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Efficient System of Artificial Oil Bodies for Functional Expression and Purification of Recombinant Nattokinase in *Escherichia coli*

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Nattokinase, a serine protease, and pronattokinase, when expressed in *Escherichia coli*, formed insoluble aggregates without enzymatic activity. For functional expression and purification, nattokinase or pronattokinase was first overexpressed in *E. coli* as an insoluble recombinant protein linked to the C terminus of oleosin, a structural protein of seed oil bodies, by an intein fragment. Artificial oil bodies were reconstituted with triacylglycerol, phospholipid, and the insoluble recombinant protein thus formed. Soluble nattokinase was subsequently released through self-splicing of intein induced by temperature alteration, with the remaining oleosin−intein residing in oil bodies and the leading propeptide of pronattokinase, when present, spontaneously cleaved in the process. Active nattokinase with fibrinolytic activity was harvested by concentrating the supernatant. Nattokinase released from oleosin−intein−pronattokinase exhibited 5 times higher activity than that released from oleosin−intein−nattokinase, although the production yields were similar in both cases. Furthermore, active nattokinase could be harvested in the same system by fusing pronattokinase to the N terminus of oleosin via a different intein linker, with self-splicing induced by 1,4-dithiothreitol. These results have shown a great potential of this system for bacterial expression and purification of functional recombinant proteins.

KEYWORDS: Artificial oil body; intein; nattokinase; oleosin; propeptide

INTRODUCTION

Current advances in recombinant DNA technology have made overproduction of target proteins in cells easily achievable. However, the task for isolating recombinant proteins in large quantity and with high purity remains challenging. In *Escherichia coli*, a massive production of heterologous proteins generally leads to the formation of inclusion bodies in cytoplasm or periplasm. To resume their active structures, protein aggregates prepared and harvested from cell lysate by centrifugation are subsequently subjected to refolding process. The refolding process generally consists of solubilization and renaturation, and its rational design is on a case-by-case basis. Consequently, the recovery yields of the processes are frequently low (*1*).

To facilitate protein purification, a variety of affinity tags have been explored and used to isolate target proteins (*2*, *3*). In general, recombinant proteins fused with a chosen tag are selectively adsorbed to a column packed with a cognate ligand of the tag and, thus, separated from the rest of the proteins in the cell extract. Recombinant proteins of high purity are eluted from the column either with an excess ligand or by a pH adjustment of the elution buffer. Separation of the target protein from the tag is executed by limited proteolytic cleavage at their linker sequence either in the column or after the elution (*4*). Preparation and operation of these affinity columns are relatively simple but expensive.

Seed oil bodies are lipid storage organelles composed of a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids (PLs) and some unique proteins (*5*−*7*). They are remarkably stable in both cells and isolated preparations as a consequence of the steric hindrance and electronegative repulsion provided by their surface proteins, particularly the unique structural protein, oleosin (*8*). Oil bodies harvested from transgenic plants and artificial oil bodies (AOBs) reconstituted with the three essential components, TAG, PL, and oleosin, have been used to develop an expression/purification system for the production of recombinant proteins (*9*, *10*). This novel technique offers a powerful and competitive alternative to the affinity chromatography conventionally used for protein purification. However, the requirement of a relatively expensive endopeptidase, for example, factor Xa or thrombin, for specific release of the target protein from its recombinant oleosin-fused polypep-

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Table 1. Primers Used in This Study

primer name	nucleotide sequence[a]
CHO15	TGCAGGCCTACAGGGCGCGTCCCATTC (*Stu*I)
CHO16	GATCCTGCAGCCTAATGAGTGAGCTAAC (*Pst*I)
CHO17	TCGAGGCCTGCGAGCTTGG (*Stu*I)
CHO18	CGCTCTGCAGCTTCCTCGCTCCACTG (*Pst*I)
CHO112	TGCGGAATTCGCGGAAAAAGCAGTACAG (*Eco*RI)
Jo6	GACCGCGGCCGCCAGTAGCGTG (*Not*I)
Jo7	TCGGGCGGCCGCAAATGGCTGAGCATTATG (*Not*I)
Jo8	AGAAACTCGAGTAAAGAAGTTTGAGAC (*Xho*I)
Mxe2	GCGAATTAAGCTTGGGCTCTTCCTGC (*Hin*dIII)
Ole8	TCTTAGATCTAATGGTGAGCATTATGGTC (*Bgl*II)
Ole9	TGAGCCATGGCAACAGGCTGCTGCTGCGAG (*Nco*I)
RC0310	TAGAGTCGACTAATTGTGCAGCTGCTTGTAC (*Sal*I)
RC0325	ACATGAATTCGCGCAAATCTGTTCC (*Eco*RI)
RC0436	CCATAGATCTGGCCGGAAAAAGCAG (*Bgl*II)
RC0437	AGGTGAATTCTTTGTGCAGCTGCTTG (*Eco*RI)
Ssp2	GGTCCCATGGTGCGCGAGTCC (*Nco*I)
Ssp3	GCCGGATCCGGCTCTTCCGTTGTG (*Bam*HI)

