the peak at 17 min (shown by an arrow). This peptide thioester **1** was found to be soluble in a neutral aqueous solution in the presence of SDS. For comparison, a peptide thioester Fmoc-ORL1(288–328)-SCH_2CH_2CO-Leu (**2**), which did not contain the Arg_5 sequence, was also synthesized. Purification of the peptide thioester **2** was achieved using the same conditions as for peptide thioester **1**. However, this peptide thioester **2** was not soluble to an aqueous solution under the conditions tested, including phosphate buffer (pH 7.6) to which either 6 M guanidium chloride, 30% TFE and 1% SDS had been added.

The original strategy for this technique, in which a number of hydrophilic amino acids are introduced to

Scheme 1 Synthetic scheme of ORL1(288–370)□ Letters in grey indicates the hydrophobic amino acid core corresponding to the putative 7th transmembrane domain of ORL1.

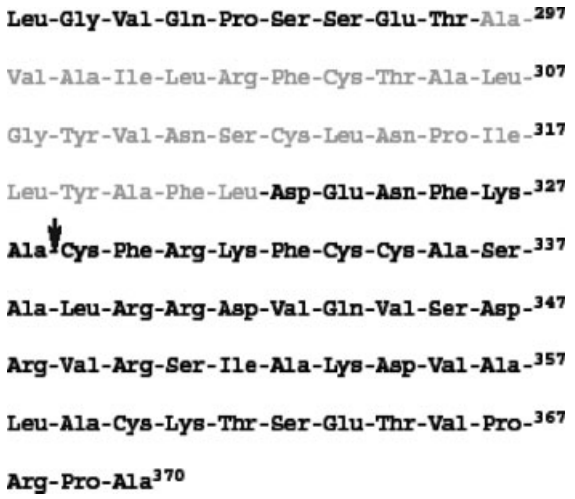

Figure 1 Amino acid sequence of ORL1(288–370). The sites of coupling are shown in bold and with arrows. The putative 7th transmembrane domain is indicated in grey.

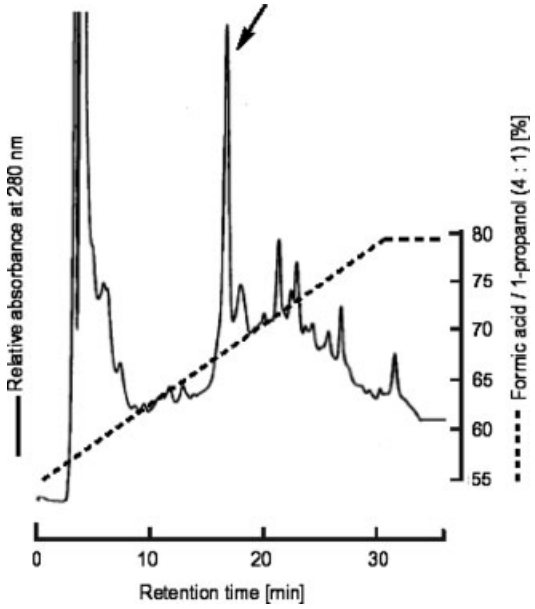

Figure 2 RP-HPLC elution profile of crude peptide thioester **1** synthesized manually using the *in situ* neutralization protocol. Column: Cosmosil 5Ph AR-300, 4.6 × 150 mm; linear gradient (25 min) from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1). Flow rate: 0.65 ml/min. Peptide thioester **1** was obtained from the peak with an arrow.

each termini of transmembrane peptide to enhance the solubility, was developed by Deber's group [21]. In our technique the Arg_5 sequence was introduced into the thioester moiety, which must function as a cation core in solution to enhance solubility and can also serve as a leaving group in the ligation reaction, in which no additional procedure is required for removing the hydrophilic sequence.

Segment Condensation by Native Chemical Ligation for the Synthesis of Fmoc-ORL1(288–370)

The *C*-terminal building block, ORL1(329–370) **(3)** was synthesized using an automated peptide synthesizer using Fmoc chemistry and the product was purified by RP-HPLC on an ODS column using 0.1% TFA aqueous acetonitrile as the eluent. For the synthesis of Fmoc-ORL1(288–370) by native chemical ligation, since peptide thioester **1** was found to be soluble in an aqueous, SDS-containing buffer, the conditions were adopted that were used for the semisynthesis of the potassium channel KcsA [17], with minor modifications. Peptide thioester **1** and peptide **3** were dissolved in pH 7.2 buffer to which 1% (35 mM) SDS, 1% (97 mM) thiophenol and 3.4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) had been added. TCEP was added to the solution to minimize disulfide bond formation [3]. All of the reactants were soluble at the time the reaction started (a precipitate began to appear over time). The

