Crystallographic analysis of the *Staphylococcus epidermidis* lipase involved in esterification in aqueous solution

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The *Staphylococcus epidermidis* lipase (SeLip, GehC) can be used in flavour-compound production *via* esterification in aqueous solution. This study reports the crystallization and crystallographic analysis of recombinant GehC (rGehC; Lys303–Lys688) with a molecular weight of 43 kDa. rGehC was crystallized at 293 K using PEG 10 000 as a precipitant, and a 99.9% complete native data set was collected from a cooled crystal at 77 K to a resolution of 1.9 Å with an overall R_{merge} value of 7.3%. The crystals were orthorhombic and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 42.07$, $b = 59.31$, $c = 171.30$ Å, $\alpha = \beta = \gamma = 90^\circ$. Solvent-content calculations suggest that there is likely to be one lipase subunit in the asymmetric unit.

1. Introduction

Esters are important flavouring agents and are often used in fruit-flavoured products (for example beverages, candies, jellies and jams), baked goods, wines and dairy products (for example cultured butter, sour cream, yogurt and cheese) (Kim *et al.*, 1998). Conventional esterification of flavour esters by chemical catalysts requires high temperatures and leads to dark-coloured products and undesired byproducts (Shaw & Lo, 1994). Enzyme-catalyzed conversion is more efficient and selective. In recent years, there has been a strong demand for natural products, including natural flavours. These conditions have created many opportunities for biocatalysis (catalysis involving the use of enzymes) to compete with traditional synthetic chemistry in flavour production (Brault *et al.*, 2014; Trbojević Ivić *et al.*, 2016). Flavour esters can be synthesized enzymatically using inexpensive natural raw materials such as fatty acids and alcohols (Manjón *et al.*, 1991). The use of lipolytic enzymes to catalyze the esterification reaction for the production of flavour esters has been investigated by many groups (Langrand *et al.*, 1988; Welsh & Williams, 1989; Karra-Chaabouni *et al.*, 1998). The esterification of alcohols and fatty acids catalyzed by lipolytic enzymes has been performed in hydrophobic organic solvents and in aqueous–organic two-phase systems (Eggers *et al.*, 1989; Goldberg *et al.*, 1990; Carta *et al.*, 1991; Monot *et al.*, 1991; Shaw *et al.*, 1991; Lima *et al.*, 1995).

The *Staphylococcus epidermidis* lipase (SeLip, GehC) is one of the few lipolytic enzymes that catalyze flavour-compound production *via* esterification in aqueous solution. The GehC gene was first cloned and characterized by Farrell *et al.* (1993).

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Table 1
Macromolecule-production information.

Source organism	<i>S. epidermidis</i> ATCC 12228 purchased from BCRC
DNA source	Template for the GehC protein cloned from the <i>S. epidermidis</i> genome
Forward primer†	GGGGCCATGGAACAAAACATATAAAAA
Reverse primer‡	GTGGTGCTCGAGTTTATTTGTTGATGTAA TTGTTC
Cloning vector	pET-28a (Novagen)
Expression vector	pET-28a (Novagen)
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MEQKQYKNNDPILVHGFNGFTDDINPSVL THYWGGDKMNIRQDLENGYEAYEASIS AFGSNYDRAVELYYIKGGRVDYGAHA AKYGHERYGKTYEGVYKDWKPGQKIHLV GHSMGGQTIHQLELLRHGNPEEVEYQK QHGGEISPLYQGGHDMVSSIITLGTTPH NGTHASDLLGNEAIVRQLAYDVGKMYGN KDSRVDFGLEHGWGLKQKPNESYIQYVNR VQNSKLWKSQDGLHDLTRDGATDLNRK TSLNPNIVYKTYTGESTHKTLAGKQKAD LNMFLPFTITGNLIGKAKEKEWRENDGL VSVISSQHPFNQKYVEATDKNQKGVQVQ VPTKHDWDHVDVFGQDSTDTKRTRDELQ QFWHGLAEDLVQSEQLTSTNK

† The NcoI site is underlined. ‡ The XhoI site is underlined.

