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Subtle control of carbon chain length in polyunsaturated fatty acid synthases

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Keywords

Polyunsaturated fatty acid, biosynthesis, enzymes, mutagenesis

ABSTRACT

Some marine bacteria synthesize docosahexaenoic acid (DHA; C₂₂) and eicosapentaenoic acid (EPA; C₂₀) by enzyme complexes composed of four subunits (A to D). We recently revealed that β -ketoacyl synthase (KSC)/chain length factor (CLF)-like domains in the “C” subunit of DHA synthase catalyzed the last elongation step (C₂₀ to C₂₂) even though their amino acid sequences are very similar to those of EPA synthase. To investigate the amino acid residues controlling the product chain length, conserved residues in the KSC/CLF-like domains in DHA synthase were replaced with corresponding EPA synthase residues. Among 12 mutants, two CLF-like domain-mutated genes completely lost DHA productivity and produced trace amounts of EPA when co-expressed with *dha-ABD* in *Escherichia coli*, whereas when co-expressed with *epa-ABD* they produced the same amounts of EPA as *epa-ABCD*. These results suggested that the product profiles were subtly controlled by several amino acid residues.

Polyunsaturated fatty acids (PUFAs, Figure 1) such as docosahexaenoic acid (DHA; C22:6 ω 3), eicosapentaenoic acid (EPA; C20:5 ω 3), and arachidonic acid (ARA; C20:4 ω 6) are indispensable fatty acids for humans and fishes and fish oils are main supply sources. Because of increasing demand, however, other resources of PUFAs are required and practical fermentative processes have been developed. [1–3]

There are two distinct PUFA biosynthetic pathways. One is the aerobic desaturase/elongase pathway and the other is the anaerobic PUFA synthase pathway. In the aerobic pathway operating in plants, fungi, microalgae, and bacteria, desaturases and elongases catalyze individual desaturation and elongation steps from oleic acid (C18:1 ω 9) to synthesize PUFAs. [3] In the anaerobic pathway occurring in eukaryotic microalgae and prokaryotic bacteria, PUFA synthases comprised of large enzyme complexes with various catalytic domains synthesize PUFAs as exemplified by microalgal DHA production (Figure S1). [4] PUFA synthase possesses acyltransferase (AT), multiple tandem acyl carrier protein (ACP), malonyl CoA transacylase (MAT), ketoacyl synthase (KS), ketoacyl reductase (KR), two types of dehydratase (polyketide synthase (PKS)-type DH and FabA-type DH), enoyl reductase (ER), and chain length factor (CLF)-like domains (Figure 1).

We previously revealed that PUFA productivities was dependent on the number of active ACPs. [5] We also revealed that two distinct dehydratases, DH_{PKS} and DH_{FabA}, controlled the formation

of the first *cis* double bonds in ARA and EPA biosynthesis. [6] We also showed that the last elongation step in EPA biosynthesis (C₁₈ to C₂₀) was catalyzed by the KS_A domain of the “A” subunit in both EPA and DHA synthases. [7] In contrast, the last elongation step for DHA biosynthesis (C₂₀ to C₂₂) was catalyzed by the KS_C/CLF-like domains of the “C” subunit in DHA synthase even though amino acid sequences of the KS_C/CLF-like domains of the “C” subunit in EPA and DHA synthases are very similar (Figure S2).

In this study, to determine the key amino acid residues controlling the product chain length, conserved amino acid residues in DHA synthase, which were suggested to face the catalytic cavities in the KS_C and CLF-like domains by docking simulations, were replaced with corresponding EPA synthase residues and the product profiles were investigated by heterologous expression in *E. coli*. Moreover, the quaternary structure of the subunits was examined with truncated KS_C and CLF-like domains.

RESULTS AND DISCUSSION

The amino acid sequences of the KS_C/CLF-like domains in DHA and EPA synthases are similar. (Figure S2). However, some amino acid residues differ and are specifically conserved in each type of enzyme. Among these, we searched for amino acid residues that might face the cavity with the catalytic Cys residue in the KS_C domain and control the product chain length by docking

simulations because we recently succeeded in converting microalgal DHA synthase into EPA synthase by substitutions of three amino acid residues in the KSc/CLF-like domains, all of which faced an estimated catalytic cavity [7]. Moreover, amino acid residues facing the cavity of the CLF-like domain, which has similarity to the KSc domain but has no conserved active residues, were also selected based on an estimated structure.

