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Purification and Characterization of Extracellular *Staphylococcus warneri* Lipase

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Abstract. The extracellular lipase of *Staphylococcus warneri* was secreted as a protein with an apparent molecular mass of 90 kDa. It was then sequentially processed in the supernatant to a protein of 45 kDa. Tryptic digestion of the crude extract resulted in a homogeneous sample containing only the 45-kDa form. Purification was achieved by hydrophobic chromatography. Purified lipase had an optimum pH of 9.0 and an optimum temperature of 25°C. The enzyme was stable within the range pH 5.0–9.0; it had a broad substrate specificity. The results of inhibition studies were consistent with the view that lipases possess a serine residue at the catalytic site.

Staphylococcal extracellular lipases have been studied with attention directed to pathogenic strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*) to establish their role in infections [4, 6, 14, 17, 20, 22]. The nonpathogenic species have been studied much less, and only the lipase of *Staphylococcus hyicus* has been well characterized [5, 7, 24, 25]. For the staphylococci isolated from meat products, few data are available on their lipases despite their participation in flavor development through their lipolytic properties. *Staphylococcus warneri*, found in French dry sausages [12], hydrolyzes pork fat at 15°C, 22°C, and at pH 6.0 [21]. When inoculated in sausage, *S. warneri* increases the free fatty acid level [13]; however, in a complex medium such as sausage, it is difficult to clearly establish its role in lipolysis for two reasons. First, lipolysis results from both endogenous and microbial lipases, and second, the free fatty acids are further metabolized to other products: alcohols, ketones, and aldehydes [1]. The enzyme of *S. warneri* was purified and characterized to evaluate its fundamental role.

Materials and Methods

Growth and production of lipase. *Staphylococcus warneri* (863) was grown in 1 L of PYS medium [11] in a 2-L flask. The medium was continuously shaken (150 rpm) and incubated at 30°C. Samples were taken after 2, 4, 6, 8, 10, 24, and 48 h of growth and centrifuged at 10^4 *g* for 15 min at 4°C. In addition, an aliquot of the supernatant after 48 h of growth was sterilized by filtration and incubated for 24 h at 4°C or 24°C. All the supernatants were kept frozen at −20°C before analysis.

Bacterial growth was assessed spectrophotometrically at 600 nm. Proteolytic activity was determined on azocasein [19]. Lipase activity was assayed on triolein with a turbidimetric method as described later.

Purification of lipase. The supernatant obtained after 16 h of growth was used for purification. Ammonium sulfate was added to the supernatant to 50% saturation, and the precipitate was collected by centrifugation for 30 min at 10^4 *g*. After dialysis in 20 mM Tris-HCl buffer at pH 8.0, the precipitate was further centrifuged at 10^5 *g*, 4°C for 1 h.

To purify the 90-kDa lipase, we chromatographed the precipitate on DEAE-Sepharose (Pharmacia) in 20 mM Tris-HCl buffer at pH 8.0. Proteins were eluted with a linear NaCl gradient (0–0.2 M) in 10-column volumes. Lipase active fraction was chromatographed on phenyl-Sepharose (Pharmacia) with a linear ethylene glycol gradient from 40% to 90% (vol/vol) in 10-column volumes. Finally, the sample was dialyzed and concentrated in an Amicon membrane CF 25.

To purify the 45-kDa protein, we incubated the precipitate with 0.1 μg of trypsin/mg of protein as described by Van Oort et al. [24], and the sample was chromatographed on phenyl-Sepharose (Pharmacia) as described above.

Lipase assays. Lipase activity was measured with a pH-Stat method. The substrate dispersion was prepared in distilled water and contained triolein 5 mM, arabic gum 5% (wt/vol), sodium deoxycholate 2 mM, and sodium chloride 50 mM. Released oleic acid was titrated continuously with 0.1 M NaOH. When triglycerides (C4–C18) were used instead of triolein, emulsions were prepared as described above but at 45°C. One enzyme unit (U) is defined as 1 μmol of acid released/min. Specific activities are expressed as U/mg of protein. The protein concentration was

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determined by the method of Bradford [2] with bovine serum albumin as standard.

Lipase activity was also measured at 334 nm and 25°C by a turbidimetric method as described previously [3], except that colipase was omitted and the pH was 8.0. Results are expressed in arbitrary unit OD/min/mg.

