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Peptide purification by affinity chromatography based on α-ketoacyl group chemistry

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Significant advances have been achieved in the fields of peptide/protein synthesis, permitting the preparation of large, complex molecules. Shortcomings, however, continue to exist in the area of peptide purification. This paper details some studies we undertook to develop a new strategy for peptide purification based on a reactivity of α-ketoacyl groups in peptides. The α-ketoacyl peptide was generated from N^{ε}-acyl-lysyl-peptide in the solid phase via a transamination reaction using glyoxylic acid and nickel(II) ion. Cleavage of the α-ketoacyl group with *o*-phenylenediamine gave the target peptide in an acceptable yield and purity. We first carried out a careful step-by-step optimization of the purification conditions using a model peptide. The strategy was then used in the purification of a transmembrane peptide that could not be effectively purified using a conventional RP-HPLC system due to the strong hydrophobicity of the peptide and its high tendency to aggregate.

Keywords: peptide purification; α-ketoacyl group; transamination; amide-bond scission

Introduction

Recent developments in peptide synthesis methodology now permit the synthesis of large and sophisticated molecules. The stepwise solid-phase peptide synthesis has recently been coupled with the fragment condensation method [1,2], which has led to the rapid and facile synthesis of polypeptides containing in excess of 100 amino acid residues. However, shortcomings in the existing strategies for peptide purification continue to exist. Although RP-HPLC is generally regarded to be one of the most effective methods for peptide purification, there are several complications inherent in the purification of peptides [3]. First, impurities from solid-phase peptide synthesis are peptides that contain failed truncated-sequences, the physical properties of which are usually similar to those of the primary product. Second, hydrophobic peptides have a tendency to adhere to RP-HPLC columns, thus greatly reducing their isolated yields. Several modifications including mobile phases (e.g. formic acid: 1-propanol: water) [4–6] and stationary phases have been reported, in attempts to address these limitations. While these modifications have shown some degree of success, significant time and effort are often required to optimize the conditions for individual purifications.

Several non-RP-HPLC separation techniques have been reported, in attempts to overcome this problem [7–12]. In most cases, a reactive group (nucleophile or electrophile) or an affinity tag (biotin or polyhistidine-tag) is introduced at the *N*-terminus of the target peptide, which permits it to be isolated from the crude sample via the formation of a covalent or non-covalent bond formation with the stationary phase. While a number of these approaches have can be useful, none are in widespread use in practical purification. This is mainly due to the harsh conditions required for tag removal (e.g. the use of aq. NaOH, piperidine or cyanogen bromide) and/or the limitations of the applicable sequence (e.g. enzymatic cleavage of specific site or only for *N*-terminal cysteine-peptide). Therefore, the development of new and more successful methodology continues to be needed. We believe that a combination of two methods operating via different chromatographic principles, such as RP-HPLC and a non-RP-HPLC method represents a promising purification strategy.

In 1964, a method for the removal of the *N*-terminal residue of a protein via transamination and scission of the resulting α-ketoacyl group was reported by Dixon [13–17]. This pioneering work, although originally developed for protein sequencing, has been applied to the removal of *N*-terminal residues from recombinant

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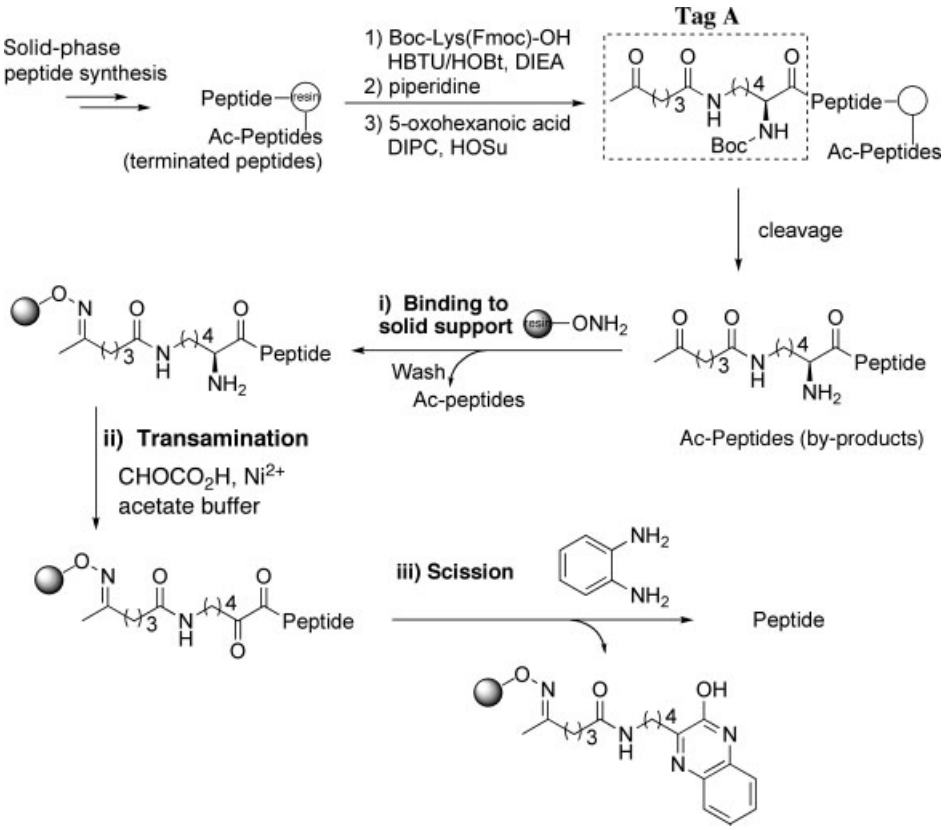
Abbreviations used: *AcOH, acetic acid; CH_3CN, acetonitrile; α-CHCA, α-cyano-4-hydroxycinnamic acid; Boc, t-butoxycarbonyl; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DIPC, diisopropylcarbodiimide; DMA, N,N-dimethylacetoamide; DMF, N,N-dimethylformamide; ESI MS, electrospray ionization mass spectrometry; Fmoc, 9-fluroenelymethoxycarbonyl; Gdn•HCl, guanidine hydrochloride; HBTU, N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HOBt, 1-hydroxybenzotriazole; HOSu, N-hydroxysuccinimide; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; β-Me, β-mercaptoethanol; MeOH, methanol; MES, 2-morpholinoethanesulfonic acid; MVP, major vault protein; NMP, 1-methylpyrolidin-2-one; ORL-1 TM7, 7th transmembrane domain of opioid receptor-like 1(288-328); o-PDA, o-phenylenediamine; Rink amide resin, 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)-phenoxy resin; RP-HPLC, reversed-phase high performance liquid chromatography; SDS, sodium dodecyl sulfate; TCEP, tris (2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; TFE, trifluoroethanol.*



