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Recombinant Production, Isotope Labeling and Purification of ENOD40B, a Plant Peptide Hormone

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Abstract

The plant peptide hormone ENOD40B was produced in a protein production strain of *Escherichia coli* harboring an induction controller plasmid (Rosetta(DE3)pLysS) as a His₆-tagged ubiquitin fusion protein. The fusion protein product was denatured and refolded as part of the isolation procedure and purified by immobilized metal ion chromatography. The peptide hormone was released from its fusion partner by adding yeast ubiquitin hydrolase (YUH) and subsequently purified by reversed phase chromatography. The purity of the resulting peptide fragment was assayed by MALDI-TOF mass spectrometry and NMR spectroscopy. The final yields of the target peptide were 7.0 mg per liter of LB medium and 3.4 mg per liter of minimal medium.

Keywords

peptide hormone; nodule development; ubiquitin fusion; MS; NMR

INTRODUCTION

The symbiosis between most legume plants and nitrogen-fixing bacteria (rhizobia) results in the formation of root nodules. Oligosaccharides produced by rhizobia, called Nod factors, are known to induce the development of nodules through the expression of several nodulin genes. These genes are classified according to the timing of their induction as early nodulin (ENOD) and late nodulin (LNOD) genes. *ENOD40*, one of the early nodulin genes, is rapidly induced in the root and nodule primordium [1–3]. When *ENOD40* is knocked out, functional nodule development is arrested; on the other hand, if *ENOD40* is overexpressed, nodulation is accelerated. These indicate that *ENOD40* plays a role in nodule development. *In vitro* translation of soybean *ENOD40* mRNA led to the discovery of two peptides: a 12-amino acid peptide (ENOD40A) and a 24-amino acid peptide (ENOD40B) [4]. Each peptide interacts with the 93-kDa subunit of sucrose synthase and activates its enzymatic activity [5]. The binding site for ENOD40A on sucrose synthase has been reported [6], but the binding site for ENOD40B is unknown. The conversion of sucrose and uridine diphosphate (UDP) into UDP-glucose and UDP-fructose catalyzed by sucrose synthase is an important

step in plant sucrose metabolism [7]. Sucrose catabolism is a key step in nitrogen fixation and is required for the nodule to develop normally [8].

It is important to understand how these peptides behave structurally, in both their free and bound states. Structural studies require a large quantity of the target, and for NMR spectroscopy, the peptide ideally should be labeled with stable isotopes. Here we present a protocol for producing recombinant ENOD40B as a ubiquitin fusion in *E. coli* Rosetta(DE3)pLysS cells and for isolating and purifying the cleaved peptide.

MATERIALS AND METHODS

Construction of the ENOD40B Expression Plasmid

The gene coding for ENOD40B (MVLEE AWRER GVRGE GAHSS HSLT) was synthesized chemically (Integrated DNA Technology, Coralville, IA, USA). The sense strand was 5'- ggt ggt atg gtg ctg gaa gaa gcg tgg cgc gaa cgc ggc gtg cgc ggc gaa ggc gcg cat agc agc cat agc ctg acc tga c3', and the antisense, 5'- tcg agt cag gtc agg cta tgg ctg cta tgc gcg cct tgc ccg cgc acg ccg cgt tgc cgc cac gct tct tcc agc acc ata cca ccg c-3'. The two DNA strands were annealed, and inserted into the vector pET-28a/ubiS, a slightly modified version of pET-28a/ubi [9] which had been previously digested with SacII and XhoI. The pET-28a/ubiS vector was constructed according to Xu *et al.* [10]. The resulting plasmid was named pET-28a/ubiS/ENOD40B.

Expression and Purification of Ubiquitin-ENOD40B Fusion Protein from LB Medium

The pET-28a/ubiS/ENOD40B plasmid was transformed into the expression host, Rosetta(DE3)pLysS (Novagen, Madison, WI). A single colony was used to inoculate 100 mL of LB medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. This culture was grown overnight in a shaking incubator at 37 °C. The next morning, the fully grown culture was used to inoculate 1 L of fresh LB medium containing the same antibiotics. The culture was grown at 37 °C, and IPTG (Gold BioTechnology, St. Louis, MO, USA) was added to a final concentration of 0.5 mM when the optical density at 600 nm reached 1.0. The culture was harvested 3 h later, and the cells were resuspended in 30 mL 10 mM TrisHCl pH 8.0. The cells were lysed by freezing and thawing, and the DNA was fragmented by sonication. The soluble fraction was retained after centrifugation at 15,000 rpm for 20 min and loaded onto a Hi-Trap Chelating HP column (5 mL) charged with Ni²⁺ ions (GE Healthcare, Piscataway, NJ, USA). The column was washed first with 20 mL Buffer A (10 mM sodium phosphate buffer pH 7.4, 10 mM imidazole, 300 mM NaCl), then 10 mL of 20% ethanol. Protein bound to the column was denatured by applying 10 mL Buffer A containing 8 M urea. On-column refolding was performed by applying 10 mL aliquots in which the urea concentration was reduced stepwise to 6, 4, 2, 1, and 0 M. The bound fraction was eluted with 10 mL Buffer B (10 mM sodium phosphate buffer pH 7.4, 400 mM imidazole, 300 mM NaCl). Ultrafiltration was used both to concentrate the solution and to exchange the buffer to 10 mM TrisHCl containing 1 M urea. A Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used to determine the amount of protein in the pooled fractions.