By homology modelling with the crystal structure of mammalian fatty acid synthase (PDB ID, 2VZ8; 25% identity with DHA synthase) [8], we estimated the structures of the KSc domain (Figure S3) and CLF-like domain (Figure S4) in the DHA synthase of *Moritella marina* and selected six amino acid residues in each domain (M229, Q239, N244, N245, H247, and K337 in the KSc domain and E665, Y670, E674, F680, N701, and R705 in the CLF-like domain) (Figures S2–S4). Then, we replaced the selected amino acids with the corresponding residues of the EPA synthase of *Photobacterium profundum*.

The mutated *dha-C* genes were co-expressed with *dha-ABD* in *E. coli* and their PUFA profiles were analyzed by GC-MS (Figures 2 and S5). Transformants harboring the mutated *dha-C* genes Q239H, N244H, N245G, or H247S produced almost the same amount of DHA (1.4~2.7 mg L⁻¹) and their PUFA profiles were the same as that of the wild type enzyme. In contrast, transformants with M229F, K337L, Y670A, E674D, F680M, or N701Q produced less DHA (0.4~0.8 mg L⁻¹) than the wild type enzyme. Moreover, transformants with E665A or R705K completely lost DHA

productivity and produced trace amounts of EPA (0.04 mg L^{-1}). These results suggested that some specific amino acid residues in the CLF-like domain mainly control the carbon chain length.

We previously showed that transformants harboring *dha-ABD* and *epa-C* produced only EPA. [7] However, the productivity was drastically decreased when compared with that of *dha-ABCD*. This result suggested that a combination of co-expressing genes is important for PUFA productivity. Therefore, we next expressed the abovementioned mutated genes with *epa-ABD*. Among the mutated genes, transformants with Y670A or F680M produced almost the same amounts of DHA, DPA, and EPA (Y670A; 0.7, 0.6, and 1.0 mg L^{-1} , F680M; 0.6, 0.3, and 0.5 mg L^{-1}). Moreover, transformants harboring E665A or R705K produced the same amount of EPA ($0.5\sim 1.0 \text{ mg L}^{-1}$) as *epa-ABCD* (0.6 mg L^{-1}) concomitant with DPA ($0.4\sim 0.8 \text{ mg L}^{-1}$) (Figure 3). The results showed that the single mutation caused complete change of the function for specifically synthesizing EPA.

Human fatty acid synthase, which possesses similar domain structures to those of PUFA synthases, forms a 540 kDa homodimer and the KS domains directly contact each other. [9] Recently, a covalent AcpP–FabB complex, an acyl carrier protein appended to a ketosynthase responsible for fatty acid biosynthesis in *E. coli*, was also predicted to form a dimer structure by X-ray crystallography, NMR, and molecular dynamics simulations. [10] We therefore examined whether the K_{Sc} and CLF-like domains of DHA synthase form a dimer structure. The whole “C” subunit is a large enzyme and is difficult to prepare as a recombinant enzyme. Therefore, we first

103 tried to prepare a truncated recombinant KSc or CLF-like domain, or KSc/CLF-like domain of
104 DHA and EPA synthases. We constructed several plasmids so that the truncated recombinant
105 enzymes were expressed with or without an *N*-terminal His-tag. Among these, the non-tagged and
106 His-tagged CLF-like domain, and His-tagged KSc/CLF-like domain of EPA synthase were
107 expressed as soluble forms, although the His-tagged KSc domain was mainly expressed as an
108 inclusion body (Figure S6). To examine the interaction between the KSc and CLF-like domains,
109 the two domains were simultaneously co-expressed in *E. coli* and then purified with a Ni²⁺-agarose
110 column. As shown in Figure 4, the non-tagged CLF-like domain was co-purified with the *N*-
111 terminal His-tagged KSc domain in the similar manner as KS and CLF-like domains of type II
112 polyketide synthase for ishigamide biosynthesis [11], suggesting that the two domains interacted
113 with each other. The interaction was also examined by a gel filtration experiment with the purified
114 enzymes. Although no peaks in agreement with the monomer molecular weight of either protein
115 were detected, two peaks corresponding to a hexamer (324 kDa) and a dimer (129 Da) structure of
116 KSc and CLF-like domains, respectively, were detected (Figure 4). After fractionation of the peaks,
117 they were confirmed to contain KSc and CLF-like domains by SDS-PAGE (Figure 4). These results
118 showed that the “C” subunit containing KSc and CLF-like domains perhaps forms a multimer
119 structure. Then, we tried to carry out the same experiments with the purified His-tagged CLF-like
120 domain and His-tagged KSc/CLF domain (Figure S6). However, these recombinants were very

unstable and easily inactivated by aggregation.

