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The use of immobilised digestive lipase from Chinook salmon (*Oncorhynchus tshawytscha*) to generate flavour compounds in milk

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ABSTRACT

The aim of this research was to determine the potential of immobilised digestive lipase from Chinook salmon (*Oncorhynchus tshawytscha*) to generate flavour compounds in milk. The lipase was immobilised on hydrophobic resin (Toyopearl® Butyl) and used to hydrolyse milk lipids in a batch reactor. The lipase was stable when immobilised and there was no significant resin fouling or enzyme inhibition between cycles. Eight cycles were achieved before the hydrolysis rate dropped significantly because of physical losses of the immobilised lipase. The immobilised lipase showed the highest specificity towards short-chain fatty acids butanoic and hexanoic acids, the main dairy product flavour and odour compounds. Based on the performance of the reactor, and the ability of the lipase to alter free fatty acid composition and sensory characteristics of milk, the immobilised salmon lipase has potential applications in developing dairy products with unique flavours.

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1. Introduction

Lipases are commonly used to generate desirable flavours in dairy products because of their unique specificities towards fatty acids (FAs) and controlled hydrolysis of milkfat triglycerides (Jooyandeh, Kaur, & Minhas, 2009). The volatile free fatty acids (FFAs) generated by lipases provide characteristic flavours in dairy products such as cheese and dairy flavour concentrates, and impart organoleptic properties of richness and creaminess (Balcão & Malcata, 1998). Fatty acids such as butanoic, hexanoic and octanoic acid are important contributors to sought-after 'buttery' flavours (Kinsella, 1975). Thus, to produce dairy products with desirable properties, it is important to identify enzymes with specificities towards these short-chain FAs. A study comparing six lipases/esterases for the release of short-chain FAs from cream lipids found that *Candida cylindracea* (*rugosa*) lipase was the most useful enzyme to enhance a buttery flavour in cream and products derived from it (Saerens, Descamps, & Dewettinck, 2008).

Lipases from the marine environment have the potential to expand the range of activities and specificities of lipases available for use in food and industrial processing. Fish are a prospective source of extractable lipases, because their digestive organs are an abundant and underused by-product of fish processing. Fish lipases may exhibit various novel activities due to the diverse environments in which fish live (e.g. at low temperatures) and in response to their diets. The lipases from some fish have higher catalytic efficiencies at lower temperatures than either their microbial or mammalian counterparts (Kurtovic, Marshall, Zhao, & Simpson, 2009). There may be an opportunity to use fish lipases in commercial dairy applications, especially if they can deliver particular and desirable flavours that are not produced by the enzymes currently in use.

We have previously demonstrated that digestive lipases from Chinook salmon (*Oncorhynchus tshawytscha*) and New Zealand hoki (*Macruronus novaezealandiae*) have the potential to produce dairy flavours. That is, they are able to release FAs and flavour volatiles from milkfat that are desirable in specialty cheeses and other 'ripened' dairy products (Kurtovic, Marshall, Miller, & Zhao, 2011). The Chinook salmon lipase released more short-chain FAs from cream than did the hoki lipase, and showed the highest specificity towards hexanoic acid. The sensory characteristics of cream treated with the salmon lipase were very similar to those of cream treated with pregastric esterase, a commercially available enzyme for use in dairy applications (Kurtovic, Marshall, Miller, et al., 2011). The salmon enzyme was targeted for further studies on its potential as a flavour-modifying agent in dairy products.

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Peptides resulting from the breakdown of residual enzymes in dairy products can contribute to bitter off-flavours (Lemieux & Simard, 1991), so an ability to immobilise the salmon lipase would avoid having the enzyme remain in the end product after lipolysis. It would also allow lipase re-use. Under the appropriate conditions, immobilisation on a lipid-like hydrophobic support (e.g. one containing butyl or hexyl groups) is highly selective for lipases in mixtures and can stabilise the structure of the enzyme. The immobilisation can also produce the effect of a lipase undergoing interfacial activation, i.e. an increase in activity (Fernandez-Lafuente, Armisen, Sabuquillo, Fernandez-Lorente, & Guisan, 1998). Such attributes are particularly useful for unstable enzymes such as Chinook salmon lipase (Kurtovic, Marshall, & Zhao, 2011). Selective hydrophobic interaction offers the opportunity of immobilisation from partially purified enzyme feedstocks and from feedstocks where the enzyme is in low concentration.

Toyopearl® is a methacrylic polymer with high mechanical and chemical stability used in several types of chromatography (e.g. size exclusion, hydrophobic interaction). The resins have high protein adsorption capacity due to the large surface area of the beads, provided by small particle size and large pores. These are typically $\leqslant$100 μm and 1000 Å, respectively. Butyl and hexyl derivatives have been used for the purification of microbial lipases (e.g. Volpato et al., 2011).

This paper describes the re-use of Chinook salmon digestive lipase, immobilised via hydrophobic interactions on Toyopearl® Butyl-650C, for the hydrolysis of milk lipids in a batch reactor system.