[a] The restriction site incorporated into each oligomer is underlined with its designated name indicated in parentheses.

tide substantially raises the processing cost and, thus, restricts its potential applications.

Inteins are self-splicing polypeptides originally identified in *Saccharomyces cerevisiae TFP1* gene (*11*), and a modification of amino acids at the splice junction of *Sce* VMA intein allowed the establishment of an in vitro splicing system (*12*). Among the inteins modified for engineering uses, *Mxe* GyrA intein (intein *M*) and *Ssp* DnaB intein (intein *S*) are particularly interesting because the separation of fusion proteins linked to these two inteins can be induced by 1,4-dithiothreitol (DTT) supplement and temperature alteration, respectively (*13*, *14*). The intein-mediated peptide cleavage occurs with the formation of thioester bond by an N−S acyl rearrangement at the N terminus of intein *M*, and DTT cleaves the thioester bond and triggers the N-terminal cleavage (*15*). In contrast, at permissive temperatures the succinimide formation at the C-terminal asparagine of intein *S* prompts the splice junction excision at the C terminus.

In this study, an attempt was made to reduce the processing cost of the AOB expression/purification system by linking oleosin and target proteins with an intein fragment to skip the utilization of an expensive endopeptidase for releasing target proteins from AOB. Nattokinase, a serine protease identified in *Bacillus subtilis* (*16*) with fibrinolytic activity, was employed as a target protein. Recombinant nattokinase released from AOB was harvested and examined for its production yield and enzymatic activity.

MATERIALS AND METHODS

DNA Manipulations. The oligomers utilized for the Polymerase Chain Reaction (PCR) are summarized in **Table 1**. Plasmids pOSP1 and pOSP2 containing N- and C-terminal fusion of intein to the oleosin gene, respectively, were constructed in several steps. The DNA fragment carrying the T7 promoter with *lacI*ts (substitution of Gly265 with Asp in *lacI*) was amplified from plasmid pET-265 (*17*) using primers CHO15 and CHO16. With the use of primers CHO17 and CHO18, the DNA containing the *bla* gene was produced from plasmid pPL450 (*18*). Subsequent ligation of the two PCR DNAs gave plasmid pWIN20. Similar to pWIN20, plasmid pJO1 was created to carry the multiple cloning site from plasmid pET29a (Novagen, Madison, WI) that was cleaved with *Xho*I−*Mlu*I and spliced with the DNA removed from pWIN20 with the same digestion. To obtain the oleosin gene, PCR was carried out with oligomers Ole8 and Ole9 priming the structural gene in pET29Ole (*19*). The resulting DNA was subcloned into pJO1 at the *Nco*I−*BamH*I site to produce plasmid pJO1-ole4. Furthermore, the PCR DNA containing intein *S* was synthesized from plasmid pTWIN1 (New England BioLabs, Beverly, MA) using primers Ssp2 and Ssp3. As a result, the PCR-amplified DNA and pJO1-ole4 subjected to the *Eco*RI−*Sal*I digestion were joined together to give plasmid pOSP1. Similarly, the oleosin gene was produced with primers Jo7 and Jo8 and subsequently subcloned into the *Not*I−*Xho*I site of pJO1 to generate plasmid pJO1-ole2. Cleaved with *Not*I and *Hin*dIII, pJO1-ole2 and the DNA containing intein *M* were spliced to yield plasmid pOSP2. This intein-carrying DNA was obtained from pTWIN1 by PCR using primers Mxe2 and Jo6.

