Journal of Industrial Microbiology & Biotechnology (1998) 21, 292–295

http://www.stockton-press.co.uk/jim

Lipase-induced hydrolysis of castor oil: effect of various metals

C Sharon, M Nakazato, HI Ogawa and Y Kato

Department of Applied Chemistry, 1-1 Sensui-cho, Kyushu Institute of Technology, Tobata-ku, Kitakyushu-shi, Japan-804-8550

The ability of an extracellular lipase from *Pseudomonas aeruginosa* KKA-5 to commence hydrolysis of castor oil in the presence of various metal chlorides, was investigated. Apart from $CaCl_2$ (commonly used for castor oil hydrolysis), $AlCl_3$ (group IIIB), $CrCl_3$ (group VIA) and $MgCl_2$ (group IIA) displayed enhanced hydrolysis capability. Specifically, our statistics show that with respect to time, when Cr^{3+} was used, hydrolysis of castor oil was four times faster than that of calcium, and 1.6 times faster with regards to Al^{3+}. The chlorides of group VIII and alkali metals had no effect on hydrolysis. Group IV metal chlorides did not enhance lipase activity and inhibited castor oil hydrolysis. The effect of metal ions from other groups on lipase activity is also reported.

Keywords: castor oil; hydrolysis; lipase; metal ions; *Pseudomonas aeruginosa*

Introduction

Seeds of *Ricinus communis* L. (family Euphorbiaceae) are a rich source of castor oil, a colourless to pale yellow viscous liquid consisting of approximately 90% ricinoleic acid [d-(+)-12-hydroxy-*cis*-9-octadecenoic acid]. Ricinoleic acid has two reactive functions, a hydroxyl group and a double bond, which lead to the formation of different derivatives of castor oil. Fatty acids derived from castor oil hydrolysis are effectively used in the food and cosmetic industries.

At the industrial level fats and oils are split using super heated steam, at high pressure and in the presence of nitrogen (ie the Colgate-Emery process). Products from this operation are often coloured and contain polymerized materials, which require extensive purification by distillation. On the other hand, lipases obtained from plants and microbes, which catalyze hydrolysis of oils and fats at the oil–water interface, yielding free fatty acids and glycerol, can also be effectively and economically used to conduct the same reaction, under mild conditions.

Lipase from seeds of *Avena sativa* L. [7] and *Pseudomonas* sp f-B-24 lipase [11] have been used for degradation of castor oil. During the course of hydrolysis, additives such as calcium ion [9], and surfactants [4] have been found useful to enhance these reactions.

In the present work we observed that many metal ions did not strongly inhibit lipase activity. Hence, apart from the usually used Ca^{2+} ions, other metal ions, belonging to different groups of the periodic table, were incorporated into the reaction mixture to investigate castor oil hydrolysis by lipase [triacylglycerol acylhydrolase (EC 3.1.1.3)], from *Pseudomonas aeruginosa* KKA-5.

Correspondence: Y Kato, Department of Applied Chemistry, 1-1 Sensui-cho, Kyushu Institute of Technology, Tobata-ku, Kitakyushu-shi, Japan-804-8550
Received 14 August 1998; accepted 22 October 1998

Materials and methods

Microorganism and cultivation *Pseudomonas aeruginosa* KKA-5 was used for production of lipase. Initially the cells were grown on Luria-Bertani plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar, pH 7.2) at 37°C for 24 h. These cultures were further inoculated into growth medium A, which contained 0.1% KH_2PO_4, 0.1% NaCl, 4% polypeptone, 0.05% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, pH 6.9 for 24 h at 30°C. To study lipase activity, 2 ml growth media A was inoculated into 100 ml lipase production medium (medium B), which contained 0.1% KH_2PO_4, 0.1% NaCl, 4% polypeptone, 0.05% yeast extract, 0.4% polyoxyethylene lauryl ether, 0.02% $MgSO_4 \cdot 7H_2O$, pH 6.9, in a 500-ml conical flask, on a rotary shaker (Biorotary Shaker BR-200L, Taiyo, Koshigaya, Japan) at 121 rpm at 30°C.

