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Short communication

Isolation, Analysis, and Expression of Lipase with Cephalosporin-C Deacetylation Activity from *Staphylococcus* sp.

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Lipase of *Staphylococcus* sp. was purified from the culture supernatant, and its molecular mass estimated to be 44 kDa by SDS-PAGE. Its optimum temperature and pH for the hydrolysis of *p*-nitrophenyl substrates was 28°C and pH 8.5, respectively. The gene encoding the lipase was cloned in *Escherichia coli* in the NH_2-teminally truncated form by using the shotgun method, and sequenced. The mature enzyme had a 49~93% amino acid sequence homology with other staphylococcal lipases. This lipase was used for the hydrolysis of the 3-O-acetate of cephalosporin-C to give an intermediate, deacetylated cephalosporin-C that is useful for further chemical elaborations.

Keywords: Cephalosporin-C, Deacetylated cephalosporin-C, Lipase, *Staphylococcus* sp.

Introduction

Lipase (EC 3.1.1.3) is a lipid-degrading enzyme widely distributed in organisms ranging from animals to bacteria. In addition to hydrolyzing esters, lipase can catalyze transesterification (Zaks *et al.*, 1984), esterification (Zaks *et al.*, 1985), and aminolysis or oximolysis (Kirchner *et al.*, 1985) under anhydrous conditions. Recently, lipases were used for deacetylation of cephalosporins (Akio *et al.*, 1994, Giacomo *et al.*, 1996, Michael *et al.*, 1997). Due to the reactivity of the â-lactamic ring, cephalosporin-C and its derivatives are ideal compounds for the mild and selective action of enzymes. Deacetylation of the 3' position of cephalosporins has received much attention, since deacetylcephalosporins were proven to be useful for the industrial production of various semisynthetic cephalosporins. The deacetylation of cephalosporins was carried out both chemically and enzymatically. However, the enzymatic route is believed to be advantageous in that it is performed at a lower temperature, which has a higher yield and lower side reactions. Cephalosporin-C deacetylase (CAH, systemic name: cephalosporin-C acetylhydrolase) was previously found to be active in various organisms, such as citrus peels (Jeffery *et al.*, 1961), *Bacillus subtilis* (Abott and Fukuda, 1975a, Abott and Fukuda, 1975b, Konecny *et al.*, 1977), acetomyces (Demain *et al.*, 1963), and fungi (Hinen *et al.*, 1976, Fujisawa *et al.*, 1975). We found that a strain of *Staphylococcus* sp. produces lipase, which converts cephalosporin-C to a deacetylated form with mild efficiency. In this work, we reported the purification, characterization, and expression of the lipase from *Staphylococcus* sp..

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Materials and Methods

Bacterial strains, plasmids, and conditions *Escherichia coli* JM109 cells were used for cloning and subcloning manipulations. *Escherichia coli* BL21(DE3) cells with DE3, a ë prophage carrying the T7 RNA polymerase gene, were used for expressing the recombinant *Staphylococcus* sp. lipase (rSL). *Staphylococcus* sp. was isolated from sewage. Plasmids pUC19 and pET28a were used for cloning and expression, respectively. All of the bacterial strains were grown in a Luria-Bertani (LB) broth. Cultures were grown in broth at 37°C with vigorous agitation. For expressing the recombinant lipase *Escherichia coli*, BL21(DE3) that harbored the pTrSL plasmid was grown at 37°C for 3 h after induction by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Assay Lipolytic activity was usually measured with *p*-nitrophenyl ester substrates (Pocker *et al.*, 1967). Enzyme activity was monitored spectrophotometrically by measuring the increase in an optical density at 405 nm, due to the formation of the *p*-nitrophenol. Alternatively, the lipase was assayed with cephalosporin-C as a substrate. Enzyme was added to the reaction mixture containing the zinc salt of the cephalosporin-C (Sigma Co. LTD.), 2 mg and 100 mM sodium phosphate (pH 7.0) in a final volume of 1.0 ml. The mixture was incubated at 30°C, and the reaction was stopped by

addition of 4.0 ml of 50% acetonitrile. The reaction was monitored at UV light 254 nm by reversed-phase high performance liquid chromatography (HPLC) on a C18 column with the mobile phase containing 3% acetonitrile (the modified method of Ahn *et al.*, 1999. Koo *et al.*, 2000).