PCR was performed to synthesize the DNA bearing the nattokinase gene with its propeptide (pronattokinase) from plasmid pTrc-proNK (Y. P. Chao) using two primer pairs, CHO112−RC0310 and RC0436−RC0437. Treated with either *Eco*RI−*Sal*I or *Bgl*II−*Eco*RI, the resulting PCR products were ligated into the corresponding site of pOSP1 and pOSP2 to produce plasmids pNK1 and pNK2, respectively. Similar to pNK1 but comprising a propeptide-free nattokinase gene, plasmid pNK3 was made by splicing pOSP1 with the structural gene of nattokinase from PCR using primers RC0310 and RC0325. Accordingly, the replacement of this PCR fragment with the pronattokinase gene in pTrc-proNK produced pTrc-NK.

Analytical Methods. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the previous method (*10*). The activity of nattokinase was determined by adding protein samples (0.01 mL) to the reaction solution (0.9 mL) consisting of 0.5% caseins in 0.1 M sodium phosphate buffer, pH 7.5. The enzymatic reaction proceeded at 37 °C for 5 min and was quenched by adding 0.1 mL of 2 N HCl. After centrifugation, the supernatant was recovered and measured at 275 nm. One CU of enzyme activity was defined as 1 μmol of tyrosine produced per minute per milliliter.

Culturing Methods. Plasmid-carrying cells were obtained by transforming the composite plasmids into *E. coli* strain BL21(DE3) (Novagene) to confer ampicillin resistance. Recombinant cells were cultured in Luria−Bertain (LB) medium (*20*), and cell growth was measured turbidimetrically at 550 nm (OD_{550}). To produce the recombinant protein, overnight culture was prepared and subsequently seeded into fresh media. The cell cultures were maintained at 37 °C and induced with 100 μM isopropyl β-D-thiogalactoside (IPTG) for protein productions upon reaching 0.5 at OD_{550}. After 4 h of induction, the cells were harvested by centrifugation and resuspended in 1 mL of 0.01 M sodium phosphate buffer, pH 7.5, for further analyses.

AOB Preparation and Protein Recovery. AOBs were prepared according to the method reported previously (*21*). The cells resuspended in 1 mL of buffer solution (reaching 10 at OD_{550}) were disrupted by French press and fractioned into supernatant and pellet parts by subsequent centrifugation. AOBs were reconstituted in 1 mL of 10 mM sodium phosphate buffer, pH 7.5, with 15 mg of TAG (canola oil from Leader Price Co., Bangkok, Thailand), 150 μg of PL, and the pellet fraction of *E. coli* cell lysate containing 550 μg of oleosin-fused recombinant polypeptides. The mixture was subjected to sonication with the amplitude set at 30% for 20 s. Subsequently, AOBs were collected after centrifugation and washed with the buffer solution. To retrieve the target protein, AOBs thus prepared were either placed at indicated temperatures or treated with 40 μM DTT for 16 h. Finally, a centrifugation was applied to segregate the oil and aqueous phases, and the protein production in each phase was analyzed by SDS-PAGE; enzyme activities were determined.

RESULTS

Expression of Nattokinase or Pronattokinase in *E. coli*. Recombinant nattokinase or its preprotein with a leading propeptide (pronattokinase) was expressed in *E. coli* under the control of *trc* promoter and induced by IPTG (**Figure 1**). The expressed nattokinase (28 kDa) or pronattokinase (37 kDa) was predominately found in the insoluble fraction of cell lysate after

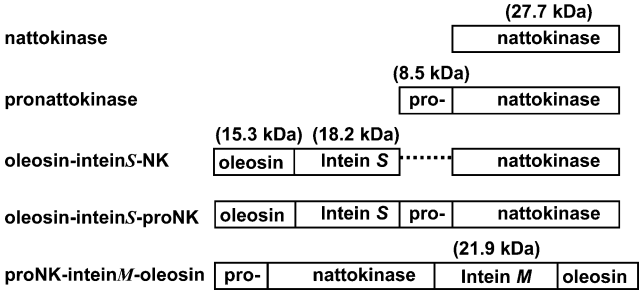

Figure 1. Schematic diagram showing all of the recombinant proteins expressed in this study. Relative positions of nattokinase, propeptide, oleosin, intein *S*, and intein *M* as well as their molecular masses are shown in each recombinant polypeptide.

