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Isolation of a phospholipase A_1-producing microorganism

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A bacterium which has phospholipase A_1 activity was isolated from soil. It is aerobic, motile, oxidase-negative and has flagella. The G + C content of the DNA was 58.1 mol%. The major isoprenoids of cell wall were Q-8 and MK-8. The main cellular fatty acids were saturated straight chain (*n*-16) and cyclic (17:Δ) fatty acids. Based on its morphological, physiological and chemotaxonomic characteristics, this organism was placed in the genus *Serratia*. Nutritional factors affecting enzyme production were explored. Xylose and ammonium sulfate were the best carbon and nitrogen sources, respectively. Ferrous ions exerted a considerable positive effect on enzyme production. The optimal pH and temperature for phospholipase A_1 production were 7.0 and 30°C, respectively.

Keywords: screening; *Serratia*; phospholipase A_1; positional specificity; nutritional factor

Introduction

Emulsifiers that are more hydrophilic than those used at present are needed in the food industry. Lyso-phospholipids such as monoacyl-*sn*-glycero-3-phosphocholine satisfy this requirement. Lyso-phospholipids also disorganize membrane structure, cause reduction in the activity of membrane enzymes and induce cell autolysis by solubilizing bacterial membranes [4,17,19]. Thus, it is probable that lyso-phospholipids can be used not only as emulsifiers but also as food preservatives.

Enzymatic production of lyso-phospholipids has been studied using lipase, which also degrades phospholipids [2,3]. Even though lipase catalyzes hydrolysis of phospholipids and is available on a large scale, the reaction rate is low and site specificity is undesirable compared to that of phospholipase A_1 and A_2 [10].

It is therefore desirable to use phospholipase A_1 and/or A_2 for production of lyso-phospholipid. Numerous phospholipases of the A_1 and A_2 type have been identified and their abilities to hydrolyze the fatty acyl ester bonds of phospholipids have been characterized. Phospholipase A_1 and A_2 hydrolyze phospholipid to lyso-phospholipid and fatty acid at the *sn-1* and *sn-2* positions, respectively [1,11,13]. Although some phospholipase A_1 has been found in microorganisms, it was mostly membrane bound [13]: a limiting factor for large scale enzyme production. Phospholipase A_1 and A_2 are obtained primarily from snake venom or porcine pancreas and are thus not readily or commercially available in the amounts required for large scale use. In an attempt to screen for microorganisms which produce phospholipase A_1 and A_2, we succeeded in isolating only one phospholipase A_1 producer. In this paper, we describe isolation of the phospholipase A_1-producing microorganism and the nutritional factors affecting enzyme production.

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Materials and methods

Materials

Soybean lecithin was kindly supplied by Nattermann GmBH, Köln, Germany. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine which was used as a substrate in the gas chromatrographic method and 60% egg yolk lecithin used as a substrate in the pH titration method were purchased from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade.

Medium

Screening medium was composed of 1 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$ and 15 g agar per 1 L distilled water. This medium was autoclaved and cooled to about 60°C. Then 10 g of soy bean lecithin and 100 mM $CaCl_2$ solution (autoclaved separately) and 5 g (0.5% w/v) of taurocholic acid were added. This mixture was emulsified by vigorous stirring for 5 min in a Waring blender (Model 32BL79, Dynamic Co, New Hartford, CT, USA). The medium was allowed to stand for 5 min to reduce foam and 20 ml was poured into each Petri dish. Phospholipase A_1 formed a transparent halo on this screening medium [5].

To produce phospholipase, a slightly modified M9 minimal salt medium was used. The basic components of M9 minimal salt medium were: 15.12 g $NaHPO_4 \cdot 12H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 0.246 g $MgSO_4 \cdot 7H_2O$ and 0.0147 g $CaCl_2 \cdot 2H_2O$ per 1 L distilled water. To study the effects of nutrients on phospholipase production, various carbon and nitrogen sources and trace elements were added to the basal medium.

Isolation of phospholipase A-producing microorganisms

Soil samples from sewage sludge, rivers and mountains in Korea were enriched aerobically at 30°C in the complex nutrient broth medium for 12 h. The culture broth was then diluted and spread on a screening medium. After 24 h at 30°C, the agar plates were examined. One loopful of cells which showed phospholipase activity on the screening

medium was inoculated into a 40-ml test tube containing 10 ml nutrient broth medium and incubated aerobically at 30°C for 12 h with shaking. Cells were harvested at the stationary phase by centrifugation at 5600 × *g* for 20 min. The enzyme activity in the supernatant medium was assayed by a pH titration method [18] as described below.

pH titration method

Phospholipase activity was assayed by titrating free fatty acids released by hydrolysis of phospholipid with 10 mM NaOH using a Fisher titration set (pH meter, titrate and burette/dispenser, Fisher, Pittsburgh, PA, USA). Phospholipid emulsion was prepared by emulsifying 2.5 g phospholipid in 100 ml water for 5 min at a maximum speed in a Waring blender. One milliliter of 300 mM $CaCl_2$ and 78 mM sodium deoxycholate solution were then added to 28 ml of phospholipid emulsion solution. Reaction temperature and pH were 50°C and pH 8.0, respectively. One unit of phospholipase activity was defined as the amount of enzyme which liberated 1 μmol fatty acid per min under the assay conditions.