Assay of lipase activity
Lipase activity was measured using 2,3-dimercaptopropan-1-ol tributyrate as the substrate (Lipase Kit S, Dainippon Pharmaceuticals Co, Osaka, Japan). One unit of lipase is defined as the amount of enzyme that liberated 1 μmol min^{-1} of fatty acid at 30°C. Specific activity is expresed as units mg^{-1} protein.

Extraction of crude lipase
On the day of maximum lipase activity in the lipase production medium, the culture was centrifuged at 9500 × *g* for 10 min at 4°C. The supernatant, used as the source of lipolytic activity, was called crude lipase.

Effects of metal chlorides on lipase activity
Potassium phosphate buffer (10 mM) containing chlorides of metal ions at 10 mM (pH 8.0) were incubated with crude lipase (21 U) at 30°C for 30 min. Lipase activity was measured using Lipase Kit S.

Hydrolysis of castor oil

Castor oil (Nacalai Tesque, Kyoto, Japan) was hydrolyzed using crude lipase preparations. The reaction mixture consisted of 0.25 g castor oil, 10 ml of 10 mM potassium phosphate buffer (pH 8.0), the crude lipase preparation (21 U) and 1 ml 0.01 M metal chloride (pH 8.0). These components were placed in 100-ml air tight Erlenmeyer bottles and incubated at 30°C on a reciprocal shaker. In the control, the reaction mixture consisted of all the above components, except that the metal chloride was replaced by adding 11 ml of 10 mM potassium phosphate buffer (pH 8.0). The reaction was stopped by adding 15 ml acetone/ethanol (1:1, v/v). Liberated fatty acids were titrated with 0.1 M alcoholic KOH, using three drops of phenolphthalein as an indicator. Banks were run by titrating the reaction mixture immediately after adding all the components.

The percentage of castor oil hydrolysis was calculated as: hydrolysis (%) = (acid value/saponification value) × 100. The saponification value of castor oil used was 181 (a value provided by the supplier).

Results

To promote lipase production, *Pseudomonas aeruginosa* KKA-5 was initially grown in medium B in the presence of different concentrations of castor oil. Media containing low concentrations of castor oil gave higher amounts of lipase (Figure 1). Effective production of lipase was observed even in the absence of castor oil. These observations suggest that the present lipase is not an inducible enzyme but constitutive, as its production occurs even in the absence of the lipid substrate and its production is inhibited by the presence of castor oil. The possibility that the fatty acids formed suppressed lipase production, however cannot be ruled out. Accordingly, in all other experiments castor oil was omitted from medium B and maximum lipase activity was observed on the fourth day of cultivation.

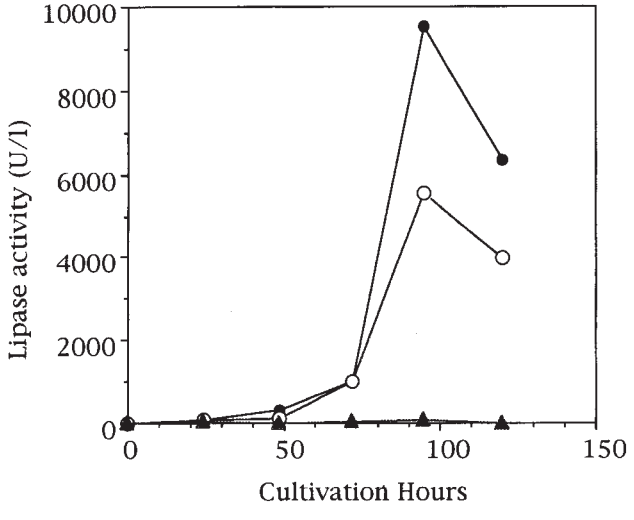

Figure 1 Lipase production in the presence of different concentrations of castor oil. ●, Control; ○, 2%; ▲, 20%.