Structure–function studies of GehC have shown that amino-acid residues Met419 and Val649, which follow the catalytic triad, affect substrate specificity and catalytic efficiency, and that Ser418 is the active site (Chang *et al.*, 2000). GehC exhibits a higher esterification yield with medium-chain fatty acids (from C8 to C14), with an optimal yield for lauric acid (Chang *et al.*, 2001). We have successfully expressed a mature fragment of recombinant GehC (rGehC; Lys303–Lys688) in *Escherichia coli* and identified the residues involved in catalysis. The amino-acid sequence of GehC is similar to that of the *S. hyicus* lipase (ShLip; PDB entry 2h1h; Tiesinga *et al.*, 2007). The C-terminal regions of these proteins are closely related: 49% of the 386 residues are identical and 60% of the differences reflect conservative changes. The significant differences in substrate specificity and enzyme catalytic efficiency between these two enzymes suggest that dissimilar amino-acid residues, particularly around the active site, are important for their biochemical properties (Chang *et al.*, 2000). To clarify the mechanism by which rGehC catalyzes esterification in aqueous solvents, rGehC was crystallized and modelled using ShLip (PDB entry 2h1h) as the initial model. The results presented in this paper mainly concern the crystallization and preliminary X-ray structural analysis of rGehC.

2. Materials and methods

2.1. Macromolecule production

GehC was cloned from the genome of *S. epidermidis* following a procedure described in detail in a previous report (Liu *et al.*, 2015). Briefly, GehC was created using NcoI and XhoI and then cloned into pET-28a (Novagen, Germany). rGehC was expressed using *E. coli* BL21(DE3) with an initial inoculated cell density of OD₅₅₀ = 0.08. When the cells reached

an OD of 0.3–0.5, isopropyl β-D-1-thiogalactopyranoside (100 μM) was added and the culture was subsequently incubated at 30°C for 4 h. The cell pellet was harvested by centrifugation (6000g, 15 min, 4°C) and resuspended in lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 40 mM imidazole pH 7.5). The cells were disrupted by sonication on ice with 1 s pulses and 2 s pauses for a total of 90 s. The supernatant containing soluble protein was obtained and filtered (0.2 μm syringe filter; Merck Millipore) after centrifugation (13 000g, 15 min, 4°C). rGehC with a His₆ tag fused to its N-terminus was purified using a HisTrap HP nickel column with an ÄKTApurifier system (GE Healthcare Life Sciences) using a buffered imidazole gradient of 40–500 mM for elution. The pure protein was collected between 150 and 300 mM imidazole. Salt was removed using a HiTrap desalting column (GE Healthcare Life Sciences) and the protein was eluted in desalting buffer (20 mM Tris–HCl, 1 mM MnCl₂ pH 7.5; Fig. 1). The purified rGehC was concentrated using an Amicon Ultra centrifugal filter unit and centrifuged at 6000g for 5 min at 4°C several times until the concentration of rGehC reached 16 mg ml^{−1}. The protein concentration was determined using protein-assay dye (Bio-Rad, USA). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Initial crystallization conditions were identified *via* the sitting-drop vapour-diffusion method with the Rigaku Wizard Classic 3 screening kit. Each solution (2 μl) from the screening kit was mixed with 2 μl purified protein solution (10 mg ml^{−1}) and allowed to equilibrate against 300 μl solution in the well at room temperature (25°C). The conditions were refined and crystals were grown from a well solution using the sitting-drop vapour-diffusion method by equilibrating a mixture of 2 μl protein solution (16 mg ml^{−1}) and 2 μl reservoir solution against 300 μl reservoir solution consisting of 100 mM HEPES, 20% (w/v) polyethylene glycol 10 000 (Sigma).

Figure 1
SDS–PAGE analysis of recombinant lipase (rGehC) cloned in pET-28a and expressed in *E. coli* Tuner (DE3) cells. Lane M, PageRuler Prestained Protein Ladder (labelled in kDa); lane 1, crude soluble extract; lane 2, pass-through from nickel column; lane 3, wash; lane 4, purified rGehC.