In conclusion, we identified several key amino acid residues in the CLF-like domain of DHA synthase for chain length control of the product. In particular, mutant genes carrying E665A or R705K, which were constructed by replacing the amino acid residue with the corresponding residue of EPA synthase, lost DHA productivity when they were heterologously expressed with *dha-ABD* in *E. coli*. In contrast, transformants harboring the mutated genes and *epa-ABD* produced the same amount of EPA as those carrying *epa-ABCD*. Moreover, truncated enzymes containing CLF-like and KSC domains were shown to form a hetero multimer. Together, these results suggest that the product profiles are subtly controlled by some amino acid residues in the CLF-like domains.

METHODS

General. All chemicals, primers, and enzymes were obtained from the same suppliers as described previously. [6]. PUFA production was analyzed by GC/MS as the same methods. [6]

Bacterial strains, media, and plasmids. *Escherichia coli* XL1-Blue (Nippon Gene Co. Ltd., Tokyo, Japan) was used for construction of plasmids (Supporting Information). An *E. coli* mutant defective in β -oxidation, BLR(DE3) Δ *fadE*, [5] was utilized for the heterologous PUFA production. *E. coli* BL21(DE3) (Nippon Gene Co. Ltd.) was used to prepare recombinant enzymes. LB broth

139 medium (Sigma-Aldrich Japan) and terrific broth (TB) medium (Becton, Dickinson and Company,
140 NJ, USA) were used for cultivation. If necessary, 1.5% agar was added into the media. Ampicillin
141 (Ap), chloramphenicol (Cm), kanamycin (Km), and streptomycin (Sm) were added to the media at
142 concentrations of 100, 30, 25, and 20 $\mu\text{g ml}^{-1}$, respectively, when needed.

143

144 **Preparation of recombinant enzymes and gel filtration.** To prepare truncated recombinant
145 enzymes, *Escherichia coli* BL21(DE3) harboring the recombinant expression plasmid pET-*epa*-
146 *C_KSc* (His-tagged KSc domain), pET-*epa*-*C_CLF* (His-tagged CLF-like domain), pCDF-*epa*-
147 *C_CLF* (non-tagged CLF-like domain), or pET-*epa*-*C_KSc*-CLF (His-tagged KSc/CLF-like
148 domain) [7] was used. For analysis of interaction between the KSc domain and CLF-like domain,
149 *E. coli* was transformed with pET-*epa*-*C_KSc* and pCDF-*epa*-*C_CLF*. The transformant was
150 cultivated at 37 °C in LB broth medium including Km for 16 h and was inoculated into 100 ml LB
151 medium containing Km. Recombinant enzymes were prepared as described previously. [6] Enzyme
152 purities and concentration were analyzed by SDS-PAGE on 8–12% gels and determined by the
153 Bradford method using bovine serum albumin as a standard. The purified enzymes were subjected
154 to gel filtration with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare UK Ltd., Little
155 Chalfont, Buckinghamshire, England) connected to an ÄKTAexplorer 10S system (GE Healthcare).
156 The column had been equilibrated with phosphate buffer (50 mM sodium phosphate, 150 mM

157 NaCl; pH 7.2) and the proteins were eluted at flow rate of 1 mL min⁻¹. Ferritin (440,000 Da),
158 aldolase (158,000 Da), conalbumin (75,000 Da), and ovalbumin (44,000 Da) were used as
159 standards for calculation of apparent molecular mass.

160
161 **Structure modelling.** The 3D structures of the KSc and CLF-like domains in subunit “C” were
162 obtained by homology modelling using the Phyre2 server with the structure of mammalian fatty
163 acid synthase (PDB ID, 2VZ8).

