Both Determinants of Allosteric and Active Sites Responsible for Catalytic Activity of Delta 12 Fatty Acid Desaturase by Domain Swapping

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Running title: Determinants of allosteric and active sites responsible for FADS12 catalytic activity

Abbreviations

EFAs, essential fatty acids; PUFAs, polyunsaturated fatty acids; FADS12, delta 12 fatty acid desaturase; OA, oleic acid (18:1^{Δ9}); LA, linoleic acid (18:2^{Δ9,12}); *G. candidum*, *Geotrichum candidum*

Abstract

Cheese lacks essential fatty acids (EFAs). Delta 12 fatty acid desaturase (*FADS12*) is a critical enzyme required for EFA biosynthesis in fermentation of the predominant strains of cheese. Previously, we identified the *FADS12* gene and characterized its function for the first time in *Geotrichum candidum*, a dominant strain used to manufacture soft cheese with white rind. In this study, we analyzed the molecular mechanism of *FADS12* function by swapping domains from *Mortierella alpina* and *G. candidum* that had, respectively, high and low oleic acid conversion rates. The results revealed three regions that are essential to this process, including regions from the end of the second transmembrane domain to the beginning of the third transmembrane domain, from the end of the third transmembrane domain to the beginning of the fourth transmembrane domain, and from the 30-amino acid from the end of the sixth transmembrane domain to the C-terminal end region. Based on our domain swapping analyses, nine pairs of amino acids including H112, S118, H156, Q161, K301, R306, E307, A309 and S323 in MaFADS12 (K123, A129, N167, M172, T302, D307, I308, E310 and D324 in GcFADS12) were identified as having a significantly effect on *FADS12* catalytic efficiency, and linoleic acid and its analogues (12,13-cyclopropanoid fatty acid) were found to inhibit the catalytic activity of *FADS12* and related recombinant enzymes. Furthermore, the molecular mechanism of *FADS12* inhibition was analyzed. The results revealed two allosteric domains, including one domain from the N-terminal region to the beginning of the first transmembrane domain and another from the 31st amino acid from the end of the sixth transmembrane domain to the C terminus. Y4 and F398 amino acid residues from *MaFADS12* and eight pairs of amino acids including G56, L60, L344, G10, Q13, S24, K326 and L344 in MaFADS12 (while Y66, F70, F345, F20, Y23, Y34, F327 and F345 in GcFADS12) played a pivotal role in *FADS12* inhibition. Finally, we found that both allosteric and active sites were responsible for the catalytic activity of *FADS12* at various temperatures, pH, and times. This study offers a solid theoretical basis to develop preconditioning methods to increase the rate at which *GcFADS12* converts oleic and linoleic acids to produce higher levels of EFAs in cheese.

Keywords

Delta 12 fatty acid desaturase; *Geotrichum candidum*; oleic acid; linoleic acid

60 Introduction

61 Mold cheese, fermented by multispecies microbial communities (Bertuzzi et al, 2018; Zhang et al, 2018), has
62 been a traditional high-fat dairy product with the unsolved problem of low essential fatty acid (EFA) levels among
63 lipids . The polyunsaturated fatty acids (PUFA) content in raw milk is relatively low in mold cheese, and its EFA level
64 accounts for only 3.5% of total fatty acids (Hageman et al, 2019). In addition, most of the predominant fungal strains
65 used to ferment this cheese do not have the ability to convert saturated fatty acids into many PUFAs or their
66 conversion rate is low. Delta 12 fatty acid desaturase (*FADS12*) plays a key role in the synthesis of EFAs during
67 fermentation. *FADS12* is a key membrane-bound desaturase that converts oleic acid [OA, 18:1^{Δ9}] to linoleic acid [LA,
68 18:2^{Δ9,12}] by introducing a double bond between carbons 12 and 13 in the carboxyl end of the substrate in the
69 biosynthesis of EFAs (Cui et al, 2016; Lee, 2016; Rodríguez-Rodríguez, 2016). As a rate-limiting enzyme, *FADS12*
70 plays an important role in the metabolism of PUFAs, and its activity directly affects the level and distribution of EFAs
71 in cheese.

72 *FADS12* has been studied as an important desaturase in fatty acid transformation for many years, with
73 experiments based on gene cloning, heterologous expression, and analysis of its catalytic mechanism (Brandstetter &
74 Ruther, 2016; Kaye et al, 2015; Kazuhiro et al, 2004; Kikukawa et al, 2013; Lamers et al, 2019; Rodríguez-Rodríguez,
75 2016; Sakamoto et al, 2017; ShanlinYu et al, 2008; Sun et al, 2016; Watanabe et al, 2004). These studies were mostly
76 focused on the preparation of PUFAs, the regulation of the proportion of omega-3/omega-6 fatty acids and
77 heterologous expression from EFAs, which could not be synthesized in the human body (Wang et al, 2016; Yan et al,
78 2013; Zhang et al, 2013; Zhang et al, 2017). However, heterologous expression of *FADS12* in different host cells often
79 resulted in significant changes in its activity, which has made it difficult to screen for *FADS12* with high activity in a
80 target strain. Therefore, it is important to study differences in the mechanisms underlying differential *FADS12* activity
81 in various species. At present, research on the catalytic mechanism of fatty acid desaturase is still in its infancy (Lee et
82 al, 2016), except that the three-dimensional structure of *FADS9* was analyzed and found to be mushroom-shaped (Bai,
83 2015; Wang, 2015) and the molecular mechanisms underlying *FADS2* substrate specificity (Shi et al, 2015) and