Effects of metal ions on lipase activity

Our preliminary studies dealt with investigating the effect of metal ions on lipase activity (Table 1). Lipase lost about 10% of its activity on incubation with Li, while its activity decreased by about 20–30% in the presence of Na, K and Cu (group IA and IB, the alkali metals). From group IIA (the alkaline-earth metals) four metals (Be, Mg, Ca and Sr), and from group IIB two non-transition metals (Cd and Hg) were selected to study their effect on lipase activity. Be^{2+}, Sr^{2+}, Cd^{2+} and Hg^{2+} slightly inhibited lipase activity, whereas Mg^{2+} and Ca^{2+} ions enhanced it. On the other hand, Ca^{2+} significantly increased enzyme activity by as much as 56% within 72 h of incubation. About 30% decrease in activity was observed on incubation with group IIIA and IIIB metals, Al and Y, respectively. The metals Zr and Pb from group IVA and IVB, respectively, inhibited lipase activity. Incubation of crude lipase in metals like Cr from group VIA, Mn from group VIIA, and Fe, Ni, Co and Pd from group VIII led to about 5–15% inhibition in activity. Contrary to our results, salts such as KCl, NaCl, $MgCl_2$, $CoCl_2$, $ZnCl_2$ and $FeCl_2$ did not stimulate lipolytic activity of oat seeds [6].

Hydrolysis of castor oil

Since lipase activity was not strongly inhibited by many of the metal chlorides tested, they were used to examine hydrolysis of castor oil using lipase as the catalyst, thereby determining whether they stimulated or inhibited the hydrolysis process.

Although Na^+ of group I did not influence castor oil hydrolysis, the other three metal chlorides of the same group strongly inhibited it (Figure 2). Maximum inhibition was found in the presence of Cu^{2+}.

Table 1 Effect of metal chlorides on lipase stability. Crude lipase was incubated in 0.01 M potassium phosphate buffer (pH 8.0) containing 10 mM of the respective metal chloride, for 30 min at 30°C. After incubation the residual activity was measured and expressed as a percentage of non-treated enzyme solution, which was taken as 100%

Metal chloride	Residual activity (%)
Control	100.0
Li	89.2
Na	81.5
K	70.0
Cu	77.7
Be	65.0
Mg	101.5
Ca	95.6
Ca (after 72 h of incubation)	156.0
Sr	79.4
Cd	72.5
Hg	70.4
Al	73.3
Y	70.0
Zr	65.7
Pb	66.8
Cr	85.7
Mn	94.7
Fe	80.9
Ni	80.6
Co	83.1
Pd	77.4

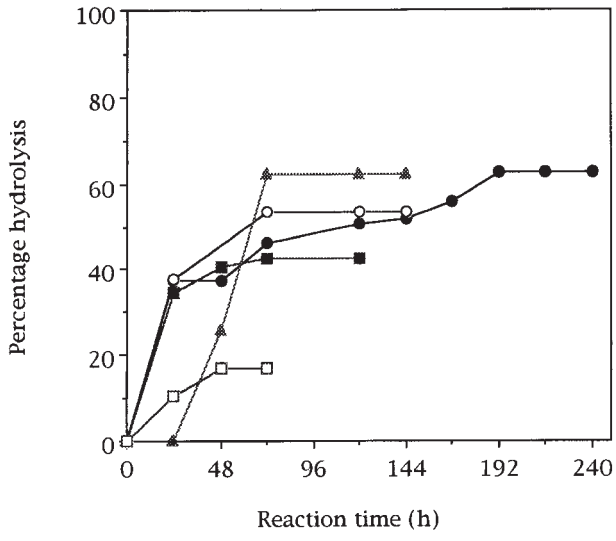

Figure 2 Effect of group IA and IB metal chlorides on the hydrolysis of castor oil. See text for reaction mixture and conditions. ●, Control; ○, Li; ■, K; □, Cu; ▲, Na.

