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Hydrophobic immobilization of a bile salt activated lipase from Chinook salmon (*Oncorhynchus tshawytscha*)

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ABSTRACT

Immobilization of a bile salt activated lipase from Chinook salmon was achieved on two hydrophobic supports. The salmon lipase immobilized on octyl-Sepharose had approximately 40-fold higher activity (on a dry weight basis) against a tributyrin emulsion than the same lipase immobilized on Lewatit VP OC 1600. It also had approximately 10-fold higher activity than *Candida antarctica* Lipase B immobilized on Lewatit (Novozym 435). Salmon lipase–octyl-Sepharose was highly active against both ghee and fish oil emulsions. Novozym 435 and salmon lipase-Lewatit had very low activities against the same fish oil emulsion. The vast discrepancies in lipolytic activity between the two forms of immobilized fish lipase, and Novozym 435, may be attributed in part to the conformation of the bound lipases, but also to the different hydrophobicities of the lipase–carrier complexes, which determine the substrate access for hydrolysis. Incubation of the salmon lipase with 40 mM sodium cholate prior to immobilization on Lewatit resulted in improved enzyme binding. The bile salt is thought to cause the lipase active site loop to open more fully and expose more hydrophobic regions on the enzyme. In contrast, when Lewatit was incubated with 40 mM cholate prior to lipase addition, significantly less binding was observed. Sodium cholate appeared to have little effect on salmon lipase immobilization on octyl-Sepharose. Immobilization was found to raise the temperature optimum of the salmon lipase against tributyrin. The thermal stability and bile salt requirement of the lipase were not affected by immobilization. The results demonstrate a successful immobilization of an active and functional fish bile salt activated lipase and its potential for low temperature modifications of lipids in emulsions.

1. Introduction

Enzymes are versatile, efficient and precise biocatalysts. Soluble lipases are easy to apply and highly useful in applications such as flavour development in food products and detergents. However, for industrial applications such as organic synthesis and modifications of fats and oils, it is necessary to immobilize these enzymes. Immobilization separates an enzyme from reaction products, allows for multiple re-use and continuous processing. Immobilization often improves the activity and stability parameters of an enzyme, as well as altering its substrate specificity and enantioselectivity [1–4].

Supports with densely packed, strongly hydrophobic, lipid-like groups (e.g. octyl-agarose) have been developed to allow preparation of immobilized lipase derivatives that are efficient catalysts in high water-activity systems [4]. Lipase attachment to the hydrophobic surface of the support is assumed to involve an extremely hydrophobic area around the active site, with the enzyme undergoing interfacial activation during immobilization. Interfacial activation is thought to involve an interaction between the lipase and substrate at a lipid/water interface causing a change in enzyme conformation which enhances activity [5]. Immobilization on a lipid-like hydrophobic support such as octyl-agarose produces the same effect. This adsorption is highly selective and provides a means to immobilize and purify at the same time [4], which is particularly useful for unstable enzymes such as Chinook salmon lipase.

Microbial lipases are of the greatest interest for biocatalysis. Commercial microbial lipases are produced extracellularly in high yield from cultures, helping to make recovery convenient and relatively economical. In addition, microbial lipases can be genetically manipulated more easily than those from animals and tend not to require additional factors such as calcium and bile salts for activity [as indicated in the review by 6]. *Candida antarctica* Lipase B (CAL-B) is one of the most versatile and commonly used lipases in biotransformations [7,8]. Novozym 435 (Novo 435, produced by

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Novozymes) is the immobilized form of CAL-B on Lewatit VP OC 1600, a macroporous acrylic polymer resin. The full immobilization protocol has not been disclosed, but the carrier is known to be of a hydrophobic nature (poly(methyl methacrylate crosslinked with divinylbenzene) [8]) and the enzyme bound to it via hydrophobic interactions. This has been demonstrated by incubating the resin with detergents and organic solvents to successfully desorb CAL-B [7,8]. Novo 435 will operate in aqueous media (i.e. emulsions), but it is most valued for its high activity in non-aqueous media such as oils and organic solvents [1,9–12], suggesting that the lipase–carrier complex also has hydrophobic character.

We have previously purified and characterized the digestive lipase from Chinook salmon, *Oncorhynchus tshawytscha* [13]. The enzyme is a carboxyl ester lipase (often called bile salt activated lipase) and requires calcium ions and sodium cholate for optimum activity. However, it should be noted that the salmon lipase can operate at a lower rate without bile salts, so these could be omitted from a food product or other application, if necessary. The versatility of bile salt activated lipases towards catalyzing many different types of reactions [6,14,15] is of significant potential benefit from an applications standpoint. The salmon lipase exhibits the highest activity against a medium chain (C10:0) ester of *p*-nitrophenol, and the activity at lower temperatures is relatively high compared to other fish and mammalian carboxyl ester lipases [13].