Enzyme purification *Staphylococcus* sp. was cultivated in a LB medium at 37°C for 24 h. After removal of the cells by centrifugation, ammonium sulfate was added to the culture supernatant to 60% saturation, and the precipitate was collected by centrifugation at 5,000 × g. The pellet was dissolved in a Tris-Cl buffer (20 mM, pH 8.0), and then dialyzed against the same buffer. The dialysate was applied to a Q-sepharose column pre-equilibrated with the same buffer. Although most of the proteins in the dialysate bound to the resin, the lipase enzyme passed through the column. The active fractions were collected and concentrated with ultracentrifugation. The concerned proteins were loaded onto a Superose column with the salty Tris-Cl buffer (mixed with 0.5 M NaCl). The active fractions were dialyzed in a Tris-Cl buffer (20 mM, pH 7.8) to a Mono-S column that was pre-equilibrated with a 0.3-0.4 M linear gradient of NaCl in the same buffer, concentrated and stored at -20°C.

DNA manipulation and recombinant DNA techniques To clone the lipase gene, a genome library of *Staphylococcus* sp. chromosomal DNA in plasmid pUC19 was constructed. Chromosomal DNA was partially digested with *Sau*3AI to obtain fragments of 4 to 6kb. Plasmid DNA, pUC19 was linearized with *Bam*HI, and chromosomal DNA fragments were added and ligated. Individual *E. coli* JM109 transformants were screened on a lipase indicator plate, 1% tributyrin-LB agar plate. To express the recombinant staphylococcal lipase, the mature lipase-encoding gene was amplified from pUSL by PCR with the oligonucleotide pair mSL-F (5'-ACAGCCATGGCTACTGTCAAAAGTAATCAATAT-3') and mSL-R (5'-GATCTCACATCTAATATGCCAGCCATTGA A-3'), which contained additional *Nco*I and *Hin*dIII restriction sites. The *Nco*I- and *Hin*dIII-digested *msl* was subcloned into the pET28a digested with the same enzymes.

Other methods Protein concentrations were determined by the method of Bradford (1976). SDS-PAGE, performed on a 12% polyacrylamide slab gel by the method described by Laemmli (1970), was used to determine the molecular mass of the enzyme.

Results and Discussions

Staphylococcus sp. lipase was purified from the culture medium by an ammonium sulfate precipitation, two ion exchange column chromatographies, and one gel filtration column chromatography. The lipase passed through the Q-sepharose column, while most of the other extracellular proteins bound to the Q-sepharose resin. About 44 kDa protein was purified to homogeneity, and after its renaturation process it showed a lipolytic activity on the overlaid tributyrin agarose plate (data not shown).

An *E. coli* transformant, forming a clear halo on the tributyrin agar plate, was selected as described previously. The recombinant plasmid (pUSL) that was isolated from this transformant had about a 4 kb insert DNA. Determination of the

