

Purification and Partial Characterization of Psychrotrophic *Serratia marcescens* Lipase

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ABSTRACT

Serratia marcescens isolated from raw milk was found to produce extracellular lipase. The growth of this organism could contribute to flavor defects in milk and dairy products. *Serratia marcescens* was streaked onto spirit blue agar medium, and lipolytic activity was detected after 6 h at 30°C and after 12 h at 6°C. The extracellular crude lipase was collected after inoculation of the organism into nutrient broth and then into skim milk. The crude lipase was purified to homogeneity by ion-exchange chromatography and gel filtration. The purified lipase had a final recovered activity of 45.42%. Its molecular mass was estimated by SDS-PAGE assay to be 52 kDa. The purified lipase was characterized; the optimum pH was likely between 8 and 9 and showed about 70% of its activity at pH 6.6. The enzyme was very stable at pH 8 and lost about 30% of its activity after holding for 24 h at 4°C in buffer of pH 6.6. The optimum temperature was observed at 37°C and exhibited high activity at 5°C. The thermal inactivation of *S. marcescens* lipase was more obvious at 80°C; it retained about 15% of its original activity at 80°C and was completely inactivated after heating at 90°C for 5 min. Under optimum conditions, activity of the enzyme was maximum after 6 min. The Michaelis-Menten constant was 1.35 mM on tributyrin. The enzyme was inhibited by a concentration more than 6.25 mM. Purified lipase was not as heat-stable as other lipases from psychrotrophs, but it retained high activity at 5°C. At pH 6.6, the pH of milk, purified lipase showed some activity and stability. Also, the organism demonstrated lipolytic activity at 6°C after 12 h. Therefore, *S. marcescens* and its lipase were considered to cause flavor impairment during cold storage of milk and dairy products.

(**Key words:** lipolytic activity, lipase enzyme, psychrotroph, *Serratia marcescens*)

INTRODUCTION

Psychrotrophic microorganisms in milk are important because they cause spoilage by altering the constituents of milk (Cousin, 1982). During growth at low temperature, many strains produce lipases and proteases that may adversely influence the quality and shelf life of most dairy products (Griffiths et al., 1981; Fox and Stepianiak, 1983; Stepianiak and Fox, 1985; Fairbairn and Law, 1986). The most frequently encountered psychrotrophs are the gram-negative genera, and *Pseudomonas fluorescens* is considered to be the most common species (Mikolajcik, 1979; Cousin, 1982).

Lipases from psychrotrophs, even at low concentration, can hydrolyze milk lipid and cause the rancid flavor in milk and dairy products that make these foods unacceptable to consumers (Deeth and Fitzgerald, 1976; Downey, 1980; Cousin, 1982; Shah, 1994). Extracellular lipases produced by psychrotrophs can withstand heat treatment of milk (Garcia et al., 1989) and may cause rancidity in UHT milk products during storage (Adams et al., 1975). Lipase production by psychrotrophs varies with the species, as does the optimum temperature, optimum pH, and enzyme specificity (Thomas and Thomas, 1973). Reports that describe purification and characterization are focused mainly on enzymes secreted by *Pseudomonas* species. Relatively few studies deal with enzymes secreted by other genera (Alichanidis, 1988).

Ray (1996) described *Serratia* species as gram-negative psychrotrophs that were able to grow and produce heat-stable extracellular lipase and protease during the refrigerated storage of foods. The early literature reported that *S. marcescens* has been isolated as a psychrophilic microorganism from raw milk (Thomas, 1958; Witter, 1961). Recently, some workers have isolated psychrotrophs from refrigerated raw milk samples; about 3 to 6% of the isolated strains were *S. marcescens* (Milliere and Veillet-Poncet, 1985; Ahmed et al., 1989; Abdou, 1997). *Serratia marcescens* was found to

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have lipolytic and proteolytic activities (Abdou and Ohashi, 1996).

Many reviews have described in some detail the isolation and properties of lipases from psychrotrophs; however, few studies have dealt with the factors affecting lipase production. Henriette et al. (1993) studied the effect of culture conditions on lipolytic production by *S. marcescens*; at 22°C the microorganism grew exponentially for approximately 6 h before entering the stationary growth phase, and the cells excreted their lipases during the stationary phase. Makhzoum et al. (1995) showed that lipase production began after about 5 to 10 h at 27°C.

Information on extracellular lipase produced by *Serratia* species is particularly limited, although some strains have been isolated from raw milk. Therefore, the objectives in this study were to focus on the lipolytic activity of *S. marcescens* and the purification and partial characterization of its extracellular lipase.