To the best of our knowledge, immobilization of an active and functional bile salt activated lipase from any source has not been demonstrated to date. This study aimed to immobilize a bile salt activated lipase from Chinook salmon on two different hydrophobic supports, octyl-Sepharose and Lewatit VP OC 1600. Catalytic performance of the immobilized salmon lipases against several emulsified lipid substrates was compared with that of Novo 435.

2. Materials and methods

2.1. General

Bile salt activated lipase was extracted from the pyloric ceca of farmed Chinook salmon (The New Zealand King Salmon Co. Ltd., Nelson, New Zealand). The ceca were frozen at −80 °C immediately after collection [13]. Ghee (clarified butter) was purchased from a local store. Fish oil extracted from New Zealand hoki (*Macruronus novaezealandiae*) was supplied by SeaDragon Marine Oils Ltd. (Nelson, New Zealand). Benzoyl-$_{DL}$-arginine-*p*-nitroanilide (BA-*p*-NA), octyl-Sepharose CL-4B (Oct-S), *p*-nitrophenyl palmitate (*p*-NPP), and tributyrin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *P*-aminobenzamidine-cellulose (*p*-ABA-cellulose) was supplied by Life Technologies (Invitrogen New Zealand Ltd., Nelson, New Zealand). Lewatit VP OC 1600 (Lew) was supplied by Lanxess (Bayer, Leverkusen, Germany) and Novozym 435 FG (Novo 435) was from Novozymes (Bagsvaerd, Denmark).

2.2. Lipase extraction and purification with p-ABA-cellulose

Frozen pyloric ceca were crushed, lyophilised, delipidated sequentially with several organic solvents, and air-dried to yield the pyloric ceca powder [13]. The ceca powder was stirred in 20 mM Tris buffer (pH 7.2) for 30 min at 21 °C. The ratio of buffer (mL) to powder (g) was 20:1. The supernatant (crude extract) was recovered by centrifugation at 10,000 × *g* for 15 min using a Beckman Avanti® J-25 I centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). This supernatant was batch loaded onto a trypsin affinity resin, *p*-ABA-cellulose, which had been pre-equilibrated with the Tris buffer then collected on a sintered glass funnel under vacuum. The ratio of crude extract (mL) to damp resin (g) was 6:1. Purification with the affinity resin was carried out to remove trypsin and, to a lesser extent, other serine proteases [16,17]. Binding was carried out at 8 °C for 1 h, using a continuous rotating mixer. The supernatant (void) was recovered by centrifugation at 3000 × *g* for 5 min. Subsequently, the resin was washed once with the Tris buffer for 5 min and the wash recovered. The void and wash were combined (*p*-ABA v-w) as the lipase solution.

2.3. Immobilization

Oct-S and Lew were washed with copious amounts of Milli-Q water, equilibrated with the Tris buffer, and collected on a sintered glass funnel under vacuum. Equal volumes of the lipase solution (mL) were mixed with each damp resin (g) in a 7:1 ratio. Binding, using a continuous rotating mixer, was carried out at 21 °C for 2 and 4.5 h for Oct-S and Lew, respectively. Each supernatant (void) was recovered by centrifugation at 3000 × *g* for 5 min. Subsequently, each resin was washed with the Tris buffer for 5 min, and the combined void-wash was retained. The resins were washed with the buffer two more times and the washes discarded. The resins were then rinsed and soaked in the Tris buffer containing 20 mM calcium chloride and 5 mM sodium cholate for 1.5 h, with gentle end-over-end mixing at 21 °C. After recovery on a sintered glass funnel, the resins were dried over silica gel under vacuum for 15 h at 21 °C. The protein loading on the supports (mg protein/g support) was calculated from the difference between lipase load and combined void-wash fractions.

2.4. Immobilization in the presence of sodium cholate

The effect of sodium cholate, a primary bile salt, on the immobilization of salmon lipase was tested in two ways: cholate was added to the lipase solution to a final concentration of 40 mM, mixed for 1 h at 21 °C, and the immobilization carried out as described in Section 2.3; alternatively, 40 mM cholate was prepared in the Tris buffer, mixed with each damp resin for 1 h at 21 °C, followed by replacement of the cholate solution with the equivalent volume of the lipase solution, and immobilization as described earlier. The standard immobilization protocol (as described in Section 2.3) was used as the control experiment, i.e. the lipase solution was mixed with the resin first, followed by addition of 40 mM cholate and further mixing for 1 h at 21 °C.

Desorption of bound lipase was attempted by mixing each resin preparation with the Tris buffer containing 0.1 M cholate for 1 h at 21 °C.

2.5. Trypsin activity assay

Throughout the course of immobilization trypsin activity was assayed at 30 °C using BA-*p*-NA as substrate [18]. The reaction mixture comprised an aliquot of the sample (25–100 μL) plus 1 mM BA-*p*-NA in 0.1 M Tris buffer (pH 8.2, containing 10 mM calcium chloride and 1% (v/v) DMSO) to make a final reaction volume of 3 mL. The sample was replaced with 20 mM Tris buffer (pH 7.2) to establish blank-rate activity. The release of *p*-nitroaniline from BA-*p*-NA was measured at 410 nm in a Unicam (model UV4) UV-visible spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) fitted with a peltier temperature controller. One unit (U) of activity was defined as 1 μmol *p*-nitroaniline released/min under the assay conditions, using the extinction coefficient of 8800 M^{-1} cm^{-1} [18].