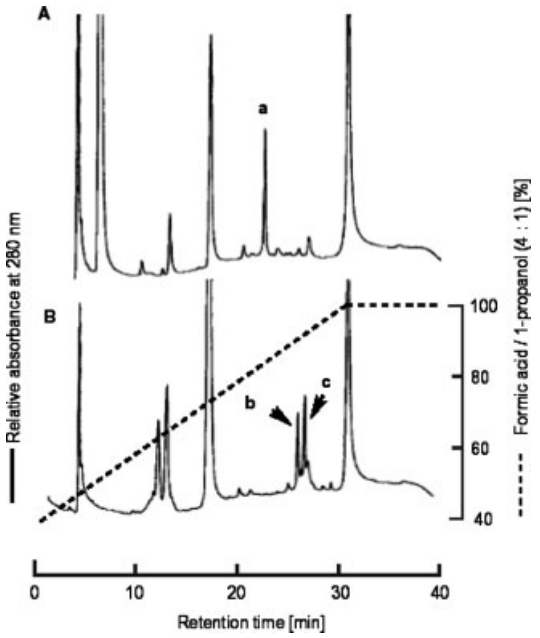

Figure 3 RP-HPLC elution profile of the ligation mixture. The ligation was carried out in a phosphate buffer at pH 7.2 to which 1% (35 mM) SDS, 1% (97 mM) thiophenol and 3.4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) had been added. A: Profile at a reaction time of 0 h. B: Profile at a reaction time of 48 h.

RP-HPLC profile of the reaction mixture is shown in Figure 3.

All the peaks that appeared on the profiles were collected and the content of each fraction was characterized by mass spectral analysis. As shown in Figure 3A (reaction time of 0 h), peak **a** was found to be peptide thioester **1**, whereas peptide **3** was not detectable at a wavelength of 280 nm. The peaks with retention times of 13 and 17 min were from thiophenol. The content of the 30 min peak could not be characterized by mass spectral analysis. The RP-HPLC elution profile of the reaction mixture at a reaction time of 48 h is shown in Figure 3B. The molecular mass of the desired product was not found in any of the peaks that appeared on the profile. The molecular mass found for peaks **b** and **c** were 5930 and 4760, respectively. The compound with a mass value of 5930 is speculated to be a molecule to which thiophenol was introduced via a disulfide bond to peptide thioester **1**. The other compound with a mass value of 4760 corresponds to a peptide phenyl thioester intermediate **(4)**, produced by the thioester exchange of peptide thioester **1** and thiophenol. These data suggest that the first thiol exchange occurred between peptide thioester **1** and thiophenol but that the second thiol exchange did not occur between peptide phenyl thioester **4** and the thiol group on the *N*-terminal cysteine residue of peptide **3**. This phenomenon may be explained by assuming that the intermediate **4** is fully incorporated into an SDS micelle due to the hydrophobic nature of phenyl thioester moiety. As a result, the subsequent thiol attack does not occur, since the site of the reaction is inside the micelle. Based on this speculation, further investigations focused on the selection of a thiol additive and the concentration of the SDS micelles. For a thiol additive, 2-mercaptoethanesulfonic acid sodium salt (MESA) was used. Due to the negatively charged and hydrophilic nature of MESA, the thioester moiety of the peptide thioester intermediate involving MESA, Fmoc-ORL1(288–328)-$SCH_2CH_2SO_3^-$ **(5)** would presumably remain outside the SDS micelle. The RP-HPLC elution profile of the ligation reaction mixture is shown in Figure 4. Figures 4A and B show the profiles of the reaction mixture at the start and at a reaction time of 48 h. All the peaks that appeared on the profile were collected, and the content of each peak was characterized by mass spectral analysis. Peak **d** was characterized as the starting material peptide **3** (molecular mass found: 5714; calcd for $[M+H]^+$: 5711.6). From peak **e**, two mass values were found, as shown in Figure 5. The values 5714.2 and 9314.4 correspond to the molecular mass of peptide thioester **1** and the desired product Fmoc-ORL1(288–370) (**6**; Calcd for $[M+H]^+$: 9310.8), respectively. Peak **f** was characterized as an