MATERIALS AND METHODS

Organism

During our study of psychrotrophic microorganisms (Abdou and Ohashi, 1996), psychrotrophic *S. marcescens* was originally isolated from raw milk, maintained on nutrient agar slope at 25°C, and stored at 4°C.

Lipolytic Activity on Media

Serratia marcescens was streaked onto spirit blue agar medium and lipase reagent (Difco Laboratories, Detroit, MI), and the plates were incubated at 6, 17, and 30°C for up to 14 d. The plates were observed after 6 h and every 12 h for the clearing of the blue or deep blue color around each streak (Marshall, 1992). Lipolytic activities of *S. marcescens*, at different degrees of temperature, were compared by measuring the width (millimeters) of areas of clearing or areas of deep blue color around the colonies.

Preparation of Crude Enzyme

Serratia marcescens was subcultured on nutrient agar slopes and then in nutrient broth at 30°C for 24 h. Aliquots of 150 μ l were inoculated into 15-ml portions of sterile 10% reconstituted skim milk (Kumura et al., 1991) and incubated at 30°C for 3 d, followed by incubation at 6°C for another 3 d. These portions were centrifuged at 20,000 $\times g$ at 4°C for 30 min. The supernatants were filtered using 0.45- μ m cellulose acetate filter units (Toyo Roshi Kaisha, Ltd., Japan). The filtrates were used as the crude enzyme in the following experiments.

Measurement of Lipase Activity

Activities of the crude lipase and chromatographic fractions were determined by the method of Saito (1979), except that the substrate was tributyrin emulsion (Fox and Stepaniak, 1983) composed of 2% gum arabic, 0.4 M NaCl, and 5 mM CaCl₂ blended for 2 min at 50°C with the appropriate concentration of tributyrin in the suitable buffer. A quantity (0.3 ml) of the enzyme preparation was added to 2 ml of the substrate solution in glass-stoppered test tubes that had been preincubated in a water bath at 37°C for 6 min at pH 8. The mixture was held for 10 min at 37°C. The enzymatic reaction was terminated by adding 7.5 ml of the extraction reagent (isopropane, 100 ml; heptane, 100 ml; 1 N H₂SO₄, 8 ml). After vigorous shaking for 2 min and standing for 1 h, the upper layer obtained was mixed with the color reagent (water 1 ml, phenol red 5 mg, Na-barbital 25 mg, ethanol 200 ml), and shaken for 30 s. The absorbance was measured at the wavelength of 420 nm (double-beam spectrophotometer UV-190^o, Shimadzu Corporation, Kyoto, Japan). Optimum assay conditions (activity calculated as 100% activity) were 2 ml of 2% tributyrin emulsion mixed with 0.3 ml of lipase preparation maintained at 37°C in buffer of pH 8 for 6 min. One lipase unit activity was defined as the μ moles of liberated free fatty acid/min at 37°C. Palmitic acid was used as a standard fatty acid. The results obtained were means of triplicates for each experiment.

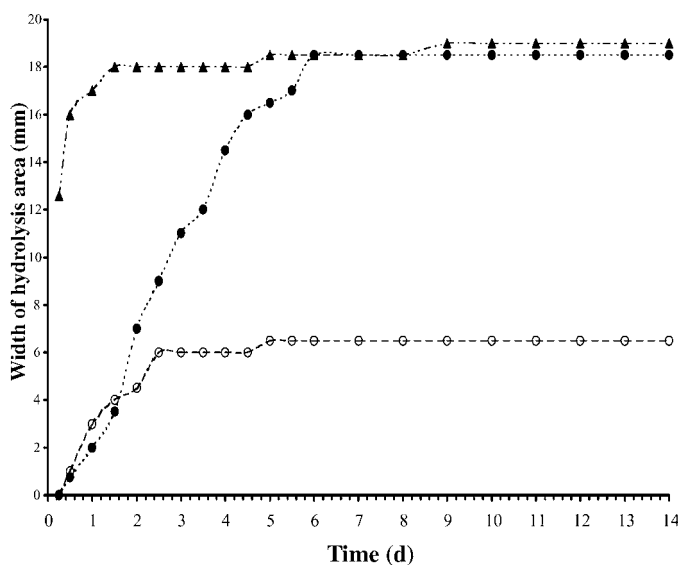


Figure 1. Lipolytic activities of *Serratia marcescens* at different temperatures on spirit blue agar media (Difco Laboratories, Detroit, MI). Width of hydrolysis area (mm) at 6°C (●), at 17°C (○), and at 30°C (▲).