2.6. Lipase activity assays during immobilization

Two methods were used to measure the lipase activity throughout the course of immobilization. The first was a spectrophotometric method, utilizing the hydrolysis of *p*-NPP in an

Table 1
Activities (U) against BA-*p*-NA (measurement of trypsin activity) for the purification of Chinook salmon lipase on *p*-ABA-cellulose.

Sample	Total volume (mL)	U/mL	Total U	Recovery (%)
Crude extract	51.0 ± 0.5	1.442 ± 0.001	73.5 ± 0.7	100
p-ABA v-w[a]	85.0 ± 0.5	0.051 ± 0.001	4.3 ± 0.1	5.9 ± 0.2

Assays were carried out at 30 °C; values are mean ± SE (n = 2).
[a] v-w = combined void-wash fractions.

aqueous medium at 30 °C and pH 8 [13]. The second method was titrimetric, utilizing the hydrolysis of tributyrin [modified from 19]. Reactions were carried out at 30 °C with 70 mL aqueous substrate emulsion (5 mM tributyrin, 0.18% (w/v) sodium caseinate and 0.015% (w/v) lecithin) in a stirred pH stat vessel (718 STAT Titrino, Metrohm, Herisau, Switzerland). Calcium chloride (2 mM) and sodium cholate (5 mM) were added at the start of the assay from 0.5 and 0.25 M stock solutions (in Milli-Q water), respectively. Each reaction was monitored for 10–45 min, depending on the sample (dry resins containing immobilized lipases required 30–45 min assays for reproducibility). The released free fatty acids were titrated with 0.01 M NaOH with the pH stat set at 8.0. The slope (mL 0.01 M NaOH/min) in the linear region of the titration curve (typically the initial rate of reaction) was used to calculate the activity. Blank-rate activity was determined in the absence of the sample. One unit of activity was defined as 1 μmol butyric acid released/min under the assay conditions.

2.7. Immobilized lipase activity against natural substrates

Immobilized lipases (dry resins) were tested for activity against a ghee emulsion and a fish oil emulsion and compared with Novo 435. The assays were titrimetric, conducted as for the tributyrin assay, except that the stat pH was either 9.0 (immobilized salmon lipases) or 6.75 (Novo 435), the optimum pH for the respective lipase. For the ghee emulsion, reactions were carried out at 35 °C with 70 mL aqueous substrate emulsion (35% ghee, 2.2% sodium caseinate, 0.4% lecithin and 0.1% gum arabic, all w/v). For the fish oil emulsion, reactions were carried out at 30 °C with 70 mL aqueous substrate emulsion containing 5% (w/v) fish oil and 5% (w/v) gum arabic [modified from 20]. Calcium chloride (2 mM) and sodium cholate (5 mM) were included in the assays for immobilized salmon lipases. Each reaction was monitored for 30–90 min. The initial rate of reaction was used to calculate the activity in all cases. Blank-rate activity was determined with the respective resin in place of the immobilized lipase sample. One unit of activity was defined as 1 μmol fatty acid released/min under the assay conditions. All assays were carried out in duplicate.

2.8. Protein determination

Protein concentrations were measured using a method based on that of Lowry et al. [21] with modifications to the concentration of reagents and incubation times. Bovine serum albumin (BSA) was used as the standard. Modified standard curves in the presence of either SDS or Triton X-100 were used when desorbing the immobilized proteins/lipase from the supports using detergents (2%, w/v).

2.9. Properties of free and immobilized lipase

Properties were assessed with the 5 mM tributyrin assay, described in Section 2.6. Samples were *p*-ABA v-w (free lipase) and salmon lipase-Oct-S (immobilized lipase). To assess the effect of pH on activity, the substrate emulsion was adjusted to the required pH (ranging from 6.0 to 10.0) prior to sample addition. To study the effect of temperature on activity, the substrate emulsion was equilibrated at temperatures from 20 to 50 °C for 10 min prior to sample addition. The effect of temperature on lipase stability was investigated by incubating the sample at various temperatures (20–60 °C) for 30 min using a digital block heater, then rapidly cooling on ice to 0 °C, prior to assay. The effect of a primary bile salt on activity was measured in the absence and presence of 5 mM sodium cholate. Calcium chloride was present in all assays. In addition to using tributyrin as substrate, activity of the immobilized lipase only was measured against the ghee emulsion (described in Section 2.7) in both the absence and presence of cholate. All assays were carried out at optimum pH for each sample.

