Influence of growth conditions on heat-stable phospholipase activity in *Pseudomonas*

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Summary. Many psychrotrophic bacteria contaminating raw milk produce phospholipase that withstands pasteurization and UHT treatments. This enzyme acts on the milk fat globule membrane and exposes triacylglycerides to the action of lipase. Phospholipase production by various isolates of *Pseudomonas* was investigated. The isolates were cultured aerobically at 8 °C in nutrient broth, McKellar's minimal salts medium, Chrisope's medium, and skim milk. Each strain produced phospholipase during the 50 h incubation. Enzyme production varied significantly ($P < 0{\cdot}001$) with strain and growth medium. Strains varied significantly ($P < 0{\cdot}001$) in their enzyme production in each medium and during the incubation time as well. Strain, incubation time, and the growth medium significantly influenced ($P < 0{\cdot}001$) heat stability of the enzyme activity. Pasteurization reduced the activity, but did not eliminate it in skim milk.

Keywords: Phospholipase, *Pseudomonas*, lipolysis, raw milk, growth media.

Phospholipase produced by spoilage organisms associated with milk is the least studied extracellular enzyme with regard to the spoilage of dairy products (Doi & Nojima, 1971; Fox *et al.* 1976; Cousin, 1982; Griffiths & Phillips, 1984; Muir, 1996). Phospholipases (EC 3.1.4.3) (phosphatidylcholine choline-phosphohydrolases) act on phospholipids and hydrolyse these to diglycerides and substituted phosphoric acid. They are often members of the phospholipase C (PLC) family (Brokerhoff & Jensen, 1974). Phospholipase isolated from *Pseudomonas fluorescens* increases lipolysis of raw milk at 30 °C (Doi & Nojima, 1971). The rate of initial lipolysis increases in washed cream incubated with lipase in the presence of both proteinase and PLC of microbial origin at pH 6·6 and 37 °C (Alkanhal *et al.* 1985).

PLC disrupts the integrity of the milk fat globule membrane (MFGM) and exposes the fat to degradation by lipase (Chrisope, 1975; Chrisope & Marshall, 1976; Alkanhal *et al.* 1985) resulting in physical degradation of the emulsion in milk (Craven & McCauley, 1992). Damage to the MFGM is important in long shelf-life products, such as whipping cream and ice cream, where functional and sensory attributes are affected (Barkworth, 1958). Additionally, PLC activity is responsible for sweet curdling, bittiness, and feathering in milk (Barkworth, 1958; Owens, 1978).

Milk does not contain native phospholipase activity (Owens, 1978; Muir, 1996). PLC activity in milk is commonly contributed by psychrotrophic bacteria during

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storage (Owens, 1978). It is produced by many genera of bacteria found in milk, including *Pseudomonas*, *Bacillus*, *Clostridium*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Flavobacterium*, *Moraxella*, *Aeromonas*, *Chromobacterium*, and *Enterobacter* (Owens, 1978; Phillips *et al.* 1981; Ivanov *et al.* 1996). The enzyme activity is extracellular and is produced during late exponential and stationary phases of growth (Chrisope, 1975). Reports regarding heat stability of the enzyme vary. Heat resistance of the enzyme in batch pasteurization conditions (62·5 °C for 30 min) is not established.

Limited studies demonstrate that growth medium influences PLC activity of *Ps. aeruginosa* and *Ps. cepacia* (Vasil *et al.* 1982; Lonon & Hooke, 1991). Ivanov *et al.* (1996) found no activity in tryptone mineral medium, while Stepaniak *et al.* (1987) found no such differences in PLC activity due to medium in *Ps. fluorescens*. The influence of growth medium on raw milk-contaminating pseudomonads is lacking. The aim of this investigation was to determine the influence of medium and strain variation for production of PLC activity in pseudomonads related to raw milk and dairy products. This was done by conducting a factorial experiment with different growth media, strain combinations and incubation times. Additionally, heat stability of the enzyme activity was determined in the cell-free supernatant.