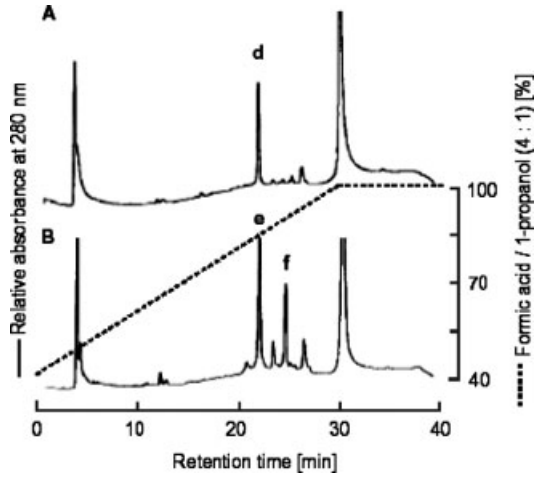

Figure 4 RP-HPLC elution profile of the ligation mixture. The ligation was performed in a buffer (pH 7.2) containing 100 mM sodium phosphate, 35 mM SDS, 60 mM MESA and 3.4 mM TCEP. A: Profile at a reaction time of 0 h. B: Profile at a reaction time of 48 h. Column: Cosmosil 5C4 AR 300 (4.6 × 150 mm); linear gradient from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1); flow rate: 0.65 ml/min.

intermediate peptide thioester, Fmoc-ORL1(288–328)-$SCH_2CH_2SO_3^-$ (**5**; found for *m/z*: 4794.9; calcd for $[M+H]^+$: 4795.5). The contents of the other peaks could not be characterized because mass values could not be obtained. The results indicate that by using MESA as a thiol additive, the ligation reaction proceeded to give product **6**. However, as shown in Figure 5, detectable amounts of peptide thioester **1** remained in the reaction mixture, even at a reaction time of

48 h, indicating that the first thioester exchange did not proceed efficiently.

Another ligation reaction, using MESA as a thiol additive, was carried out in the SDS concentration of 7 mM. Since the critical micelle concentration (CMC) of SDS is 8 mM [22], micelles are not formed at this concentration. The RP-HPLC elution profile of the ligation reaction mixture is shown in Figure 6. Figures 6A and 6B show the profiles of the reaction mixture for reaction times of 3 h and 24 h, respectively. All of the peaks that appeared on the profile were collected, and each peak was characterized by mass analysis. From peak **g**, two mass values were found (Figure 7A), 5718.4 and 9320.1, corresponding to the starting material peptide thioester **1** and the product **6**, respectively. Peak **h** was characterized as peptide thioester intermediate **5** (found for m/z: 4801.3). From peak **i**, only one mass value, 9310.4 was found, which corresponded to the molecular mass of the desired product **6**. As shown in Figure 7B, the peak corresponding to the peptide thioester intermediate **5** disappeared completely. On the other hand, application of thiophenol as a thiol additive, instead of MESA, in the SDS concentration of 7 mM did not allow the ligation reaction to be completed though peptide thioester **1** was converted to a corresponding phenyl thioester within 24 h. In Figure 8, the RP-HPLC elution profiles of the reaction mixture are shown. Figures 8A and 8B are the profiles of the reaction times of 0 h and 24 h, respectively. Peaks **j** and **k** were characterized as the starting material peptide thioester **1** and the desired product **6**, respectively. Peak **m** was characterized as peptide phenyl thioester intermediate **4** (found for m/z: 4764.0). These results indicate that using thiophenol as a thiol additive led to the reduction of the ligation reaction efficiency in this system probably due to the hindrance that the phenyl thioester moiety was taken inside the hydrophobic core of the aggregate of peptides and/or the aggregate with SDS in aqueous surroundings.

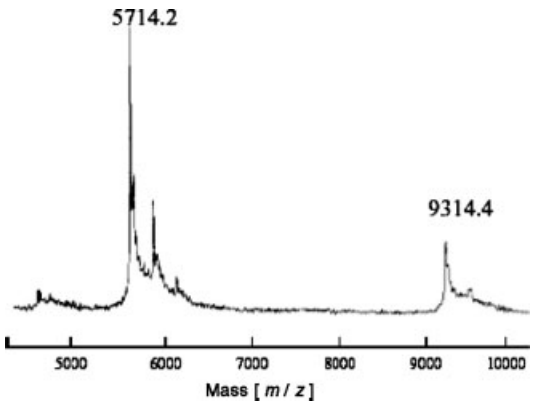

Figure 5 Result of the mass spectral analysis of peak **e** on Figure 4.