Scheme 1. General scheme for peptide purification by utilizing the reactivity of an α-ketoacyl group.

proteins [18] as well as to the preparation of protein building blocks for chemical ligation [19]. This unique chemistry of the α-ketoacyl group prompted us to develop a new strategy for peptide purification. In this paper, we described the details of the developed procedure including the scope and limitations of the method. The procedure involves the following three-reaction sequence (Scheme 1): (i) selective binding of a full length keto-peptide to an aminooxy-functionalized resin via oxime formation; (ii) transamination of the α-amino group to the ketone using glyoxylic acid and nickel(II) ion; and (iii) selective scission of the α-ketoacyl group by treatment with *o*-phenylenediamine. Having established suitable conditions for the model system, the strategy was then applied to the purification of a transmembrane peptide that cannot be effectively purified by conventional RP-HPLC.

Materials and Methods

Amino acid derivatives and HOBt were purchased from the Peptide Institute Inc. (Osaka, Japan); glyoxylic acid and 5-oxohexanoic acid were obtained from TCI (Tokyo, Japan); NovaPEG amino resin, amino PEGA resin, NovaSyn TG amino resin, and NovaSyn TG acetal resin were from Novabiochem (Tokyo, Japan); and HBTU, TFA, DIEA, DCM, TFE, Rink amide-PEG-polystyrene resin, and DIPC were from Watanabe Chemical. Co. Ltd. (Hiroshima, Japan). All other chemicals and solvents of special grade were obtained from Nacalai Tesque (Kyoto, Japan) and were used without further purification.

All peptides were identified by mass number and amino acid composition. The amino acid compositions of peptides were determined using an L-2000 amino acid analyzer (Hitachi Ltd, Tokyo, Japan) after hydrolysis by treatment with constant boiling point hydrochloric acid for 24 h at 110 °C. MALDI-TOF MS data was collected on an Autoflex (Brucker Daltonics, Bremen, Germany) using α-CHCA as a matrix under the positive ionization and in the linear mode. ESI MS data were collected on a LCQ DECA XP^{plus} (Thermo Finnigan, San Jose, US) under positive ionization. All RP-HPLC runs were carried out on a Hitachi analytical HPLC system, which utilized an ELITE Lachrom instrument (L2130 pumps and a L2400 UV detector), and an ERC-3215α degasser. Details of RP-HPLC conditions can be found in the figure captions. All reactions were carried out at room temperature under air unless otherwise noted.

Peptide Synthesis

Peptides were synthesized on an ABI 433A (Applied Biosystems, Foster City, CA, US) using Fmoc-chemistry on a Rink amide resin, except as otherwise noted. The peptide chain was elongated using *MonPrev* protocols (single or double coupling and acetylation after each coupling step). The acetyl-capping step is needed to prevent the incorporation of the purification tag onto failed-truncated-peptides. 5-Oxohexanoic acid was manually coupled to the target peptide. 5-Oxohexanoic acid (237 µl, 1.0 mmol, 8 equiv) in NMP (3 ml) was pre-mixed with DIPC/HOSu (1 mmol, 8 equiv each) and vortexed for 20 min. The resulting solution was then added to the resin (0.25 mmol), followed by agitation for 6 h, and the progress of the reaction was monitored by the Kaiser test. Following the coupling, the resin was drained and washed with NMP (5 × 4 ml), DCM (5 × 4 ml), and ether (5 × 4 mL), and dried *in vacuo* overnight. Cleavage of the keto-peptides from resin (100 mg) was performed with a cleaving cocktail comprised

of TFA/thioanisole/phenol/water = 850 µl/50 µl/50 µl/50 µl for 90–120 min. The use of 1,2-ethanedithiol and triisopropylsilane should be avoided. Following filtration of the resin, the peptide was precipitated by adding ether and dried *in vacuo*.