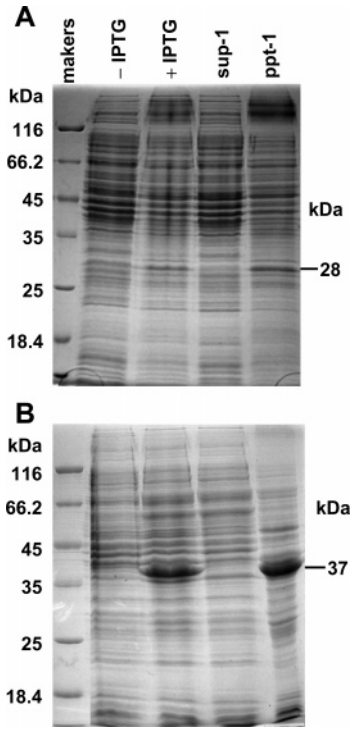

Figure 2. SDS-PAGE of nattokinase and pronattokinase expressed in *E. coli*. With or without IPTG induction, total proteins of *E. coli* expressing nattokinase (**A**) and pronattokinase (**B**) were extracted, fractionated into supernatant (sup-1) and precipitate (ppt-1), and resolved in SDS-PAGE. The positions of nattokinase (28 kDa) and pronattokinase (37 kDa) are indicated. The markers of molecular masses are β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp981 (25 kDa), and β-latoglobulin (18.4 kDa).

centrifugation (**Figure 2**), and no enzymatic activity was detected in either nattokinase or pronattokinase thus harvested (data not shown).

Functional Expression and Purification of Nattokinase via AOB System. For functional expression and purification, nattokinase (NK) or pronattokinase (proNK) was first overexpressed in *E. coli* as a recombinant protein fused to the C terminus of oleosin by a linker polypeptide, intein *S* (**Figure 1**). The overexpressed recombinant protein, oleosin−intein *S*-NK or oleosin−intein *S*-proNK, was predominately found in the insoluble fraction of cell lysate after centrifugation (**Figures 3A** and **4A**). AOBs were reconstituted with the insoluble pellet of cell lysate consisting of mainly oleosin−intein *S*-NK or oleosin−intein *S*-proNK. After centrifugation, AOB formed a milky "scum" on the top, with the supernatant (sup-2) relatively

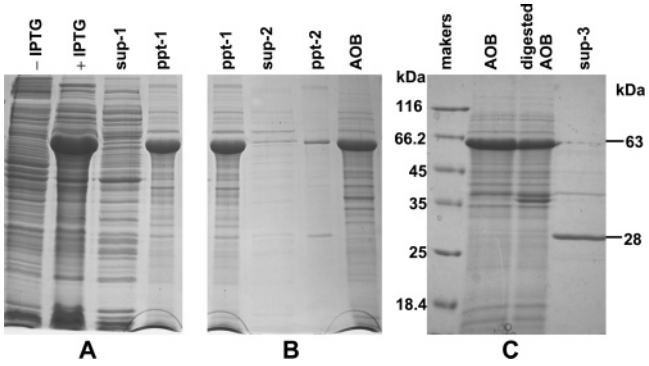

Figure 3. Analyses of oleosin–intein *S*-NK overexpressed in *E. coli.* (**A**) Total proteins of *E. coli* containing oleosin–intein *S*-NK were extracted, fractionated into supernatant (sup-1) and precipitate (ppt-1), and resolved in SDS-PAGE. (**B**) AOBs were constituted with the pellet fraction (ppt-1) of *E. coli* cell lysate containing oleosin–intein *S*-NK. After constitution, three fractions, supernatant (sup-2), precipitate (ppt-2), and AOB, were obtained by centrifugation at 10000*g* for 15 min and resolved in SDS-PAGE. (**C**) AOBs constituted with oleosin–intein *S*-NK were induced for self-splicing of the intein linker by elevating the temperature from 4 to 25 °C, then fractionated into oil-body layer (digested AOB) and supernatant (sup-3) by centrifugation, and resolved in SDS-PAGE. The positions of oleosin–intein *S*-NK (63 kDa) and nattokinase (28 kDa) are indicated.