SDS–gel electrophoresis and zymography. SDS–PAGE was performed with 10% (wt/vol) gels as described by Laemmli [10]. The proteins of the supernatants were precipitated with five volumes of cold acetone and solubilized in the sample buffer. The molecular mass standards (BioRad) were trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), serum albumin (66.2 kDa), and phosphorylase B (97 kDa). Proteins were stained with Coomassie brilliant blue G250 or with silver by use of a stain kit (Amersham).

For tributyrin zymography, unstained SDS–polyacrylamide gel was washed for 30 min in 2.5% Triton X-100 to remove SDS. The gel was laid on a 1.3% agar gel prepared in 20 mM Tris HCl buffer, pH 8.0, containing 0.4% of tributyrin. Lipase activity was visualized by a zone of clearing after 2–3 h at 25°C.

Determination of the isoelectric point. Two-dimensional electrophoresis was carried out according to the method of O'Farrell [15]. Isoelectric focusing was performed with 5% carrier ampholytes (LKB) ampholines (2 parts pH 5–7, 3 parts pH 3–10). The second dimension was a standard SDS-PAGE performed according to Laemmli [10]. The zymography was performed as described above.

Acylglycerol specificity. The enzyme was incubated with triolein, diolein, monoolein as test substrates at a final concentration of 10 mM. The reaction mixture was as described for the pH-Stat method. Sequential dilutions of the substrates were done to determine V_m and K_m values.

Influence of pH and temperatures. Lipase activity was measured at different pH values (5.0–10.0) at 25°C and at various temperatures (15°–50°C) at pH 9.0 with triolein as substrate by titration with a pH-Stat method.

To investigate lipase stability, the enzyme was preincubated at different pHs and temperatures, and the residual activity assayed by the turbidimetric method at pH 8.0 and 25°C.

Effect of ions and inhibitors. Lipase was preincubated during 1 h at 30°C in 20 mM Tris-HCl buffer, pH 8.0, with various ions or inhibitors. Residual activity was assayed by the turbidimetric method at pH 8.0 and 25°C but without $CaCl_2$ in the reaction mixture.

Results

Time course of growth and lipase. The kinetics of lipase production paralleled those of growth, reaching a maximum after 10 h (Fig. 1). No proteolytic activity was detected on azocasein substrate (data not shown).

The production of extracellular proteins was followed by SDS–PAGE (Fig. 2). We observed an evolution of the protein pattern with time. Up to 24 h of growth, the primary contributor to the lipase activity was a 90-kDa form on the basis of zymographic evidence (Fig. 2A,B, lanes 1, 2, 3, 4). This protein was clearly dominant, and its concentration

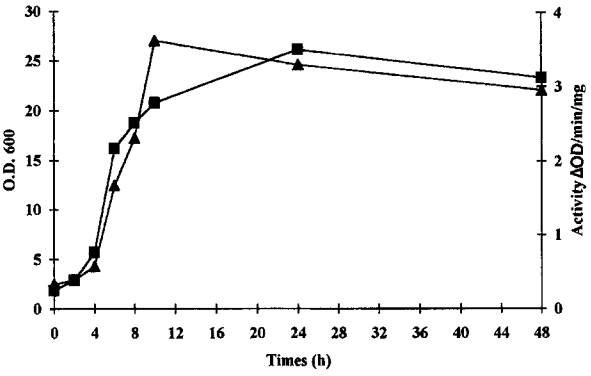


Fig. 1. Growth of *Staphylococcus warneri* in PYS medium and lipase production. ■ O.D. 600; ▲ lipase activity.