84 catalytic activity (Shi et al, 2018a) were analyzed in our previous study. However, studies on fatty acid desaturase in
 85 dominant strains of cheese have not yet been undertaken. Previously, our laboratory identified the *FADS12* gene in a
 86 *Geotrichum candidum* (*G. candidum*) genome sequence submitted to the GenBank database (LOCUS:
 87 CCBN010000001) by Casaregola, S. The function of this gene has been verified by the addition of substrates *in vitro*,
 88 and, although the conversion rate of the enzyme encoded by this gene has been determined, its ability to transform OA
 89 and LA substrates was found to be low (Luo et al, 2019).

90 In studies focusing on the catalytic mechanism of membrane-bound enzyme function, reciprocal domain
 91 swapping has commonly been used to locate regions or sites that affect activity (Aranko et al, 2013; Gammons et al,
 92 2016; Haywood et al, 2018; Liu et al, 2012; Marcin et al, 2015; Meesapyodsuk & Qiu, 2014; Park et al, 2010; Qin et al,
 93 2016; Sun et al, 2017; Vendome et al, 2011). We also used this method in the previous study of *FADS2* to locate the
 94 domains and sites of *FADS2* activity, but the conversion rates of recombinant *FADS2*s could not reach that of the
 95 wild-type. Therefore, we wondered whether there were any components (Bichi et al, 2012; Chen et al, 2016; Pedrono
 96 et al, 2018; Ralston et al, 2014) that hindered the synthesis of unsaturated fatty acids. R. Jeffcoat and M. R. Pollard
 97 (Jeffcoat & Pollard, 1977) pointed out in 1977 that there was an accumulation of stearic acid
 98 [8-(2-octyl-1-cyclopropenyl) octanoic acid, a C18 cyclopropenoid fatty acid] and apparent loss of OA in both tissues
 99 and lipids in cows' milk. They showed that cyclopropenoid acid was an inhibitor of fatty acid desaturase. This is a
 100 structural analogue of other fatty acids such as 12,13-cyclopropenoid fatty acid, which might also inhibit the activity
 101 of *FADS12* and prevent the conversion of OA to LA. If so, this could help to explain the low *FADS12* activity in *G.*
 102 *candidum* (Luo et al, 2019) and provided a new clue to study the catalytic mechanism of fatty acid desaturase.

103 In the present study, key domains and amino acid sites for *FADS12* catalytic activity were located by domain
 104 swapping. In addition, the product (LA) and its analogues (12,13-cyclopropenoid fatty acid) were found to inhibit the
 105 catalytic activity of *FADS12*. Furthermore, the allosteric domains and amino acid sites that affect *FADS12* catalytic
 106 activity were identified by domain swapping. Finally, key allosteric and active domains were shown to stabilize
 107 recombinant *FADS12* at various temperatures, pH, and times.

108 **Materials and methods**

109 **Domain swapping between MaFADS12 and GcFADS12**

110 The *MaFADS12* (GeneBank accession AB020033) gene from *Mortierella alpina* was synthesized for domain
 111 swapping; *GcFADS12* (GeneBank accession MH198047) was amplified from *G. candidum* in our previous screen.
 112 *Pichia pastoris* was used for heterogeneous expression and catalytic activity determinations for *MaFADS12*,
 113 *GcFADS12*, and each chimera. Plasmid pPICZαA was used for expression of each chimera. LB agar plates and YPD
 114 medium was used as described previously (Shi et al, 2015). YPDS + Zeocin medium consisted of 1% yeast extract,
 115 2% peptone, 2% glucose, 1 M sorbitol, and 100 µg/ml Zeocin. The recombinant plasmids expressing the amplified
 116 *MaFADS12* and *GcFADS12* genes were designated pPICZαA-*MaFADS12* and pPICZαA-*GcFADS12*, respectively.

117 To identify which structural elements play functional roles in *MaFADS12* and *GcFADS12* catabolism, both
 118 enzymes were divided into nine sections based on the transmembrane topology (hydrophilic regions and hydrophobic
 119 regions) of *FADS12* as follows: Section 1: from the N-terminal end region to the beginning of the first transmembrane
 120 domain (cytoplasmic region); Section 2: the first and the second transmembrane regions; Section 3: from the end of
 121 the second transmembrane domain to the beginning of the third transmembrane domain (cytoplasmic region); Section
 122 4: the third transmembrane region; Section 5: the end of the third transmembrane domain to the beginning of the
 123 fourth transmembrane domain (cytoplasmic region); Section 6: the fourth transmembrane region; Section 7: the end of
 124 the fourth transmembrane domain to the beginning of the fifth transmembrane domain (cytoplasmic region); Section 8:
 125 the fifth and the sixth transmembrane regions; Section 9: the end of the sixth transmembrane domain to the C-terminal
 126 end region. In second-level domain swapping, section 5 and section 9 were divided into small sections as follows:
 127 Section 5-1: from the end of the third transmembrane domain to the 18th amino acid of section 5; Section 5-2: from
 128 the 19th amino acid of section 5 to the beginning of the fourth transmembrane domain; Section 9-1: from the end of
 129 the sixth transmembrane domain to the 30th amino acid of section 9; Section 9-2: from the 31th amino acid to the 54th
 130 (for *MaFADS12*) and 52th (for *GcFADS12*) amino acid of section 9; Section 9-3: from the 54th (for *MaFADS12*) and
 131 52th (for *GcFADS12*) amino acid to the C-terminal end region.. All swapped recombinant genes were generated by

overlap extension PCR using the primers listed in **Supplemental Table S1** and the PCR protocol described previously (Shi et al, 2015). The hybrid genes were ligated into the pPICZαA plasmid and transformed into competent Top10 cells.