3. Results and discussion

3.1. Lipase immobilization on hydrophobic supports

The removal of trypsin/serine proteases with *p*-ABA-cellulose was necessary to prevent the proteolytic degradation of lipase during immobilization. This step was crucial for the retention of lipase activity in the immobilized form, since protease inhibitors (e.g. benzamidine), bile salts and glycerol were omitted from the extraction buffer. Although these compounds protect the lipase from inactivation [13,22,23], they were not included in the buffer as they can interfere with the hydrophobic interactions necessary for lipase binding (data not shown).

The activity results are divided into two parts. Based on the activities against BA-*p*-NA, trypsin – the most abundant digestive protease in Chinook salmon – was removed almost completely by the affinity resin (Table 1).

With *p*-NPP as substrate, low free-lipase activity was obtained in Oct-S and Lew v-w fractions, indicating that the lipase had bound to both supports (Table 2). However, the immobilized salmon lipases also exhibited very low activities against this synthetic substrate. Our studies suggest that conformational changes in the enzyme and/or increased hydrophobicity following immobilization adversely affected the substrate access. Similarly, the activity of Novo 435 against *p*-NPP was <2-fold that of salmon lipase-Oct-S.

Tributyrin appeared to be a more appropriate substrate to measure the amount of lipase (activity) bound on the supports, especially Oct-S (Table 2). When immobilized on this resin, the estimated total recovery of salmon lipase activity (including the unbound and bound lipase) was >60%. In contrast, it seems that the conformational orientation of the bound lipase and the hydrophobic character of the Lew resin did not favour either *p*-NPP or tributyrin. The change in properties of the fish lipase due to the immobilization meant that it was difficult to estimate the amount of lipase bound on the Lew resin with either of these substrates. Salmon lipase-Oct-S had higher activity against tributyrin than the same lipase immobilized on Lew or Novo 435. On a dry weight basis, this activity was approximately 40- and 10-fold higher than those of salmon lipase-Lew and Novo 435, respectively.

Considerably more protein bound to Oct-S than Lew (207.0 and 60.1 mg/g of dry resin, respectively). However, this is only true for the dry forms of the immobilized lipases. Due to its agarose backbone, Oct-S is considerably more hydrophilic than Lew and swells to approximately 12 times its dry weight when wet, whereas Lew absorbs a lot less water. Thus, in a practical sense where damp

Table 2
Lipase activities (U) against *p*-NPP and tributyrin for the immobilization of Chinook salmon lipase on two hydrophobic supports (Oct-S and Lew).

Sample	Total volume (mL)	U/mL or g[a]		Total U		Recovery (%)	
		p-NPP	Tributyrin	*p*-NPP	Tributyrin	*p*-NPP	Tributyrin
Load	42.5 ± 0.5	1.737 ± 0.002	13.2 ± 0.2	73.8 ± 0.9	561 ± 9	100	100
Oct-S v-w[b]	63.0 ± 0.5	0.054 ± 0.001	3.1 ± 0.4	3.40 ± 0.07	190 ± 20	4.6 ± 0.1	35 ± 4
Lew v-w	67.0 ± 0.5	0.053 ± 0.001	0.20 ± 0.05	3.52 ± 0.04	13 ± 3	4.8 ± 0.1	2.3 ± 0.5
Salmon lipase-Oct-S (dry)	0.490 ± 0.005 g	0.42 ± 0.04	330 ± 2	0.21 ± 0.02	163 ± 2	0.28 ± 0.03	29.0 ± 0.6
Salmon lipase-Lew (dry)	2.310 ± 0.005 g	0.0024 ± 0.0002	8.6 ± 0.4	0.006 ± 0.001	20 ± 1	0.010 ± 0.001	3.5 ± 0.2
Novo 435 (dry)[c]	–	0.77 ± 0.09	32 ± 2	–	–	–	–

Oct-S/Lew damp weight was 6.0 g; assays were carried out at 30 °C; values are mean ± SE (n = 2).
[a] U/mL for liquid samples; U/g for immobilized lipases.
[b] For all v-w samples, v-w = combined void-wash fractions.
[c] Included for comparison.

resins are used (i.e. in our experiments), higher protein loading was observed for Lew (25.9 compared to 17.3 mg/g of damp resin for Oct-S). These results are in agreement with the results for CAL-B binding onto octyl-agarose and Lew [7].

The complete removal of salmon proteins with either 2% Triton X-100, a non-ionic detergent, or 2% SDS, an anionic detergent, was not achieved with either support. SDS-PAGE of the detergent-eluted fractions did not show evidence of any lipase (data not shown). This suggests a relatively tight binding of the salmon lipase on both carriers. The tight binding is a desirable characteristic that would allow multiple reuse cycles with minimal leaching of the enzyme.

Bile salt activated lipases appear to bind more strongly than microbial lipases as CAL-B was desorbed completely from octyl-agarose and Lew using 1–2% Triton X-100 [7]. Bile salt activated lipases are relatively large and complex proteins. They have a large hydrophobic patch surrounding the active site and two bile salt binding sites, one of which is a large active site loop [22,24,25]. When the loop is in a closed form, the large hydrophobic patch is not completely shielded [24]. In contrast, microbial lipases have a smaller active site lid that upon closure completely covers the hydrophobic residues around the active site. They also lack any bile salt binding sites [24,26,27]. The structural differences between the two types of lipases are likely to result in stronger hydrophobic interactions between the salmon lipase and the hydrophobic supports compared with CAL-B.