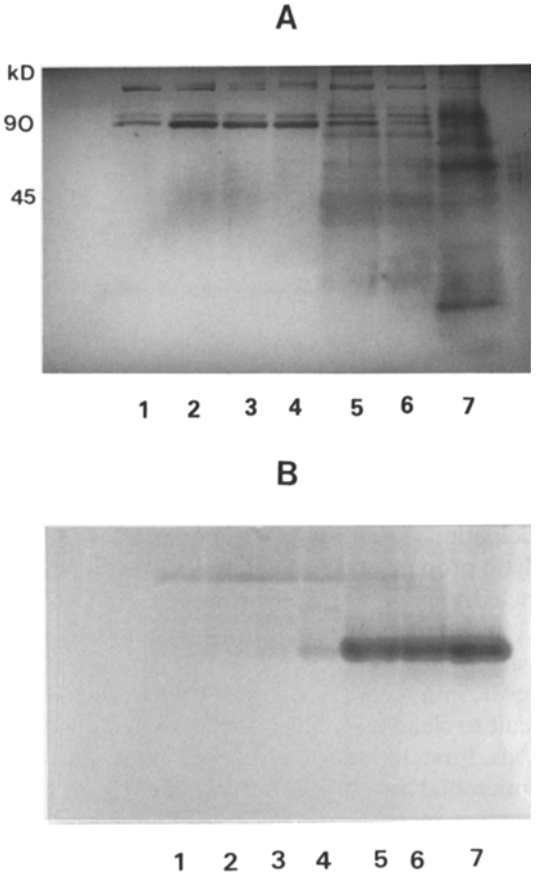


Fig. 2. SDS–PAGE of the culture supernatants of the experiment shown in Fig. 1. (A) Protein stained with silver. (B) Lipase activity detected with tributyrin. Lane 1, supernatant after 6 h growth, T_{6h}; lane 2, T_{8h}; lane 3, T_{10h}; lane 4, T_{24h}; lane 5, T_{48h}; lanes 6 and 7, supernatant at T_{48h}, incubated 24 h at 4°C or at 24°C respectively.

increased after 6 h of growth. After 48 h, however, the main tributyrin-degrading activity corresponded to a molecular mass of approximately 45 kDa (Fig. 2B, lanes 5, 6, 7). The activity of the 90-kDa lipase was

also detected in the supernatant after 48 h of growth (lane 5) and after 24 h incubation at 4°C (lane 6), but not at 24°C (lane 7). Examination of secreted proteins in silver-stained gel showed a decrease of the 90-kDa protein band in the supernatants after 48 h of growth (Fig. 2A, lanes 5, 6, 7). In these samples, proteins between 90 kDa and 45 kDa were present. A diffuse and broad activity between the 90 and the 45 kDa was consistently present after 8 h of growth (Fig. 2B).

Purification of the lipase. The 90-kDa lipase protein was dominant in the ammonium sulfate precipitate of the 16-h supernatant (Fig. 3, lane 2), although some activity was also detected for the low-molecular-weight lipase (data not shown). A partial purification of the 90-kDa protein was obtained after DEAE chromatography (Fig. 3, lane 4). However, during hydrophobic chromatography on phenyl-Sepharose, only the 45 kDa was eluted (Fig. 3, lane 5). We also tried to purify the 90-kDa lipase by Mono Q chromatography followed by gel filtration. Again only the low-molecular-weight lipase was detected (data not shown). To produce a homogeneous lipase comprising only the low molecular weight, the ammonium sulfate precipitate was digested by trypsin (Fig. 3, lane 7). The 45-kDa lipase was finally purified by hydrophobic chromatography (lane 8). The purification is summarized in Table 1.

Isoelectric point. After the bidimensional electrophoresis, three protein bands with a molecular weight of 45 kDa were detected. Each was active on tributyrin gel. The isoelectric points were at pH 6.8, 7.1, and 7.4.

Effect of pH and temperature. The *S. warneri* lipase (45 kDa) had a basic pH optimum (Fig. 4). Between pH 5.0 and 6.0, the activity was around 1% of the maximum activity at pH 9.0. The lipase was stable within the range pH 5.0–10.0 (Fig. 4).

The enzyme was very active between 20° and 40°C, with an optimal temperature of approximately 25°C (Fig. 5). Heating at 70°C for 30 min totally inactivated the enzyme.

Effect of different ions and inhibitors. The activity was strongly inhibited by cobalt and zinc, whereas calcium and manganese enhanced it (Fig. 6, A).

The activity was not affected by thiol-binding reagents such as PCMB, E64, nor by the aspartic proteinase inhibitor (pepstatin A) (Fig. 6, B). The chelating agent 1–10 phenanthroline did not inhibit the activity, but EDTA did. Activity was completely inhibited by the serine protease inhibitors: 3–4 DCI and pefabloc. With PMSF, also a serine protease inhibitor, residual activity was around 70% after 1 h incubation, and 40% after 2 h incubation (data not shown).