164 165 REFERENCES

- 166 (1) Winwood, R. J. (2013) Recent developments in the commercial production of DHA and EPA
167 rich oils from micro-algae, *OCL*. 20, D604.
- 168 (2) Xue, Z., Sharpe, P. L., Hong, S., Yadav, N. S., Xie, D., Short, D. R., Damude, H. G., Rupert,
169 R. A., Seip, J. E., Wang, J., Pollak, D. W., Bostick, M. W., Bosak, M. D., Macool, D. J.,
170 Hollerbach, D. H., Zhang, H., Arcilla, D. M., Bledsoe, S. A., Croker, K., McCord, E. F., Tyreus,
171 B. D., Jackson, E. N., Zhu, Q. (2013) Production of omega-3 eicosapentaenoic acid by
172 metabolic engineering of *Yarrowia lipolytica*. *Nat. Biotechnol.* 31, 734–740
- 173 (3) Sakuradani, E., Ando, A., Shimizu, S., Ogawa, J. (2013) Metabolic engineering for the
174 production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. *J.*

175 *Biosci. Bioeng.* 116, 417–422.

176 (4) Metz, J. G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R.,
 177 Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., Browse, J. (2001)
 178 Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and
 179 eukaryotes. *Science* 293, 290–293.

180 (5) Hayashi, S., Satoh, Y., Ujihara, T., Takata, Y., Dai, T. (2016) Enhanced production of
 181 polyunsaturated fatty acids by enzyme engineering of tandem acyl carrier proteins. *Sci. Rep.*
 182 6, 35441.

183 (6) Hayashi, S., Satoh, Y., Ogasawara, Y., Maruyama, C., Hamano, Y., Ujihara, T., Dai, T. (2019)
 184 Control mechanism for *cis* double-bond formation by polyunsaturated fatty-acid synthases.
 185 *Angew. Chem. Int. Ed.* 58, 2326–2330.

186 (7) Hayashi, S., Naka, M., Ikeuchi, K., Ohtsuka, M., Kobayashi, K., Satoh, Y., Ogasawara, Y.,
 187 Maruyama, C., Hamano, Y., Ujihara, T., Dai, T. (2019) Control mechanism for carbon-chain
 188 length in polyunsaturated fatty-acid synthases. *Angew. Chem. Int. Ed.* 58, 6605–6610.

189 (8) Maier, T., Leibundgut, M., Ban, N. (2008) The crystal structure of a mammalian fatty acid
 190 synthase. *Science* 321, 1315–1322.

191 (9) Herbst, D. A., Townsend, C. A., Maier, T. (2018) The architectures of iterative type I PKS and
 192 FAS. *Nat. Prod. Rep.* 35, 1046–1069.

- (10) Milligan, J. C., Lee, D. J., Jackson, D. R., Schaub, A. J., Beld, J., Barajas, J. F., Hale, J. J., Luo, R., Burkart, M. D., Tsai, S. C. (2019) Molecular basis for interactions between an acyl carrier protein and a ketosynthase. *Nat. Chem. Biol.* *15*, 669–671.
- (11) Du, D., Katsuyama, Y., Shin-ya, K., Ohnishi, Y. (2018) Reconstitution of a type II polyketide synthase that catalyzes polyene formation. *Angew. Chem. Int. Ed.* *57*, 1954–1957.

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxx.

Detailed experimental procedures, Supporting Information Figures S1–S6, Supporting Information Table S1 (PDF)

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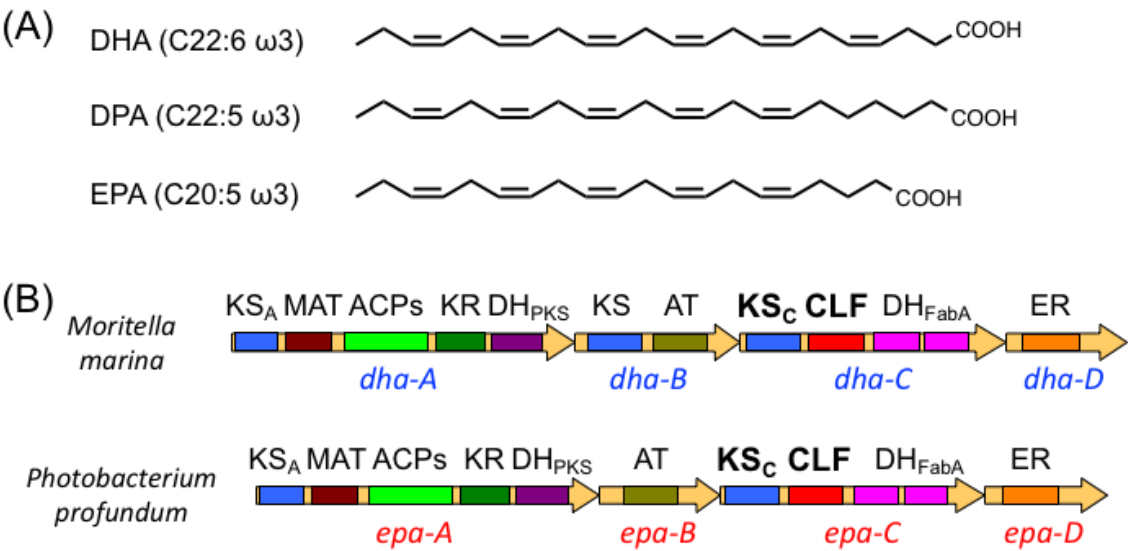
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214 **CONFLICT OF INTEREST**