Alkaline-earth metal chlorides of group II exhibited different effects on castor oil hydrolysis. For example, compared to the control, Hg^{2+} ions caused maximum inhibition, whereas Be^{2+}, Sr^{2+} and Cd^{2+} ions caused lesser inhibition. About 80% castor oil hydrolysis was achieved in the presence of Ca^{2+}, within 192 h. With respect to Mg^{2+}, after incubation of the reaction mixture for 192 h, castor oil showed 87% hydrolysis (Figure 3). It must be noted that in the control, in the same period of time, only 60% castor oil hydrolysis was attained.

Chlorides of both non-transition metals had different effects on hydrolysis. Addition of Al^{3+} to the hydrolysis mixture enhanced the rate of hydrolysis, ie 71% hydrolysis was attained within 120 h of incubation, whereas in the same period of time only 50% hydrolysis was obtained in the control. Y^{3+} did not promote or inhibit hydrolysis (Figure 4).

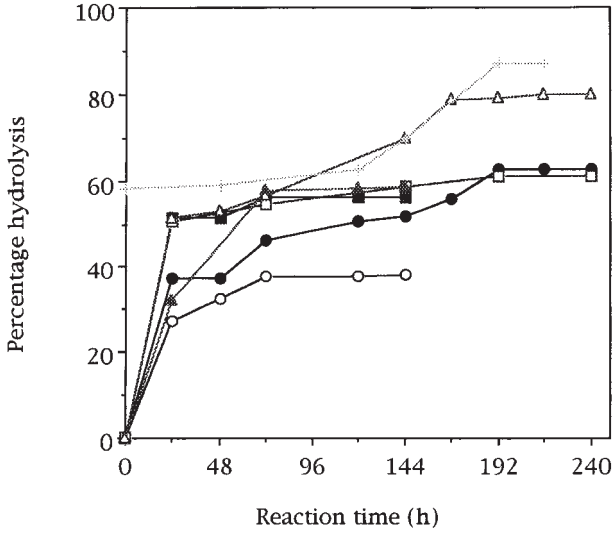

Figure 3 Effect of group IIA and IIB metal chlorides on the hydrolysis of castor oil. See text for reaction mixture and conditions. ●, Control; ○, Hg; ■, Be; □, Sr; ▲, Cd; △, Ca; ⊣⊢, Mg.

Both the metals of group IVA and IVB, Zr and Pb, resulted in a noticeable decrease in hydrolysis. As these heavy metal ions inhibited lipase activity it also was likely that they altered the enzyme conformation, presumably by displacing the native ions from their position, consequently limiting hydrolysis. Similar to other *Pseudomonas* species [1,2,10], *P. aeruginosa* KKA-5 has also been proved to be a metalloenzyme producer [8].

The chalcogen ($CrCl_3$) of group VIA was another metal which also promoted hydrolysis of castor oil. Within 48 h of incubation, about 37% castor oil hydrolysis was recorded in the control, whereas in the presence of Cr^{3+} about 73% hydrolysis was observed.

$MnCl_2$ inhibited hydrolysis. Three chlorides of the transition elements (group VIII) considered in this work, namely iron, nickel and cobalt, neither inhibited nor promoted lipase-catalyzed hydrolysis of castor oil (Figure 5), while palladium ions slightly inhibited it.

In all the cases mentioned above, an increase in incubation time had no significant impact on the percentage of hydrolysis.

Discussion

Effective lipase production from *P. aeruginosa* KKA-5 was recorded even in the absence of castor oil, contrary to the results of Pabai *et al* [5], who proposed higher lipase production in the presence of butter fat. Since lipases work at the oil–water interface, a large amount could have remained bound to the oil added to the culture medium. This could account for low lipase activity with increasing castor oil concentration.