3.2. Effect of sodium cholate on salmon lipase immobilization

As Oct-S contains 40 mM of octyl groups [28], the equivalent concentration of sodium cholate was used to test its effect on lipase binding to the hydrophobic supports. When the salmon lipase was incubated with 40 mM sodium cholate prior to immobilization on Lew, slightly better binding was seen compared to binding in the absence of cholate. With tributyrin as substrate, 19 and 25% of applied lipase activity were measured in the respective unbound fractions. However, cholate had less influence on the lipase binding to Oct-S where not much difference was seen between the cholate treatments. These results suggest that cholate does not interfere with lipase immobilization on the hydrophobic resins. On the contrary, the bile salt's interaction with the lipase appears to pull the active site loop more open, thus exposing more hydrophobic residues around the lipase active site, which are involved in interactions with the resin. Bile salt binding to and stabilization of the loop in an open conformation has been demonstrated by Wang et al. [25]. The improved access to the lipase's hydrophobic regions has a greater effect on binding to Lew since this resin has a less substrate-like surface as opposed to the presence of octyl groups on Oct-S. The octyl groups can act like a substrate analogue and trigger the change in conformation of the active site loop to a fully open form [4], whereas Lew does not appear to cause this. Very little lipase (approximately 6 and 10% of bound activity) was desorbed from Oct-S and Lew resins, respectively, after incubation with 0.1 M sodium cholate for 1 h, indicating that the lipase bound directly to the resin and not through cholate.

When Lew resin was incubated with 40 mM cholate prior to lipase addition, significantly less binding (50% unbound activity) was measured compared to binding in the absence of cholate (25% unbound). However, this effect was not seen when Oct-S was pre-incubated with cholate where similar levels of binding were obtained independent of the cholate treatment. The presence of cholate did not appear to block the enzyme's access to the hydrophobic groups on Oct-S. In contrast, it appears that cholate molecules attached to Lew via hydrophobic interactions, which limited the number of binding sites available for the lipase. Cholate acts as a surfactant and can form strong interactions with hydrophobic supports [29]. The hydrophobic interaction attaches the cholate onto Lew in a different conformation than that seen with covalent binding, such as in a cholate affinity resin [30]. The orientation of cholate on the resin is also likely to impair its ability to form aggregated lipase complexes [25]. Approximately 20 and 10% of bound lipase activity was desorbed from Lew and Oct-S resins, respectively, after incubation with 0.1 M sodium cholate for 1 h. This confirmed that the lipase was immobilized via hydrophobic interactions with the resins rather than affinity binding.

3.3. Immobilized lipase activity against natural lipid substrates

The catalytic performance of the immobilized salmon lipases was examined with two lipids with very different fatty acid profiles, and compared with that of Novo 435. Of all the natural fats, milkfat/ghee is the most complex and contains a relatively high proportion (11%, w/w) of saturated, short chain fatty acids [31,32]. In contrast, the majority of fatty acids in hoki oil are long chain, with a considerable proportion (18–22%, w/w) of n-3 PUFAs [33]. The percentage of ghee in the emulsion (35%) was equivalent to the fat content in the standardized dairy cream sold in New Zealand. For a representative substrate containing a considerable proportion of short chain fatty acids, ghee emulsion was used instead of cream as the fat does not separate in the assay mixture. The assay with ghee was carried out at 35 °C, the minimum temperature required to prevent the substrate from solidifying. A 35% fish oil emulsion made in the same manner as the ghee emulsion resulted in very low or no activity with all the samples. The optimized final emulsion contained 5% fish oil and gum arabic.

With ghee as substrate, the specific activity of the immobilized CAL-B (Novo 435) was higher than that of the salmon lipase immobilized on Lew, but lower than that of the fish enzyme immobilized on Oct-S (Table 3). When examined for the chain length selectivity against saturated fatty acids, Novo 435 was most active against C12–C16 fatty acids [11]. Thus, ghee may not be

Table 3
Activity (U) of the immobilized Chinook salmon lipases and Novo 435 against ghee and fish oil emulsions.

Lipase	U/g	
	Ghee	Fish oil
Salmon lipase-Oct-S (dry)	69 ± 4	47 ± 2
Salmon lipase-Lew (dry)	29 ± 3	5 ± 1
Novo 435 (dry)	44 ± 5	2.5 ± 0.4

Assays were carried out at 35/30 °C (ghee/fish oil); values are mean ± SE (n = 2).

an optimal substrate for Novo 435 as it contains a relatively high proportion of short chain fatty acids (C4:0–C8:0).