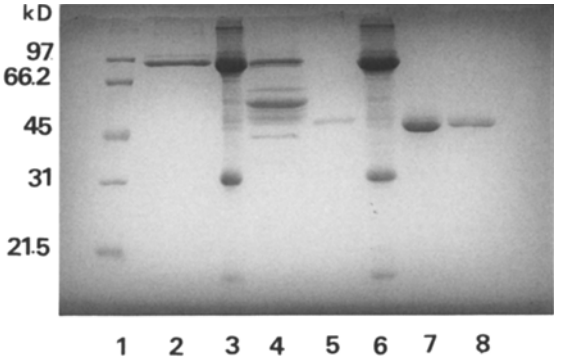


Fig. 3. SDS–PAGE of molecular weight markers (lane 1); 16-h supernatant (lane 2); ammonium sulfate precipitate (lanes 3, 6); DEAE eluate (lane 4); phenyl Sepharose eluate (lanes 5, 8); products of proteolysis of ammonium sulfate precipitate (lane 7). Proteins were stained with Coomassie blue.

Activity on various triglycerides. *Staphylococcus warneri* lipase hydrolyzed a wide range of substrates from C4 to C18:1 (Fig. 7). The maximum activity was recorded with medium chain length fatty acids: C8, C10, and C12.

Acylglycerol specificity. The lipase had high catalytic activity against triolein and diolein emulsions with similar V_m values (Table 2). Monoolein micelles with bile salt were hydrolyzed at a lower rate. K_m values were similar for the three oleins.

Discussion

The production of extracellular lipase by *Staphylococcus warneri* is correlated with growth. As described for *S. aureus*, *S. hyicus*, and *S. epidermidis* [4, 16, 24], the lipase of *S. warneri* is secreted in the supernatant as a prolipase with an apparent molecular weight of 90 kDa. This prolipase is processed in the supernatant, producing a mature 45-kDa lipase. Lipases produced by other staphylococcal species have similar molecular masses (43–46 kDa). The prolipase and the intermediate forms have full lipolytic activity. Rollof and Normark [16] showed that a metallocysteine protease is probably responsible for this processing. Farrell et al. [4] measured a proteinase activity in the supernatant of *S. epidermidis*. By contrast, no azocasein proteolytic activity was found in the supernatant of *S. warneri*, so it is not known whether the conversion to 45 kDa was caused by a protease undetected in our assay condition or by an autolytic activity of the lipase.

Table 1. Flow sheet for purification of *Staphylococcus warneri* extracellular lipase

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification (fold)
Culture supernatant	295	81	23940	100	1
Ammonium sulfate precipitate	20.2	707	14260	59.7	87
Proteolytic digestion products	14.2	800	11360	47.7	99
Phenyl Sepharose	0.95	1860	1770	7.4	229

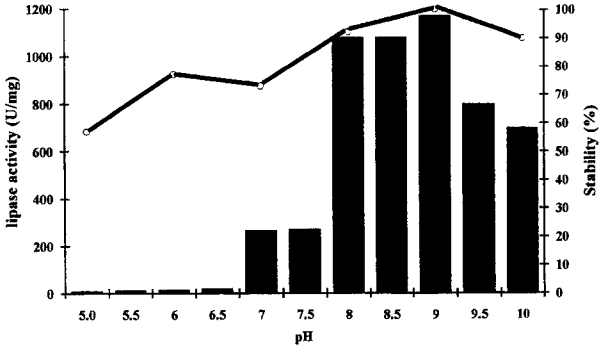


Fig. 4. Effect of pH on the activity and stability of *S. warneri* lipase. ▮ Activity was measured at different pH values and 25°C with the pH-Stat method. ❍ Activity was measured after preincubation of lipase solution at different pH values during 1 h at 4°C. The assay method was turbidimetric at pH 8.0 and 25°C. Data are expressed as percentage of original activity at pH 8.0, 25°C.