Expression and Purification of Ubiquitin-ENOD40B Fusion Protein from Minimal Medium

E. coli Rosetta(DE3)pLysS containing the pET-28a/ubiS/ENOD40B was grown at 37 °C in a 5 mL LB medium inoculated from a single colony. 1 mL of the fully grown culture was used as an inoculum for 100 mL of the minimal medium and grown overnight at 37 °C. The fully grown culture was used in turn as an inoculum for 900 mL minimal medium, and the culture was grown at 37 °C. For uniform ¹⁵N-labeling, 1 g of ¹⁵NH₄Cl per liter culture was

provided as the sole nitrogen source. The fusion protein was isolated and purified as described above.

Purification of ENOD40B

To the purified ubiquitin-ENOD40B fusion protein, β -mercaptoethanol and yeast ubiquitin hydrolase (YUH) were added to final concentrations of 1 mM and 0.1 mg/mL, respectively. YUH was prepared according to Moon *et al.* [9]. The mixture was incubated at 37 °C overnight. The reaction mixture was loaded directly onto a Resource RPC column (GE Healthcare, Piscataway, NJ, USA), and a 20%–60% acetonitrile gradient was applied during chromatography on an HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The ENOD40B fraction was pooled and lyophilized. The final product was analyzed by MALDI-TOF mass spectrometry directly after lyophilization.

NMR Spectroscopy

The NMR sample contained 1 mM [^{15}N]-ENOD40B in 10 mM sodium acetate buffer pH 3.2 and 10% D_2O . The ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectrum was collected at 25 °C on a Varian 800 MHz spectrometer. The raw data contained 2048 and 256 complex points in t_2 and of t_1 , respectively. The NMRPipe software package[11] was used for data processing. The final spectrum contained 1024 and 256 real points in t_2 and of t_1 , respectively.

RESULTS AND DISCUSSION

Construction of Expression Plasmids

The gene coding for ubiquitin-ENOD40B was inserted into a (His) $_6$ - containing vector to facilitate purification of the fusion protein. The ubiquitin fusion system, which was similar to that described by Moon *et al.* [9], enabled extensive column washing with high concentration denaturant so that non-specifically binding proteins were washed away.

Expression and Purification of Ubiquitin-ENOD40B Fusion Protein

The expressed protein appeared as two bands, a minor band corresponding to ubiquitin and a major band corresponding to ubiquitin/ENOD40B fusion protein (Fig. 1, lane 8). The fusion protein apparently was cleaved by a protease present in the *E. coli* cells as described in the preparation of piscidin, an antimicrobial peptide [12]. The final protein yields (fusion protein plus ubiquitin alone) were 40 mg per liter of LB medium and 18 mg per liter of minimal medium. The ratio between the full fusion protein and ubiquitin molecule as measured by ImageJ software (NIH, USA) was 2:1. Thus, the amount of ubiquitin/ENOD40B fusion protein was around 67% of the total mass.

Purification of ENOD40B Peptide

The ENOD40B peptide was efficiently clipped off of ubiquitin by YUH. Unlike other widely used proteases, such as thrombin and TEV protease, only a very small amount of YUH (50 μg) was required to cleave 10–20 mg of the ubiquitin fusion protein. Following cleavage, the entire reaction mixture was loaded onto a Resource RPC column in 1 mL aliquots on an HP1100 HPLC system. The volume of the cleavage reaction mixture was 10 mL, and reversed phase chromatography was repeated 10 times. The peptide eluted at ~28% acetonitrile. All ENOD40B-containing fractions were pooled and lyophilized (Fig. 2). The final yield of ENOD40B was around 7.0 or 3.4 mg per liter of LB or of minimal medium, respectively. MALDI-TOF mass spectrometry was used to assay the purified peptide (Fig. 3). MALDI-TOF showed a major peak at 2694.18 Da, which is in good agreement with the theoretical molecular weight of 2694.9 Da.