It has been demonstrated that Novo 435 is efficient at both cleaving and synthesizing triglycerides (TGs) containing long chain unsaturated fatty acids from sunflower, cod liver and microalgal oils in non-aqueous media [10,12,34]. However, in our study Novo 435 was unable to hydrolyze the TGs in a fish oil emulsion. This was also the case for salmon lipase-Lew. Significant activity against the emulsion was only achieved with salmon lipase-Oct-S. There are several reasons for the difference in activity between Oct-S- and Lew-bound enzymes. These are likely to include micelle size in the emulsified substrate and enzyme conformation. However, it also seems reasonable to propose that the hydrophobicity or hydrophilicity of the resins play a significant part.

The conformation of the bound lipase and its orientation on the carrier directly affect the catalytic performance. For salmon lipase-Oct-S, the attachment likely involves the large hydrophobic patch surrounding the active site of the lipase, which requires the active site loop to open fully [24]. The lipase may undergo a partial interfacial activation during immobilization [4] because the hydrophobic groups on the resin act as a substrate analogue. The bound lipase conformation would therefore favour a relatively higher activity against the substrates. In addition, Oct-S is more hydrophilic than Lew so the emulsified lipid substrate will have a higher affinity towards Oct-S in an aqueous medium [3]. This is in agreement with salmon lipase-Oct-S having a higher activity against the emulsified substrates (specifically tributyrin and fish oil) than salmon lipase-Lew.

For salmon lipase–Lew, the hydrophobic or hydrophilic character of the resin is likely to have a greater influence on the catalytic performance than the bound lipase conformation. Physical entrapment [8] and weaker hydrophobic adsorption [7,8] without interfacial activation are the likely modes of attachment of salmon lipase on Lew. Lew consists of a crosslinked polymer and lacks the well-defined and dense planar hydrophobic surface created by the octyl residues in Oct-S [4]. Although the attachment to Lew likely involves the same hydrophobic patch around the active site of the lipase as involved in binding to Oct-S, the absence of 'substrate analogues' in Lew results in a less active conformation of the bound lipase. Furthermore, Lew, the more hydrophobic carrier, does not favour the partition of the emulsified substrate from the aqueous reaction medium to the resin, which could explain the lower activities observed in our study.

3.4. Effect of immobilization on pH and thermal properties and bile salt requirement of Chinook salmon lipase

Immobilization may alter the properties of an enzyme and therefore its use in applications so it was important to determine if the salmon lipase had different properties as a result of the immobilization process. Of the two immobilized salmon lipases, only salmon lipase-Oct-S was investigated as it had higher activity against tributyrin, as well as the natural substrates (Tables 2 and 3).

The free form of salmon lipase was most active within a narrow pH range of 8.0–8.5 (Fig. 1). Similarly, the highest activity for the immobilized lipase was measured at pH 8.0–9.0. A previous study on porcine pancreatic lipase reported an increase in pH optimum after immobilization [35].

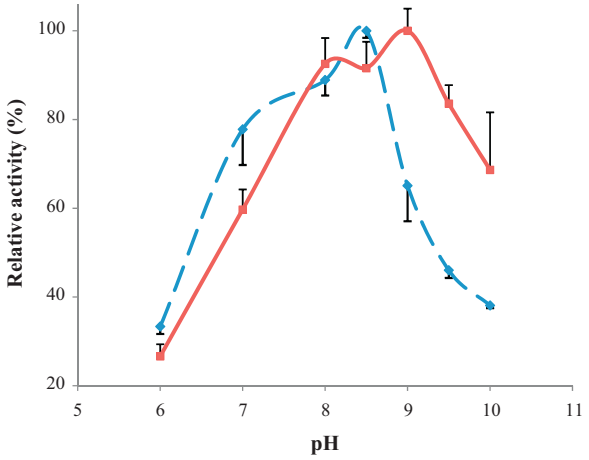

Fig. 1. Activity of free (– – –) and immobilized (—) Chinook salmon lipase against tributyrin as a function of pH. Duplicate measurements were taken at each pH.

An increase in the temperature optimum of the immobilized lipase was seen compared with the free form (Fig. 2). The immobilized lipase had the highest activity at 45 °C, whereas the free form was most active at 35 °C. The increase in temperature optimum is likely due to the stabilization of enzyme structure (i.e. decreased enzyme flexibility), and has been reported for many immobilized enzymes [35]. Although both the free and immobilized forms have high hydrolytic activity (>60%) at lower temperatures, the immobilized form is less sensitive to temperature changes up to the optimum temperature for hydrolysis.

Improved thermal stability of an enzyme is often one of the outcomes of the immobilization process [1,3]. In the present study, the thermal stability of the salmon lipase appeared unchanged upon immobilization (Fig. 3). Both forms of the lipase lost a significant amount of activity when incubated at temperature above 30 °C. The thermal properties of the immobilized salmon lipase make it particularly useful for low temperature applications. An example of such an application is the production of long chain, highly unsaturated fatty acids, where mild reaction conditions are necessary to minimize the oxidation of fatty acids [34].