In sections 3, 5, and 9-1, 18 mutants of the *MaFADS12* gene were constructed, with amino acids substituted with the corresponding residues in the *GcFADS12* gene (H112K, Q117G, S118A, H156N, M157L, T158Q, K159R, Q161M T298S, K301T, R306D, E307I, G308T, A309E, Q313A, L317A/C318A, V320I and S323D). Then, 18 corresponding mutants of the *GcFADS12* gene were constructed with reciprocal amino acids from the *MaFADS12* gene (K123H, G128Q, A129S, N167H, L168M, Q169T, R170K, M172Q, S299T, T302K, D307R, I308E, T309G, E310A, A314Q, A318L/A319C, I321V and D324S). Mutation primers are listed in **Supplemental Table S1**.

Each chimeric and wild-type desaturase gene was induced by 0.5 mM *cis*-OA at 28°C for 12 h under previously described conditions (Shi et al, 2018a). Then, the expression level of each chimeric desaturase in *Pichia pastoris* was determined by western blotting analysis as described previously (Shi et al, 2015); each sample loading volume was adjusted to the same level according to the western blot results to determine desaturase activity of each chimera. Chimeric desaturase activity were analyzed by gas chromatography (GC) as described previously (Shi et al, 2016; Shi et al, 2018b).

Feedback inhibition of LA and its analogues

To examine *FADS12* inhibition, 0.00 mM, 0.05 mM, 0.10 mM, 0.15 mM, 0.20 mM, 0.25 mM, 0.30 mM, 0.35 mM, 0.40 mM, 0.45 mM and 0.50 mM of 12,13-cyclopropenoid fatty acid was selected as the product analogue to add to the culture medium with 0.5 mM *cis*-OA. LA, in concentrations corresponding to those of 12,13-cyclopropenoid fatty acid, was selected to verify feedback inhibition of *FADS12*. Meanwhile, the conversion rates of chimera G3, G5, G9, G3/5/9-1, chimera M3, M5, M9, and M3/5/9-1 were determined at various concentrations of 12,13-cyclopropenoid fatty acid.

Allosteric domain swapping between chimera M3/5/9-1 and chimera G3/5/9-1

To identify which allosteric domains are functionally involved in the catalytic activities of *MaFADS12* and

156 *GcFADS12*, the corresponding sections 1, 2, 4, 6, 7, 8, and 9-2/3 in chimeras M3/5/9-1 and G3/5/9-1 were
 157 systematically exchanged to construct recombinant swap genes that were generated by overlap extension PCR. In
 158 sections 1 and 9-2/3, 26 mutants derived from the chimeric M3/5/9-1 gene were constructed, in which amino acids
 159 were substituted with the corresponding residues in the chimeric G3/5/9-1 gene (Q34V, L35V, E37Q, R44L, E45D,
 160 C46A, H50E, F52Y, E53K, G56Y, L57V, R58K, G59S, L60F, G334D, V336I, H337E, A341L, L344F, F345V, Q347R,
 161 G10F, Q13Y, S24Y, K326F and L344F). Then, 28 corresponding mutants derived from the chimeric G3/5/9-1 gene
 162 were constructed, in which amino acids were substituted with the corresponding residues in the chimeric M3/5/9-1
 163 gene (V44Q, V45L, Q47E, L54R, D55E, A56C, E60H, Y62F, K63E, Y66G, V67L, K68R, S69G, F70L, D335G, I337V,
 164 E338H, L342A, F345L, V346F, R348Q, Y4A, F20G, Y23Q, Y34S, F327K, F345L and F398A). Mutation primers are
 165 listed in **Supplemental Table S1**.

166 **Determination of chimeric FADS12 properties**

167 Chimera M3/5/9-1, chimera G3/5/9-1, chimera A1 M1/9-2/3, chimera A1 G1/9-2/3, *MaFADS12*, and *GcFADS12*
 168 were selected to determine catalytic properties, including induction temperatures (18°C, 23°C, 28°C, 33°C, and 38°C
 169 at pH 7.0 with a 12-h induction), pH (6.0, 6.5, 7.0, 7.5, and 8.0 at 28°C with a 12 h induction), and induction times (4
 170 h, 8 h, 12 h, 16 h, 20 h, and 24 h at pH 7.0 and 28°C).

171 **Molecular docking of fatty acids on the binding site of FADS12**

172 The Discovery Studio 4.3 program (Accelrys Inc., San Diego, CA, USA) was used to optimize *FADS12* by
 173 adding hydrogen atoms and removing water molecules, and the Molegro. Docker 4.0 program (Molegro ApS, Aarhus,
 174 Denmark) was used to prepare the *FADS12* structure for docking calculations.