Castor oil on hydrolysis yields free fatty acids. On addition of metal ions, the fatty acids form their respective metal salts. In the case of divalent ions, it is likely that the cations can form di-salts and salts. They inhibit lipase activity [3,4] and also obstruct the hydrolysis reaction. Fatty acids can be removed from the oil–water interface by

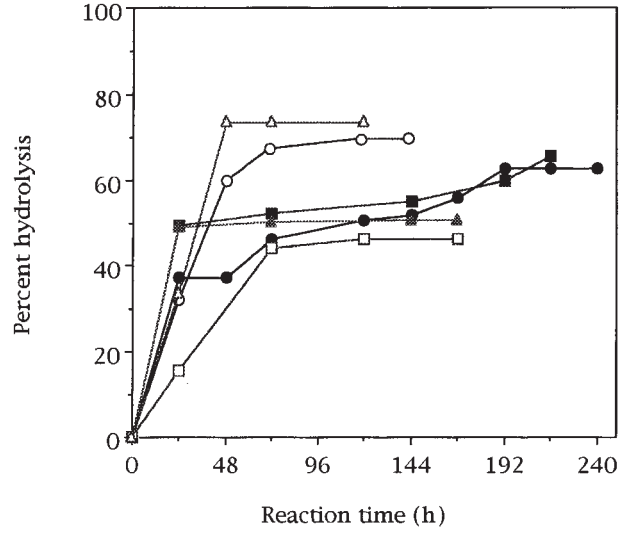

Figure 4 Effect of group IIIA, IIIB, IVA, IVB and VIAVIIA metal chlorides on the hydrolysis of castor oil. See text for reaction mixture and conditions. ●, Control; ○, Al; ■, Y; □, Zr; ▲, Pb; △, Cr.

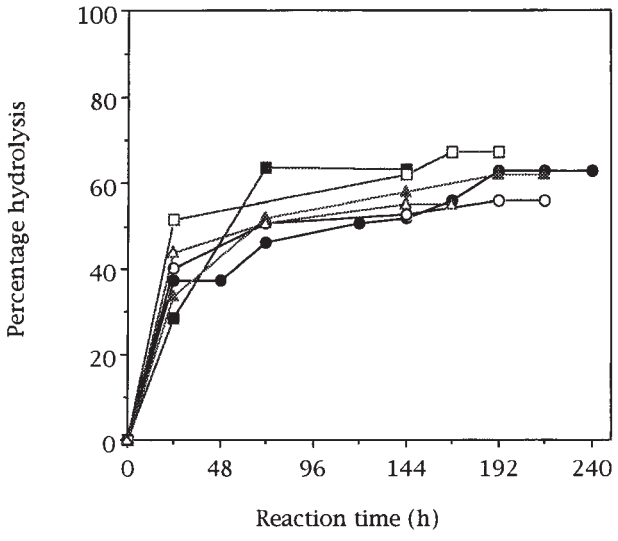

Figure 5 Effect of group VIIA and VIII metal chlorides on the hydrolysis of castor oil. See text for reaction mixture and conditions. ●, Control; ○, Mn; ■, Fe; □, Ni; ▲, Co; △, Pd.

addition of metal ions, so that the lipase can act freely on other oil molecules. Fatty acid removal could thus act as the rate controlling step [9]. On the other hand, monovalent cations cannot form di-salts. Consequently, reutilizing lipase for further castor oil hydrolysis was less effective with monovalent cations (group I) than with the divalent cations (Mg^{2+} and Ca^{2+}). Trivalent cations (Al^{3+} or Cr^{3+}) also showed the capability to hydrolyze, not because they formed salts with three molecules of acid but because they could possibly form di-salts. The percentage of hydrolysis was lower in the case of Al^{3+} or Cr^{3+}, presumably due either to a steric effect or to the fact that they partially inhibited lipase activity. Al^{3+} and Cr^{3+}, though capable of forming salts, could not be as effective as Mg^{2+} and Ca^{2+}, with respect to the percentage of hydrolysis. Moreover, Mg^{2+} and Ca^{2+} form planar salts which lead to less steric effect, as compared to the others. Al^{3+}, Cr^{3+}, Mg^{2+} and Ca^{2+} ions therefore are proposed to stimulate lipase-catalyzed hydrolysis of castor oil, not only by activating the lipase but also by possibly removing fatty acids from the oil–water interface, so that lipase can act freely on the oil molecules. It could be possible to extract the metal salts of fatty acids from the reaction mixture by separation in a separating funnel. The lower phase containing the metal salts could then be reutilized, thereby reducing disposal problems. As Li^+, Na^+ and K^+ are strong alkalis, the enzyme may not have been stable in their presence. Other divalent cations like Hg^{2+} could inhibit lipase due to their toxicity.