Determination of positional specificity of phospholipase A

To determine the positional specificity of phospholipase A, we used the GC method described in our previous paper, using synthetic phospholipid, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, as a substrate [5]. Fatty acids in the reaction mixture were analyzed without derivatization by the method of Kwon *et al* [7].

Identification of organism

Routine tests for identification of the organism were performed according to the 'Manual of Methods for General Bacteriology' [14]. API 20E and VITEK systems (bioMèrieuk SA, Lyon, France) were used for physiological characters. The DNA base composition was determined by reversed-phase HPLC by the method of Tamaoka and Komagata [16]. Fatty acid and isoprenoid quinone composition were analyzed as described by Suzuki and Komagata [15] and Kroppenstedt [6], respectively.

Results

Isolation of phospholipase A_1-producing Serratia sp MK1

Out of approximately 500 colonies grown on screening agar plates at 30°C, 38 strains had phospholipase A activity as shown by a transparent halo around the colonies. Using these selected strains, phospholipase activities in culture medium supernatant were further evaluated for their ties to hydrolyze phospholipid and for their positional specificities. As a result, one bacterium, which showed the highest lipolytic activity on phospholipid, was chosen by the pH titration method using phospholipid emulsion as a substrate. To identify the positional specificity between phospholipase A_1 and A_2, we used 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine as a substrate and the reaction was traced by gas chromatographic analysis. Phospholipase from the isolated strain liberated palmitic acid preferentially (Figure 1). Phospholipase A_1 and phospholipase A_2 preferentially produce palmitic and linoleic acids, respectively from this substrate. The positional specificity of the phospholipase from the isolated strain was therefore concluded to be A_1.

To further identify the isolated strain, its morphological and physiological characteristics were studied. They were as follows: gram-negative, motile, oxidase-negative, tryptophanase-negative. The G + C content was 58.1 mol% by analysis of the nucleotides in the P1 digest. The major isoprenoids were Q-8 and MK-8. This strain contained mainly saturated (*n*-16), mono-unsaturated (16:1) straight chain and cyclic (17:Δ) fatty acids in the cells. Cells were short rods about 0.9 μm × 2.3 μm. Considering these characteristics, the isolate was identified as *Serratia* sp according to Bergey's Manual of Systematic Bacteriology and named *Serratia* sp MK1.

Production of phospholipase A_1 from the isolated strain

The composition of the growth medium is an important factor in the rate of enzyme production by a microorganism. Previous reports indicate that each type of microorganism may require a different medium to produce enzyme at its maximum level [9,20]. In our studies with *Serratia* sp a variety of carbon, nitrogen and trace elements were tried in an effort to increase phospholipase A_1 activity. The effect of carbon sources on the production of phospholipase A_1 is presented in Table 1. Xylose produced the highest lipolytic activity among the carbon sources tested. Statistical analysis of the results showed that there was a significant difference in the level of enzyme activity when sucrose or xylose was used as a carbon source, but they showed similar growth regardless of the carbon source. The optimum xylose concentration to produce phospholipase A_1 was 5 g L^{-1} (data not shown). We also tested the effects of

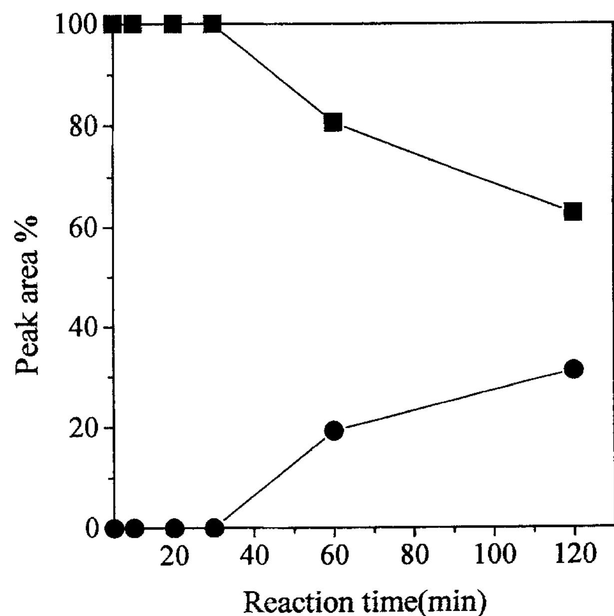

Figure 1 Time course for hydrolysis of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine: % of palmitic acid (■), and linoleic acid (●) among the liberated total free fatty acids.