Preparation of Aminooxy-functionalized Resin

NovaPEG amino resin, Amino PEGA resin, or NovaSyn TG amino resin (typically 0.2–0.6 mmol of amino group/g of dry resin, 0.5 g, 1 equiv) was washed with NMP (5 ml, 3 × 3 min) and then allowed to swell in the presence of NMP (5 ml) for overnight. The manual couplings of Fmoc-NH$(CH_2)_5$COOH (5 equiv) was carried out using HBTU/HOBt/DIEA (5 : 5 : 10 equiv) in DMF (1.5–4 ml) for 3 h. Fmoc removal was achieved by treatment with 20% piperidine in NMP (v/v, 5 ml 3 min and 5 ml 15 min). Boc-NHOCH_2COOH [20] was then coupled using DIPC/HOSu/Et_3N (5 : 5 : 5 equiv) in DMF (1.5–4 ml) for 3 h, with a 5 min preactivation period. After washing with DCM (5 × 3 ml) and MeOH (3 × 3 ml), the resin was dried *in vacuo*. The Boc group was removed by treatment with TFA/DCM = 1/1 (v/v, 3 ml for 100 mg dry resin) for 25 min and washed with DCM (5 × 3 ml), CH_3CN (5 × 3 ml), and water/DMF = 1 : 1 (v/v, 5 × 3 ml). The resulting deprotected aminooxy-functionalized resin was used in next step within 24 h. The loading of aminooxy-group on the resin was assumed to be the same as the amino group of starting resin (0.2–0.6 mmol of aminooxy-group/g of dry resin).

Purification of Peptide with α-Ketoacyl Strategy

Binding to the solid support

The following describes a typical procedure: A crude tag-peptide (0.01 mmol) was dissolved in 0.1 M aqueous acetate buffer/DMF = 4 : 1, adjusted to pH 4.5 using pH paper to a final concentration of 1–5 mM. If the peptide is insoluble, it is usually first dissolved in DMSO or TFE, acetate buffer is then added to a final concentration of 0.1–5 mM, the pH of the solution was adjusted at 4.5 with 2 M aqueous AcOH. The addition of Triton X-100 (final concentration 0.1 mM), SDS (0.5 mM), or Gdn•HCl (2 M) was helpful for hydrophobic peptides. For peptides containing cysteine, 200 mM β-Me was used to prevent disulfide bond formation. A peptide solution was added to the aminooxy-resin (2–5 equiv) and gently agitated for several hours. The progress of the binding was monitored by analysis of the supernatant of the reaction by analytical RP-HPLC.

Washing

The following describes a typical procedure: The resulting resin was extensively washed with 0.1 M aqueous acetate buffer at pH 4.5/DMF = 4 : 1 containing 0.1 mM Triton X-100 and 200 mM β-Me (8 × 3 ml), NMP (5 × 3 ml), TFE (5 × 3 ml) then 2 M acetate buffer at pH 5 (5 × 3 ml).

Solid-phase transamination

The following describes a typical procedure: 50 wt% of aqueous glyoxylic acid (112 µl, 1 mmol, 100 equiv.) was added to a peptide-resin (0.01 mmol, 1 equiv) in 1 ml of 2.25 M acetate buffer at pH 5, followed by gentle agitation for 5 min. A 1 M aqueous solution of nickel(II) sulfate (12 µl, 0.012 mmol, 1.2 equiv) was then added to the resin, followed by gentle agitation for 1–2 h.

Quantification of solid-phase transamination

The following describes a typical procedure: During a transamination reaction (*vide supra*), a portion of the resin (0.5 µmol) was placed in a filtered tube and washed with 0.1 M aqueous acetate buffer at pH 4.5/DMF = 4 : 1 (5 1 mL), 0.5 M aqueous EDTA solution (5 × 1 ml), and water/CH_3CN = 1 : 1 (5 × 1 ml). A solution of 2 M *O*-methylhydroxylamine hydrochloride in water/DMF = 4 : 1 unbuffered (375 µl, molar ratio: *O*-methylhydroxylamine/peptide = 1500 : 1) was added to the resin. The sealed tube was shaken at 60 °C for 2 h, or at rt for 16 h. The eluted material was then analyzed by RP-HPLC.

Solid-phase scission reaction

The following describes a typical procedure: An α-ketoacyl-peptide-resin (0.01 mmol, 1 equiv) was washed with 0.1 M aqueous acetate buffer at pH 4.5/DMF = 4 : 1 (5 × 3 ml), DMF (5 × 3 ml), 0.5 M aqueous EDTA solution (5 × 3 ml), then 2 M acetate buffer at pH 4.0 (5 × 3 ml). Solid *o*-PDA (27 mg, 0.25 mmol, 25 equiv) was then added to the resin in 2–3 ml of degassed 2 M acetate buffer at pH 4.0 and gently agitated under argon and in the dark at 37 °C overnight. The resin was carefully washed with CH_3CN/water = 1 : 1 (5 × 1 ml), and the eluted material was analyzed by RP-HPLC.