175 **Results**

176 **Locating key domains and amino acid sites associated with FADS12 catalytic activity**

177 Based on a multiple sequence alignment of the *FADS12*s and their C18:1 catalysis efficiencies (**Fig. S1**),
 178 *MaFADS12* with highest catalytic activity and *GcFADS12* with lowest catalytic activity were selected for domain
 179 swapping. The results of domain swapping showed that the catalytic efficiencies of chimera M3 (*MaFADS12*

aa111–120 replaced by *GcFADS12* aa122–131), chimera M5 (*MaFADS12* aa150–190 replaced by *GcFADS12* aa161–197), and chimera M9 (*MaFADS12* aa295–400 replaced by *GcFADS12* aa296–412) were $12.6 \pm 0.7\%$, $15.9 \pm 0.6\%$, and $11.3 \pm 1.6\%$ respectively, much lower than those of wild-type *MaFADS12* ($87.6 \pm 0.6\%$). Conversely, whereas *GcFADS12* exhibited a catalytic efficiency of $20.4 \pm 1.0\%$, those of chimera G3 (*GcFADS12* aa122–131 replaced by *MaFADS12* aa111–120), chimera G5 (*GcFADS12* aa161–197 replaced by *MaFADS12* aa150–190), and chimera G9 (*GcFADS12* aa296–412 replaced by *MaFADS12* aa295–400) were increased to $44.3 \pm 0.5\%$, $28.2 \pm 0.5\%$, and $46.5 \pm 4.0\%$, respectively (**Figure 1**). Furthermore, section 9 was divided into three ‘second-level’ sections to identify key functional areas that were then systematically exchanged between *MaFADS12* and *GcFADS12*. The results of an analysis of the second-level chimeras showed that the catalytic efficiency of chimera M9-1 (aa295–325 in *MaFADS12* replaced by aa296–326 in *GcFADS12*) was decreased to $5.6 \pm 1.9\%$, compared to that exhibited by *MaFADS12* (**Figure 1**). Likewise, replacement of aa296–326 (section 9-1) of *GcFADS12* with aa295–325 of *MaFADS12* resulted in significantly increased ($36.6 \pm 1.0\%$) catalytic efficiency (**Figure 1**). Replacement of the other two second-level sections (sections 9-2 and 9-3) had no significant impact on the catalytic efficiency of *FADS12*. Key functional areas, namely sections 3, 5, and 9-1, were exchanged simultaneously between *MaFADS12* and *GcFADS12*, and our results showed that the catalytic efficiency of chimera M3/5/9-1 decreased to $5.6 \pm 0.6\%$, whereas that of chimera G3/5/9-1 reached $56.3 \pm 2.3\%$, exhibiting either lower or higher efficiencies than those of the other corresponding section (i.e., the catalytic efficiencies of chimeras M3, 5, and 9-1 in *MaFADS12* were lower than those of chimeras G3, 5, and 9-1 in *GcFADS12*).

To further identify key amino acid sites associated with *FADS12* catalytic activity, a series of constructs with mutations in sections 3, 5, and 9-1 were generated (**Figure 2**). Conversion efficiencies of these constructs were shown to be greatly reduced in single mutants H112K, S118A, H156N, and Q161M to a rate of $23.6 \pm 1.6\%$, $25.6 \pm 2.5\%$, $20.9 \pm 3.9\%$, and $22.6 \pm 2.1\%$, respectively, for *MaFADS12* with mutant sections 3 and 5 (**Figure 2**). Conversely, catalytic efficiencies were significantly enhanced in single mutants K123H, A129S, N167H, and M172Q ($35.2 \pm 1.3\%$, $43.0 \pm 2.1\%$, $32.6 \pm 3.5\%$, and $36.3 \pm 2.7\%$, respectively, for *GcFADS12* with mutant sections 3 and 5) (**Figure 2**).

The conversion efficiencies of the section 9-1 *MaFADS12* mutants K301T, R306D, E307I, A309E, and S323D and the corresponding *GcFADS12* mutants T302K, D307R, I308E, E310A, and D324S showed significant differences from those of wild-type *FADS12* (**Figure 2**). Targeted mutagenesis of other points within sections 3, 5 and 9-1 did not induce any major changes in *FADS12* catalytic activity.

Inhibition of FADS12 catalytic activity by 12,13-cyclopropenoid fatty acid

To explain why the increase in activity of chimeras with key regions and sites described above was not significant, we considered that there may be reaction components that inhibited activity of the enzyme. Therefore, we explored feedback inhibition. By adding LA or 12,13-cyclopropenoid fatty acid (an LA analogue) as suspected inhibitors of enzyme activity or a control (with neither fatty acid), we showed that both LA and 12,13-cyclopropenoid fatty acid inhibited *FADS12* activity (**Figure 3a, b, c**). This result was confirmed by the effect of LA or 12,13-cyclopropenoid fatty acid on *GcFADS12* activity at a low concentration of LA or 12,13-cyclopropenoid fatty acid with substrate in the medium. *MaFADS12* activity did not significantly change when OA and 12,13-cyclopropenoid fatty acid or OA and LA were added, whereas *GcFADS12* activity decreased with an increase in 12,13-cyclopropenoid fatty acid or LA. When the concentration of 12,13-cyclopropenoid fatty acid or LA reached 0.5 mM, *GcFADS12* activity decreased from 20.4 % to less than 3 %, which confirmed that these compounds strongly inhibit *GcFADS12*.