Table 1 Effect of different carbon sources on phospholipase A_1 production by *Serratia* sp MK1

Carbon sources[a,b]	Enzyme activity (units ml^{-1})[c]	Biomass (g L^{-1})
Mannitol	11.4 ± 0.2	1.34
Sucrose	1.2 ± 0.1	1.46
Glycerol	10.3 ± 0.4	1.28
Fructose	15.0 ± 0.4	1.43
Glucose	1.4 ± 0.3	1.34
Arabinose	14.9 ± 0.8	1.4
Xylose	16.5 ± 0.5	1.43
Lactose	3.7 ± 0.3	1.63
Galactose	14.1 ± 1.2	1.4

[a]5 g L^{-1} added.
[b]The culture medium was M9 minimal salt medium. Nitrogen source was $(NH_4)_2HPO_4$ and the culture conditions were pH 7.0 and 30°C.
[c]Maximum phospholipase activity was shown at late exponential growth phase.

phospholipid, fatty acid, triglyceride and non-ionic surfactant on phospholipase A_1 production. These materials showed negligible effects on phospholipase A_1 production (data not shown).

Various organic and inorganic nitrogen sources were also tested. Although organic nitrogen sources were good for cell growth, phospholipase activity was very low. Among the inorganic nitrogen sources tested, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ showed similar high phospholipase activities (Table 2).

The effects of trace elements on the production of phospholipase A_1 were studied (Table 3). All components of the trace element mixture had a positive effect on phospholipase production. $FeSO_4$ especially enhanced the production of phospholipase. The optimum concentration of $FeSO_4$ was 1 μM (data not shown).

In addition to culture medium, physical parameters such as incubation temperature and initial medium pH also affected phospholipase production. As shown in Figure 2, the optimum growth temperature and pH for phospholipase production were 30°C and pH 7, respectively. Finally, the comparative behavior of phospholipase A_1 production by

Table 2 Effect of different nitrogen sources on phospholipase A_1 production by *Serratia* sp MK1

Nitrogen source[a,b]	Enzyme activity (units ml^{-1})[c]	Biomass (g L^{-1})
NH_4Cl	15.4 ± 0.22	1.35
$(NH_4)_2SO_4$	17.0 ± 0.71	1.4
$(NH_4)_2HPO_3$	16.1 ± 0.98	1.36
NH_4NO_3	11.0 ± 1.12	0.91
Peptone	9.7 ± 0.56	1.67
Yeast extract	4.7 ± 0.23	2.05
Tryptone	11.0 ± 0.43	2.21
Casitone	8.0 ± 0.87	2.09

[a]NH_4Cl (1 g L^{-1}) equivalent nitrogen was added and in the case of organic nitrogen sources, 5 g L^{-1} of each organic nitrogen source were used.
[b]The culture conditions were the same as Table 1. Fructose (5 g L^{-1}) was used as a carbon source.
[c]Maximum phospholipase activity was shown at late exponential growth phase.

Table 3 Effect of trace elements on phospholipase A_1 production by *Serratia* sp MK1

Addition[a]	Enzyme activity (units ml^{-1})[b]	Biomass (g L^{-1})
None	17.0	1.35
$FeSO_4$	31.6	1.51
$ZnSO_4$	17.0	1.37
$MnSO_4$	22.3	1.57
$CuSO_4$	20.2	2.42
$Co(NO_3)_2$	23.6	1.55
H_3BO_3	9.0	1.37

[a]Each metal ion was added at 1 μM concentration.
[b]The culture medium was M9 minimal salt medium. Xylose and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources, respectively. Culture conditions were 30°C and pH 7.0.

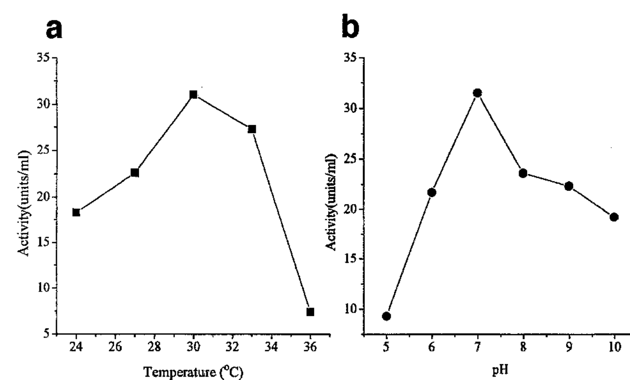

Figure 2 Effect of cultivation temperature (a) and initial medium pH (b) on phospholipase A_1 activity. The culture medium was M9 minimal salt medium. Xylose and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources, respectively. Culture pH was 7.0 in (a) and culture temperature was 30°C in (b).