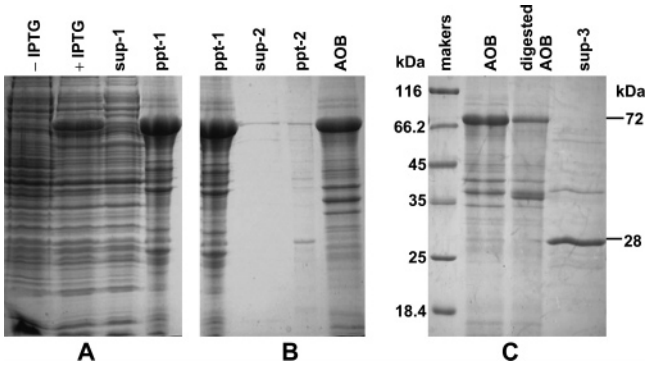

Figure 4. Analyses of oleosin–intein *S*-proNK overexpressed in *E. coli.* (**A**) Total proteins of *E. coli* containing oleosin–intein *S*-proNK were extracted, fractionated into supernatant (sup-1) and precipitate (ppt-1), and resolved in SDS-PAGE. (**B**) AOBs were constituted with the pellet fraction (ppt-1) of *E. coli* cell lysate containing oleosin–intein *S*-proNK. After constitution, three fractions, supernatant (sup-2), precipitate (ppt-2), and AOB, were obtained by centrifugation at 10000*g* for 15 min and resolved in SDS-PAGE. (**C**) AOBs constituted with oleosin–intein *S*-proNK were induced for self-splicing of the intein linker by elevating the temperature from 4 to 25 °C, then fractionated into oil-body layer (digested AOB) and supernatant (sup-3) by centrifugation, and resolved in SDS-PAGE. The positions of oleosin–intein *S*-proNK (72 kDa) and nattokinase (28 kDa) are indicated.

transparent with nearly no visible pellet (ppt-2). In company with other insoluble bacterial proteins, oleosin−intein *S*-NK or oleosin−intein *S*-proNK was primarily present in the AOB fraction (**Figures 3B** and **4B**). These AOBs were extremely stable and maintained their integrity for several days (data not shown) in a similar pattern as observed in AOBs reconstituted with TAG, PL, and other oleosin-fused proteins (*10*, *19*). Release of nattokinase or pronattokinase from AOBs was achieved via self-splicing of the intein linker induced by elevating the temperature from 4 to 37 °C. Followed by centrifugation, mature nattokinase was found predominately in the supernatant (sup-3) in both expression strategies, whereas oleosin−intein *S* remained in AOBs (**Figures 3C** and **4C**). The leading propeptide

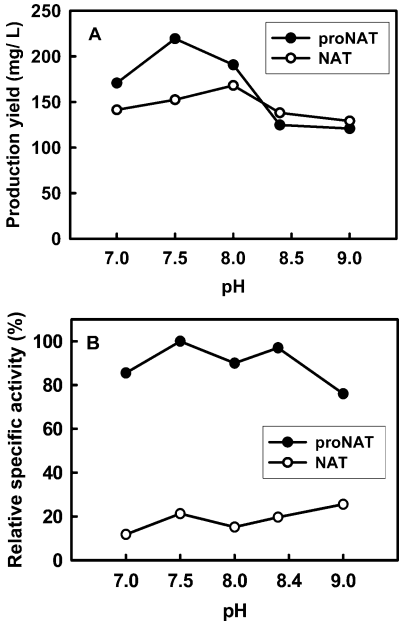

Figure 5. Production of nattokinase via AOBs at different pH values. Production yield (**A**) and relative specific activity (**B**) of nattokinase harvested from AOBs constituted with oleosin–intein *S*-NK (○) or oleosin–intein *S*-proNK (●) were determined at pH ranging from 7 to 9.