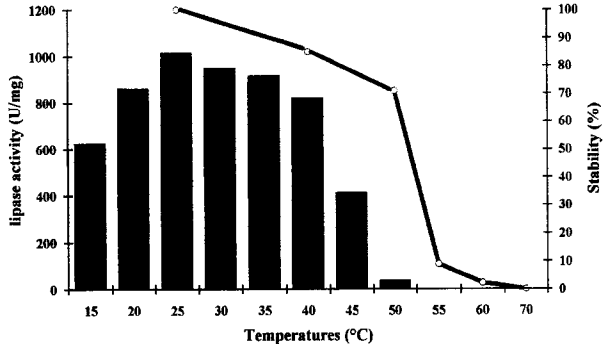
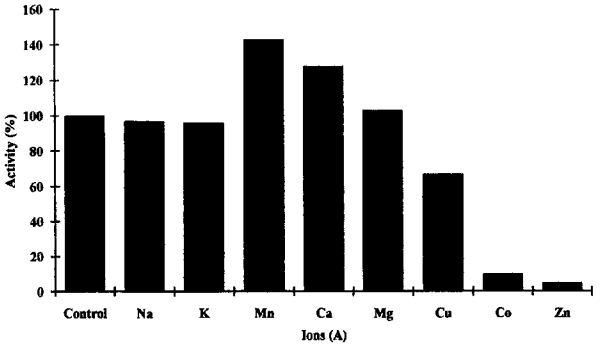


Fig. 5. Effect of temperature on the activity and stability of *S. warneri* lipase. ▮ Activity was measured at pH 9.0 and at various temperatures with the pH-Stat method. ❍ Activity was measured after preincubation of lipase solution at various temperatures for 30 min. The assay method was turbidimetric at pH 8.0 and 25°C. Data are expressed as percentage of original activity at pH 8.0, 25°C.



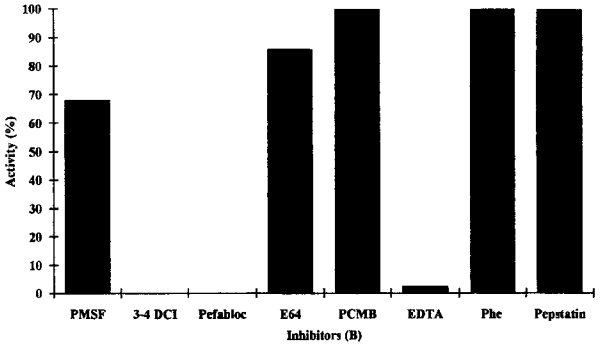


Fig. 6. Effect of some ions (A) and inhibitors (B) on lipase activity. The enzyme was incubated in 20 mM Tris-HCl buffer, pH 8.0, containing various ions or inhibitors for 1 h at 30°C. Activity was then determined with the turbidimetric method at pH 8.0, 25°C. $CaCl_2$ was excluded from the reaction mixture. The final concentration of the ions was 2 mM. The final concentration of the inhibitors was as follows: phenylmethanesulfonyl fluoride (PMSF), 10 mM; 3,4-dichloroisocoumarin (3–4 DCI), 1 mM; Pefabloc, 10 mM; L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane, N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]- agmatine (E64) 0.01 mM; para-chloromercuribenzoate (PCMB), 0.01 mM; pepstatin A, 0.001 mM; ethylenediamine tetraacetic acid (EDTA), 10 mM; 1,10-phenanthroline (Phe) 1 mM.

To purify the lipase of *S. warneri*, we harvested the culture supernatant after 16 h of growth, at which time a large proportion of the enzyme was in the 90-kDa form. However, we could not purify this prolipase because of its conversion to the 45-kDa form during purification. The same results were observed for *S. epidermidis*, *S. aureus*, and *S. hyicus* lipases [4, 16, 24]. To produce a homogeneous sample of the mature lipase, Van Oort et al. [24] performed a tryptic digestion. The same treatment of crude *S. warneri* lipase also yielded one active protein of molecular mass 45 kDa. This mature enzyme was purified by hydrophobic chromatography as described for other strains of *S. aureus* [8, 9, 22].

The 45-kDa lipase of *S. warneri* has neutral isoelectric points and in this respect differs from the

