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Synthesis of ethyl butanoate by a commercial lipase in aqueous media under conditions relevant to cheese ripening

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A fruity flavour note is traditionally regarded as a defect in cheese varieties such as Cheddar (Bills et al. 1965; McGugan et al. 1975; Horwood et al. 1987). However, fruitiness is an attribute of other cheese varieties such as Parmesan and Parmigiano Reggiano (Dumont et al. 1974; Meinhart & Schreier, 1986). It is well accepted that esters such as ethyl butanoate and ethyl hexanoate cause the fruity flavour described as apple-like or pineapple-like in raw milk and cheeses (Bills et al. 1965; Engels et al. 1997; Friedrich & Acree, 1998). The development of fruity flavour is often attributed to the esterification of free fatty acids and ethanol by esterases from lactic acid bacteria and psychrotrophic pseudomonads (Hosono et al. 1974; Morgan, 1976).

Commercial lipases are added to the cheese milk for some cheese varieties such as some hard Italian cheeses to promote lipolysis (El Soda, 1997). In other cheeses such as mould-ripened varieties, the ripening microbial flora provide elevated lipases for lipolysis during cheese ripening (Gripon, 1997). Lipolysis is the hydrolysis of milkfat to release free fatty acids, which contribute directly to cheese flavour in these cheese varieties and, probably, to a lesser extent in other varieties (Fox & Wallace, 1997). Lipases are also used in the production of enzyme-modified cheeses (EMC) to generate cheese flavour (Kilcawley et al. 1998).

Although the primary function of lipases is to hydrolyse triacylglycerols and hydrophobic fatty acid esters, the same enzymes can catalyse the synthesis of esters under certain conditions. Lipases can catalyse ester synthesis by esterification ($R_1COOH+R_2OH \rightarrow R_1COOR_2+H_2O$), alcoholysis ($R_1COOR_2+R_3OH \rightarrow R_1COOR_3+R_2OH$), acidolysis ($R_1COOR_2+R_3COOH \rightarrow R_3COOR_2+R_1COOH$) and/or transesterification ($R_1COOR_2+R_3COOR_4 \rightarrow R_1COOR_4+R_3COOR_2$) (Malcata et al. 1992). Acidolysis and transesterification are employed to structurally modify lipids to produce nutritionally functional fats and oils (Willis et al. 1998). Ester synthesis by lipases via alcoholysis is also known (Briand et al. 1994, 1995; Lecointe et al. 1996) and numerous reports are available in the literature on the esterification of alcohols and acids by lipases under low-water conditions such as in organic media containing little water (see a review by Yahya et al. 1998).

In this short communication, we report the synthesis of the ester ethyl butanoate by a commercial fungal lipase (Palatase 20 000 L) in aqueous media under conditions that are relevant to cheese ripening. We characterized the nature of the reaction with a view to understanding and controlling the development of fruity flavour in cheeses and enzyme-modified cheese products.

Materials and Methods

Fungal lipase

Palatase 20 000 L (referred to as Palatase below) is a purified 1,3-specific lipase from *Rhizomucor miehei* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and contains no significant side activities, according to the manufacturer. The enzyme is a commercial product developed for applications in the food industry. The lipase was obtained in liquid form from Novo Nordisk A/S, Bagsvaerd, Denmark and has 20 000 Lipase Units/g with a pH optimum of 7·0.

Synthesis of ethyl butanoate

Ethyl butanoate was synthesized in a 5·0 ml reaction mixture comprising 80 mM-potassium phosphate, pH 7·0, 50 mM-ethanol and 33 mM-tributyrin, unless specified otherwise. The tributyrin substrate was prepared as described elsewhere (Holland & Coolbear, 1996). The reaction was started by adding 0·1 ml of appropriately diluted enzyme preparation (250 to 1000 fold dilution, i.e. rate-limiting concentrations up to 10 units) to the above reaction mixture pre-incubated at 30 °C for 5 min. A 1·0 ml sample was immediately removed and added to a test tube containing diethyl ether and extracted (see below). The remaining reactants were incubated at 30 °C for up to 40 min and samples were taken at intervals and extracted for ethyl butanoate analysis as described below. In experiments in which the water activity (a_w) of the reaction mixture was decreased, this was achieved by incorporating

