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Method for Bacterial Expression and Purification of Sesame Cystatin via Artificial Oil Bodies

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A method was developed for production of sesame cystatin, a thermostable cysteine protease inhibitor. Sesame cystatin was first expressed in *Escherichia coli* as an insoluble recombinant protein fused to oleosin, a unique structural protein of seed oil bodies, by a short hydrophilic linker peptide. Stable artificial oil bodies were constituted with triacylglycerol, phospholipid, and the insoluble oleosin−cystatin fusion protein. After centrifugation, the oleosin−cystatin fusion protein was exclusively found in the artificial oil bodies. Proteolytic cleavage with papain, a cysteine protease effectively inhibited by cystatin, separated soluble cystatin from oleosin that was firmly embedded in the artificial oil bodies. After recentrifugation, papain that coexisted with cystatin in the collected supernatant was denatured by incubating at 55 °C for 30 min. The insoluble denatured papain was removed by one more centrifugation, and the expressed cystatin of high yield and purity was harvested simply by concentrating the ultimate supernatant. Comparable inhibitory activity toward papain was observed between the expressed cystatin and the native one purified from sesame seeds. This method is presumably applicable to production of other protease inhibitors whose target proteases are economically available.

KEYWORDS: Artificial oil bodies; cystatin; papain; protease inhibitor; oleosin

INTRODUCTION

Cystatins are natural inhibitors of cysteine proteases first identified from animal sources and have been investigated extensively in their physiological functions and reversible interaction with their target enzymes (*1*). Homologous cystatin-like proteins found in plants were classified as a new subfamily termed phytocystatins on the basis of their sequence uniqueness (*2*−*4*). The roles of phytocystatins were proposed to regulate endogenous cysteine proteases taking parts in various biological activities, e.g., mobilizing stored reserves during embryogenesis and seed germination (*5, 6*) and responding to biotic and abiotic stresses (*7*). They may also be involved in defensive mechanisms to protect plants from invasion of pests employing cysteine proteases as their digestive enzymes in gut. In general, phytocystatins are thermally stable and have been used in food processing for inhibition of autolysis and gel softening of mackerel (*8, 9*) and considered for biotechnological applications in protecting plants from pathogens, nematodes, and insect invasion (*2, 10*).

Plant seed oil bodies are lipid storage organelles preserving energy for seed germination and subsequent seedling growth (*11, 12*). An oil body of 0.5−2 μm in diameter contains a triacylglycerol (TAG) matrix surrounded by a monolayer of phospholipids (PLs) and some unique proteins (*13*−*15*). Oil bodies are remarkably stable in both cells and isolated preparation as a consequence of the steric hindrance and electronegative repulsion provided by their surface proteins, particularly the unique structural protein oleosin (*16*). Stable artificial oil bodies (AOBs) can be technically constituted with the three essential constituents, TAG, PL, and oleosin according to their relative proportions in native oil bodies (*17*−*19*).

Seed oil bodies extracted from transgenic plants expressing oleosin-fused proteins have been demonstrated to be adequate carriers for the production of recombinant proteins (*20*). On centrifugation, the desired protein fused to oleosin on the surface of oil bodies may be easily separated from the rest of the cellular extract as an oily scum, which floats on the top. The resuspended oil bodies are subjected to specific proteolytic cleavage at the designed linker sequence to separate the desired protein from oleosin. The desired protein is harvested with high purity from the supernatant after one more centrifugation. Definitely, the

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stability of the introduced recombinant construct and the expression level of the desired protein in transgenic plants should be evaluated prior to commercial applications. In addition, the requirement of a relatively expensive endopeptidase for releasing the desired protein from oleosin evidently restricts the realistic applications of this oil-body affinity purification system.

In this study, we intended to design a protocol for production of sesame cystatin. Sesame cystatin was first expressed as an oleosin-fused protein in *Escherichia coli*, affinity-purified by formation of AOBs, released from oleosin by proteolytic cleavage with papain, a relatively inexpensive endopeptidase, and finally harvested by concentration of the ultimate supernatant. The functional activity of the expressed cystatin was also examined in comparison with that of native cystatin purified from sesame seeds.