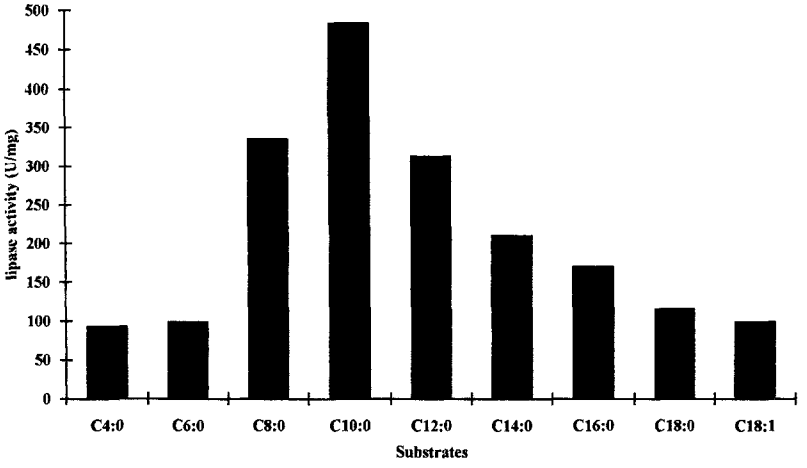


Fig. 7. Substrate specificity of the lipase from *S. warneri*. Activity was measured with the pH-Stat method at 45°C, pH 9.0. $C_{4:0}$ tributyrin; $C_{6:0}$ tricaproin; $C_{8:0}$ tricaprylin; $C_{10:0}$ tricaprin; $C_{12:0}$ trilaurin; $C_{14:0}$ trimyristin; $C_{16:0}$ tripalmitin; $C_{18:0}$ tristearin; $C_{18:1}$ triolein.

Table 2. V_{max} and K_m values for *S. warneri* lipase with triolein, diolein, and monoolein as substrate. V_{max} and K_m values were calculated from double-reciprocal plots

	V_{max} (U/mg)	K_m (mmol/L)
Triolein	1960	1.6
Diolein	1920	1.8
Monoolein	658	1.5

acidic (pH 4.1) or alkaline (pH 9.6, 9.9) isoelectric points of some strains of *S. aureus* [9, 14].

Like most lipases, *S. warneri* has an alkaline pH optimum. Its activity is weak between pH 5.0 and 6.0, the normal pH range of sausages. However, during sausage manufacturing the incubation of enzyme and substrate occurs over at least 1 month, so the lipolysis by *S. warneri* will be significant. Indeed, when *S. warneri* was grown in fatty tissue at pH 6.0 for 1 month, lipolysis was observed [21].

Staphylococcus warneri lipase has a low optimum temperature (25°C) compared with the other lipases of staphylococci. The fact that the lipase of *S. warneri* is very active at relatively low temperatures (15°–25°C) explains the participation of this strain in the lipolysis during sausage ripening [13]. *S. warneri* lipase was inactivated after heating at 70°C for 30 min; but it must be stressed that heating at 100°C for 3 min in a buffer containing SDS and 2-mercaptoethanol did not completely inactivate the enzyme. Heat resistance has also been noticed for *S. aureus*, *S. hyicus*, and *S. epidermidis* lipases [4, 16, 24].

Staphylococcus warneri lipase, typical of extracellular staphylococcal lipases, is characterized by its ability to hydrolyze a wide range of substrates. It hydrolyzes triglycerides esterified with short-chain and long-chain fatty acids, but it preferentially degrades tricaprin (C10). For *S. aureus*, trilaurin (C12) was the preferential substrate [14]. The lipase of *S. warneri* hydrolyzed triolein and diolein emulsions at the same rate, but monoolein micelles at a lower rate. This difference could, however, be due to the different forms of substrate, emulsified or micellar [18].

In contrast to *S. hyicus* [24] and *S. aureus* lipases [23], *S. warneri* lipase was not dependent on calcium for its enzymatic activity. However, this cation, and also manganese, stimulated lipase activity as for some strains of *S. aureus* [14, 17, 22]. The zinc inactivated the lipase of *S. warneri* and *S. aureus* L1 [22]. The mechanism of these ion-specific effects remains to be determined, but Van Oort et al. [24] suggest that they modify the lipase structure rather than act specifically at the catalytic center of the protein.

The active centers of mammalian and microbial lipases consist of the triad, Ser-His-Asp [7]. All these catalytic centers have an active serine. This was also demonstrated for *S. aureus*, *S. epidermidis*, and *S. hyicus* lipases [18, 4, 7]. This was also true for *S. warneri* lipase, because two serine-active reagents led to a complete loss of activity, while a third, PMSF, also reduced activity.

In conclusion, the results obtained on *S. warneri* lipase compare well with those of other staphylococcal lipases. The main difference lies in its high activity between 15°C and 25°C. This property and the stability and activity of the enzyme at low pH show that *S. warneri* does participate in lipolysis in sausage.

