Characterization of Lipases from *Staphylococcus aureus* and *Staphylococcus epidermidis* Isolated from Human Facial Sebaceous Skin

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Two staphylococcal lipases were obtained from *Staphylococcus epidermidis* S2 and *Staphylococcus aureus* S11 isolated from sebaceous areas on the skin of the human face. The molecular mass of both enzymes was estimated to be 45 kDa by SDS-PAGE. S2 lipase displayed its highest activity in the hydrolysis of olive oil at 32°C and pH 8, whereas S11 lipase showed optimal activity at 31°C and pH 8.5. The S2 lipase showed the property of cold-adaptation, with activation energy of 6.52 kcal/mol. In contrast, S11 lipase’s activation energy, at 21 kcal/mol, was more characteristic of mesophilic lipases. S2 lipase was stable up to 45°C and within the pH range from 5 to 9, whereas S11 lipase was stable up to 50°C and from pH 6 to 10. Both enzymes had high activity against tributyrin, waste soybean oil, and fish oil. Sequence analysis of the S2 lipase gene showed an open reading frame of 2,067 bp encoding a signal peptide (35 aa), a pro-peptide (267 aa), and a mature enzyme (386 aa); the S11 lipase gene, at 2,076 bp, also encoded a signal peptide (37 aa), pro-peptide (255 aa), and mature enzyme (399 aa). The two enzymes maintained amino acid sequence identity of 98–99% with other similar staphylococcal lipases. Their microbial origins and biochemical properties may make these staphylococcal lipases isolated from facial sebaceous skin suitable for use as catalysts in the cosmetic, medicinal, food, or detergent industries.

**Keywords:** *Staphylococcus*, lipase, oil hydrolysis

A group of enzymes used widely in industrial and household conversion processes are the lipases (E.C. 3.1.1.3). Enzymes belonging to this group are biocatalysts in the hydrolysis of triacylglycerols into free fatty acids and glycerols. These enzymes exhibit high substrate specificity according to their diverse chemo-, regio-, and enantio-selective properties [9]. Lipases are also selective in their recognition of fatty acid species and can be used for interesterification reactions to perform cocoa butter substitutions and in the production of specialty fats or biodiesel [9, 15, 20].

Among the microbial lipases, staphylococcal lipases are classified as belonging to family I, subfamily 5 [10]. They are produced as pre-pro-enzymes in which the pre-region acts as a signal peptide, and are secreted as precursors to form a mature protein of approximately 400 amino acid residues after cleavage of the peptide bond between the pro-region and mature enzyme by a specific protease.

Staphylococcal lipases have been applied industrially to produce flavor esters. Lipases from *Staphylococcus xylosus* play a role in aroma production in fermented food [23]. *S. xylosus* is commonly used in lipolytic starter cultures for fermented meat products such as sausages and ham [11]. *Staphylococcus capitis* lipase has been used in a hair treatment formulation to suppress dandruff and itching [24]. These properties have led to the adoption of staphylococcal lipases for catalytic processes in the food and medicinal industries.

The endogenous role of lipases in *Staphylococcus* bacteria can be pathogenic in nature as well as to metabolize lipids, and the opportunistic pathogen *Staphylococcus aureus* can produce a lipase interfering with phagocytosis of human granulocytes [17]. An immune response towards *S. aureus* lipase is also reported [3].

On the other hand, their detailed three-dimensional structures, as well as their specific pathogenic mechanisms, have not yet been addressed. Further intensive biochemical and structural studies on these lipases are necessary for their cost-effective industrial application.

Over 200 different genera have been identified from human skin [7]. Corynebacteria, staphylococci, and propionibacteria...
compri the major portion of the microbiota of normal human skin. Staphylococci in particular are commonly found on sebaceous skin, which includes areas such as the alar crease, back of the scalp, upper chest, and back. Staphylococci isolated from such sebaceous areas are likely to produce lypolytic enzymes and to metabolize sebum. Human sebaceous skin is therefore a suitable site to isolate lipase-producing staphylococcal strains.