Figure 2

Crystals of lipase (rGehC) obtained using the sitting-drop vapour-diffusion method. The crystals shown are approximately $305 \times 55 \times 18 \mu\text{m}$ in size.

Crystals appeared within 2 d and the largest crystal in the needle clusters grew to dimensions of approximately $305 \times 55 \times 18 \mu\text{m}$ (Fig. 2). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

X-ray data were collected using synchrotron radiation with a crystal-to-detector distance of 200 mm. The oscillation width and exposure time for each frame were 1° and 16 s, respectively. Crystallographic data integration and reduction were performed using the *HKL-2000* program package (Otwinowski & Minor, 1997). Crystallographic data-collection statistics for GehC are listed in Table 3.

3. Results and discussion

We characterized the substrate-affinity and enzyme-kinetic properties of rGehC (Supplementary Table S1). The results show that rGehC has the strongest affinity ($K_m = 0.52 \pm$

Table 2
Crystallization.

Method	Vapour diffusion
Plate type	24-well sitting-drop plate (Hampton Research)
Temperature (K)	298
Protein concentration (mg ml^{-1})	16
Buffer composition of protein solution	20 mM Tris-HCl pH 7.5, 1 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
Composition of reservoir solution	100 mM HEPES-NaOH pH 7.5, 20% PEG 10 000
Volume and ratio of drop	1:1; 2 μl reservoir solution mixed with 2 μl purified protein solution
Volume of reservoir (μl)	300

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL15A1, National Synchrotron Radiation Research Center, Hsinchu, Taiwan
Wavelength ($\text{\AA}$)	1.00000
Temperature (K)	298
Detector	Rayonix MX300HE CCD area detector
Crystal-to-detector distance (mm)	200.00
Rotation range per image ($^\circ$)	1
Total rotation range ($^\circ$)	180
Exposure time per image (s)	16
Space group	$P2_12_12_1$
a, b, c ($\text{\AA}$)	42.071, 59.306, 171.302
α, β, γ ($^\circ$)	90, 90, 90
Mosaicity ($^\circ$)	0.55–0.96
Resolution range ($\text{\AA}$)	30.00–1.90 (1.97–1.90)
Total No. of reflections	250176
No. of unique reflections	34640
Completeness (%)	99.9 (100.0)
Multiplicity	7.2 (7.3)
$\langle I/\sigma(I) \rangle$	20.746 (3.697)
R_{merge} (%)	9.5 (55)
Overall B factor from Wilson plot ($\text{\AA}^2$)	19.34

0.03 mM), but exhibits the lowest turnover number ($k_{\text{cat}} = 79.18 \pm 1.2 \text{ s}^{-1}$), towards the long-chain substrate *p*NP-C14. The calculated k_{cat} values decrease as the acyl-chain length of

Figure 3

Typical X-ray diffraction pattern of *S. epidermidis* lipase (rGehC). The arrow indicates 1.9 $\text{\AA}$ resolution.