2. Materials and methods

2.1. Substrate and other chemicals

Fresh milk (homogenised, 3.3% fat; Anchor, Auckland, New Zealand) was purchased from a local supermarket. Toyopearl® Butyl-650C was sourced from Tosoh Bioscience (Tosoh Corporation, Tokyo, Japan). P-aminobenzamidine-cellulose (*p*-ABA-cellulose) was supplied by Life Technologies (Thermo Fisher Scientific, Nelson, New Zealand). All solvents and other reagents were of chromatography or analytical grade.

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

Frozen pyloric ceca of farmed Chinook salmon (The New Zealand King Salmon Co. Ltd, Nelson, New Zealand) were crushed, lyophilised, and delipidated sequentially with several organic solvents, and then air-dried to yield the pyloric ceca powder. These procedures were conducted as described previously (Kurtovic, Marshall, Zhao, & Simpson, 2010). 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 crude extract was centrifuged at 10,000*g* for 15 min (Beckman Avanti® J-25 I, Beckman Coulter, Fullerton, CA, USA). The supernatant was batch-loaded onto a trypsin affinity resin, *p*-ABA-cellulose, which had been pre-equilibrated with the Tris buffer and then collected on a sintered glass funnel under vacuum. The ratio of the supernatant (mL) to damp resin (g) was 7:1. Purification with the affinity resin was carried out to remove trypsin and, to a lesser extent, other serine proteases that could potentially hydrolyse the lipase (Cohen, Gertler, & Birk, 1981; Yang et al., 2009). Binding was carried out at 8 °C for 1.5 h, using a continuous rotating mixer at 40 rpm. The unbound fraction (flow-through) was recovered through a sintered glass funnel. Subsequently, the resin was washed once with Milli-Q water and the wash water was recovered. The flow-through and wash were combined as the lipase solution.

2.3. Immobilisation

Toyopearl® Butyl-650C was washed with Milli-Q water, and collected on a sintered glass funnel under vacuum. The lipase solution (mL) was mixed with the damp resin (g) at a ratio of 10:1. Binding was carried out at 21 °C for 2 h, using a continuous rotating mixer at 40 rpm. The flow-through was recovered through a sintered glass funnel, the resin was washed with Milli-Q water twice, and the combined flow-through and washes were retained. The resin was washed with Milli-Q water several more times and the washes were discarded. The fully drained, damp resin was recovered as the immobilised lipase preparation. The protein loading on the support (mg protein/g support) was calculated from the difference between the protein amount in lipase load and that in combined flow-through and wash fractions.

2.4. Lipase assays

Lipase activity against tributyrin was measured as described previously (Kurtovic, Marshall, & Zhao, 2011). Reactions were carried out at 30 °C and pH 8.

Lipase activity against milk was measured titrimetrically with a modified method based on the above assay against tributyrin (Kurtovic, Marshall, & Zhao, 2011). Reactions were carried out at 30 °C with 70 mL milk and 0.3 g immobilised lipase, in a stirred pH-stat vessel (718 STAT Titrino, Metrohm, Herisau, Switzerland). Calcium chloride (2 mM) and sodium cholate (5 mM) were included in the assay. Each reaction was monitored for 15–30 min. The released FFAs were titrated with 0.01 M NaOH with the pH-stat set at 8.0 (pH of milk was 6.6–6.7). 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 lipase activity. Blank-rate activity was determined in the absence of enzyme. One unit (U) of activity was defined as 1 μmol FA released per minute under these assay conditions. Assays were carried out in duplicate.

2.5. Protein assay

Protein concentrations were measured using the method of Lowry, Rosebrough, Farr, and Randall (1951) with some modifications to the concentration of reagents and incubation times. Bovine serum albumin (BSA) was used as the standard.

2.6. Hydrolysis of milk lipids

We constructed a batch reactor consisting of a glass container placed inside a jacketed reaction vessel connected to a circulating water bath. Pre-warmed milk (50 mL) was added to the glass container, followed by calcium chloride (2 mM) and sodium cholate (5 mM), and then the pH was adjusted to 8.0. The hydrolysis reaction was started by adding the enzyme (2.00 g immobilised lipase) to the milk and maintaining the mixture at 30 °C for 6 h, followed by a gradual decrease to 21 °C over the next 12 h, stirring continuously with an overhead propeller. The temperature and pH of the mixture were monitored using a combined pH/temperature electrode. A temperature of 30 °C was chosen for the first 6 h of milk lipolysis as the stability of immobilised salmon lipase is compromised above 30 °C (for $\geqslant$30 min), although the temperature optimum is around 45 °C (Kurtovic, Marshall, & Zhao, 2011). Thus, 30 °C represented a compromise between a relatively high rate of activity and stability. Stirring was stopped after 18 h and the resin was allowed to settle for 2 h at 21 °C. At this point, a sample of milk was collected for solid-phase micro extraction (SPME)–GC–MS analysis (Section 2.8). The reaction container was incubated at 4 °C for 4 h, and then another sample was collected to analyse

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