The immobilized fish lipase was soaked in the buffer containing calcium ions and cholate before drying. Despite this treatment,

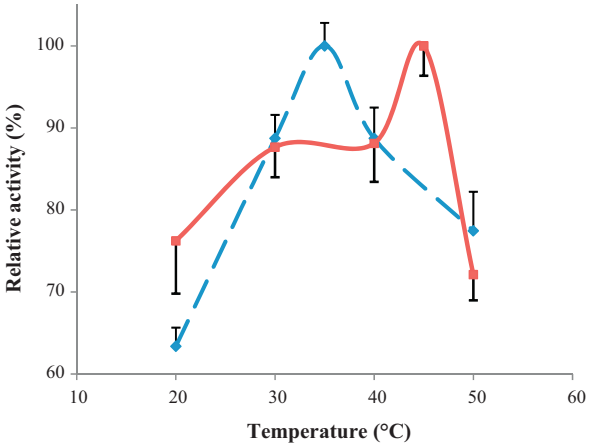

Fig. 2. Activity of free (– – –) and immobilized (—) Chinook salmon lipase against tributyrin as a function of temperature. Duplicate measurements were taken at each temperature.

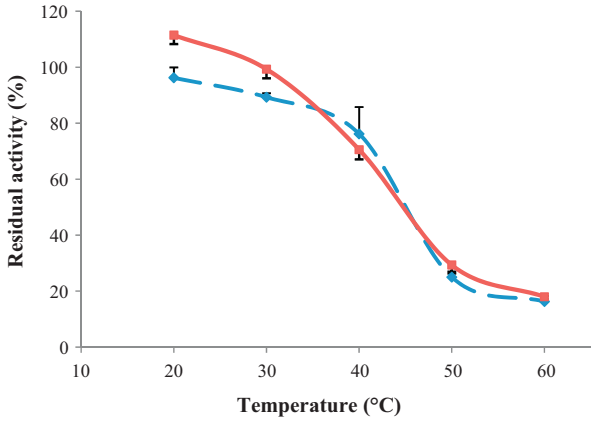

Fig. 3. Effect of temperature on the stability of free (– – –) and immobilized (—) Chinook salmon lipase when incubated in duplicate for 30 min prior to assay. Residual activities are relative to the un-incubated sample and were measured against tributyrin.

it was found that the immobilized salmon lipase required additional bile salts for optimum activity against tributyrin. However, the activity in the absence of cholate was still high for both the free and immobilized forms (74–76% of the cholate-inclusive activity). The high activity in the absence of cholate is not unexpected as bile salt activated lipases are known to have basal activity against smaller substrates, such as the short chain monoacid TGs [15].

Cholate was also required for optimum activity of the immobilized salmon lipase in a ghee emulsion. In the absence of cholate the activity was 55–60% of the optimum. The relatively lower activity of the immobilized lipase in the absence of cholate against ghee, compared with that against tributyrin, is due to lack of basal activity against the bulkier, water-insoluble TGs in ghee.

4. Conclusions

This study presents a successful immobilization of Chinook salmon bile salt activated lipase. When immobilized on Oct-S, the lipase had higher activity against tributyrin than the same lipase immobilized on Lew, and CAL-B immobilized on Lew. Salmon lipase-Oct-S also had high activity against both ghee and fish oil emulsions. Salmon lipase–Lew showed moderate activity against the ghee emulsion, but minimal activity against the fish oil emulsion, indicating that the active site had a less open conformation than in salmon lipase-Oct-S. The discrepancies in activities between the two forms of immobilized fish lipase were attributed in part to the conformation of the bound lipase, but also to the different hydrophobicities of the lipase–carrier complexes, which determine the substrate access for hydrolysis.

Sodium cholate had different effects on salmon lipase immobilization on Lew, depending on the order in which it was included. When the resin was incubated with cholate prior to lipase addition, significantly less binding occurred. The cholate molecules were likely attached to Lew via hydrophobic interactions and limited the number of binding sites available for the lipase. However, when the lipase was incubated with cholate prior to immobilization, slightly better binding was seen compared to binding in the absence of cholate. The bile salt's interaction with the lipase appears to cause the active site loop to assume a more open conformation, thus exposing more hydrophobic residues around the lipase active site, which are involved in interactions with the resin.

Sodium cholate appeared to have little effect on salmon lipase immobilization on Oct-S. The cholate molecules did not block hydrophobic groups on the resin necessary for lipase binding. The octyl groups on the resin appeared to have a similar effect to cholate with regards to the active site loop opening, such that the addition of cholate did not improve lipase binding.

Immobilization raised the temperature optimum of the salmon lipase against tributyrin, but did not affect the thermal stability of the enzyme. In addition, the bile salt requirement of the lipase was not affected by immobilization.

The immobilized fish lipase (salmon lipase-Oct-S) hydrolyzed natural substrates containing a wide range of fatty acids. This versatility in substrate utilization and activity at low temperatures show that Chinook salmon lipase immobilized on Oct-S has potential for applications which require the modification of lipids in aqueous media.