HSQC Spectrum of ^{15}N -Labeled ENOD40B Peptide

The HSQC spectrum showed 24 strong signals with uniform linewidth and intensity (Fig. 4). From the peptide sequence, one predicts a maximum of 23 signals from backbone and 1 from the sidechain indole group. The position of the peak at (131.5 ppm ^{15}N , 10.0 ppm ^1H) is characteristic for the sidechain indole of Trp, and was assigned to Trp-7 of ENOD40B.

CONCLUSIONS

The plant peptide hormone ENOD40B was produced recombinantly by a ubiquitin fusion system and purified successfully. Even though the fusion protein suffered partial proteolysis, sufficient peptide was recovered for further experiments. The major advantage of the ubiquitin fusion system over others stems from the small size of ubiquitin, which provides a higher relative amount of the target peptide and easier recovery and refolding from inclusion bodies. NMR and X-ray structural studies of this peptide are now in progress.

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REFERENCES

1. Crespi MD, Jurkevitch E, Poiret M, d'Aubenton-Carafa Y, Petrovics G, Kondorosi E, Kondorosi A. enod40, a gene expressed during nodule organogenesis, codes for a nontranslatable RNA involved in plant growth. *Embo J.* 1994; 13:5099–5112. [PubMed: 7957074]
2. Kouchi H, Hata S. Isolation and Characterization of Novel Nodulin cDNAs Representing Genes Expressed at Early Stages of Soybean Nodule Development. *Mol Gen Genet.* 1993; 238:106–119. [PubMed: 7683079]
3. Kouchi H, Takane K, So RB, Ladha JK, Reddy PM. Rice ENOD40: isolation and expression analysis in rice and transgenic soybean root nodules. *Plant J.* 1999; 18:121–129. [PubMed: 10363365]
4. Charon C, Sousa C, Crespi M, Kondorosi A. Alteration of enod40 expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *Plant Cell.* 1999; 11:1953–1965. [PubMed: 10521525]
5. Rohrig H, Schmidt J, Miklashevichs E, Schell J, John M. Soybean ENOD40 encodes two peptides that bind to sucrose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:1915–1920. [PubMed: 11842184]
6. Rohrig H, John M, Schmidt J. Modification of soybean sucrose synthase by S-thiolation with ENOD40 peptide A. *Biochem Bioph Res Co.* 2004; 325:864–870.
7. Thummler F, Verma DPS. NODULIN-100 OF Soybeans Is The Subunit Of Sucrose Synthase Regulated By The Availability Of Free Heme In Nodules. *J. Biol. Chem.* 1987; 262:14730–14736. [PubMed: 2889731]
8. Gordon AJ, Minchin FR, James CL, Komina O. Sucrose synthase in legume nodules is essential for nitrogen fixation. *Plant Physiol.* 1999; 120:867–877. [PubMed: 10398723]
9. Moon WJ, Hwang DK, Park EJ, Kim YM, Chae YK. Recombinant expression, isotope labeling, refolding, and purification of an antimicrobial peptide, piscidin. *Protein Expr Purif.* 2007; 51:141–146. [PubMed: 16935005]
10. Xu X, Jin F, Yu X, Ren S, Hu J, Zhang W. High-level expression of the recombinant hybrid peptide cecropinA(1-8)-magainin2(1-12) with an ubiquitin fusion partner in *Escherichia coli*. *Protein Expr Purif.* 2007; 55:175–182. [PubMed: 17572103]

11. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
12. Moon WJ, Hwang DK, Park EJ, Kim YM, Chae YK. Recombinant expression, isotope labeling, refolding, and purification of an antimicrobial peptide, piscidin. *Protein Expr Purif*. 2007; 51:141–146. [PubMed: 16935005]

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Figure 1.

Purification of ENOD40B produced from 1 L LB medium as monitored by 12% SDS-PAGE. Lane 1, size marker; lane 2, whole cell lysate; lanes 3 and 4, supernatant and pellet of cell lysate, respectively; lane 5, flowthrough fraction of supernatant from the HiTrap Chelating HP column; lane 6, fraction eluted by 20% ethanol; lane 7, fraction eluted by Buffer A containing 8M urea; lane 8, fraction eluted by Buffer B (fusion protein); lane 9, fusion protein after YUH cleavage.

Figure 2.
HPLC elution profile from the Resource RPC column. The peak corresponding to ENOD40B is marked with an arrow. The acetonitrile gradient is also shown.

Figure 3. MALDI-TOF spectrum of purified syndecan ENOD40B. Single and double charged molecular ions are seen at $m/z = 2694$ and 1348 , respectively.

Figure 4. $[\text{}^1\text{H}-^{15}\text{N}]$ HSQC spectrum of ENOD40B. The NMR sample contained 1 mM $[\text{}^{15}\text{N}]$ -ENOD40B in 10 mM sodium acetate buffer pH 3.0 and 10% D_2O . The spectrum was collected at 25 °C on a Varian Inova 800 MHz-NMR spectrometer.