MATERIALS AND METHODS

Construction of pET29OleCys. The cDNA fragment encoding the first 140 amino acids of sesame 15 kDa oleosin (accession no. AF091840) was constructed in the fusion expression vector pET29a (Novagen), by use of a *Nco*I site in the polylinker of the vector. The resulting plasmid, pET29Ole, was digested with *Bam*HI and *Hin*dIII; the desired cleaved fragment was isolated and purified with a Geneclean III kit (Carlsbad). A short sequence encoding a hydrophilic linker was introduced to the 5′-end of a sesame cystatin (accession no. AF240007) by polymerase chain reaction (PCR) with a forward primer, AAG TTG GAT CCC ATC GAA GGA AGA ATG AGCT ACT CTA GGG GGC GTC CAT, containing a *Bam*HI site (underlined), and a reverse primer, ATC GAA GCT TAA GAG TGA TCA AGA TCC ACC TTC TT, containing a *Hin*dIII site (underlined). The desired PCR product was purified, digested with *Bam*HI and *Hin*dIII, and then ligated at 16 °C overnight with the cleaved fragment from the pET29Ole plasmid. The resulting plasmid, pET29OleCys, was used to transform *E. coli* DH5α competent cells on an LB agar plate containing kanamycin at 50 μg/mL, and the accuracy of plasmid construction was also confirmed by direct sequencing.

Overexpression of an Oleosin−Cystatin Fusion Protein. The recombinant plasmid pET29OleCys was transformed into *E. coli* BL21 (DE3). Overexpression of the recombinant fusion protein, oleosin−cystatin, was induced by adding 0.1 mM isopropyl β-D-thiogalactoside (IPTG) in a bacteriophage T7 RNA polymerase/promoter system. After 3 h of induction at 37 °C, the *E. coli* cells were harvested, lysated by sonication in 10 mM sodium phosphate buffer, pH 7.5, fractionated into supernatant and pellet by centrifugation, and then subjected to analyses by SDS−PAGE and western blotting. Nonfusion cystatin was also expressed as a positive control by use of the pET29Cys plasmid, constructed in a similar manner, and used to generate antibodies in rabbit as reported earlier (*21*). Antibodies against oleosin were raised in chicken by use of sesame 15 kDa oleosin purified from sesame oil bodies as described previously (*22*).

Constitution of AOBs with TAG, PL, and Oleosin−Cystatin. Seed oil bodies were purified from sesame (*Sesamum indicum* L.) according to the protocol developed by Tzen et al. (*23*). TAGs were obtained from sesame oil bodies by chemical extraction as reported previously (*17*); dioleoyl phosphatidylcholine, the major PL found in sesame oil bodies, was purchased from Sigma. AOBs were constituted in 1 mL of 10 mM sodium phosphate buffer, pH 7.5, with 15 mg of TAG, 150 μg of PL, and the pellet fraction of *E. coli* cell lysate containing 550 μg of oleosin−cystatin. At first, PL dissolved in chloroform was placed at the bottom of the eppendorf tube, and the chloroform was allowed to evaporate in a chemical hood overnight. After evaporation, TAG and oleosin−cystatin were incorporated, followed by sonication with a 3-mm diameter probe in an Ultrasonic Processor GE 601 at an amplitude of 30% for 20 s. The sample was then cooled in an ice bucket for 5 min. The sonication was repeated two more times to generate AOBs.

Stability of AOBs Observed in a Light Microscope. AOBs constituted with oleosin−cystatin were suspended in 50 mM sodium phosphate buffer, pH 7.5. The stability and integrity of the suspended AOBs were observed under a Nikon type 104 light microscope at different time intervals.

Release of Cystatin from AOBs by Proteolytic Cleavage. To release cystatin from the oleosin−cystatin fusion protein, AOBs were treated with 5 units of papain in a reaction buffer of 1 mL containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.5. The reaction mixture was gently agitated on a shaker (30 rpm) at room temperature for 30 min, and then centrifuged to separate soluble cystatin from oleosin that remained embedded in AOBs. The collected supernatant was incubated at 55 °C for 30 min to denature papain, and the insoluble denatured papain was removed by centrifugation. The released cystatin was harvested by concentrating the ultimate supernatant through a Centriprep-30 membrane and a Centriprep-10 membrane, and its purity was checked by SDS−PAGE.