MATERIALS AND METHODS

Strains and media

Seventeen isolates of *Pseudomonas* were used in this study (Table 1). Working cultures were prepared from frozen culture stocks stored at −70 °C in reconstituted non-fat dry milk containing 200 ml glycerol/l. An inoculum was freshly prepared from a 24 h culture ($A_{620} = 0{\cdot}2$) by harvesting the cells with centrifugation at 6000 ***g*** for 10 min at 4 °C. The inoculum was washed three times with sterile saline and resuspended in sterile saline to an A_{620} of 0·2. This was used to inoculate each sterile medium (1% v/v). The media used in this study were nutrient broth (Difco Laboratories, Detroit, MI 48232, USA), McKellar's minimal medium with the lipase inducer added (McKellar & Cholette, 1984), UHT skim milk (Gossner Foods, Logan, UT 84321, USA), and Chrisope's medium (Chrisope *et al.* 1976). Inoculated media (500 ml) were incubated at 10 ± 2 °C with shaking at 200 rpm for at least 50 h.

The lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 M HCl to pH 4·6 to precipitate the caseins. The whey fraction was prepared by centrifugation (7000 ***g*** for 10 min at 4 °C) and filtration through Whatman No. 1 filter paper (Whatman International, Maidstone, Kent ME16 0LS, UK). The filtered whey was neutralized to pH 7·0 using 1 M HCl, filter-sterilized through a 0·22 μm syringe filter (Gelman Sciences, Ann Arbor, MI 48103, USA) and stored at −70 °C until used. It was added (10 ml/l) to sterile McKellar's minimal medium.

Sampling

Samples (10 ml) were drawn from each medium at 24 and 50 h and divided into two sub-samples. One was heated to 62·5 ± 0·5 °C and held at that temperature for 30 min in a water bath and the other was not given a heat treatment. These two incubation periods correspond to the late exponential and stationary phases of growth, respectively (Blake *et al.* 1996). These two sub-samples were subsequently referred to as heated and non-heated, respectively. The measurements were done in duplicate.

Table 1. *Strains used to study phospholipase C*

Organism	Strain	Source or reference
Pseudomonas fluorescens	38	Craven & McCauley, 1992
Ps. fluorescens	CHA96	Laville *et al.* 1992
Ps. fluorescens	CHA0	Laville *et al.* 1992
Ps. fluorescens	CHA89	Laville *et al.* 1992
Ps. fluorescens	3	Craven & McCauley, 1992
Ps. fluorescens	RO98	Drew & Manners, 1985
Ps. fluorescens	B52	Rowe & Gilmour, 1983
Ps. fluorescens	RO13	Drew & Manners, 1985
Ps. fluorescens	AFT29	Dring & Fox, 1983
Ps. fluorescens	AFT36	Dring & Fox, 1983
Ps. fluorescens	948	ATCC†
Ps. fluorescens	31732	ATCC†
Ps. fluorescens	LS107d2	Johnson *et al.* 1992
Pseudomonas fragi	71	Craven & McCauley, 1992
Pseudomonas putida	345	Craven & McCauley, 1992
Pseudomonas sp.	113	Craven & McCauley, 1992
Pseudomonas xanthophilia	RO28	Drew & Manners, 1985

† American Type Culture Collection, Manassus, VA 20110, USA.

Total plate counts

Total plate counts were determined at both 24 and 50 h before heat treatment of the culture using the standard spread plate method with nutrient agar (Richardson, 1985).

Phospholipase assay

A lecithin agar diffusion assay was used to determine PLC activity (Chrisope *et al.* 1976). Petri plates containing 20 ml of lecithin agar were used to make four wells of 4 mm diam. The plates were incubated overnight at 30 °C prior to addition of the sterile cell supernatant to check sterility. Heated and non-heated culture supernatants (30 μl) were dispensed into each well and the plate was covered with the lid. The sides of the plate were sealed with Parafilm and incubated upright at 30 °C for 24 h. Zones of opacity formed due to hydrolysis of phosphotidylcholine were measured. Two measurements taken at right angles to each other were averaged. A negative control consisting of sterile medium was subtracted from the sample value. Plate counts were used to calculate activity obtained per cell (eqn 1). Enzyme activity was expressed as activity units/cell (U), which was defined as opacity zone diameter (mm) obtained per cell in 24 h.