Literature Cited

1. Berdagué JL, Monteil P, Montel MC, Talon R (1993) Effects of starter cultures on the formation of flavour compounds in dry sausage. Meat Sci 35:?75–287
2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
3. Carrere J, Serre G, Vincent C, Croute F, Soleilhavoup JP,

Thouvenot JP, Figarella C (1987) Human serum pancreatic lipase and trypsin 1 in aging: enzymatic and immunoenzymatic assays. J Gerontol 42:315–317

4. Farrell AM, Foster TJ, Holland KT (1993) Molecular analysis and expression of the lipase of *Staphylococcus epidermidis*. J Gen Microbiol 139:267–277
5. Götz F, Popp F, Korn E, Schleifer KH (1985) Complete nucleotide sequence of the lipase from *Staphylococcus hyicus* in *Staphylococcus carnosus*. Nucleic Acids Res 13:5895–5906
6. Hedström SA, Nillsson-Ehle P (1983) Trioleoylglycerol lipolysis by *Staphylococcus aureus* strains from recurrent furunculosis, pyomyositis, impetigo and osteomyelitis. Acta Pathol Microbiol Immunol Scand Sect B91:169–173
7. Jäger S, Demleitner G, Götz F (1992) Lipase of *Staphylococcus hyicus*: analysis of the catalytic triad by site-directed mutagenesis. FEMS Microbiol Lett 100:249–254
8. Jürgens D, Huser H (1981) Large-scale purification of staphylococcal lipase by hydrophobic interaction chromatography. J Chromatogr 216:295–301
9. Jürgens D, Huser H, Brunner H, Fehrenbach FJ (1981) Purification and characterization of *Staphylococcus aureus* lipase. FEMS Microbiol Lett 12:195–199
10. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685
11. Lechner M, Märkl H, Götz F (1988) Lipase production of *Staphylococcus carnosus* in a dialysis fermentor. Appl Microbiol Biotechnol 28:345–349
12. Montel MC, Talon R, Cantonnet M, Fournaud J (1992) Identification of *Staphylococcus* from French dry sausage. Lett Appl Microbiol 15:73–77
13. Montel MC, Talon R, Berdagué JL, Cantonnet M (1993) Effects of starter cultures on the biochemical characteristics of French dry sausages. Meat Sci 35:229–240
14. Muraoka T, Ando T, Okudo H (1982) Purification and properties of a novel lipase from *Staphylococcus aureus* 226. J Biochem 92:1933–1939
15. O'Farrell PH (1975) High resolution two dimensional electrophoresis of proteins. J Biol Chem 25:4007–4021
16. Rollof J, Normark S (1992) In vivo processing of *Staphylococcus aureus* lipase. J Bacteriol 174:1844–1847
17. Rollof J, Hedström SA, Nillsson-Ehle P (1987a) Purification and characterization of lipase from *Staphylococcus aureus*. Biochim Biophys Acta 921:364–369
18. Rollof J, Hedström SA, Nillsson-Ehle P (1987b) Positional specificity and substrate preference of purified *Staphylococcus aureus* lipase. Biochim Biophys Acta 921:370–377
19. Sarath G, De la Motte RS, Wagner FW (1989) Protease assay methods. In: Beynon RJ, Bond JS (eds) Proteolytic enzymes, a practical approach. Oxford, New York, Tokyo: IRL Press, pp 25–54
20. Shah DB, Wilson JB (1965) Egg yolk factor of *Staphylococcus aureus*. J Bacteriol 89:949–953
21. Talon R, Montel MC, Gandemer G, Viau M, Cantonnet M (1992) Lipolysis of pork fat by *Staphylococcus warneri*, *S. saprophyticus* and *Micrococcus varians*. Appl Microbiol Biotechnol 38:606–609
22. Tyski S, Hryniewicz W, Jeljaszewicz J (1983) Purification and some properties of the staphylococcal extracellular lipase. Biochim Biophys Acta 749:312–317
23. Vadehra DV (1974) Staphylococcal lipases. Lipids 9:158–165
24. Van Oort MG, Deveer AMTJ, Dijkman R, Tjeenk ML, Verheij HM, De Haas GH, Wenzig E, Götz F (1989) Purification and substrate specificity of *Staphylococcus hyicus* lipase. Biochemistry 28:9278–9285
25. Vogel RF, Gaier W, Hammes WP (1990) Expression of the lipase gene from *Staphylococcus hyicus* in *Lactobacillus curvatus* Lc2-c. FEMS Microbiol Lett 69:289–292