Trap experiment

The following describes a typical procedure: A peptide solution, after the scission reaction, was added to the aminooxy-resin (10 equiv) and aldehyde resin (30 equiv, from NovaSyn TG acetal resin) and gently agitated in the dark overnight. The progress of the reaction was monitored by analyzing the supernatant of the reaction by analytical RP-HPLC.

Purification of model peptide 1

Peptide 1 was purified as described above. Recovery yield 86% (based on the amount of target peptide in the crude sample by quantitative amino acid analysis); ESI MS found *m/z* 835.5, calcd 835.4 $[M + H]^+$; Amino acid analysis: $Glu_{1.06}Gly_{2.00}Ala_{2.00}Leu_1Tyr_{0.83}Arg_{1.01}$.

Purification of MVP(811–830)

This peptide was purified as described above, except that all reactions were carried out under an argon atmosphere, to prevent oxidation of the methionine residue. Recovery yield 72% (based on the amount of the tag-target peptide in the crude sample by quantitative amino acid analysis); ESI MS found (reconstituted) 2069.9, calcd 2069.5 [M]; Amino acid analysis: $Thr_{1.01}Ser_{1.89}Glu_{3.04}Gly_{2.04}Ala_{2.06}Val_2Met_{0.88}Leu_{4.20}Lys_{1.90}$.

Purification of ORL-1 TM7

A crude tag-ORL-1 TM7 (95 mg, from 0.02 mmol of dry resin) was suspended in a 10 ml of 50 mM aqueous MES buffer at pH 5.5/DMA/CH_3CN/TFE = 3 : 1 : 1 : 1 containing 1 M Gdn·HCl, 0.1 mM Triton X-100, and 100 mM β-Me. The peptide solution was then added to the aminooxy-resin (0.08 mmol) and gently agitated for 72 h. During the course of the agitation, all of the precipitate (peptide) dissolved. Washing and transamination was performed as described above. For the scission reaction, an α-ketoacyl-peptide-resin (0.01 mmol, 1 equiv) was treated

with *o*-PDA (27 mg, 0.25 mmol, 25 equiv) in 2 ml of degassed 1 M acetate buffer at pH 4.0/DMA/TFE = 3 : 1 : 1 containing 0.1% of Triton X-100 and gently agitated under argon and in the dark at 37 °C for 24 h. The excess *o*-PDA and the by-products generated from *o*-PDA were removed by the aminooxy-resin and aldehyde-resin as described above. The eluted material was analyzed by RP-HPLC. Yield 0.31 mg, 0.47% (based on the starting resin with quantitative amino acid analysis); MALDI-TOF MS found *m/z* 4449.3, calcd 4449.2 $[M + H]^+$; Amino acid analysis: $Asp_{4.14}Thr_{1.98}Ser_{2.70}Glu_{3.01}Gly_{2.49}Ala_{5.34}Cys_{0.65}Val_3Ile_{1.92}Leu_{6.32}Tyr_{1.20}Phe_{2.78}Lys_{0.96}Arg_{1.18}$.

Results and Discussion

We envisioned a new strategy for peptide purification, as shown in Scheme 1. The key functional group, an α-ketoacyl amide, could be prepared by the oxidation of α-hydroxyl amides using pyridinium dichromate (PDC) [21], a coupling reaction between α-keto acids and amines [22] or the transamination of α-amino group of peptides [13–17]. We decided to use the transamination method because of its simplicity and the fact that it is a clean reaction that has broad applicability. Our approach began with the synthesis of the precursor of purification tag **A** (Scheme 1) at the *N*-terminus of the target-peptide. The precursor tag **A** was easily prepared by standard peptide synthesis using the commercially available Boc-Lys(Fmoc)-OH and 5-oxohexanoic acid. The transamination and successive scission of peptide/protein in the solution phase have been extensively investigated by Dixon's group [13–17]. In our experience, however, a reaction in the solid phase often behaves differently from that in a solution phase. Therefore, we undertook a careful step-by-step optimization of the conditions for a solid-phase reaction using a model peptide (Table 1).

Binding to Solid-phase Support

It is known that aminooxy groups react selectively with aldehydes and ketones to generate oximes, that the resulting oximes are stable under slightly acidic conditions and that the reactions proceed favorably, even in aqueous solvents [23–26]. We decided to use this reaction to selectively isolate the target from the crude sample. A similar approach was recently employed in the purification of oligosaccharides [27] and peptides [10].

When the crude Tag-peptide 1 was treated with aminooxy-resin in a mixture of DMF and 0.5 M acetate buffer at pH 4.5, the peptide became quantitatively bound to the resin by oxime formation within several hours (Figure 1a and b). As expected, the reaction between aminooxy-groups and ketones proceeded at pH 4.5 (0.5 M acetate buffer), 5.5 (0.1 M MES buffer), and 6.8 (0.25 M phosphate buffer). The binding rate was dependent on the peptide concentration and the quality of the resin used. When a larger excess of resin (10 equiv) was used, the reaction was reached completion within 1 h. The same dependence was observed for previously published data by Rose's group for thiazolidine ring formation between aldehyde-functionalized resins and *N*-terminal cysteinyl-peptides [28].