Moreover, the effects of 12,13-cyclopropenoid fatty acid on the activity of chimeras G3, G5, G9, G3/5/9-1, M3, M5, M9, and M3/5/9-1 (shown by domain swapping) were analyzed. Our results showed that activities of four *GcFADS12* mutant enzymes, namely chimeras G3, G5, G9, and G3/5/9-1) decreased to half that of *GcFADS12* at a concentration as low as 0.05 mM of 12,13-cyclopropenoid fatty acid. With an increase in concentration, the activity of these chimeras showed a slight decline (**Figure 3d**). This confirmed that 12,13-cyclopropenoid fatty acid affected the activities of these four chimeras, and that the concentration of LA or 12,13-cyclopropenoid fatty acid was not the real reason for the change in enzyme activities. However, the activities of the four chimeras from *MaFADS12* (chimeras M3, M5, M9, and M3/5/9-1) remained unchanged in the presence of 12,13-cyclopropenoid fatty acid at various concentrations (**Figure 3e**). These results indicated that there may be “sensitive” regions for 12,13-cyclopropenoid

fatty acid or LA in the structure of *GcFADS12* (“sensitive” regions, meaning regions that were changed by the product and that affected binding between *GcFADS12* and the substrate), and that 12,13-cyclopropenoid fatty acid does not change the corresponding binding regions in *MaFADS12*.

Allosteric domains and amino acid sites also affect FADS12 catalytic activity

To explain the effects of 12,13-cyclopropenoid fatty acid and LA on *GcFADS12* activity, we speculated that the product changed the structure outside the active domains of *GcFADS12*, whereas the product did not change or changed only to a small extent the corresponding region in *MaFADS12*. To locate allosteric domains, we swapped domains other than those with localized active regions (i.e., chimeras 1, 2, 4, 6, 7, 8, and 9-2/3). The results showed that the rates of conversion to OA by chimeras Al M1 and Al G1 derived from section 1 of *MaFADS12* and *GcFADS12* were $0.5 \pm 0.2\%$ (10 times lower than that of chimera M3/5/9-1) and $75.2 \pm 0.3\%$ (about 20% higher than that of chimera G3/5/9-1), respectively (**Figure 4a**). In addition, the rates of conversion to OA chimeras Al M9-2/3 and Al G9-2/3 derived from section 9-2/3 of *MaFADS12* and *GcFADS12* were $0.3 \pm 0.1\%$ (22 times lower than that of chimera M3/5/9-1) and $73.3 \pm 3.5\%$ (about 18% higher than that of chimera G3/5/9-1) (**Figure 4a**). This result suggested that there were two regions in *MaFADS12*, section 1 and section 9-2/3 that housed allosteric domains. Moreover, the conversion rates of chimeras Al M1/9-2/3 and Al G1/9-2/3, which included the section 1 and 9-2/3 domains of *MaFADS12* and *GcFADS12*, respectively, were $0.1 \pm 0.0\%$ and $80.7 \pm 5.6\%$, which were higher than those observed with single domain swapping (**Figure 4a**). This confirmed that these two regions were the root cause of feedback inhibition.

To further define key sites of feedback inhibition in the two regions, we first selected sites near conserved regions in the allosteric domain for site-directed mutagenesis swaps in *MaFADS12* and *GcFADS12*. Our results showed that the conversion rates of mutants G56Y and L60F with section 1 from *MaFADS12* were reduced to $0.4 \pm 0.1\%$ and $1.3 \pm 0.2\%$, respectively, whereas the conversion rates of the corresponding mutants Y66G and F70L with section 1 from *GcFADS12* increased to $79.0 \pm 4.1\%$ and $75.0 \pm 2.5\%$, respectively (**Figure 4b**). In addition, the conversion rates of mutant L344F with section 9-2/3 from *MaFADS12* and the corresponding F345L mutant with section 9-2/3 from

252 *GcFADS12* significantly changed, reaching $0.9 \pm 0.2\%$ and $71.2 \pm 0.5\%$, respectively (**Figure 4b**).

253 Based on the results of this site-directed mutagenesis swap, tyrosine and phenylalanine, aromatic amino acids
 254 with a benzene ring structure, were implicated in *FADS12* catabolism. We inferred that amino acids with large steric
 255 hindrances such as benzene rings had a greater impact on product feedback inhibition. Subsequently, other aromatic
 256 amino acids in sections 1 and 9-2/3 of *GcFADS12* were selected for exchange with corresponding sites in *MaFADS12*.
 257 Our results showed that the conversion rates of all *MaFADS12* mutants, including G10F, Q13Y, S24Y, K326F, and
 258 L344F, were reduced by an order of magnitude compared with those of chimera M3/5/9-1 ($0.8 \pm 0.2\%$, $0.8 \pm 0.3\%$, 0.6
 259 $\pm 0.2\%$, $0.4 \pm 0.2\%$, and $0.3 \pm 0.2\%$, respectively), whereas the conversion rates of Y4A, F398A, and corresponding
 260 mutants from *GcFADS12*, namely F20G, Y23Q, Y34S, F327K, and F345L, increased by about 20% compared with
 261 those of chimera G3/5/9-1, reaching $78.2 \pm 2.5\%$, $72.2 \pm 2.1\%$, $71.2 \pm 2.0\%$, $77.2 \pm 1.5\%$, $78.9 \pm 2.5\%$, $75.2 \pm 1.2\%$,
 262 and $76.2 \pm 1.5\%$, respectively (**Figure 4c**). This result indicated that the aromatic amino acids in sections 1 and 9-2/3
 263 of *GcFADS12* caused feedback inhibition that affected the catalytic activity of *FADS12*.