$$\text{PLC activity (U)} = (\text{Average zone (mm)}/\text{Average plate count}) \times 10^8 \qquad (1)$$

Statistical analyses

Measurements were analysed by analyses of variance in a $17 \times 4 \times 2 \times 2$ (eqn 2) factorial design using the Minitab statistical software (Release 9.1, Minitab, State College, PA 16801, USA). The three-way and four-way interaction mean squares were pooled to obtain the error term since no replication was done (personal communication, Dr D. Sisson, Utah State University, Department of Mathematics and Statistics, 1998) (eqn 3). Least square difference (Moore & McCabe, 1989) tests were used to calculate the differences between means ($\alpha < 0{\cdot}05$).

$$Y_{ijklm} = \mu + S_i + M_j + H_l + SM_{ij} + T_k + ST_{ik} + MT_{jk} + TH_{kl} + SH_{il} + MH_{jl} + \text{Error} \qquad (2)$$

$$\text{Error} = SMT_{ijk} + SMH_{ijl} + STH_{ikl} + MTH_{jkl} + SMTH_{ijkl} + d_{ijklm} \qquad (3)$$

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Table 2. *ANOVA for the analysis of phospholipase C production*

Treatment	df	*P* value
Medium (M)	3	< 0·001
Strain (S)	16	< 0·001
M × S	48	< 0·001
Incubation time (T)	1	< 0·001
M × T	3	0·7892
S × T	16	< 0·001
Error	48	
Heat treatment (H)	1	< 0·001
M × H	3	0·0924
S × H	16	< 0·001
T × H	1	0·0196
Error†	115	

† The error term was summed over duplication, three-way and four-way interactions.

μ = population estimate; S = strain; M = medium; T = incubation time; H = heat treatment; Y = phospholipase activity, d = duplication; capital double or triple letters = interaction between the individual factors.

RESULTS

Influence of medium, strain, incubation time, and heat treatment

The medium, strain, incubation time, and heat treatment significantly influenced ($P < 0{\cdot}001$) PLC activity (Table 2). The strain by incubation time, the strain by heat treatment, and the incubation time by heat treatment interactions also significantly influenced ($P < 0{\cdot}001$–$0{\cdot}0196$) PLC activity.

PLC activity was not significantly different in McKellar's broth, nutrient broth, and skim milk, while growth in Chrisope's medium produced significantly lower PLC activity ($1{\cdot}85 \pm 0{\cdot}6$ U/cfu) compared with growth in the other three media ($> 3{\cdot}35 \pm 0{\cdot}6$ U/cfu).

Individual strains produced significantly different ($P < 0{\cdot}001$) amounts of PLC activity that varied from 0 to $7{\cdot}78 \pm 0{\cdot}26$ U/cfu (Table 3), producing five significantly distinct groups. Little or no enzyme activity was observed in *Pseudomonas* sp. 113, *Ps. putida* 345. *Ps. fragi* 71 produced the most activity ($7{\cdot}78 \pm 0{\cdot}26$ U/cfu).

Increasing incubation time significantly increased ($P < 0{\cdot}001$) PLC activity (Table 2). The average enzyme activity detected at 24 h was $2{\cdot}04 \pm 0{\cdot}4$ U/cfu, and increased 110% at 50 h ($4{\cdot}22 \pm 0{\cdot}4$ U/cfu). Once the enzyme was produced, pasteurizing the culture supernatant significantly decreased activity ($P < 0{\cdot}001$) (Table 2). After pasteurization, about 47% of the activity remained independent of the strain. The average PLC activity for all strains and media decreased from $4{\cdot}26 \pm 0{\cdot}4$ U/cfu before the heat treatment to $1{\cdot}99 \pm 0{\cdot}4$ U/cfu following pasteurization.