Several additives were tolerated in the reaction, including 0.5 mM SDS and 0.1 mM Triton X-100, 2 M Gdn•HCl and 200 mM β-Me. It should also be noted that other solvent systems gave comparable results [e.g. a mixture of acetate buffer at pH 4.5/organic solvents (DMA, DMF, NMP, 1,4-dioxane, TFE, or CH_3CN, organic solvent up to 75% v/v)]. Quantitative binding under identical conditions was also observed when the polarity of the oxime bond was reversed using a reaction between aminooxy-peptide and a ketone or aldehyde functionalized-resin (data not shown). These additives, co-solvents and the reverse polarity of oximes would be advantageous for slightly soluble peptides and peptides with a high tendency toward aggregation.

Table 1. Amino acid sequences of the purified peptides

Peptide	Sequence
Model peptide 1	GLAEYGAR-NH_2
MVP(811–830)	AVAGPEMQVK LLQSLGLKST-NH_2
ORL-1 TM7	LGVQPSSETA VAILRFCTAL GYVNSCLNPI LYAFLDENFK A-NH_2

Solid-phase Transamination

We subsequently explored the transamination reaction of the α-amino group in the precursor of purification tag **A** to produce an α-ketoacyl group for a successive scission reaction. Although considerable progress has been made recently in the development of transamination reactions, to our knowledge, only two such reactions have been applied to the *N*-terminal residues of resin-bound peptides. Meldal and co-workers reported on a reaction involving copper ion-catalyzed conditions with glyoxylate as an electrophile in an aqueous acetate buffer at pH 5.5–6.0 [29]. More recently, Francis and co-workers investigated a reaction catalyzed by the biological cofactor pyridoxal 5′-phosphate as a catalysis [30]. Conditions for optimizing the solid-phase transamination of the α-amino group in our tag system were investigated next.

An oxime-peptide-resin was treated with glyoxylic acid in an acetate buffer in the presence of a metal ion, i.e. conditions based on Dixon's method [13–17]. The progress of the solid-phase transamination was initially quantified using the following two methods: (i) measurement of the uptake of dansylhydrazine from a supernatant solution by the resin-bound ketone for ketone formation [31] and (ii) a quantitative ninhydrin test [32], to determine the consumption of amino groups in the case of model peptide 1. While these processes showed some degree of success, more than 1 day was required to develop the quantitative information, and a large amount of resin was needed to obtain a reliable value when low-loading resins were employed. Therefore, we developed a simple method to follow the progress of the reaction, as shown in Figure 2a. At an appropriate time of transamination reaction, a portion of the resin was treated with a solution of (large excess) 2 M *O*-methylhydroxylamine hydrochloride in water/DMF (4 : 1) unbuffered at 60 °C for 2 h (or at rt for 16 h). An α-ketoacyl group generated transamination was trapped with *O*-methylhydroxylamine to give an oxime, and concomitant *transoximation* between *O*-methylhydroxylamine and the oxime at the resin-peptide linkage, to afford oxime-peptides in the solution phase. The mild release of peptide, followed by RP-HPLC analysis, provided an accurate result that could be directly related to the progress of transamination. The reactant peptide was eluted as a double peak corresponding to the *syn-anti* isomers of the oxime bond (Figure 2b). Similarly, the transaminated α-ketopeptide was eluted as two sets of double peaks, due to the presence of an additional oxime bond (Figure 2c and d). The formation of α-ketoacyl peptide and the consumption of α-amino peptide were calculated based on the quantitative

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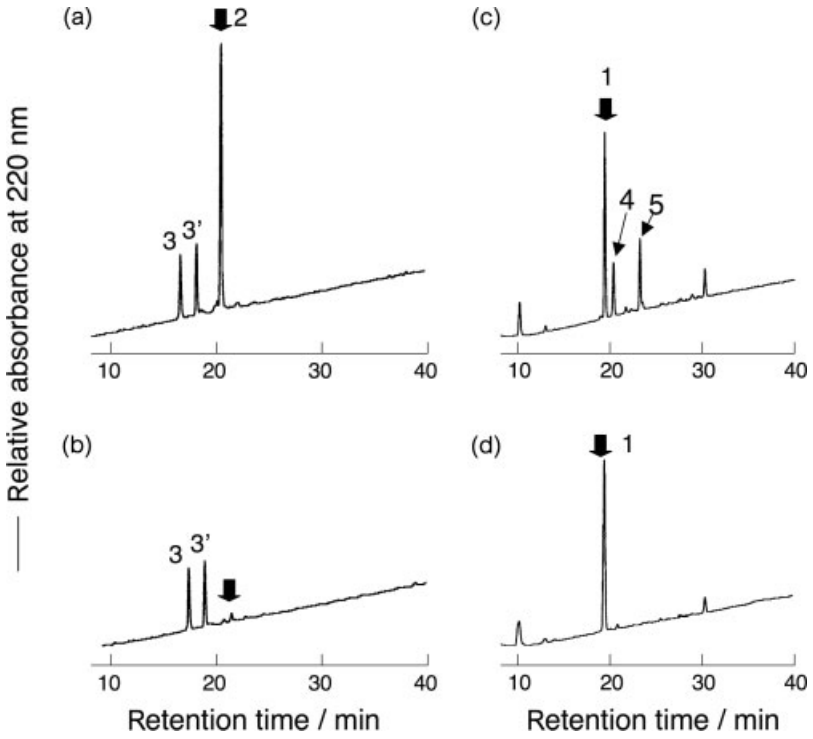