Serratia sp MK1 in an Erlenmeyer flask was studied using the initial complex medium (nutrient broth) and optimized medium. Maximum extracellular phospholipase A_1 production was reached at the late exponential growth and early stationary phase (Figure 3). Using modified M9 minimal salt medium, 31 units ml^{-1} of enzyme activity was obtained. This was 7.5 times higher than the 4 units ml^{-1} found in the initial complex medium. A further increase could probably be obtained by the use of experimental designs that allow the study of interactions among the medium constituents, as well as more profound studies of regulation.

Discussion

Serratia sp MK1 produced phospholipase A_1 capable of hydrolysis of the *sn-1* ester bond in phospholipids. Linoleic acid liberation also commenced after 1 h (Figure 1). This resulted from the acyl migration; 2-linoleoyl-*sn*-glycero-3-phosphocholine which was produced by the action of phospholipase A_1, was converted into 1-linoleoyl-*sn*-glycero-3-phosphocholine (data not shown). The 1-linoleoyl-*sn*-glycero-3-phosphocholine thus produced was, in turn, hydrolyzed by the phospholipase A_1 [10].

Phospholipase A_1 production by *Serratia* sp MK1 was

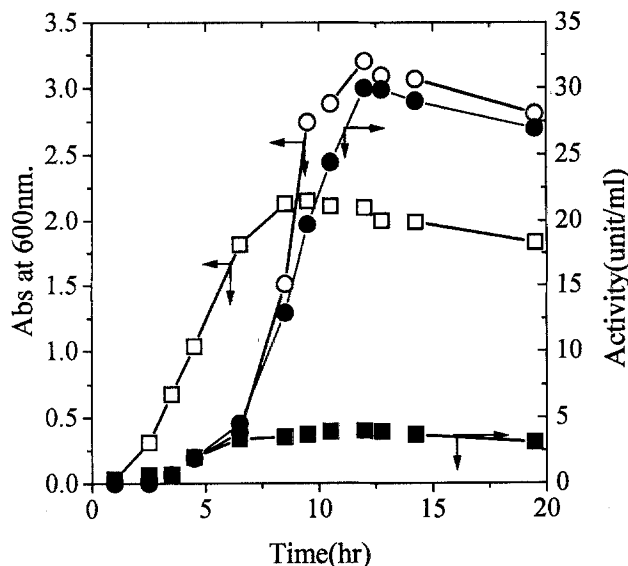

Figure 3 Comparative kinetics of phospholipase production by *Serratia* sp MK1. Cell density in initial complex (□) and optimized (○) media. Phospholipase A_1 activity in initial complex (■) and optimized (●) media. The media compositions are as follows (per liter): nutrient broth 8 g, in the case of initial complex medium and $Na_2HPO_4 \cdot 12H_2O$ 15.12 g; KH_2PO_4 3 g; NaCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.246 g; $CaCl_2$ 0.0147 g; $FeSO_4$ 500 μg; $(NH_4)_2HPO_4$ 1.143 g; Xylose 5 g, in the case of optimized medium.

enhanced by optimization of the medium as well as the physical (pH, temperature) parameters of the fermentation process. These optimized parameters resulted in an increase in phospholipase A_1 activity by 7.5 times over the initial complex medium.

As expected, phospholipase A_1 production by *Serratia* sp MK1 was influenced by components of the culture medium. With respect to carbon source, our strain was different from other organisms in its utilization of carbon sources. Glucose [8,20] or sucrose [12] have been found suitable for many strains to produce lipase and protease. However, we found that xylose was the best carbon source for the organism isolated in this study.

$(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ were better nitrogen sources for production of phospholipase A_1 for our strain. Pal *et al* showed that there is large difference of enzyme activity when different salts of NH_4 are used as nitrogen sources [9]. Henriette *et al* found casamino acids were the best nitrogen source for protease and lipase synthesis by *Serratia marcescens* [20].

The effect of ferrous ions on phospholipase A_1 production by our strain was very interesting. Although growth remained almost the same as without ferrous iron, phospholipase A_1 activity increased considerably. Pal *et al* reported that ferrous ions had an accelerating effect on the lipase yield [9]. However, the role of ferrous ions in increasing enzyme activity was not clear. Studies of the role of ferrous ions in increasing enzyme activity are under way.

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