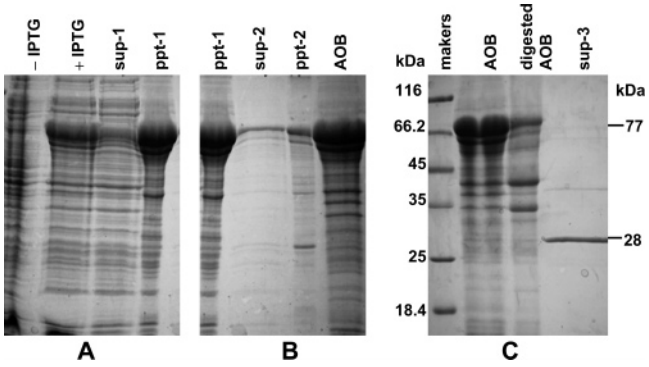

Figure 6. Analyses of proNK–intein *M*-oleosin overexpressed in *E. coli.* (**A**) Total proteins of *E. coli* containing proNK–intein *M*-oleosin were extracted, fractionated into supernatant (sup-1) and precipitate (ppt-1), and resolved in SDS-PAGE. (**B**) AOBs were constituted with the pellet fraction (ppt-1) of *E. coli* cell lysate containing proNK–intein *M*-oleosin. After constitution, three fractions, supernatant (sup-2), precipitate (ppt-2), and AOB, were obtained by centrifugation at 10000*g* for 15 min and resolved in SDS-PAGE. (**C**) AOBs constituted with proNK–intein *M*-oleosin were induced for self-splicing of the intein linker by adding DTT, then fractionated into oil-body layer (digested AOB) and supernatant (sup-3) by centrifugation, and resolved in SDS-PAGE. The positions of proNK–intein *M*-oleosin (77 kDa) and nattokinase (28 kDa) are indicated.

in pronattokinase seemed to be cleaved spontaneously after being separated from oleosin−intein *S*. Soluble nattokinase of high yield was harvested by concentrating the ultimate supernatant.

Production Yield and Optimal pH of Expressed Nattokinase. Production yields of recombinant nattokinase in the two expression strategies (via fusion proteins of oleosin−intein *S*-NK and oleosin−intein *S*-proNK) were similar at pH conditions ranging from 7 to 9 (**Figure 5A**). Production of recombinant nattokinase at pH <6 was not examined as AOBs tended to aggregate in acidic conditions, presumably due to the neutralization of surface negative repulsion (data not shown). On the the basis of the hydrolytic activity toward casein, the specific

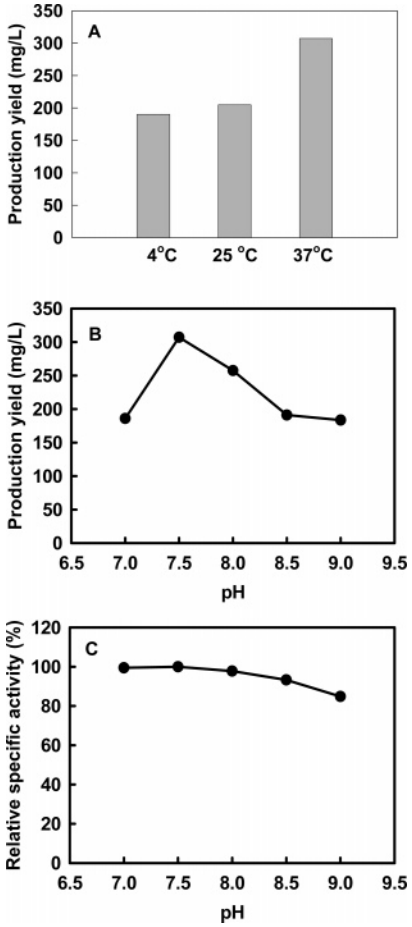

Figure 7. Production of nattokinase via AOBs constituted with proNK–intein *M*-oleosin. (**A**) Production yield of nattokinase from AOBs constituted with proNK–intein *M*-oleosin was examined at 4, 25, and 37 °C (pH 7.5). Production yield (**B**) and relative specific activity (**C**) of this recombinant nattokinase were determined at pH ranging from 7 to 9.

activity of the expressed nattokinase released from oleosin−intein *S*-proNK was ∼5 times higher than that released from oleosin−intein *S*-NK (**Figure 5B**). The optimal pH for nattokinase production was pH 7.5 via the AOB expression/purification system using oleosin−intein *S*-proNK as a recombinant carrier. As expected, fibrinolytic activity was observed in the recombinant nattokinase (data not shown).