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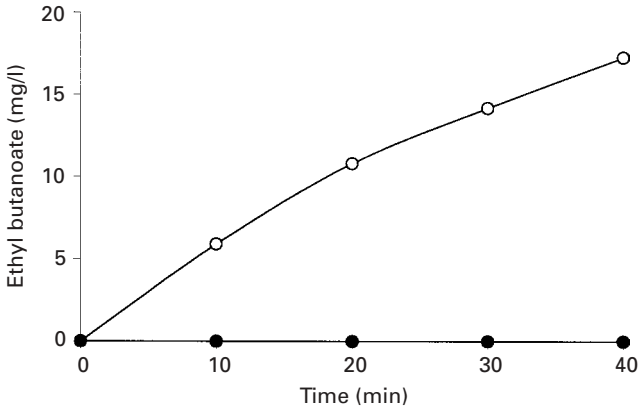

Fig. 1. Ethyl butanoate synthesis by Palatase at a_w 0·83 in phosphate buffer (PEG 200 as the humectant). ○, 50 mM-ethanol and 66 mM-tributyrin; ●, 50 mM-ethanol and 50 mM-butanoic acid.

polyethylene glycol (PEG) 200 (up to 62 ml/100 ml) or NaCl (up to 7·5 g/100 ml) into the potassium phosphate stock solution. The water activity was determined as previously described (Liu et al. 1998). In some experiments, the pH and the substrate concentration were varied according to a particular experiment as indicated in the text.

Ethyl butanoate was also synthesized in a cheese-based medium. This was carried out in a 9 ml reaction mixture consisting of 4 g cheese-based medium and 10 mM-ethanol. The reaction was initiated with the addition of 40 µl of undiluted enzyme preparation and the mixture was incubated at 30 °C for 4 h. A control containing no added ethanol was also included. The cheese-based medium was prepared from young Cheddar cheese (two-week old) (75%), emulsifying salts (1·5% each of trisodium citrate and disodium phosphate), proteolytic enzymes (0·2%) and water (22%), using a similar procedure to that of Sutherland (1975). The ingredients were mixed, pasteurised and cooled before the addition of proteolytic enzymes. After incubation at 37 °C for 4 h, the cheese medium was heated to 95 °C for 5 min to inactivate the residual enzyme activity. The finished product had a gross composition (% w/w on a wet weight basis) of 57·3% moisture, 21·3% fat, 2·4% salt-in-moisture and 2·5% total nitrogen (1·0% non-protein nitrogen).

Analysis of ethyl butanoate and butanoic acid

One millilitre of sample taken from a reaction mixture was immediately added to a 16-ml Kimax screw-capped test tube containing 2 ml of diethyl ether and 1 ml of internal standard (180 mg/l ethyl acetate in water). Extraction was performed by shaking vigorously for approximately 2·5 min, followed by centrifugation at 1260 ***g*** for 5 min (Heraeus Megafuge 1.0). The top solvent layer was transferred to a gas–liquid chromatography (GLC) vial containing a small amount of oven-dried anhydrous sodium