The objectives of this research were to measure lipase activity from Staphylococcus strains isolated from human facial skin, to characterize the lipases responsible, and to analyze their genetic sequence.

**MATERIALS AND METHODS**

**Screening for Lipase Activity**

Microbes isolated from human facial sebaceous skin were grown on tributyrin [1% (v/v)] and tricaprylin [1% (v/v)] agar plates containing 1× gum arabic solution, 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar. Gum arabic stock solution (10×) contained 10% (w/v) gum arabic, 200 mM NaCl, and 50 mM CaCl2. *Staphylococcus epidermidis* S2 and *Staphylococcus aureus* S11, which formed distinct clear zones around their colonies after 24 h incubation at 37°C, were selected for further study.

**Production and Concentration of S2 and S11 Lipases**

*S. epidermidis* S2 and *S. aureus* S11 strains were cultivated in 800 ml of LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37°C for 20 h with shaking at 200 rpm. Extracellular enzymes were separated from bacterial cells by centrifugation (7,000 × g, 10 min) at 4°C. Supernatants containing extracellular lipases were collected and added with ammonium sulfate to 30% saturation. Centrifugation (10,000 × g, 10 min) was performed to remove non-protein polymers and protein aggregates from supernatants. Addition of ammonium sulfate was continued until 70% saturation. Protein precipitates were collected by centrifugation (10,000 × g, 10 min), dissolved in distilled water, and dialyzed with SpectraPor 4 membrane (Spectrum Labs, USA) to remove ammonium sulfate. The dialysates were concentrated by an ultrafiltration kit using an Amicon PLGC 47 mm membrane with the cut-off size of 10,000 MW (Millipore, USA).

**Lipase Activity Assay and Estimation of Protein Concentration**

An olive oil emulsion containing 1% (v/v) olive oil (Sigma, USA) and 1% gum arabic was prepared by blending in a Waring blender (model 51BL31) at maximum speed for 2 min. Lipase activity was measured at 37°C using the pH-STAT method. Substrate emulsions were adjusted to pH 8.0 before the addition of enzyme. Reactions were initiated after addition of an appropriate amount of enzyme (0.5 – 5 U). Titration of free fatty acids with 10 mM of NaOH solution was performed during the reaction to maintain the pH of the reaction at 8.0 for 5 min. The hydrolysis rate for lipase conversion of olive oil into free fatty acids was measured with a 718 Titirino pH titrator (Metrhythm, Switzerland). The amount of enzyme catalyzing the release of 1 μmol fatty acid per minute was defined as one lipase unit.

Protein concentration was measured using a Bradford assay kit (Bio-Rad Lab., USA), and was calculated relative to a standard curve of bovine serum albumin.

**Molecular Mass Determinations**

SDS-PAGE and zymograms were performed to determine the molecular masses of S2 and S11 lipases. SDS-PAGE was performed using polyacrylamide gels (10%) as described by Laemmli ([13]). Proteins were stained with Coomassie Brilliant Blue R-250. Gels used for zymograms were washed with 50 mM Tris-HCl (pH 8.0) containing 1% Triton X-100 for 10 min with shaking. A second 10 min wash step was performed with 50 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100; and a final 10 min wash was performed with distilled water. Renatured proteins were checked for activity by attaching gels to a tricaprylin agar plate and incubating at 37°C for 2 h.

**Effects of Temperature on Lipase Activity and Stability**

The optimal reaction temperatures of S2 and S11 lipases were determined by assaying their hydrolytic activities toward olive oil at various temperatures (10 – 60°C) using the pH-STAT method. Lipase temperature stability was examined by their pre-incubation at various temperatures for 30 min before assay, with a pH-STAT instrument, for optimal temperature.

