(Supporting Information)

Expressed Protein Ligation of 5-Enolpyruvylshikimate-3-phosphate (EPSP) Synthase: An Application to a Protein Expressed as an Inclusion Body

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Materials and Methods

Construction of plasmids. For the construction of the C-terminal intein-fused N-terminal fragment (**1** at Figure 1) expression vector, the N-terminal gene fragment of the EPSP synthase was amplified from pWS250 by the polymerase chain reaction (PCR) with T7 promoter primer and EPSPSN (Table 1).[1] The amplified gene fragment which encodes residues 1-237 was then purified and digested with *Xba*I and *Sap*I. The gel-purified insert was ligated into the pTWIN1 vector digested with the same enzymes. The ligation mixture was transformed into XL-1 Blue (Invitrogen, Carlsbad, CA, USA). Plasmids were isolated from a few colonies and the correct inserts were identified by restriction enzyme digestion and DNA sequencing. For the N-terminal intein-fused C-terminal fragment (**2'** at Figure 1), the C-terminal gene fragment (residues 238^{CYS}-427) was amplified from the same template with two primers (Table 1). The fragment was digested with *Sap*I and *Bam*HI, gel-purified and ligated into the pTWIN1 vector cut with the same enzymes, and the sequences were confirmed as described above.

For the expression vector of *E. coli* methionine aminopeptidase, the gene fragment of MAP was amplified from the BL21 genomic DNA using primers (Table 1) and ligated into a pCRII vector (Invitrogen, CA, USA). The insert was further manipulated to make it compatible with other expression vectors; the insert was digested with *Eco*RI and filled in with *pfu* DNA polymerase (Stratagene). The pACYC184 vector was digested with *Hind*III, filled in and digested with *Hinc*II. The insert was subsequently ligated into the digested pACYC184. A plasmid containing the insert in a clockwise direction was chosen and used throughout the experiment.

Protein Expression and Purification. For the preparation of EPSPSN-thioester tagged fragment, pHKN was transformed into the ER2566 strain. Four liters of LB medium containing 100 μg/L ampicillin was inoculated with a freshly grown culture and incubated in a shaker at 37 °C until the OD600 0.5-0.6. Overexpression of the fusion protein was induced by addition of IPTG to a final concentration of 0.4 mM. Then, the cultures were transferred to a 22 °C shaker and incubated for a further 9 hours. The cells were harvested by centrifugation at 8,000 rpm, 10 min, and 4 °C. The harvested cells were kept frozen until use. The fusion protein was purified by a chitin affinity column. The cell pellet was resuspended in buffer A (20 mM TrisHCl, pH 8.5, 500 mM NaCl, and 1 mM EDTA) with DNase I, and lysed by sonication. The lysate was clarified by centrifugation at 15,000 rpm, 15 min, and 4 °C. The supernatant was loaded onto the chitin column equilibrated with buffer A at a flow rate of 1 mL/min. The column was washed thoroughly with 6 bed volumes of buffer A. To produce the thioester-tagged protein (EPSPSN-thioester), the column was flushed quickly with 18 mL of buffer B (buffer A with 50 mM MESNA). The chitin column was stopped and left overnight for the completion of the cleavage. The thioester-tagged protein was eluted using two bed volumes of buffer B. The eluted proteins were concentrated to 1 mM by ultrafiltration.

For the preparation of $EPSPSC^{CYS}$ fragment, the plasmid encoding the C-terminal fragment and intein fusion protein was transformed into the ER2566 strain. The cells were grown and induced as above. This fusion protein was expressed as an inclusion body, which was not refolded under the various conditions utilized. Alternatively, the EPL active C-terminal fragment was prepared from two different strains. The plasmid that harbors Met-$EPSPSC^{CYS}$ (Met-238^{CYS}-427) was transformed into the BL21(DE3) and the BL21(DE3) harboring pHK623 which encodes the methionine aminopeptidase (MAP). The cells were grown in LB medium containing 50 μg/L kanamycin, or 50 μg/L ampicillin and 17 μg/L chloramphenicol depending on the plasmids they had. The cells were grown at a 37 °C shaker until OD600=0.6, then induced with IPTG to a final concentration of 0.4 mM. The protein was overexpressed ~30% of total cell protein as an inclusion body. The cells were then incubated for an additional 5 hours. Cells were pelleted by centrifugation and kept at −70 °C prior to use.

Inclusion bodies of $EPSPSC^{CYS}$ protein were prepared as follows: the harvested cell pellet was suspended in buffer C (50 mM TrisHCl, pH 7.5, 1 mM β-mercaptoethanol, and 0.5% Trition X 100) with lysozyme (5 mg/mL) and DNase I (0.01 mg/mL), lysed by sonication, and stirred for 30 min at 4 °C. The lysate was clarified by centrifugation at 10,000

rpm, 10 min, and 4 °C. The pellet was resuspended in buffer C by sonication while the soluble fraction was discarded. The suspension was spun down under the same conditions. This step was repeated once again followed by washing step: the pelleted inclusion body was suspended in buffer C containing 1 M GdHCl, sonicated, and spun down as described above. This step was repeated a total of three times. The white pellet was then washed with distilled water to remove the detergent, and centrifuged. Buffer D (50 mM TrisHCl, pH 7.8, 1 mM EDTA, 20 mM β-mercaptoethanol and 6 M GdHCl) was added to dissolve the inclusion body pellet. The solution was stirred overnight at room temperature, and centrifuged at 15,000 rpm, 15 min and 4 °C. The supernatant was collected and concentrated. The purified protein was then transferred to the NVDF membrane after SDS-PAGE and sequenced to determine the presence of the first methionine.

References

1. Shuttleworth, W. A.; Evans, J. N. S. *Biochemistry* **1994**, *33*, 7062.