Figure 1. Analytical RP-HPLC chromatograms of the purification of the model peptide 1 at different stages. (a) crude Tag-peptide 1 before purification; (b) supernatant of a 6 h binding reaction; (c) elution by a scission reaction; (d) after trapping with scavenger resins. **1**: peptide 1; **2**: tag-peptide 1; **3** and **3′**: *N*-terminal acetylated by-products; **4**: unknown by-product; **5**: 2,3-diaminophenazine (*o*-PDA dimer), [Column: Cosmosil 5C18-AR-II (4.6 × 150 mm); Eluent: A: water containing 0.1% TFA(v/v), B: CH_3CN containing 0.1% TFA (v/v); Linear gradient 5–45% B over 40 min; Flow rate 0.5 ml/min; Detection at 220 nm].

amino acid analysis of each of the peptide derivatives (Figure 2e). The results were consistent with the results obtained the two above-mentioned methods [31,32]. Under optimal conditions, the transamination proceeded to completion within 1–2 h using a solution of glyoxylic acid (100 equiv) and nickel(II) sulfate (1.2 equiv) in 2 M acetate buffer at pH 5.5 (Figure 2e).

For the reaction, the amount of nickel ion used was found to be critical for an efficient reaction. Efficient conversions were achieved when the resin was treated with more than 1 equivalent of nickel (II) sulfate. Although based on the reaction mechanism, the reaction is generally thought to proceed in the presence of a catalytic amount of metal ion, our attempts under these conditions resulted in a low reaction rate and a prolonged reaction time, with by-products being produced.

Solid-phase Scission Reaction

The best scission results were obtained with *o*-phenylenediamine (*o*-PDA) in 2 M acetate buffer pH 4.0 at 37 °C under argon (Figure 1c), and the desired product **1** was isolated in 86% recovered yield based on the starting Tag-peptide in the crude sample (Figure 1a). Other nucleophilic reagents that were tested (3,4-diaminobenzoic acid [18], 4-chloro-*o*-phenylenediamine, 4-methyl-*o*-phenylenediamine, 2-aminobenzylamine, 2-aminophenol, 2-aminothiophenol, thiosemicarbazone, and thiourea) afforded even poorer yields.

Similar to the formation of the oxime bond (*vide supra*), the scission reaction also proceeded smoothly in a mixture of acetate buffer at pH 4.0 and organic solvents (DMA, DMF, NMP, 1,4-dioxane, TFE, or CH_3CN, organic solvent up to 40% v/v). The reaction tolerated additives (0.5 mM SDS, 0.1 mM Triton X-100, 2 M Gdn•HCl, or 10 mM TCEP•HCl) in acetate buffer at pH 4.0/DMA (3 : 1, v/v). The addition of a variety of organic solvents and additives had no adverse effects on the reaction of the model peptide 1. However, the yield of hydrophobic peptides increased, resulting from an increase in the solubility of the peptides (see later).

After confirming the recovery of target peptide 1, the next issue addressed was the removal of by-products **4** and **5** in Figure 1c, and the excess *o*-PDA that was added for the scission reaction. An analysis of peak **5** indicated that the product is 2,3-diaminophenazine, a dimer of *o*-PDA (Figure 3a, **5**). The spontaneous formation of the *o*-PDA dimer in aqueous solution has been reported previously [33], and the mass number and ^{1}H-NMR data was consistent with the reported values [34]. For product **4**, NMR data could not be acquired due to the limited amount of sample. Based on ESI MS data (found $m/z = 147.3$), however, we speculate that one of the possible structures is 2-quinoxalinone (Figure 3a, **4**). We attempted a trapping experiment, based on the hypothesis that these by-products would be captured by the solid phase by scavenger resins, as shown in Figure 3b. The brownish-orange colored scission eluent was treated with scavenger resins (aminooxy-resin 10 equiv and aldehyde-resin 30 equiv) in 1 M acetate buffer at pH 4.5 for 18 h, which resulted in a colorless solution. RP-HPLC analysis of the supernatant of the reaction revealed that the undesired by-products were completely eliminated (Figure 1d). Peptide 1 was isolated in excellent purity and 86% recovered yield.

Scope of the α-Ketoacyl Strategy

In the next step, we examined the effect of the *N*-terminal residue of target peptides on this purification strategy. The