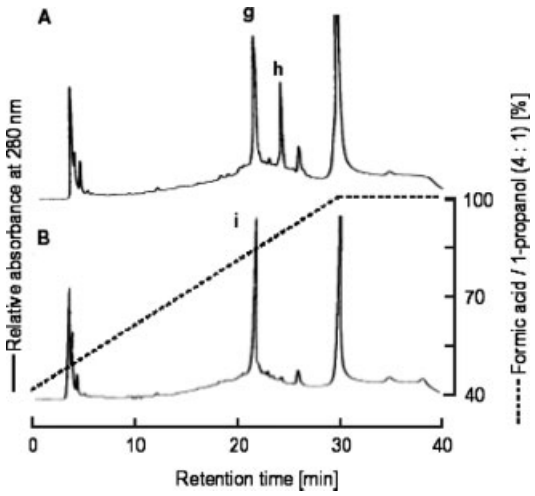

Figure 6 RP-HPLC elution profile of the ligation mixture. The ligation was carried out in a phosphate buffer at pH 7.2 to which 7 mM SDS, 60 mM MESA and 3.4 mM TCEP were added. A: Profile at the reaction time of 3 h. B: Profile at the reaction time of 24 h. Column: Cosmosil 5C4 AR 300 (4.6 × 150 mm); linear gradient from formic acid–water (2 : 3) to formic acid–1-propanol (4:1); flow rate: 0.65 ml/min.

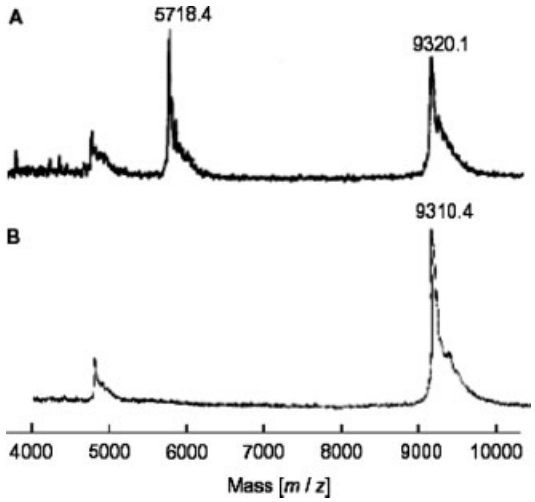

Figure 7 MALDI-TOF mass spectra of peaks **g** and **i** on Figure 6. A: Peak **g**. B: Peak **i**.

These results serve to emphasize that the detergent molecule and the thiol additive are significant factors in the synthesis of membrane proteins via native chemical ligation. In the case mentioned above, adjusting the SDS concentration to slightly below the CMC might have reduced the extent of electrostatic repulsion between SDS and MESA, thus permitting the efficient first thioester exchange to give peptide thioester intermediate **5**, which led to the completion of the reaction process. On the other hand, the use of SDS concentrations above the CMC (Figures 4 and 5) gave a poor yield, which can be attributed to the MESA molecule not being accessible in the proximity to the thioester moiety of peptide thioester

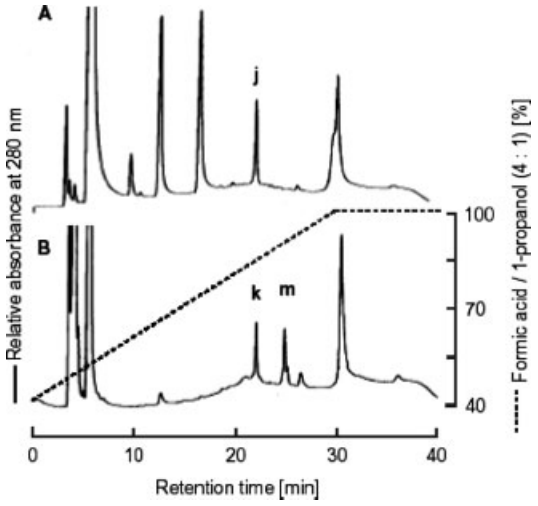

Figure 8 RP-HPLC elution profile of the ligation mixture. The ligation was carried out in a phosphate buffer at pH 7.2 to which 7 mM SDS, 97 mM thiophenol and 3.4 mM TCEP were added. A: Profile at the reaction time of 0 h. B: Profile at the reaction time of 24 h. Column: Cosmosil 5C4 AR 300 (4.6 × 150 mm); linear gradient from formic acid–water (2 : 3) to formic acid–1-propanol (4 : 1); flow rate: 0.65 ml/min.

1 due to electrostatic repulsion against the negative charge on the surface of the SDS micelle. Changing the charge on the micelle surface, via the use of hexadecyltrimethylammonium bromide (HDTA) as a detergent, represents another approach to this problem. However, hydrolysis of the thioester was predominant over the first thioester exchange, thus leading to a poor yield (data not shown).

CONCLUSIONS

The conditions for native chemical ligation in the presence of a detergent were optimized for the synthesis of a specific membrane protein. The results presented herein provide a general strategy for the selection of detergents for ligation chemistry, in terms of concentration and charge. The introduction of the Arg_5 sequence to the thioester moiety of the peptide thioester building block is generally applicable to the preparation of transmembrane peptide thioesters. The findings provide the basis for the synthesis of membrane protein and extend the use of ligation chemistry for protein preparation.

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