215 The authors declare no conflict of interest.

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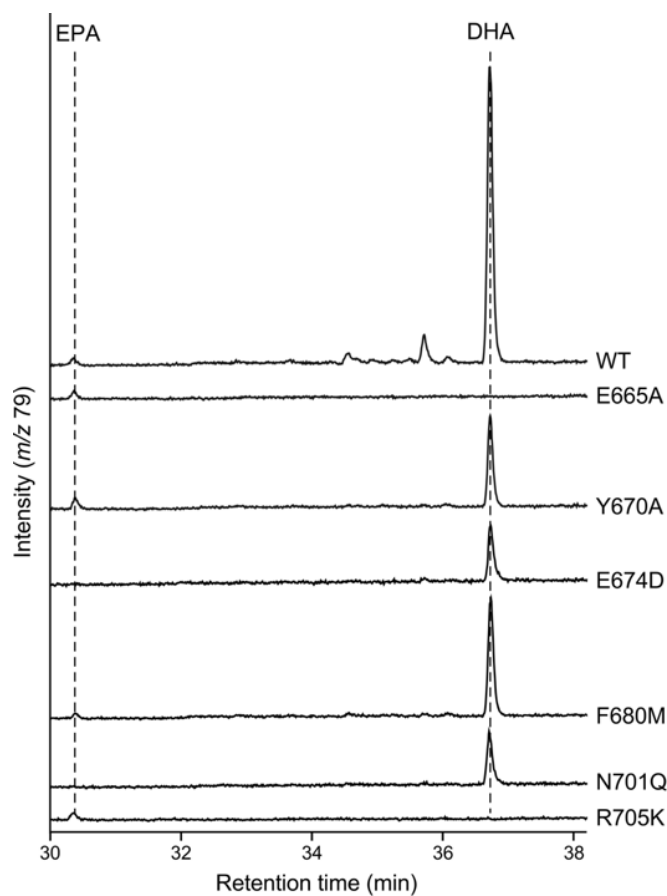


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220 **Figure 1.** (A) Chemical structures of DHA, DPA, and EPA. (B) Domain organizations of bacterial
221 PUFA synthases. KS: β -ketoacyl synthase, MAT: malonyl CoA transacylase, ACP: acyl carrier
222 protein, KR: β -ketoacyl reductase, DH_{PKS}: polyketide synthase (PKS)-type dehydratase, AT:
223 acyltransferase, CLF: chain length factor-like domain, DH_{FabA}: FabA-type dehydratase, ER: enoyl
224 reductase.
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227

228

229 **Figure 2.** GC-MS analysis (traced at m/z 79) of products produced by *E. coli* expressing Dha-ABD
230 and CLF-like domain mutants of Dha-C. The traces show the wild-type (WT) and E665A, Y670A,
231 E674D, F680M, N701Q, and R705K mutants (from top to bottom).