264 **Key allosteric and active domains with FADS12-stabilizing properties**

265 Based on these results, we determined that there are both active and allosteric regions in *MaFADS12* that are
 266 associated with high catalytic activity, and that product feedback inhibition was weak with this enzyme because there
 267 were no aromatic amino acids in the allosteric region so there was little steric hindrance. To determine whether this
 268 conclusion was valid under different conditions, the conversion rates of *MaFADS12*, *GcFADS12*, and their
 269 recombinant enzymes at various induction temperatures, pH, and times were determined. Our results showed that the
 270 conversion rates of *MaFADS12*, chimera M3/5/9-1, and chimera A1 G1/9-2/3 did not change significantly at different
 271 induction temperatures, whereas those of *GcFADS12*, chimera G3/5/9-1, and chimera A1 M1/9-2/3 fluctuated with
 272 temperature (**Figure 5a**). The same conclusion can be drawn about substrate conversion efficiencies at various pH and
 273 induction times (**Figure 5b, c**). From this result, it is apparent that the conversion efficiencies of *FADS12*s containing
 274 allosteric regions (i.e., *MaFADS12*, chimera M3/5/9-1, and chimera A1 G1/9-2/3) were not influenced by induction
 275 temperature, pH, or time, but for *FADS12*s without allosteric regions, even if they contained active regions (i.e.,

GcFADS12, chimera G3/5/9-1, and chimera A1 M1/9-2/3), their conversion rates were unstable with a change in induction temperature, pH, or time (**Figure 5**). Based on these results, we knew that two key allosteric and active domains stabilize *FADS12* properties.

Discussion

The present study indicates that *FADS12* cytoplasmic regions extending from the end of the second transmembrane domain to the beginning of the third transmembrane domain (section 3), the end of the third transmembrane domain to the 18th amino acid of section 5 (section 5-1), and the end of the sixth transmembrane domain to the 30th amino acid of section 9 (section 9-1) critically mediates *FADS12* catalytic activity (**Figure 1**). In these three small regions, 10 sites (His112, Ser118, His156, Gln161, Lys 301, Arg306, Glu307, Ala 309, Gln313 and Ser323) in *MaFADS12* and 10 sites (Lys123, Ala129, Asn167, Met172, Thr302, Asp307, Ile308, Glu310, Ala314 and Asp324) in *GcFADS12* were identified as key sites affecting *FADS12* catalytic activity. From the results of molecular docking experiments with these sites and the substrate, we learned that all key sites were close to the substrate, and, in the enzyme pocket, these sites associated with the substrates by van der Waals and conventional hydrogen bonds to form a stable structure (**Figure 6a**). The results of molecular docking experiments were in full agreement with the observed *FADS12* conversion rates of *FADS12* (**Figure 6b**).

The conversion rate of chimera G3/5/9-1, constructed by superimposition of three small active domains (sections 3, 5-1, and 9-1), reached $56.3 \pm 2.3\%$, which was 30% lower than that of wild-type *MaFADS12*. We speculated that this was due to feedback inhibition by the product. To exclude the effect of products on the conversion rate, 12,13-cyclopropanoid fatty acid was used as a product analogue; this confirmed that 12,13-cyclopropanoid fatty acid inhibited *GcFADS12* activity, but had no significant effect on *MaFADS12* activity. There are likely allosteric regions in *MaFADS12* that are less sensitive to the product, whereas this allosteric region in *GcFADS12* is highly sensitive to it. In addition, the minimal steric hindrance of OA and maximal steric hindrance of LA and 12,13-cyclopropanoid fatty acid in various twisted structures were analyzed, and diameters at which the said hindrance may interfere were 5.91Å, 7.21Å, and 6.72Å respectively (**Figure 6c**). By comparing the pocket size of the active region of *FADS12* with the

steric hindrance distance of the product or its analogues, we inferred that it was difficult for LA to be released from the pocket due to steric hindrance after the catalysis of the OA substrate and feedback inhibition by the product (**Figure 6d**).

Allosteric regions were again found to be located in chimeras M3/5/9-1 and G3/5/9-1 after domain swapping. The results of this study showed that the activities of chimeras A1 G1 and A1 G9-2/3, both of which were derived from chimera G3/5/9-1 by exchanging the first and last regions, respectively, were close to those of wild-type *MaFADS12*; and the activity of chimera A1 G1/9-2/3, which was derived by exchanging the first and last regions, was similar to that of wild-type *MaFADS12* (**Figure 4a**). Based on the simulated structure of *MaFADS12*, these two segments can be considered "flexible regions," which change during the catalytic reaction to release the product (**Figure 6d**). In these two regions, once an amino acid with a benzene ring structure was mutated in *GcFADS12*, its activity significantly increased, whereas activity in the corresponding site of *MaFADS12* significantly decreased (**Figure 4b, c**). From the docking results, we inferred that macromolecule groups such as a benzene ring at the first and last regions in *FADS12* hindered product release (**Figure 6e**).