the substrate increases, while rGehC displays lower affinity for *p*NP-C8 than for *p*NP-C4 ($K_m = 1.59 \pm 0.24$ mM) and *p*NP-C10 ($K_m = 1.02 \pm 0.04$ mM). Therefore, the catalytic efficiency (k_{cat}/K_m) is highest towards *p*NP-C4 (836.60 ± 8.3 s⁻¹ mM⁻¹; Supplementary Table S1), which is in agreement with previous results for substrate specificity (Chang *et al.*, 2000). In order to obtain further insights into the catalytic mechanism of GehC, we crystallized rGehC for further structure determination. The rGehC crystal chosen for this study diffracted to a resolution of 1.9 Å (Fig. 3) and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 42.071$, $b = 59.306$, $c = 171.302$ Å, $\alpha = \beta = \gamma = 90^\circ$. A Matthews coefficient of 2.37 Å Da⁻¹ was calculated using *MATTHEWS_COEF* from *CCP4* (Matthews, 1968; Winn *et al.*, 2011) and suggested that there was one molecule in the asymmetric unit, with a solvent content of 48.23%. A homology search for the rGehC structure was performed using the *Phyre*² server (<http://www.sbg.bio.ic.ac.uk/~phyre/>). The model-selection standard was selected based on identity and coverage values. The sequence-alignment search indicated that rGehC shares high identity with other lipases from *Staphylococcus* (Supplementary Fig. S1). For example, residues 303–688 of rGehC have 49% sequence identity to ShLip. ShLip (PDB entry 2hih; Tiesinga *et al.*, 2007) was chosen as the initial model because its confidence was 100%, and a total of 387 residues were modelled. The first molecular-replacement trial was performed using *AutoMR* in *PHENIX* (Adams *et al.*, 2010). The modelled core has an α/β -hydrolase fold, with a central parallel β -sheet surrounded by loops. The molecular-replacement method was then applied to the model with *Phaser-MR* in *PHENIX* (Adams *et al.*, 2010), using reflections in the 30.0–1.9 Å resolution range. A single and unambiguous solution for one lipase molecule in the asymmetric unit was obtained in the rotation and translation functions, yielding a final R_{work} of 0.29 and an R_{free} of 0.33. The structural refinement of rGehC is currently in progress.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Brault, G., Shareck, F., Hurtubise, Y., Lépine, F. & Doucet, N. (2014). *PLoS One*, **9**, e91872.
- Carta, G., Gainer, J. L. & Benton, A. H. (1991). *Biotechnol. Bioeng.* **37**, 1004–1009.
- Chang, R.-C., Chou, S.-J. & Shaw, J.-F. (2000). *J. Am. Oil Chem. Soc.* **77**, 1021–1026.
- Chang, R.-C., Chou, S.-J. & Shaw, J.-F. (2001). *J. Agric. Food Chem.* **49**, 2619–2622.
- Eggers, D. K., Blanch, H. W. & Prausnitz, J. M. (1989). *Enzyme Microb. Technol.* **11**, 84–89.
- Farrell, A. M., Foster, T. J. & Holland, K. T. (1993). *J. Gen. Microbiol.* **139**, 267–277.
- Goldberg, M., Thomas, D. & Legoy, M. D. (1990). *Enzyme Microb. Technol.* **12**, 976–981.
- Karra-Chaabouni, M., Pulvin, S., Touraud, D. & Thomas, D. (1998). *J. Am. Oil Chem. Soc.* **75**, 1201–1206.
- Kim, J., Altreuter, D. H., Clark, D. S. & Dordick, J. S. (1998). *J. Am. Oil Chem. Soc.* **75**, 1109–1113.
- Langrand, G., Triantaphylides, C. & Baratti, J. (1988). *Biotechnol. Lett.* **10**, 549–554.
- Lima, F. V., Pyle, D. L. & Asenjo, J. A. (1995). *Biotechnol. Bioeng.* **46**, 69–79.
- Liu, C.-H., Chen, P. T., Chiang, C.-J., Shaw, J. F. & Chao, Y.-P. (2015). *J. Taiwan Inst. Chem. Eng.* **53**, 1–5.
- Manjón, A., Iborra, J. L. & Arocas, A. (1991). *Biotechnol. Lett.* **13**, 339–344.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Monot, F., Borzeix, F., Bardin, M. & Vandecasteele, J.-P. (1991). *Appl. Microbiol. Biotechnol.* **35**, 759–765.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Shaw, J.-F. & Lo, S. (1994). *J. Am. Oil Chem. Soc.* **71**, 715–719.
- Shaw, J.-F., Wang, D.-L. & Wang, Y. J. (1991). *Enzyme Microb. Technol.* **13**, 544–546.
- Tiesinga, J. J. W., van Pouderoyen, G., Nardini, M., Ransac, S. & Dijkstra, B. W. (2007). *J. Mol. Biol.* **371**, 447–456.
- Trbojević Ivić, J., Veličković, D., Dimitrijević, A., Bezbradica, D., Dragačević, V., Gavrović Jankulović, M. & Milosavić, N. (2016). *J. Sci. Food Agric.* **96**, 4281–4287.
- Welsh, F. W. & Williams, R. E. (1989). *J. Food Sci.* **54**, 1565–1568.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.