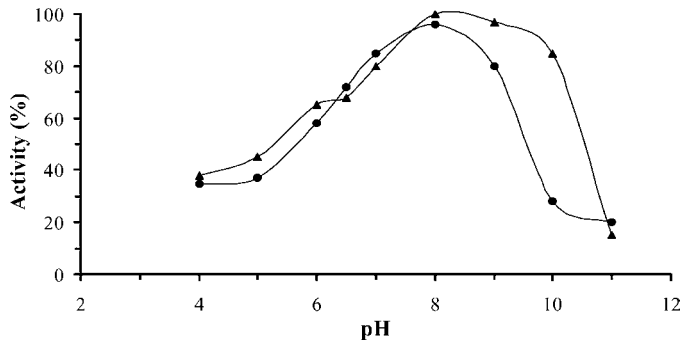


Figure 2. Effect of pH on *Serratia marcescens* purified lipase. Optimum pH for lipase on 2% tributyrin in universal buffers at pH 4 to 11 at 37°C (▲). Stability of lipase held for 24 h in universal buffers pH 4 to 11 at 4°C (●)

Measurement of Protein Concentration

The protein concentration was determined by the method of Bradford (1976) using BSA (Sigma Chemical Co., St. Louis, MO) as the standard.

Purification Procedures

Step 1: CM-cellulose chromatography. The crude enzyme was first applied to a column (20 × 2.5 cm) of CM-cellulose (Sigma Chemical Co.), which was pre-equilibrated with 10 mM sodium phosphate buffer pH 7.5. The lipase was allowed to bind to the gel for 2 h at 4°C and was eluted with a linear gradient of Triton X-100 (0 to 1%; 200 ml). The flow rate was 60 ml/h, and fractions of 5 ml were collected.

Step 2: DEAE-cellulose chromatography. The lipase-rich fractions were pooled and applied to a column (15 × 1.5 cm) of DEAE-cellulose (Sigma Chemical Co.), which was pre-equilibrated with 10 mM sodium phosphate buffer at pH 7.5. The lipase was allowed to bind to the gel for 1 h at 4°C. The column was washed thoroughly with the same buffer to remove unabsorbed material, which included Triton X-100 and was eluted with a linear gradient of NaCl (0.05 to 1 M; 300 ml) in the same buffer at a flow rate of 40 ml/h. The eluate was collected in 5-ml fractions.

Step 3: gel filtration. The concentrate was then applied to a column (100 × 2.5 cm) of Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden), which was pre-equilibrated with 10 mM sodium phosphate buffer pH 7.5 and eluted with the same buffer. The lipase-rich fractions were pooled, stored at 2°C, and used as the purified lipase.

Characterization of the Purified Lipase

Optimum pH. Tributyrin emulsions (Fox and Stepaniak, 1983) were prepared in universal buffers (Stauffer,

1989) covering the pH range 4 to 11. The enzyme was diluted in the buffers and assayed separately in each substrate emulsion of the same pH.

pH stability. Portions of the purified lipase were held at 4°C for 24 h in universal buffers for use at pH 4 to 11. The described assay was carried out for each lipase portion to obtain its stability.

Optimum temperature. Lipase activity of the enzyme was assayed at temperatures ranging from 5 to 75°C. The substrate emulsions were held at the respective temperature for 5 min before the addition of the enzyme.

Thermal stability. Portions of the purified lipase were held at temperatures ranging from 50 to 100°C for 5 min in culture tubes. Heating time did not include the time to attain the required temperature. The assay was carried out for each treatment to obtain lipase stability.

Optimum reaction time. Lipase activity of the enzyme was assayed under optimum reaction conditions at incubation times ranging from 2 to 20 min.

Enzyme kinetics. The purified lipase was incubated with various concentrations of tributyrin substrate, and the final substrate concentration ranged from 0.2 to 15 mM. The Michaelis-Menten constant was calculated by the double reciprocal plot of Lineweaver and Burk (1934).