The substrate conversion rate is determined by both active and allosteric regions. With changes in the induction temperature, pH, or time, the substrate conversion rates of recombinant *FADS12* containing a "flexible region" (i.e., *MaFADS12*, chimera M3/5/9-1, and chimera A1 G1/9-2/3) showed few significant changes, indicating that the flexible region plays a role in product release, even when the overall three-dimensional structure of the enzyme changed with external conditions. However, the conversion rate of chimera G3/5/9-1 with an active region but no allosteric region was not stable under different conditions, suggesting that this flexible region is the key domain to maintaining *FADS12* stability (**Figure 5**). Here, the three-dimensional structure of the two regions could be analyzed by X-ray diffraction in the future, which will help elucidate how the two regions combine with the substrate.

Conclusions

The present study elucidated the molecular mechanism underlying the catalytic activity of *FADS12* from *M. alpina* and *G. candidum*. A catalytic product of *FADS12* was shown to be an inhibitor of *FADS12* catabolism; also, for

the first time, a product analogue as found to inhibit this enzyme. The molecular mechanism underlying *FADS12* inhibitory activity was analyzed based on the presence of allosteric domains and amino acid sites affecting *FADS12* catalytic activity. These studies indicated that both allosteric and active domains can stabilize *FADS12* in various induction conditions. This study provides a theoretical basis for improving essential fatty acid contents in dominant strains in fermented cheese.

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473 **Figure legends**

474 **Figure 1** (a): Skeleton map of the amino acid sequences of MaFADS12 (red box) and GcFADS12 (blue box), which
 475 were divided into 9 sections showed with dotted lines. The braces indicate their trans-membrane sequences. Section 5
 476 was divided into 2 sections and section 9 was divided into 3 sections with dotted lines. (b): phobius posterior
 477 probabilities for MaFADS12 and GcFADS12. (c): Relative substrate conversion efficiency of each FADS12 chimera
 478 expressed in *Pichia.pastoris*, determined by adding LA substrate. Map of each FADS12 chimera constructed by
 479 reciprocal section swapping was exhibited below each FADS12 chimera name. Substrate conversion efficiency of each
 480 chimera was classified into two groups: *, conversion rate of chimera MX > chimera GX; ***, conversion rate of
 481 chimera MX < chimera GX. (d): Relative substrate conversion efficiency of each second-level chimera in section 9
 482 expressed in *Pichia.pastoris* determined by adding LA substrate. (e): Gas chromatogram of fatty acids for the
 483 conversion rate of MaFADS12 (e1), chimera M3 (e2), chimera M5 (e3), chimera M9 (e4), chimera M3/5/9-1 (e5),
 484 GcFADS12 (e6), chimera G3 (e7), chimera G5 (e8), chimera G9 (e9) and chimera G3/5/9-1 (e10). (f): Gas
 485 chromatogram of fatty acids for the conversion rate of chimera M9-1 (f1) and chimera G9-1 (f2).

486

487 **Figure 2** Relative substrate conversion efficiencies of each mutant from section 3, 5-1 and 9-1. The mutants (red
 488 words under red column) of H112K, Q117G, S118A, H156N, M157L, T158Q, K159R, Q161M T298S, K301T, R306D,
 489 E307I, G308T, A309E, Q313A, L317A/C318A, V320I and S323D from MaFADS12 were corresponding to the
 490 mutants (blue words under blue column) of K123H, G128Q, A129S, N167H, L168M, Q169T, R170K, M172Q, S299T,
 491 T302K, D307R, I308E, T309G, E310A, A314Q, A318L/A319C, I321V and D324S from GcFADS12, respectively.
 492 Substrate conversion efficiency of each mutant was classified into two groups: *, conversion rate of mutant from
 493 MaFADS12 > mutant from GcFADS12; ***, conversion rate of mutant from MaFADS12 < mutant from GcFADS12.
 494 (a): each mutant from section 3 and 5-1; (b): each mutant from section 9-1.

495

Figure 3 Feedback inhibition of LA and its analogues for MaFADS12 and GcFADS12. (a): Map of catalytic reaction of MaFADS12 by LA addition, 12, 13-cyclopropenoid fatty acid addition and control. (b): Map of catalytic reaction of GcFADS12 by LA addition, 12, 13-cyclopropenoid fatty acid addition and control. (c): Relative substrate conversion efficiency of MaFADS12 and GcFADS12 by addition of 0.5 mM *cis*-OA/various concentrations of 12, 13-cyclopropenoid fatty acid and 0.5 mM *cis*-OA/various concentrations of LA, respectively. (d): Relative substrate conversion efficiency of chimera G3, G5, G9 and G3/5/9-1 by addition of 0.5 mM *cis*-OA/various concentrations of 12, 13-cyclopropenoid fatty acid. (e): Relative substrate conversion efficiency of chimera M3, M5, M9 and M3/5/9-1 by addition of 0.5 mM *cis*-OA/various concentrations of 12, 13-cyclopropenoid fatty acid. Substrate conversion efficiency with LA addition had removed the level of added LA.