**Effects of pH on Lipase Activity and Stability**

The optimal pHs for S2 and S11 lipase activity were determined by assaying their hydrolytic activities toward olive oil or p-nitrophenyl caprylate (pNPC) at various pHs (pH 6–10) using pH-STAT and spectrophotometry, respectively. The activity of the S11 lipase was determined spectrophotometrically using pNPC, as accurate titration of fatty acids released was difficult to determine above pH 9 by pH-STAT. The result of pNPC assay for S11 lipase were normalized with the result of the pH-STAT assay. The stability of the lipases at various pHs was examined by pre-incubating 25 μl (corresponding to about 2 U) of the S2 and S11 lipases in 225 μl of 0.1 M sodium acetate (pH 4–6), 0.1 M potassium phosphate (pH 6–7.5), 0.1 M Tris-HCl (pH 7.5–9), 0.1 M KCl-glycine-KOH (pH 9–10), or 0.1 M potassium phosphate (pH 10–12) for 30 min and assaying with a pH-STAT machine at their optimal temperature.

**Analysis of Substrate Specificity**

Tributyrin, tricaprylin, olive oil, soybean oil, sunflower oil, fish oil (Sigma, USA), home waste cooking oil, and waste soybean oil (National Fisheries Research and Development Institute, Busan, South Korea) were selected for use in substrate emulsions. S2 and S11 lipase activity toward various substrates was measured using the pH-STAT method at their optimal temperature and pH.

**PCR Cloning of S2 and S11 Lipase Genes**

To obtain the S2 and S11 lipase genetic sequence, four primers were designed based on the 5'- and 3'-terminal sequences of the *S. epidermidis* 9 (GenBank: M95577) and *S. aureus* B56 lipase genes (GenBank: AY028918) ([5, 12]). The primer sequences for S11 (AF and AR) and S2 (EF and ER) were as follows: AF, 5'-GGA CAT ATG TTA AGA GGA CAA GAA-3'; AR, 5'-CTT GGA TTC ATA CTT GCT TTC AAT TGT GT-3'; EF, 5'-GGA CCA TGG TGA AGA CAA GAC AAA A -3'; ER, 5'-TCC GGA TCC ATT TTA TTT GIT GAT GAT AAT TG-3'.

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PCR conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 41°C for 0.5 min, and extension at 72°C for 1.5 min, and a post-extension step of 5 min at 72°C.

PCR products were inserted into pGEM-T vectors (Promega, USA) and the recombinant vectors were transformed by electroporation into E. coli XL1-Blue cells. Purified plasmids were sequenced with primers directed against the T7 and SP6 promoters.

Lipase Gene-Sequence Analysis
The genetic sequences of the S2 and S11 lipases were analyzed for homology to other organisms by NCBI BLAST. The DNA sequences were translated into amino acid sequences by using the EditSeq application from the DNASTAR program. Amino acid sequences were aligned against other known staphylococcal lipases using the ClustalW method in the DNASTAR MegAlign application. Protein divergence (in millions of years since species divergence) was calculated based on amino acid substitutions using cytochrome S as a "molecular clock" [14]. Protein divergence ($\pi$) at each node in the phylogenetic tree (Fig. 5) was calculated according to the formula $\pi = \frac{\Sigma \pi_{ij}}{2n}$, where $\pi_{ij}$ is the pairwise divergence between the $i^{th}$ protein in one branch and the $j^{th}$ protein in the other.

RESULTS

Isolation of Lipase-Producing Staphylococcal Strains
We isolated microbial strains from human facial sebaceous skin as follows. The condition of sample source was oily face skin with some acne on the face skin. The oily part of the face (forehead and cheek) was swabbed using a cotton bath. Then, the cotton bath was streaked on an LB agar plate and incubated overnight at 37°C. Approximately 14 colonies showing different morphologies were selected and cultivated on Rhodamine B agar plate at 37°C for 48 h. Then 9 colonies forming orange fluorescent halos under UV irradiation were finally selected. We assessed their ability to form clear zones around colonies grown on tributyrin (TBN) (Fig. 1A and 1B) and tricaprylin (TCN) LB plates. Of these strains, the two colonies showing the largest halos were selected and designated as S2 and S11. 16S rRNA analysis identified these strains as Staphylococcus epidermidis and Staphylococcus aureus, respectively. The GenBank accession numbers for the 16S rRNAs of these bacteria are JN245969 and JN245970, respectively.