Purification of Endogenous Cystatin from Sesame Seeds. Endogenous cystatin in the soluble proteins extracted from mature sesame seeds was incubated with papain-coupled resins as described previously (*21*). The cystatin-bound resins were equilibrated with 50 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1% Brij 35, packed into a column, and washed with 50 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl and 10% (v/v) glycerol. Affiliated cystatin proteins were eluted with 50 mM K_3PO_4, pH 11.5, containing 0.5 M NaCl and 10% (v/v) glycerol. The eluent containing cystatin was adjusted to pH 7.4 with 5 M sodium formate buffer, pH 3.0, and concentrated by Amicon Ultra-15 (Millipore).

Papain Inhibitory Activity Assay. Cysteine protease inhibitory assay was performed according to the methods described by Barrett (*24*) and Abe et al. (*25*) with papain as the target enzyme and *N*-benzoyl-l-arginine-2-naphthylamide (BANA) (Sigma) as its substrate with some modifications. Papain, 10 μg, was first incubated with various amounts of cystatin in 250 μL of assay solution containing 0.1 M sodium phosphate, pH 6, 1 mM EDTA, and 2 mM β-mercaptoethanol at 37 C for 5 min. Subsequently, proteolytic reaction was initiated by adding 0.1 mL of 1 mM BANA at 37 °C and terminated by adding 0.5 mL of 2% HCl/ethanol after reaction for 10 min. Color was developed by adding 0.5 mL of 0.06% *p*-dimethylaminocinnamaldehyde (Sigma) in ethanol for 15 min, and the absorbance of the mixture was measured at 540 nm. Inhibitory activity was detected as a decreased amount of substrate-hydrolyzing activity of papain.

RESULTS

Overexpression of an Oleosin−Cystatin Fusion Protein in *E. coli*. Cystatin was overexpressed in *E. coli* as a recombinant protein fused to oleosin, a unique oil-body structural protein, by a hydrophilic linker peptide of Ile-Glu-Gly-Arg. The oleosin−cystatin fusion protein of 37 kDa was predominantly found in the insoluble fraction of cell lysate (**Figure 1**). Accuracy of expression was confirmed by western blots; the 37 kDa fusion protein could be recognized by antibodies against sesame 15 kDa oleosin and 22 kDa cystatin, respectively.

Constitution of AOBs with TAG, PL, and the Oleosin−Cystatin Fusion Protein. AOBs were constituted with TAG, PL, and the insoluble pellet of cell lysate largely containing the oleosin−cystatin fusion protein. The oleosin−cystatin fusion protein, as well as other insoluble bacterial proteins, was almost entirely present in the oil-body fraction after centrifugation (**Figure 2**). The sizes of AOBs were mostly 0.5−3 μm as observed under a light microscope (**Figure 3**). These AOBs were extremely stable and maintained their integrity at least for 24 h at 4 °C.

Release of Cystatin from AOBs. Separation of cystatin from oleosin embedded in AOBs was achieved by proteolytic cleavage with papain (**Figure 4**). After proteolytic digestion followed by centrifugation, cystatin was found predominantly in the supernatant while oleosin remained in AOBs as detected in SDS−PAGE and western blotting. Papain that coexisted with