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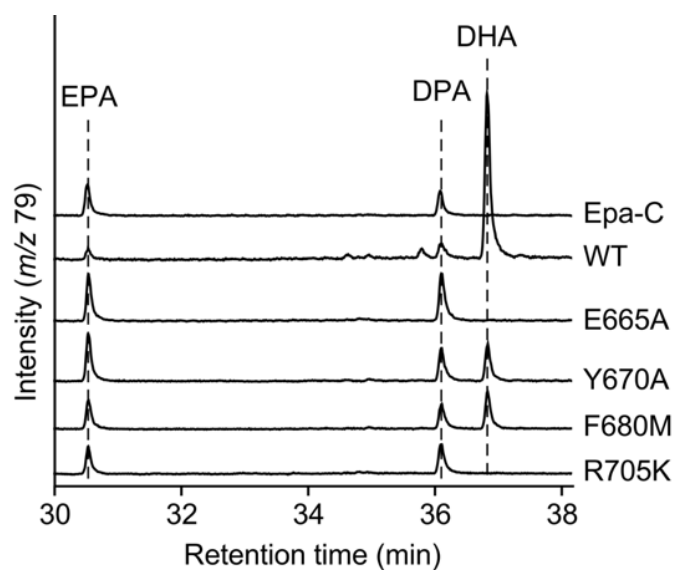


Figure 3. GC-MS analysis (traced at m/z 79) of products produced by *E. coli* expressing Epa-ABD and Epa-C or CLF-like domain mutants of Dha-C. The traces show Epa-C, Dha-C wild-type (WT), E665A, Y670A, F680M, and R705K mutants of Dha-C (from top to bottom).

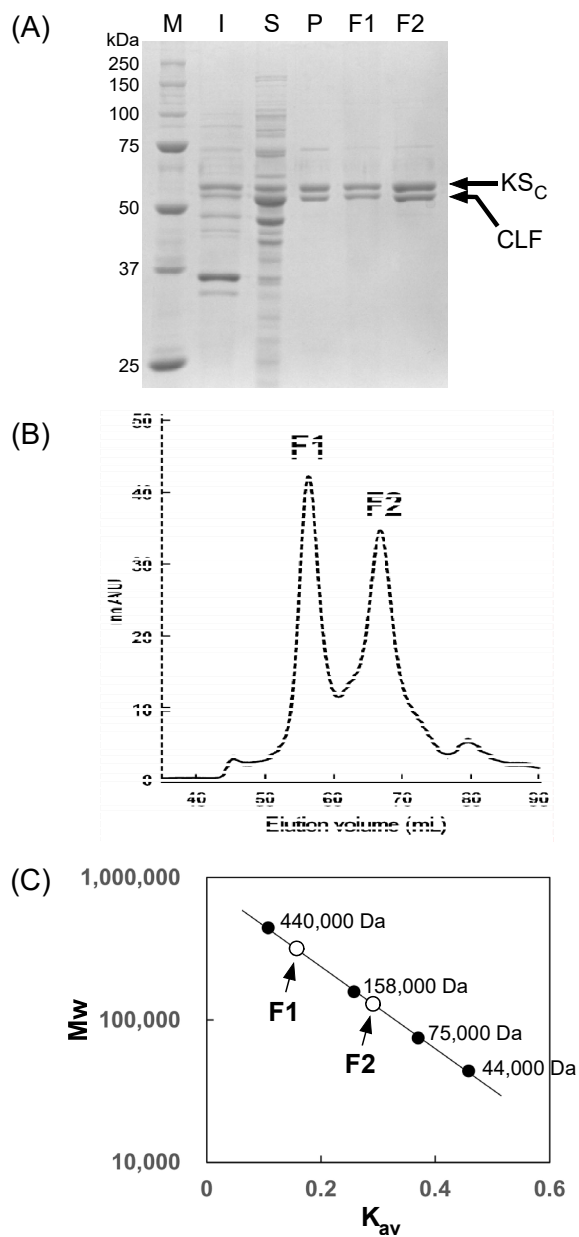


Figure 4. Analysis of interaction between truncated KS_C and CLF-like domains of EPA synthase.

(A) SDS-PAGE analysis of co-expressed His-tagged KS_C and non-tagged CLF-like domains (58.6 and 53.6 kDa) in *E. coli*. I, insoluble fraction; S, soluble fraction; P, purified fraction obtained by Ni^{2+} -agarose chromatography; F1 and F2, F1 and F2 fractions shown in (B). (B) Gel filtration

246 chromatography (traced at 280 nm) of the co-purified His-tagged KSc and non-tagged CLF-like
247 domains. The two major fractions are shown as F1 ($K_{av} = 0.155$) and F2 ($K_{av} = 0.295$). (C)
248 Calculation of molecular weights of proteins involved in the F1 and F2 fractions (open circles)
249 with standards; ferritin (440,000 Da), aldolase (158,000 Da), conalbumin (75,000 Da), and
250 ovalbumin (44,000 Da) (closed circles).