Expression of Pronattokinase Linked to the N Terminus of Oleosin by Intein *M*. To test if a target protein for functional expression/purification via AOB system could be fused to the N terminus of oleosin, pronattokinase was fused to oleosin via a different linker, intein *M*, the self-splicing of which could be induced by the inclusion of DTT (**Figure 1**). The recombinant protein was overexpressed, harvested, and assembled into and released from the AOBs as described above for fusing this protein to the C terminus of oleosin (**Figure 6**). Again, mature nattokinase was obtained from the concentrated ultimate supernatant, with the leading propeptide cleaved spontaneously. Production yields of recombinant nattokinase released from AOBs were examined at 4, 25, and 37 °C (**Figure 7A**). As relatively effective production was obtained at 37 °C, subsequent evaluation of nattokinase expression at various pH conditions was made at this temperature. The optimal pH for the production of recombinant nattokinase in this strategy was pH 7.5 (**Figure 7B**,**C**).

DISCUSSION

An improved artificial oil body-based system, as exemplified by the production of nattokinase in *E. coli* in the current study, has been developed for bacterial expression and purification of functional recombinant proteins. In this system, a target protein was first overexpressed as an insoluble oleosin−intein-fused polypeptide, collected from the pellet of cell lysate simply by centrifugation, assembled into AOBs, and released from AOBs via self-splicing of the intein linker and then harvested by concentrating the ultimate supernatant. Compared to the previous AOB system using an expensive protease for specific cleavage (*10*), the release of target protein via intein self-splicing induced by temperature alteration or DTT supplementation significantly reduces the processing cost. In addition, releasing target proteins from AOBs by DTT supplementation may favor the isolation of oxygen-labile proteins. A target protein, for example, nattokinase as demonstrated in this study, could be linked to either the N or C terminus of oleosin for functional production with equal production yields of ~300 mg/L and recovery yields of 60%. Therefore, either fusion strategy may be selected for functional expression, depending upon the possible existence of structural constraints in target proteins. Apparently, this improved system provides an easy and cost-effective process for protein expression and purification with high efficiency.

It has been well recognized that propeptide functions as an intramolecular chaperone and is essential for guiding the correct folding of the linked protein to its mature form (*22*, *23*). After fulfilling its mission, the propeptide is removed autoproteolytically and degraded subsequently to ensure the active function of the mature protein (*24*, *25*). The significance of propeptide as well as its autocleavage has also been observed in this study. The considerably higher specific activity of mature nattokinase released from oleosin−intein *S*-proNK compared with that of nattokinase released from oleosin−intein *S*-NK (**Figures 3** and **4**) suggests that the pronattokinase immobilized on the AOB surface might fold into an optimal structure ready for autocleavage of its propeptide as soon as it is separated from AOBs by the intein-mediated self-splicing. This structural organization may be considered to be similar to its pre-proprotein ready for autocleavage of its propeptide after the removal of presequence. A similar observation of propeptide autocleavage in a recombinant protein after a renaturation process was reported previously in the histidine-tagged prosubtilisin immobilized on Talon resin (*26*).

The conventional strategy for protein refolding in vitro generally includes solublization of the protein aggregates followed by protein renaturation. In principle, preventing unfolded peptide intermediates from interactions is necessary to ensure efficient refolding. The recent development of two major approaches, the addition of chemical additives known as chaotropic agents and the adsorption of denatured proteins onto a solid matrix, is aimed to renature proteins at high concentrations (*27*). The former gives the repulsive force, whereas the latter provides spatial constraints to reduce intermolecular collisions. Following these processes, a final removal of excessive denaturants is required to promote the protein refolding. Utilization of AOBs for functional expression of recombinant proteins seems to combine both approaches. Owing to its hydrophobicity and heterogeneity, the fusion partner oleosin in this study entraps the target protein into insoluble aggregates, a state favorable for concentrating the target protein, reducing the risk of proteolytic attack and facilitating the harvesting process. Furthermore, the insoluble oleosin-fused protein is directly solublized by forming AOBs without the aid of extra denaturant, and thus the final removal of excessive denaturant is no longer required. It seems to be most likely that the hydrophobic patch in oleosin may function like the additives in separating the unfolded intermediates. Upon sonication, the water-repelling regions of oleosin are readily incorporated into AOB, and the unfolded target protein fused to the water-accessible N or C terminus of oleosin is rendered to AOB surfaces, where it undergoes the refolding process.

ABBREVIATIONS USED

AOB, artificial oil body; DTT, 1,4-dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; PL, phospholipid; TAG, triacylglycerol.

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