Determination of Lipase Molecular Mass
S. epidermidis S2 and S. aureus S11 were cultured and the culture supernatants were partially purified by ammonium sulfate precipitation, dialysis, and ultrafiltration. The concentrated enzymes were loaded into polyacrylamide gels for separation and zymogram analysis. TCN zymograms demonstrated formation of a single distinct band in each lane (Fig. 1D). The molecular mass of each enzyme was estimated at approximately 45 kDa by comparison of marker size and zymogram results (Fig. 1C and 1D).

Effects of Temperature and pH on Lipase Activity and Stability
The S. epidermidis S2 lipase activity against olive oil reached its optimum at 32°C, but it could stably retain activity relatively up to 45°C after 30 min incubation at various temperatures; activity decreased beyond pre-incubation at 50°C. S11 lipase showed optimal activity at 31°C and was stable up to 50°C pre-incubation (Fig. 2A and 2B).

In contrast to S2 lipase, which exhibited diminished stability above 45°C and activity above 50°C, the activity of S11 lipase increased upon high-temperature pre-incubation. Although the activity continued to increase with pre-incubation of up to 50°C, it declined rapidly beyond 55°C.

We calculated the lipase activation energy with the Arrhenius equation according to their activity at various temperatures (Fig. 2C and 2D). The activation energy of S2 lipase was 6.52 kcal/mol in the temperature range from
A higher activation energy, of 21 kcal/mol, was calculated for the S2 lipase.

The activity of S2 lipase was optimal at pH 8 and retained more than 60% of its maximum activity between 10 to 32°C. A higher activation energy, of 21 kcal/mol, was calculated for the S2 lipase.
pH 5 and pH 9 (Fig. 3). The S11 lipase had optimal activity at pH 8.5 and was stable from pH 6 to pH 10.

**Analysis of Substrate Specificity**

We tested S2 and S11 lipase activity against a panel of oils. Both lipases showed relatively high hydrolytic activity against tributyrin, waste soybean oil, and fish oil in comparison with the other tested substrate (Fig. 4). The S2 lipase had the lowest activity against sunflower oil, and the S11 against tricaprylin.

**PCR Cloning of S2 and S11 Lipase Genes**

We PCR-amplified the *S. epidermidis* S2 and *S. aureus* S11 lipase genes with primers designed to amplify *S. epidermidis* 9 and *S. aureus* B56 lipase sequences. PCR product sizes were confirmed to be 2.1 kbp by agarose gel electrophoresis.

Total sequences composed of 2,067 nucleotides were obtained for the S2 lipase gene (Supplementary Fig. S1; GenBank Accession No. JN245971), which was found to encode a signal peptide (35 aa), a pro-peptide (267 aa), and a mature enzyme (386 aa). The S11 lipase was encoded by a 2,076 bp region and also comprised a signal peptide (37 aa), a pro-peptide (255 aa), and a mature enzyme (399 aa; Supplementary Fig. S2; GenBank Accession No. JN245972). The protein domains were determined by comparing the amino acid sequence of S2 and S11 lipases with other known staphylococcal lipases [5, 12].

The S2 lipase gene had 98% identity with that of *S. epidermidis* strain ATCC 12228, whereas the S11 lipase gene was 99% identical to that of *S. aureus* subsp. aureus NCTC 8325. Nucleotide sequence analysis showed some differences between the *S. epidermidis* S2 lipase gene and that of *S. epidermidis* ATCC 12228. About 42 single nucleotide substitutions have resulted in 97.4% amino acid sequence homology. The lipase gene from *S. aureus* S11 showed minimal nucleotide substitution; only six single nucleotide substitutions were observed, but three deletions can additionally be observed between S11 and *S. aureus* subsp. aureus NCTC 8325.

We translated lipase sequences to determine the protein composition and analyzed them for similarity to known staphylococcal lipases by BLASTp. The alignment showed a similarity of 97.8% to a lipase from *S. epidermidis* RP62A for S2 lipase, and a similarity of 99.4% with the glycerol ester hydrolase of *S. aureus* subsp. aureus N315 for S11 lipase (Table 1).