Further studies on the immobilization of fish bile salt activated lipases are in progress, relating in particular to the improvement of catalysis in an oil medium for the production of free fatty acids and alkyl esters.

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References

[1] H.C. Holm, D. Cowan, Eur. J. Lipid Sci. Technol. 110 (2008) 679–691.
[2] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill, C.H. Amundson, J. Am. Oil Chem. Soc. 67 (1990) 890–910.
[3] U. Hanefeld, L. Gardossi, E. Magner, Chem. Soc. Rev. 38 (2009) 453–468.
[4] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernandez-Lorente, J.M. Guisan, Chem. Phys. Lipids 93 (1998) 185–197.
[5] U. Derewenda, A.M. Brzozowski, D.M. Lawson, Z.S. Derewenda, Biochemistry 31 (1992) 1532–1541.
[6] I. Kurtovic, S.N. Marshall, X. Zhao, B.K. Simpson, Rev. Fish. Sci. 17 (2009) 18–40.
[7] Z. Cabrera, G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Palomo, J.M. Guisan, J. Mol. Catal. B: Enzym. 57 (2009) 171–176.
[8] B. Chen, J. Hu, E.M. Miller, W.C. Xie, M.M. Cai, R.A. Gross, Biomacromolecules 9 (2008) 463–471.
[9] M. Adamczak, U.T. Bornscheuer, W. Bednarski, Eur. J. Lipid Sci. Technol. 111 (2009) 800–813.
[10] L. Deng, X. Xu, G.G. Haraldsson, T. Tan, F. Wang, J. Am. Oil Chem. Soc. 82 (2005) 341–347.
[11] I. Karabulut, G. Durmaz, A.A. Hayaloglu, J. Agric. Food Chem. 57 (2009) 7584–7590.
[12] A. Robles Medina, L. Esteban Cerdan, A. Gimenez Gimenez, B. Camacho Paez, M.J. Ibanez Gonzalez, E. Molina Grima, J. Biotechnol. 70 (1999) 379–391.
[13] I. Kurtovic, S.N. Marshall, X. Zhao, B.K. Simpson, Fish Physiol. Biochem. 36 (2010) 1041–1060.
[14] D.Y. Hui, P.N. Howles, J. Lipid Res. 43 (2002) 2017–2030.
[15] C.-S. Wang, J.A. Hartsuck, Biochim. Biophys. Acta 1166 (1993) 1–19.
[16] T. Cohen, A. Gertler, Y. Birk, Comp. Biochem. Physiol. B: Comp. Biochem. 69 (1981) 639–646.
[17] F. Yang, W.-J. Su, B.-J. Lu, T. Wu, L.-C. Sun, K. Hara, M.-J. Cao, Food Chem. 116 (2009) 860–866.
[18] B. Erlanger, N. Kokowsky, W. Cohen, Arch. Biochem. Biophys. 95 (1961) 271–278.
[19] Committee on Codex Specifications, Food Chemicals Codex, The National Academies Press, Washington, DC, 1981, p. 493.
[20] P. Desnuelle, M. Constantin, J. Baldy, Bull. Soc. Chim. Biol. 37 (1955) 285–290.
[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[22] L. Blackberg, O. Hernell, FEBS Lett. 323 (1993) 207–210.
[23] I. Kurtovic, S.N. Marshall, B.K. Simpson, Comp. Biochem. Physiol. B: Comp. Biochem. 143 (2006) 432–440.
[24] S.A. Moore, R.L. Kingston, K.M. Loomes, O. Hernell, L. Blackberg, H.M. Baker, E.N. Baker, J. Mol. Biol. 312 (2001) 511–523.
[25] X. Wang, C.-S. Wang, J. Tang, F. Dyda, X.C. Zhang, Structure 5 (1997) 1209–1218.
[26] K.-E. Jaeger, B.W. Dijkstra, M.T. Reetz, Annu. Rev. Microbiol. 53 (1999) 315–351.
[27] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627–662.

[28] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernandez-Lafuente, J. Huguet, J.M. Guisan, Biotechnol. Bioeng. 58 (1998) 486–493.
[29] M.J. Armstrong, M.C. Carey, J. Lipid Res. 23 (1982) 70–80.
[30] C.S. Wang, Anal. Biochem. 105 (1980) 398–402.
[31] V.M. Balcão, F.X. Malcata, Biotechnol. Adv. 16 (1998) 309–341.
[32] H. Lindmark Mansson, Food Nutr. Res. 1821 (2008), doi:10.3402/fnr.v52i0.
[33] C.H. McLean, K.R. Bulling, J. Food Lipids 12 (2005) 313–326.
[34] G. Haraldsson, Rit Fiskideildar 16 (1999) 97–105.
[35] F. Kartal, A. Akkaya, A. Kilinc, J. Mol. Catal. B: Enzym. 57 (2009) 55–61.