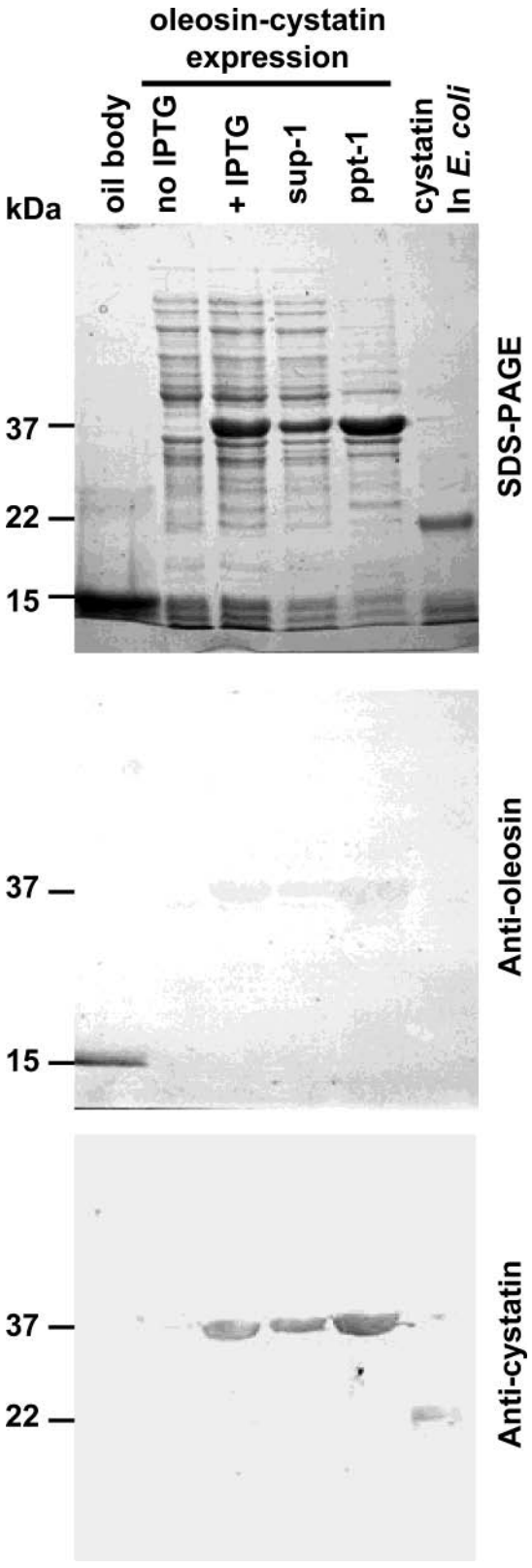

Figure 1. SDS–PAGE and western blotting of the oleosin–cystatin fusion protein overexpressed in *E. coli*. Total proteins of *E. coli* harvesting pET29OleCys before or after IPTG induction were extracted, fractionated into supernatant and precipitate, resolved in SDS–PAGE, and subjected to immunodetection with antibodies against sesame 15 kDa oleosin or 22 kDa cystatin. Sesame oil-body proteins and nonfusion cystatin in *E. coli* lysate were used as positive controls. The sizes of oleosin–cystatin (37 kDa), cystatin (22 kDa), and oleosin (15 kDa) are indicated on the left.

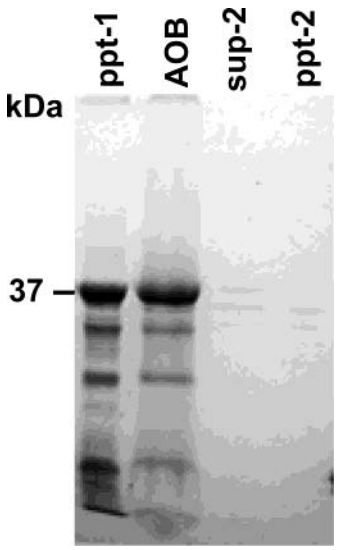

Figure 2. SDS–PAGE of protein distribution in different centrifugal fractions after constitution of AOBs. AOBs were constituted with TAG, PL, and the pellet fraction (ppt-1) of *E. coli* cell lysate containing oleosin–cystatin. After constitution, three fractions, artificial oil body (AOB), supernatant (sup-2), and precipitate (ppt-2) were obtained by centrifugation and resolved in SDS–PAGE. The molecular mass of oleosin–cystatin fusion protein is indicated on the left.