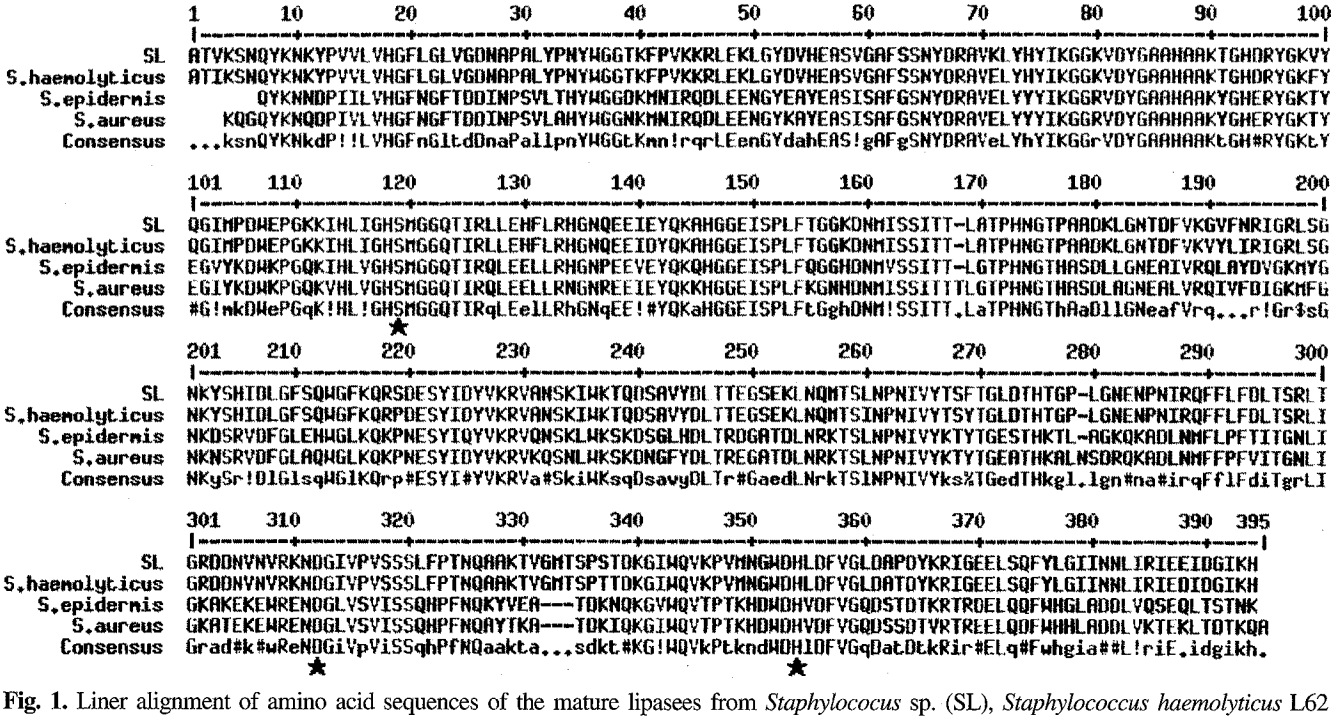

Fig. 1. Liner alignment of amino acid sequences of the mature lipasees from *Staphylococus* sp. (SL), *Staphylococcus haemolyticus* L62 (GI: 6648932), *Staphylococcus epidermis* (GI: 2981225), *Staphylococcus aureus* (GI: 2981225). In this alignment, the numbering starts at the N-terminal residue of the mature sequences. The putative active site residues, serine-histidine-aspartic acid, are marked with asterisks.

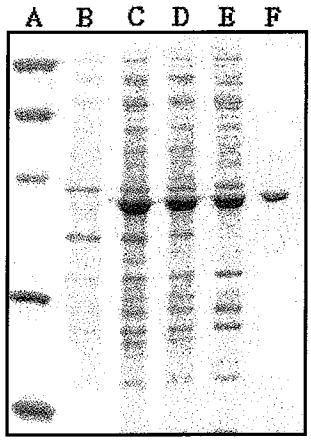

Fig. 2. SDS-PAGE of the recombinant SL at different stages of purification. From the left to right: lane A, marker proteins, phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trysin inhibitor (21.5 kDa); lane B, whole cells not induced; lane C, whole cells induced with IPTG; lane D, sample after cell-lysis; lane E, sample after ammonium sulfate precipitation; lane F, sample after chromatographies.

nucleotide sequence of the DNA fragment did reveal a significant homology with a *Staphylococcus haemolyticus* L62 lipase gene (Oh *et al.*, 1999). On the basis of the sequence homology, this lipase was cloned in the NH_2-terminally truncated form. A sequence analysis showed that this lipase was composed of a truncated pro-peptide (132 aa), and a mature enzyme (393 aa). The mature enzyme has a 49-93% amino acid sequence homology with other staphylococcal lipases (Fig. 1). This lipase-producing *Staphylococcus* sp. may be a variant strain of *S. haemolyticus* L62.