Molecular mass. The molecular mass of the purified lipase was estimated by SDS-PAGE assay. Phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa) were used as molecular mass markers (Bio-Rad Laboratories, Richmond, CA). The SDS-PAGE assay was conducted according to the procedure of Laemmli (1970). Samples were loaded on a 10% polyacrylamide gel containing 0.1% SDS with stacking gel. Electrophoresis was performed at 30 mA constant cur-

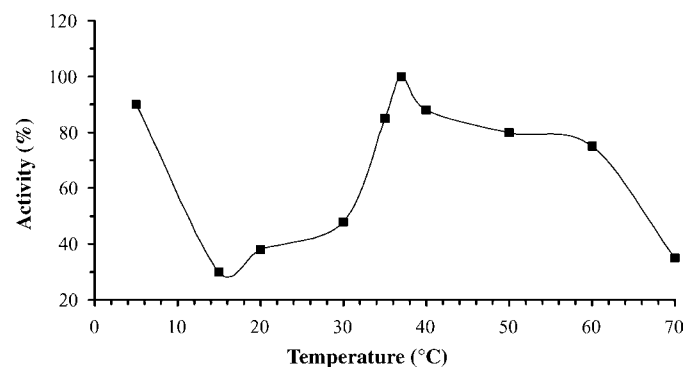


Figure 3. Effect of temperature on the activity of *Serratia marcescens* purified lipase on 2% tributyrin at pH 8.

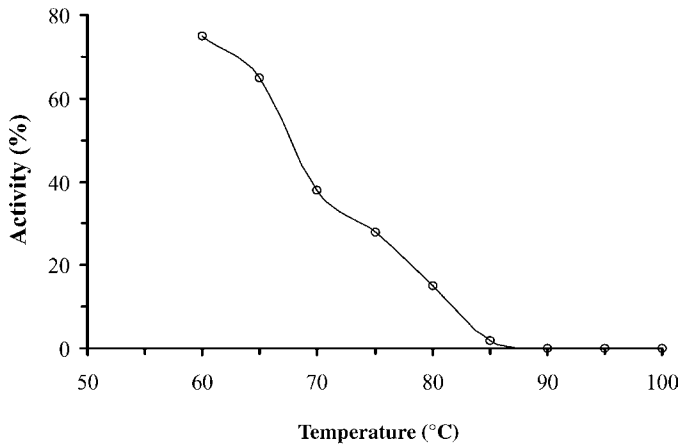


Figure 4. Heat stability curve for *Serratia marcescens* purified lipase, held at temperature range from 50 to 100 °C for 5 min.

rent. Gels were stained with silver stain (Wako Pure Chemical Industries, Ltd., Japan).

RESULTS AND DISCUSSION

Lipolytic Activity of *Serratia marcescens* on Medium

The widths of hydrolysis areas were measured to compare the lipolytic activities of *S. marcescens* at different temperatures. At 30°C, the lipolytic activity of *S. marcescens* appeared after 6 h with a large discoloration area and the widest area observed after 1.5 d (Figure 1). At 6°C, the area of discoloration appeared after 12 h and increased in width very rapidly and showed its widest area after 6 d. At 17°C, the activity began after 12 h, but the areas of hydrolysis were smaller than that at 30°C and 6°C. *Serratia marcescens* showed apparent low activity at 17°C. The higher activity at 30°C may

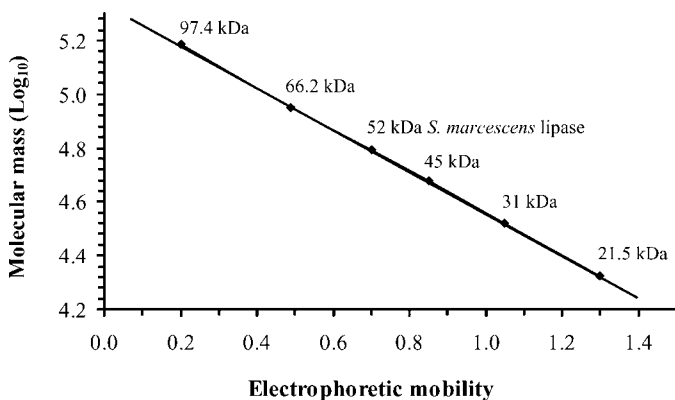


Figure 5. Molecular mass estimation of *Serratia marcescens* purified lipase by using SDS-PAGE assay.

be attributed to maximum growth of the organism and the subsequent increased lipase secretion as it was observed by Makhzoum et al. (1995) during their study of lipase production by *P. fluorescens*. On the other hand, Herbert (1981) reported that many psychrotrophs compensate for growth at low temperature by synthesizing increased quantities of enzymes; Stead (1986) found that lipase production was greatest at 8°C for many *P. fluorescens* strains and concluded that increasing the temperature above 8°C had a depressing effect on lipase production. *Serratia marcescens* may have the same pattern of increasing synthesis of lipase at low temperature and depressing production above 8°C, thus showing activity at 6°C higher than that at 17°C.