Figure 4 (a): Relative substrate conversion efficiencies of each FADS12 chimera constructed from chimera M3/5/9-1 and chimera G3/5/9-1 expressed in *Pichia.pastoris*, determined by adding LA substrate. Map of each chimera constructed by reciprocal section swapping was exhibited below each FADS12 chimera name. Substrate conversion efficiency of each chimera was classified into two groups: *, conversion rate of chimera M3/5/9-1 and chimera G3/5/9-1; ***, conversion rate of chimera AI MX < chimera M3/5/9-1 and chimera AI GX > chimera G3/5/9-1 chimera GX. (b) and (c): Relative substrate conversion efficiency of each mutant from chimera M3/5/9-1 and chimera G3/5/9-1, determined by adding LA substrate. The mutants (red words under red column) of Q34V, L35V, E37Q, R44L, E45D, C46A, H50E, F52Y, E53K, G56Y, L57V, R58K, G59S, L60F, G334D, V336I, H337E, A341L, L344F, F345V, Q347R, G10F, Q13Y, S24Y, K326F and L344F from chimera M3/5/9-1 were corresponding to the mutants (blue words under blue column) of V44Q, V45L, Q47E, L54R, D55E, A56C, E60H, Y62F, K63E, Y66G, V67L, K68R, S69G, F70L, D335G, I337V, E338H, L342A, F345L, V346F, R348Q, Y4A, F20G, Y23Q, Y34S, F327K, F345L and F398A from chimera G3/5/9-1, respectively. Substrate conversion efficiency of each mutant was classified into two groups: *, conversion rate of chimera M3/5/9-1 and chimera G3/5/9-1; ***, conversion rate of mutants from chimera M3/5/9-1 < chimera M3/5/9-1 and mutants from chimera G3/5/9-1 > chimera G3/5/9-1.

520

521 **Figure 5** Relative substrate conversion efficiencies of chimera M3/5/9-1, chimera G3/5/9-1, chimera AI M1/9-2/3,
522 chimera AI G1/9-2/3, MaFADS12 and GcFADS12 at various induced temperature (a), pH (b) and induced time (c).
523 Active domain and Allosteric domain in chimera M3/5/9-1, chimera G3/5/9-1, chimera AI M1/9-2/3, chimera AI
524 G1/9-2/3, MaFADS12 and GcFADS12 indicated with “+”, none with “-”.

525

526 **Figure 6** (a): H-bonds and hydrophobic interactions between LA and MaFADS12 in a 2D docking model. (b): LA
527 interacted with the amino acid residues located within the active site of MaFADS12 pocket in 3D docking model. (c):
528 The molecular structure of OA, LA and 12,13-cyclopropenoid fatty acid with diameters perpendicular to the direction
529 of the substrate entering the pocket of FADS12. (d): Predicted binding mode of LA docked into key amino acids of
530 MaFADS12. The binding location of MaFADS12 interacting with LA was shown as molecular surface structures; (e):
531 Predicted binding mode of LA docked into key amino acids mutated to tyrosine and phenylalanine in MaFADS12
532 pocket.

533

534 **Fig. S1** Multiple Sequence alignment of deduced amino acids for GcFADS12 with FADS12s from other species,
535 including *Mortierella alpina*, MaFADS12, *Mucor circinelloides*, McFADS12, *Cannabis sativa*, CsFADS12, *Arachis*
536 *hypogaea*, AhFADS12, *Santalum acuminatum*, SaFADS12, *Spinacia oleracea*, SoFADS12, *Camelina sativa*,
537 CmFADS12, *Fusarium verticillioides*, FvFADS12, *Candida parapsilosis*, CpFADS12, *Exocarpos cupressiformis*,
538 EcFADS12 using DNAMAN software. Black line indicate domain boundaries for reciprocal section swapping. CA,
539 catalytic activity.

540

541 **Fig. S2** Gas chromatogram of fatty acids in *Pichia.pastoris* carrying MaFADS12 (a), GcFADS12 (b) genes and each
542 substitution including H112K (c), S118A (e), H156N (g), Q161M (i), K301T (k), R306D (m), E307I (o), A309E (q),
543 Q313A (s) and S323D (u) from MaFADS12 and K123H (d), A129S (f), N167H (h), M172Q (j), T302K (l), D307R (n),

544 I308E (p), E310A (r), A314Q (t) and D324S (v) from GcFADS12 with 0.5 mM *cis*-OA addition at 28°C for 12 h
545 induction.

546

547 **Fig. S3** Gas chromatogram of fatty acids in *Pichia.pastoris* carrying MaFADS12 (a), GcFADS12 (b) genes and each
548 substitution including G56Y (c), L60F (e), L344F (g), G10F (i), Q13Y (k), S24Y (m), K326F (o) and L344F (q) from
549 MaFADS12 and Y66G (d), F70L (f), F345L (h), F20G (j), Y23Q (l), Y34S (n), F327K (p), F345L (r), Y4A (s) and
550 F398A (t) from GcFADS12 with 0.5 mM *cis*-OA addition at 28°C for 12 h induction.

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552

Figure 1

Figure 2

Figure 3

Figure 4

a

b

c

Figure 5

a

b

c

d

e

Figure 6