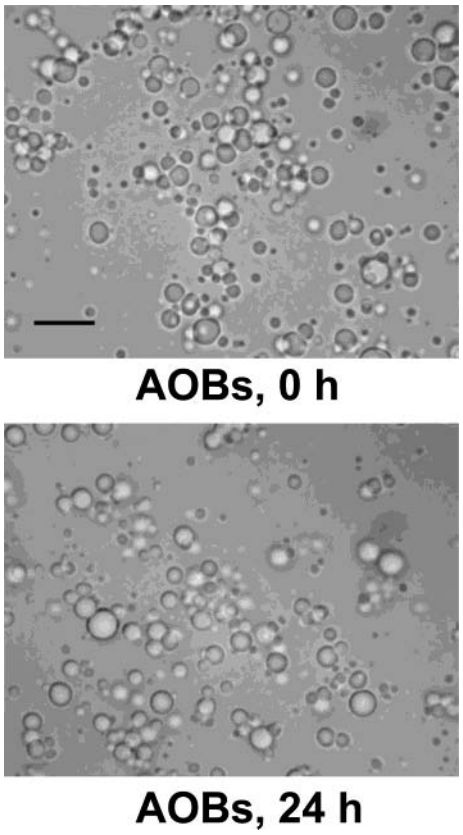

Figure 3. Light microscopy of AOBs. AOBs were observed either right after constitution (top) or after storage at 4 °C for 24 h (bottom). Both photos are at the same magnification. Bar represents 10 μm.

cystatin in the supernatant was denatured by heating at 55 °C and removed by one more centrifugation (data not shown). Soluble recombinant cystatin of high yield and purity was harvested by concentrating the ultimate supernatant. According to protein staining with Coomassie Blue in SDS−PAGE

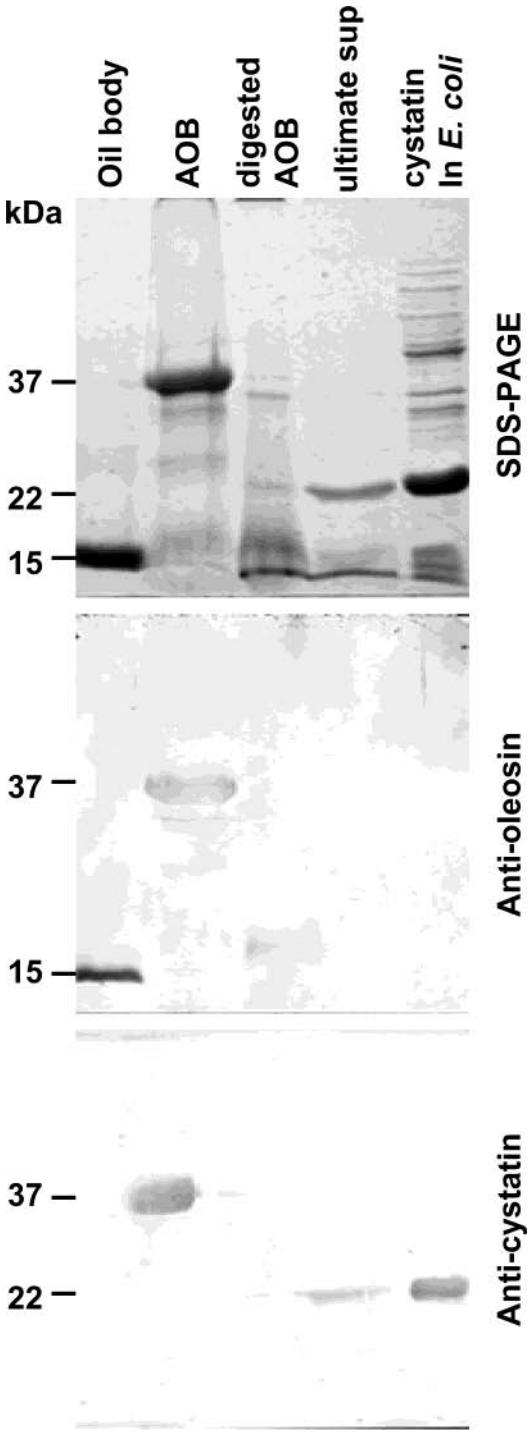

Figure 4. SDS–PAGE and western blotting of cystatin purified from AOBs. AOBs constituted with oleosin–cystatin were digested with papain, fractionated into oil-body layer (digested AOB) and supernatant (ultimate sup) by centrifugation, resolved in SDS–PAGE, and subjected to immunodetection with antibodies against sesame 15 kDa oleosin or 22 kDa cystatin. Sesame oil-body proteins and nonfusion cystatin in *E. coli* lysate were used as positive controls. The sizes of oleosin–cystatin (37 kDa), cystatin (22 kDa), and oleosin (15 kDa) are indicated on the left.

analysis, the expressed cystatin was purified to apparent homogeneity in the current protocol.