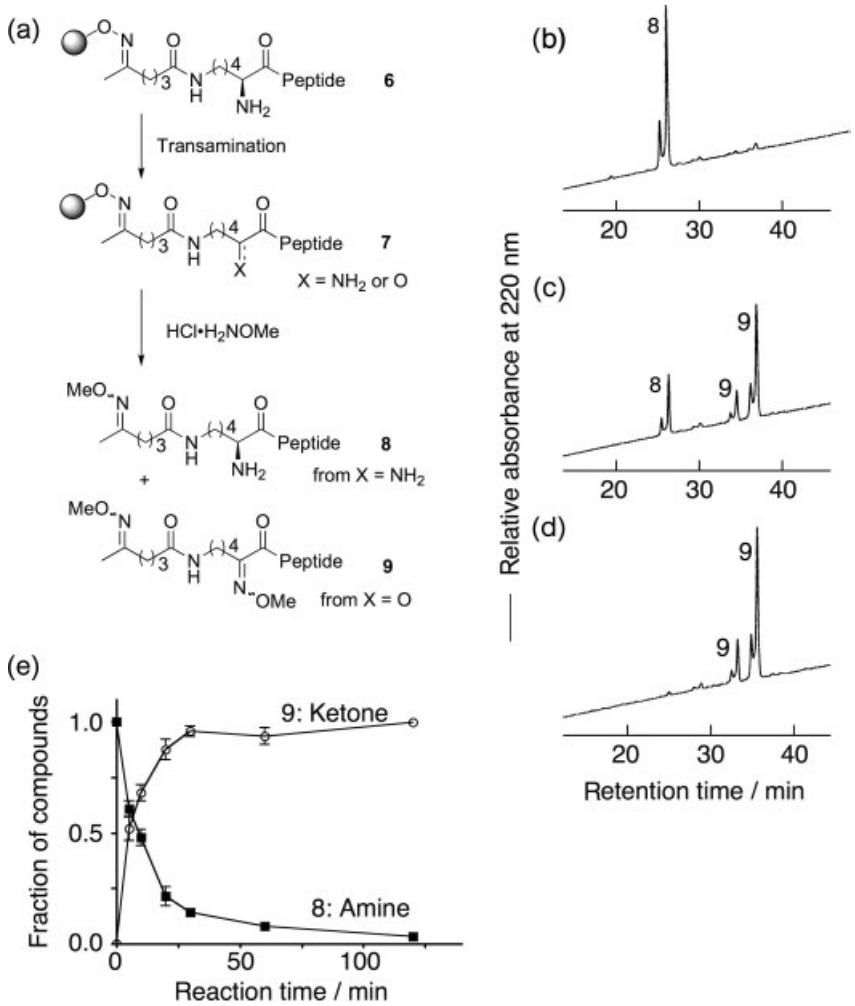

Figure 2. Quantification of solid-phase transamination. (a) General scheme for the transoximation method. RP-HPLC chromatograms obtained throughout the course of the transamination followed by *O*-methoxyamine treatment. (b) 0 min; (c) 10 min and (d) 60 min; [HPLC conditions were the same as those employed in Figure 1, except that the flow rate was 1.0 ml/min.] (e) Generation of ketone and consumption of amino group during transamination. Data points were determined in duplicate or triplicate and error bars indicate the standard deviation.

established conditions were applied to a series of model peptides: Tag-**X**LAEYGAR-NH_2 containing a range of *N*-terminal residues (X = Leu, Ser, Met, Lys, Gln, Glu, His, and Cys). We confirmed that all of the *N*-termini except Cys gave the target peptide in more than 75% recovered yield. The *N*-terminal Cys-peptide tends to afford the desired peptide, but with several by-products present (data not shown). Hence, it cannot serve as an optimal target for this purification strategy.

Application of α-Ketoacyl Strategy to Practical Peptide Purifications

Having developed efficient purification conditions for model peptides, we set out to apply this method to practical peptide purification. We chose to synthesize and purify a *C*-terminal region of the major vault protein: MVP(811–830) and the 7th transmembrane domain of opioid receptor-like 1 (288–328): ORL-1 TM7 (Table 1). Figure 4a shows an analytical RP-HPLC of the crude MVP(811–830) without the purification-tag. Using the α-ketoacyl purification strategy (binding, transamination, scission and trapping), the complete purification of the peptide was achieved (Figure 4b and c) in 72% recovered yield based on the amount of tagged-target peptide in the crude sample.

Figure 5a shows an analytical RP-HPLC of the crude ORL-1 TM7 without the purification-tag. A MALDI-TOF MS analysis revealed that the mass number of the target peptide was observed in all peaks marked in Figure 5a, and another mass number was also found in the each spectrum. We speculate that the target peptide formed aggregates with by-products even in a mixture of formic acid/1-propanol/water, and co-eluted with by-products in the form of oligomers due to the strong hydrophobicity and high tendency for aggregation, which is sometimes observed in transmembrane peptides. These peptides are not easily purified by conventional RP-HPLC. Result for the purification of this problematic peptide using the α-ketoacyl strategy are depicted in Figure 5b–d. During the binding step, we could not quantify the binding of the target peptide to the solid support, because of the presence of large amounts of interferences (salt, Triton X-100 and β-ME) for MALDI-TOF MS and by-products (Ac-peptides) in the binding sample, the flow-through and washing fractions. The target peptide (marked in Figure 5a) were disappeared after the 72 h binding reaction (Figure 5b), therefore we speculated that the binding reaction was completed by the time and moved on to next step without quantitative information. Although the eluted sample after trapping step was not as pure as that achieved in the former