The optimum temperature of the lipase was 28°C. The lipase showed the highest activity toward *p*-nitrophenyl carprylate (C8) among the synthetic substrates tested (C2-C16; data not shown). The lipase showed high activity at pH 8.5, when the activity differs somewhat depending on the various incubation buffers used. The lipase SL (staphylococcal lipase) was fairly stable for 24 hours from pH 5 to pH 11.

When pTSL (*msl* subcloned into pET28a) was expressed in *E. coli* BL21 (DE3) by IPTG induction, a new protein of about 43.7 kDa was produced. Using sonication for 5 min, lysates were prepared from 4 h induced cells grown at 37°C. After ultracentrifugation, the supernatant was precipitated with ammonium sulfate to a 50% saturation, then the precipitated proteins were applied to a Q-sepharose column. The lipase rSL passed through the Q-sepharose column (Fig. 2). The process of purification of rSL was followed as described in Materials and Methods.

We investigated the ability of lipase SL to deacetylate cephalosporin-C. Two mg of cephalosporin-C, dissolved in 1 ml of a 50 mM sodium phosphate buffer (pH 7.0, 1 mM $CaCl_2$), and 50 μg of lipase SL was added. The degree of conversion was estimated after 40 h at 28°C by analytical HPLC. Retention times of cephalosporin-C and deacetylated cephalosporin-C were 5.6 min and 3.2 min, respectively. The degree of conversion was about 35% (Fig. 3)

The cloned lipase gene suggests that the staphylococcal lipase is produced in a preproenzyme secretion, and processed rapidly to the 43.7 kDa mature enzyme in this staphylococcal strain. In our experiment, neither prepro- nor proenzyme bands could be detected by Coomasie staining, or activity staining of the SDS-PAGE gel.

The deacetylation of cephalosporin-C is of considerable interest, since deacetyl cephalosporin-C are valuable intermediates

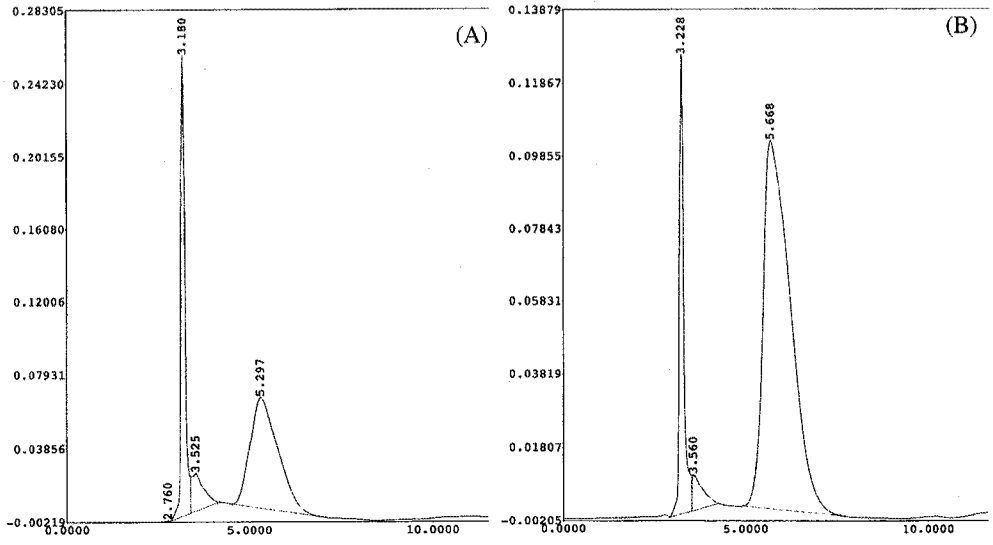

Fig. 3. Reversed-phase HPLC elution pattern of degradation products of cephalosporin-C by the lipase, SL from *Staphylococcus* sp. A, products of SL-digested cephalosporin-C; B, products of equal volume of SL-not-digested cephalosporin-C and SL-digested cephalosporin-C.

in synthesizing therapeutically useful cephalosporin antibiotics. Since SL is involved in the conversion of cepjalosporin-C into deacetylated cephalosporin-C, this lipase will be applied in the development of cephalosporin antibiotics.

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