**Fig. 4.** Substrate specificity of S2 and S11 lipases.

Hydrolytic activities of S2 and S11 lipases were measured against various substrates.

**Fig. 5.** Phylogenetic tree of staphylococcal lipases.

Phylogenetic relationships of S2 and S11 lipases toward various staphylococcal lipases were analyzed using ClustalW in the MegAlign application. Protein divergence was calculated with the formula $\pi = \Sigma \pi_{ij}/2n$, where $\pi_{ij}$ is the pairwise divergence between the $i^{\text{th}}$ protein in one branch and the $j^{\text{th}}$ protein in the other.
We reasoned that staphylococci isolated from human skin might produce lyolytic enzymes capable of metabolizing sebum. We characterized the S2 and S11 lipases, isolated respectively from *S. epidermidis* and *S. aureus* harvested from the surface of human facial skin, for their biochemical properties. Their optimal hydrolytic activity occurred at a temperature of approximately 31–32°C, which is consistent with their habitat on the surface of the skin; normal adult human skin has an average temperature of 32.5°C [8].

S2 lipase has the low activation energy usually possessed by cold-adapted enzymes. This value is lower than the activation energies of lipases from Alaskan or Antarctic psychrotrophic bacteria, which have been calculated at approximately 14 kcal/mol, or 28 kcal/mol [2, 22]. Each enzyme is stable in a fairly wide pH distribution. The S11 lipase was most active close to pH 8.5, but stable up to pH 10, whereas S2 lipase was most active at pH 8 and stable to pH 9. Lipases with activity at alkaline pH can be used as detergent additives to remove hydrophobic dirt. However, other criteria, such as broad activity on a variety of fats and lipids, sufficient solubility in water to soak into fabrics, and compatibility with proteases present in detergent formulations, must be considered for such applications [16].

Both lipases exhibited high activity in the hydrolysis of short-chain triacylglycerols (Fig. 4), including tributyrin, which contains a C4 acyl group. In contrast, the activity of both enzymes toward tricaprylin, with a C8 acyl group, was poor compared with other tested substrates such as waste soybean oil and fish oil. Although the exact composition of waste soybean oil is unknown, fish oil contains high levels of eicosapentanoic acid (EPA), docosahexaenoic acid (DHA), palmitic acid (C16:0), and palmitoleic acid (C16:1), as shown in Table 2.

We analyzed S2 and S11 lipase homology against that of other staphylococcal lipases with the MegAlign application.
in DNASTAR (Table 1), compiling a phylogenetic tree (Fig. 5).

The biochemical properties of S. epidermidis S2 lipase were quite different from those of S. epidermidis RP62A lipase despite the high conservation of amino acid sequence. The S. epidermidis RP62A lipase has optimal activity at pH 6 rather than the pH 8 optimum exhibited by S2 lipase. The enzymes also differ in stability: S2 lipase is not stable at pH 4, but RP62A lipase retains its activity at pH 4 after 2.5 h incubation. Nevertheless, S2 lipase can hydrolyze a broader range of substrates than RP62A, including olive oil; the latter was found to be inactive against an olive oil emulsion [21].

Comparison of the biochemical properties of S. aureus S11 lipase with lipase from S. aureus subsp. aureus N315 could not be conducted, as the biochemical properties of the latter have not been reported. Instead, we compared S11 with lipase from S. simulans, to which S11 lipase is also highly homologous (99.7%).

S. simulans lipase showed optimal activity at pH 8.5 and 37°C. Interestingly, although the two lipases demonstrated identical pH optima, they differed with respect to temperature preference. Enzyme pH stability was disparate with respect to each other and other known staphylococcal lipases despite substantial homology. S11 lipase’s stability in a wide range of pHs, including alkaline pHs, may be indicative of its potential as an additive in detergents. Functional expression of these enzymes in a heterogeneous host may achieve their economical and practical applications to cosmetic, medicine, food, or detergent manufacture.

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References