To date, to enhance the rate of hydrolysis, most researchers have used calcium ion. In the present work we have demonstrated that, regardless of the fact that Al^{3+} or Cr^{3+} slightly inhibited lipase activity, their presence in the reaction mixture can be more effective than calcium in promoting castor oil hydrolysis. Specifically, the data show that with respect to time, the hydrolysis of castor oil is four times faster than with calcium, when Cr^{3+} is used, and is 1.6 times faster with Al^{3+}. In the present work we also propose that Mg^{2+} stimulates castor oil hydrolysis more efficiently than Ca^{2+} ions.

The lipase from *P. aeruginosa* KKA-5, in crude form could efficiently hydrolyze castor oil. This lipase thus may have a future in biotechnological applications. With this in mind, we therefore propose that the concentration of lipase and the metal ion activation used must be considered.

Acknowledgements

Thanks are due to Professor Maheshwar Sharon, Indian Institute of Technology, India, for his valuable discussions and to Dr Madhuri Sharon, Excel Industry, India, for her advice in the preparation of this manuscript. This research was partly supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, Sports and Culture, Japan.

References

1 Bozoglu F, HE Swaisgood and DM Adams. 1984. Isolation and characterization of a heat-stable lipase produced by *Pseudomonas fluorescens* MC 50. J Agric Food Chem 32: 2–6.
2 Dring R and PF Fox. 1983. Purification and characterization of a heat-stable lipase from *Pseudomonas fluorescens* AFT 29. J Food Sci Technol 7: 157–171.
3 Gilbert EJ, JW Drozd and CW Jones. 1991. Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginosa* EF-2. J Gen Microbiol 137: 2215–2221.
4 Isobe K, T Akiba and S Yamaguchi. 1988. Crystallization and characterization of lipase from *Penicillium cyclopium*. Agric Biol Chem 52: 41–47.
5 Pabai F, S Kermasha and A Morin. 1996. Use of continuous culture to screen for lipase-producing microorganisms and interestification of butter fat by lipase isolates. Can J Microbiol 42: 446–452.
6 Piazzo GJ, A Bilyk, US Schwartz and M Haas. 1989. Lipolysis of olive oil and tallow in an emulsifier-free two-phase system by the lipase from oat seeds (*Avina sativa* L.). Biotechnol Lett 11: 487–492.
7 Piazza GJ and HM Farrell Jr. 1991. Generation of ricinoleic acid from castor oil using the lipase from ground oat (*Avina sativa* L.) seeds as a catalyst. Biotechnol Lett 13: 179–184.
8 Sharon C, S Furugoh, T Yamakido, HI Ogawa and Y Kato. 1998. Purification and characterization of a lipase from *Pseudomonas aeruginosa* KKA-5 and its role in castor oil hydrolysis. J Ind Microbiol Biotechnol 20: 304–308.
9 Wang YJ, JY Sheu, FF Wang and JF Shaw. 1988. Lipase-catalyzed oil hydrolysis in the absence of added emulsifier. J Ferment Technol 65: 23–29.
10 Yamamoto K and N Fujiwara. 1988. Purification and some properties of a castor-oil hydrolyzing lipase from *Pseudomonas* sp. Agric Biol Chem 52: 3015–3021.
11 Yamamoto K and N Fujiwara. 1995. The hydrolysis of castor oil using a lipase from *Pseudomonas* sp f-B-24: positional and substrate specificity of the enzyme and optimum reaction conditions. Biosci Biotech Biochem 59: 1262–1266.