Interactions and phospholipase C activity

PLC activity was significantly influenced ($P < 0{\cdot}001$) by the interaction between medium and strain (Table 4). In milk, the activity ranged from 0 to 7·47 U/cfu with strain CHA0 producing the highest activity. In nutrient broth, the highest activity was produced with *Ps. fragi* 71. When this strain was grown in other media, the activity varied from 6·21 to 11·20 U/cfu. Three strains failed to produce significant

Table 3. *Influence of strain on phospholipase activity. Strains with the same letter indicate no significant difference*

Strain	Total PLC activity (U/cfu)†
Pseudomonas sp. 113	0·00[a]
Pseudomonas putida 345	0·04[a]
Pseudomonas xanthophilia RO28	0·11[a]
Pseudomonas fluorescens 38	0·90[a]
Ps. fluorescens 31732	1·74[b]
Ps. fluorescens LS107d2	1·98[b]
Ps. fluorescens RO98	2·09[b]
Ps. fluorescens RO13	2·14[b]
Ps. fluorescens CHA89	2·24[b]
Ps. fluorescens CHA96	3·55[c]
Ps. fluorescens B52	3·86[c]
Ps. fluorescens 948	4·68[cd]
Ps. fluorescens AFT36	5·13[d]
Ps. fluorescens AFT29	5·20[d]
Ps. fluorescens CHA0	5·59[d]
Ps. fluorescens 3	6·15[d]
Pseudomonas fragi 71	7·78[e]

† Standard error of the mean (SEM) = 0·26, LSD = 1·2.

Table 4. *Interaction between heat treatment and growth medium on phospholipase activity (U/cfu) of strains. Comparison of the growth medium columns and the strain rows indicates the growth medium × strain interaction. Comparison of the heat treatment columns with the strain rows indicates the heat treatment by strain interaction. The data are sorted by activity produced in milk*

	Growth medium†				Pasteurization	
Strain‡	CB	MB	NB	M	Yes	No
Pseudomonas fluorescens 38	0·44	1·44	1·71	0	0·41	1·34
Pseudomonas sp. 113	0	0	0	0	0	0
Pseudomonas putida 345	0·16	0	0	0	0·10	0
Pseudomonas xanthophilia RO28	0·46	0	0	0	0	0·23
Ps. fluorescens 31732	0·80	1·39	3·05	1·71	0·63	2·84
Ps. fluorescens LS107d2	1·55	1·07	3·34	1·97	0·46	3·51
Ps. fluorescens CHA89	2·61	1·19	2·04	3·14	1·59	2·89
Ps. fluorescens RO98	0·67	1·32	3·22	3·15	0·77	3·42
Ps. fluorescens RO13	0·48	2·66	2·05	3·37	0·31	3·99
Ps. fluorescens CHA96	3·93	2·88	3·50	3·89	3·14	3·96
Ps. fluorescens 3	2·84	10·3	6·85	4·65	4·22	8·07
Ps. fluorescens B52	0	4·02	6·18	5·25	2·69	5·04
Ps. fluorescens AFT36	2·85	5·80	5·56	6·30	2·94	7·31
Ps. fragi 71	6·21	11·2	7·46	6·31	5·48	10·1
Ps. fluorescens 948	1·67	4·69	5·81	6·56	2·79	6·60
Ps. fluorescens AFT29	2·82	4·15	7·17	6·64	3·32	7·10
Ps. fluorescens CHA0	3·95	4·84	6·08	7·47	4·81	6·36

† CB = Chrisope's broth, MB = McKellar's broth, NB = nutrient broth, M = milk.
‡ LSD (M × S) = 2·4; LSD (S × H) = 0·1.

amounts of PLC activity in any medium tested. These data highlight the complex interaction between the medium and the strain for production of PLC activity.

PLC activity increased significantly ($P < 0·001$) during the incubation time (data not shown) for all strains tested, except *Pseudomonas* spp. 113 and *Ps. putida* 345 where no activity was observed. The activity increased more than double in *Ps. fluorescens* CHA0, CHA89, CHA96, RO98, AFT29, AFT36 and *Ps. fragi* 71.

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Heat resistance to pasteurization of PLC activity produced by each strain varied significantly (Table 4). Each supernatant retained an average of 34·9 % activity after pasteurization. *Ps. fluorescens* CHA96 exhibited the most heat-stable activity (79·1 % residual activity), while *Ps. fluorescens* RO13 was found to be least heat-stable (7·7 % residual activity).