Papain Inhibition of the Expressed Cystatin. To examine whether the recombinant cystatin was functionally active, both endogenous cystatin purified from sesame seeds and the expressed cystatin harvested from *E. coli* were subjected to inhibitory activity assay with papain as a target protease. The

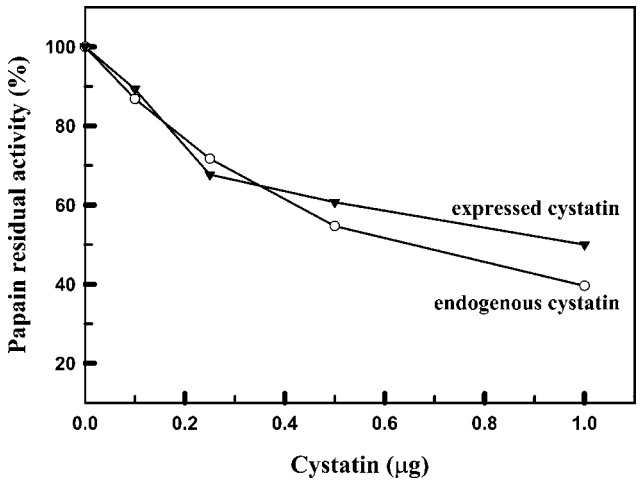

Figure 5. Inhibition of papain activity by the endogenous and expressed cystatins. Endogeous cystatin purified from sesame seeds and the expressed cystatin harvested from *E. coli* were subjected to inhibitory assay. Inhibition is expressed as the residual activity of papain with BANA as the substrate.

result showed that the expressed cystatin was able to inhibit papain with inhibitory activity similar to that of endogenous cystatin (**Figure 5**).

DISCUSSION

Endogenous cysteine proteases in seafood and meat were found activated during meat or surimi processing and might cause deleterious effects of textural destruction responsible for meat softening and surimi gel weakening (*8*). Inclusion of chicken cystatin during surimi processing effectively eliminated protein decomposition (*9*). However, the low abundance of cystatins in living organisms limits their potential applications. Economically, overexpression of functionally active cystatins in microorganisms seems to be a feasible solution. Unfortunately, cystatins of animal origin are frequently glycosylated and/or composed of intradisulfide bonds, and thus their functional expression in microorganisms is relatively complicated. In contrast, phytocystatins possessing neither glycosylation nor intradisulfide bonds are presumably suitable for bacterial expression on a large scale for commercial utilization. In a previous study, we have demonstrated the functional expression of sesame cystatin in *E. coli* with equivalent inhibitory activity compared with the native cystatin purified from sesame seeds (*21*). In this study, we combined the effectiveness of bacterial recombinant expression and the simplicity of oil-body affinity purification to develop a method for production of functional sesame cystatin. Compared with the papain-coupling affinity purification used in our previous expression of sesame cystatin, this new protocol displayed equivalent purification efficiency at a lower cost by replacing the expensive papain-coupled column with AOBs.

During the process of producing cystatin via AOBs, it was assumed that folding of the recombinant cystatin was first tangled with the hydrophobic fusion oleosin and thus became insoluble aggregates in the pellet of *E. coli* lysate. Constitution of AOBs attracted the hydrophobic oleosin portion into the TAG matrix, thus leaving cystatin alone for self-refolding right after its physical segregation from oleosin. As cystatin self-folded into its correct conformation on the surface of AOB, inhibitory activity might be recovered concurrently. Functional soluble cystatin was then easily released from AOBs after papain digestion.

In the oil-body affinity purification (*20*), the requirement of a relatively expensive endopeptidase, e.g., factor Xa or thrombin, for specific release of a desired protein from its recombinant oleosin-fused polypeptide substantially raises the processing cost and thus restricts its potential applications. Only pharmaceutical proteins of high market value may be economically produced by this purification system. Nevertheless, based on the successful production of sesame cystatin in this study, the oil-body purification system seems to be suitable for commercially producing various protease inhibitors by using their inexpensive target endopeptidases to replace the expensive specific protease required for releasing the desired proteins from oleosins that are firmly embedded in oil bodies.

ABBREVIATIONS USED

AOBs, artificial oil bodies; BANA, *N*-benzoyl-L-arginine-2-naphthylamide; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thio-β-D-galactoside; PL(s), phospholipid(s); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAG(s), triacylglycerol(s)

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