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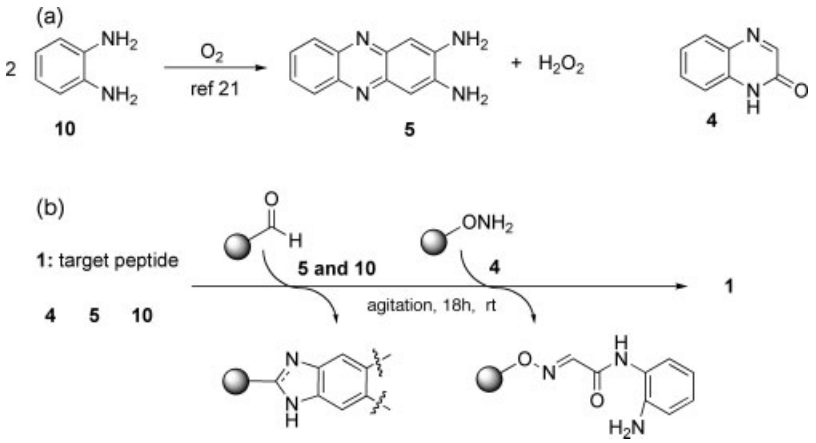

Figure 3. Trapping of by-products. (a) Possible structures of by-products formed during the purification steps. (b) General scheme for a scavenge reaction.

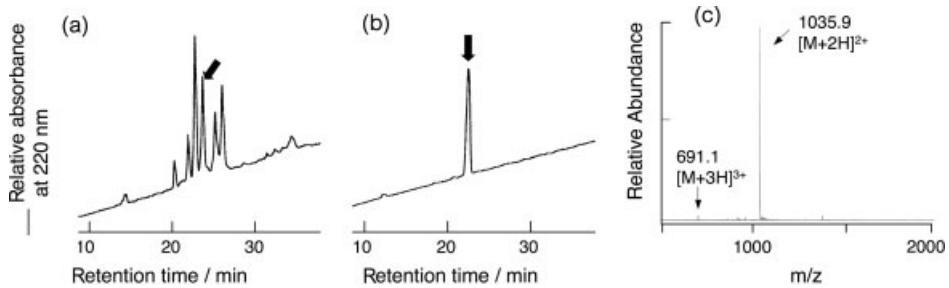

Figure 4. Purification of MVP(811–830). Analytical RP-HPLC chromatogram of (a) crude peptide and (b) after purification using the α-ketoacyl strategy. (c) ESI MS of the purified peptide. [HPLC conditions were the same as in Figure 1, except that a 10–70% B was used for 40 min].

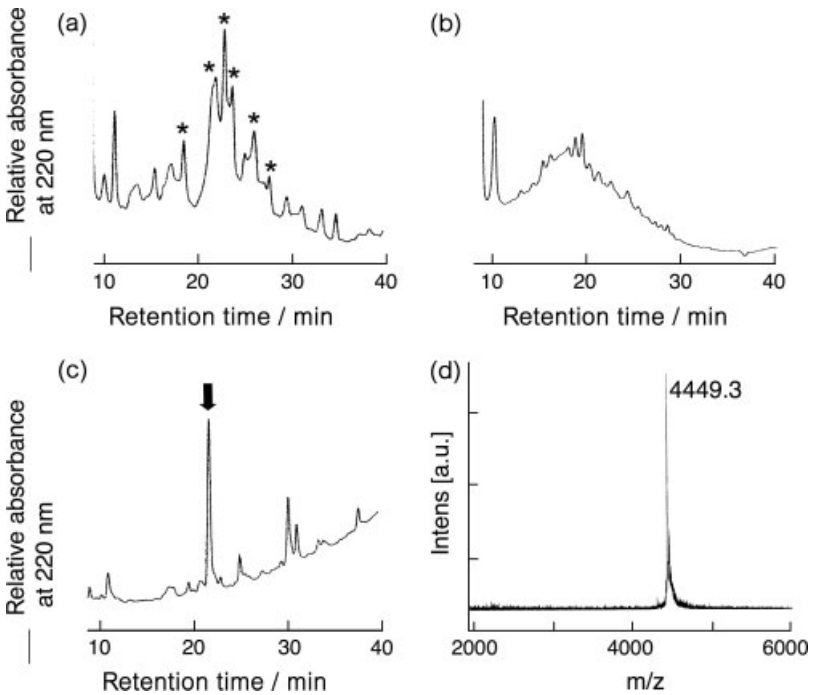

Figure 5. Purification of ORL-1 TM7. Analytical RP-HPLC chromatogram of (a) crude peptide, (b) combined flow-through and washing fractions after 72 h binding, and (c) after purification using the α-ketoacyl strategy. (d) MALDI-TOF MS of the peak at 23 min. [Column: Cosmosil 5C4-AR-300 (10 $\times$ 250 mm); Eluent A: formic acid/water = 2 : 3, B: formic acid/propanol = 4 : 1; Linear gradient 70–100% B over 45 min; Flow rate 2.5 ml/min; Detection at 280 nm].

purification, the target peptide was enriched significantly and eluted as a sharp peak (Figure 5c and d). This finding indicates that the combination of our α-ketoacyl method and a second purification procedure, such RP-HPLC, is a powerful purification strategy for the isolation and purification of membrane peptides.

Conclusions

In conclusion, we report on the details of a new strategy for the purification of peptides, by taking advantage of the reactivity of an α-ketoacyl group. The key advantage of this strategy is its broad applicability, in that both hydrophilic and hydrophobic peptides are amenable, utilizing commercially available building blocks and readily available resins.

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