Purification of Lipase

A representative purification profile is summarized in Table 1. On the CM-cellulose column, the highest lipase activity was eluted very early in fractions 6 to 20; specific activity was increased 3.06-fold, and about 93% of its activity was recovered. Active fractions were applied to the DEAE-cellulose, and the lipase activity was highest between 0.23 and 0.35 M NaCl in fractions 21 to 32. Active fractions, after concentration, were further purified by gel filtration. The purified enzyme was increased by about 20-fold, and recovery was about 45%. The purified lipase showed only one protein band on SDS-PAGE.

Characterization of the Purified Lipase

Optimum pH. The optimum pH was likely between 8 and 9. The pH activity curve (Figure 2) showed that the enzyme reached about 73% of its maximum activity at pH 6.6. The purified lipase from *Serratia grimsii* exhibited an optimum pH over the range 8 to 9 (Abdou, 1997), while that from *S. marcescens* Sr41 8000 showed maximum activity at about pH 8 (Matsumae and Shibata, 1994). Some previous investigators (Landaas and Solberg, 1978; Adams and Brawley, 1981; Fox and Stepaniak, 1983) showed that the optimum pH for lipases from different psychrotrophs was 8, but others (Driessen and Stadhouders, 1974; Law et al., 1976) showed that the pH optima were 6.5 and 8.75.

pH stability. The purified lipase showed maximal stability at pH 8 (Figure 2). Under pH 6.6, this lipase lost about 30% of its activity after holding at 4°C for 24 h. *Serratia marcescens* Sr41 8000 lipase was stable between pH 6 to 9 (Matsumae and Shitani, 1994); also, that of *S. grimsii* was stable over the pH range of 7 to 9 (Abdou, 1997). Other lipases showed stability within the pH range of 5.5 to 9 (Fox and Stepaniak, 1983; Kumura et al., 1993).

Table 1. Purification of the extracellular lipase from *Serratia marcescens*.

| Purification step | Total activity (LU) ¹ | Specific activity (LU/mg) ² | Recovered activity (%) | Purity index |
|-------------------|----------------------------------|--|------------------------|--------------|
| The crude lipase | 380.6 | 0.107 | 100 | 1 |
| CM-Cellulose | 355.4 | 0.166 | 93.38 | 3.06 |
| DEAE-Cellulose | 192.4 | 1.25 | 50.55 | 12.44 |
| Sephadex G-150 | 172.9 | 1.88 | 45.42 | 20.88 |

¹Lipase unit.² Lipase unit/mg protein.

Optimum temperature. The effect of temperature on the lipase activity (Figure 3) showed that optimum temperature for the enzyme was 37°C, and about 90% of its maximum activity occurred at 5°C. That of *S. grimisii* exhibited almost the same activity at low temperature (Abdou, 1997). This high activity at low temperature was observed by some workers; Fox and Stepanski (1984) found that purified lipase of *P. fluorescens* strain AFT 36 exhibited about 15% of its maximum activity at 4°C, Stepanski et al. (1987) reported that *P. fluorescens* P1 lipase showed about 30% at 4°C and declared that high activities at low temperatures have been found typical characteristics for many lipases from psychrotrophs. Roy (1981) suggested that *S. marcescens* and *P. fluorescens* lipases are probably more stable at low temperatures.

Fox and Stepanski (1983) concluded that the stability of lipase enzyme for some psychrotrophs over a wide pH range coupled with its high activity at low temperatures suggest that the enzyme would remain stable for long periods in all dairy products and could have serious implications during their cold storage.

Thermal stability. The lipase of *S. marcescens* showed some stability (Figure 4) at 65°C, but the least residual activity was at 85°C. It was completely inactivated at 90°C. *S. marcescens* lipase is not susceptible to low heat inactivation and is not as thermostable as other lipases from psychrotrophs.

Optimum reaction time. The enzyme reached its maximum activity after 6 min of incubation under optimum reaction conditions (data are not shown).

Enzyme kinetics. Using the double reciprocal plot of Lineweaver and Burk (1934), the enzyme had Michaelis-Menten constant on trybutyrin of 1.35 mM. The enzyme was inhibited by a concentration more than 6.25 mM.

Molecular mass. The molecular mass was estimated to be 52 kDa (Figure 5). Matsumae and Shibatani (1994) found that the molecular mass for *S. marcescens* Sr41 8000 lipase was 62 ± 2 kDa, while that of *S. grimisii* was 57 kDa (Abdou, 1997). Lipases from *P. fluorescens* had a molecular mass of 55, 52, 32 kDa (Bozoglu, 1984; Kumura et al., 1993; Stepanski and Fox, 1985).

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