Heat resistance of PLC activity also significantly increased with incubation time ($P = 0{\cdot}019$; Table 2). As the incubation time increased, production of heat-stable PLC activity also increased (data not shown) for all strains tested.

DISCUSSION

Medium, strain, incubation time and heat treatment significantly influenced PLC activity (Table 2). Four of six two-way interactions significantly influenced PLC activity as well. Growth in Chrisope's medium produced the least amount of PLC activity, despite being designed to maximize this activity. This medium is a combination of peptone and yeast extract as the N source and was optimal for PLC production with *Ps. fluorescens* 178, as reported by Chrisope (1975). It is interesting to note that a medium developed for optimum PLC production in one strain was not optimal for others used in this investigation. Conversely, growth in skim milk resulted in the maximum PLC activity in 38 % of the strains. Milk was therefore an adequate medium for PLC production. Therefore, it safe to assume that milk or processed dairy products that contain pseudomonads will contain PLC activity.

With these findings in mind, and coupled to milk composition variation with diet and stage of lactation (Muir, 1996), it is reasonable to suspect that PLC activity in milk will vary due to factors beyond the scope of this work. For example, it would be interesting to determine the relationship of PLC activity and milk composition in systems that have continuous milk production and in those that have a seasonal milk supply (such as New Zealand and Australia). No studies in the literature were found that made this connection.

All but one strain produced PLC activity (Table 3) in the growth media used. The amount produced by each strain varied significantly depending on the medium used for growth (Table 4). Strain *Ps. fragi* 71 produced the most activity irrespective of medium and growth time, while strain *Pseudomonas* sp. 113, *Ps. putida* 345 and *Ps. xanthophilia* RO28 produced little or no activity in any condition (Table 3). Milk was one of the better media for PLC production for most of the strains (Table 4). These findings indicate that, irrespective of which strain contaminates milk, PLC activity may be produced and is available to cause spoilage. Hence, it is important to minimize the amount of PLC activity left in finished dairy products.

The influences of incubation time and heat treatment were investigated as mechanisms to reduce the amount of PLC activity in the raw milk that enters the processing plant. Activity increased significantly during incubation to 50 h, but also varied significantly with strain. Longer incubation produced more PLC activity, and the most activity was present in *Ps. fragi* 71 after 50 h incubation (data not shown). Enzyme production at 24 and 50 h incubation corresponded to the late exponential and stationary phases of growth, respectively (Blake *et al.* 1996). PLC activity is noted to be a stationary phase metabolite in *Ps. aureofaciens* (Sonoki & Ikezawa, 1975), but the present investigation observed PLC activity earlier than stationary phase, suggesting that expression may be regulated differently. This interaction indicates that the longer the raw milk was stored, the more PLC activity it would

contain, but the absolute amount of activity present would depend on the milk composition and the strains growing in milk. If these organisms produce PLC activity in exponential phase, control of growth may be one of the only mechanisms for control of this enzyme. Additionally, long shelf-life products contaminated with pseudomonads (via post-processing contamination), which is common (Craven & McCauley, 1992), may contain elevated levels of PLC activity that degrade the product and change the functionality (Barkworth, 1958; Chrisope & Marshall, 1976).

Heat treatment significantly reduced PLC activity; however, elimination of the enzyme activity was unsuccessful irrespective of the raw milk holding time or incubation time. Therefore, milk should be protected from pseudomonad contamination before and after processing to reduce the level of PLC in dairy products. Traditional temperature-processing strategies will not eliminate, but will reduce, the amount of enzyme in milk after processing (Ivanov *et al.* 1996). Additionally, the effect of heat treatment varied significantly with strain (Table 4) and the length of incubation. A higher proportion of activity was retained following heat treatment of the 50 h culture supernatant compared with the 24 h supernatant. This may be due to the effect of increased protein concentration in the supernatant, which may have offered protection to the enzyme from heat denaturation. It can also be speculated that some other protective metabolite may be produced at 50 h, or an additional PLC enzyme with different characteristics from the one produced at 24 h.

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