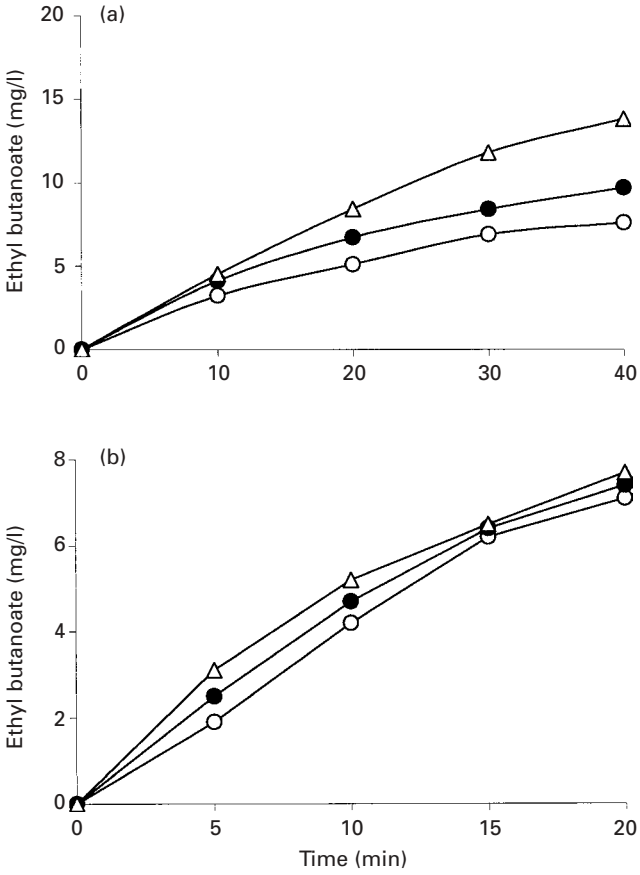

Fig. 2. Effect of a_w (PEG 200 as the humectant) and NaCl on ethyl butanoate synthesis by Palatase from 33 mM-tributyrin and 50 mM-ethanol in phosphate buffer. (a) a_w: ○, a_w 0·99 (control, no PEG 200 added); ●, a_w 0·93; △, a_w 0·83. (b) NaCl: ○, control (a_w 0·99, no NaCl added); ●, 3 g/100 ml (a_w 0·97); △, 6 g/100 ml (a_w 0·95).

sulphate and analysed for ethyl butanoate by GLC as described below.

Following sample extraction, ethyl butanoate was separated and analysed using GLC. The analysis was performed using a Shimadzu GC-15A gas chromatograph with a fused silica capillary column (liquid phase, DB-1; length, 30 m; i.d., 0·25 mm; film thickness, 1·0 µm) (J&W Scientific, CA, USA). The oven was temperature programmed at 45 °C for 5 min, followed by increasing the temperature to 50 °C at 5 deg C/min, then to 270 °C at 20 deg C/min, and held at 270 °C for 8 min. A split injection was performed at a ratio of 5 : 1 and 3 µl of sample was injected. The injector and flame ionization detector temperatures were 250 °C and 275 °C, respectively. Other conditions were: carrier gas (He) at 0·9 ml/min; H_2 and air at 0·6 kg/cm^2; makeup gas (N_2) at 2 kg/cm^2. The FID output signal was recorded and processed using appropriate software (Shimadzu CLASS-VP™ Chromatography Data System Version 4.2 from Shimadzu, MD, USA). The ethyl butanoate in the samples was identified and quantified by comparison with injections of known amounts of pure

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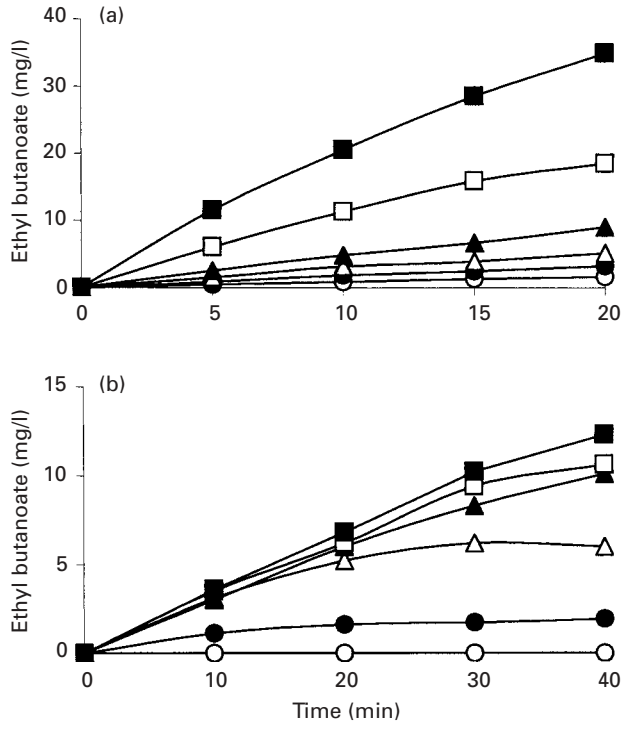

Fig. 3. Effect of substrate concentration on ethyl butanoate synthesis by Palatase at a_w 0·83 (PEG 200 as the humectant) in phosphate buffer. (a) 33 mM-tributyrin and various amounts of ethanol: ○, 3·1 mM; ●, 6·3 mM; △, 12·5 mM; ▲, 25 mM; □, 50 mM; ■, 100 mM. (b) 50 mM-ethanol and various amounts of tributyrin: ○, 0 mM; ●, 4·1 mM; △, 8·3 mM; ▲, 16·5 mM; □, 33 mM; ■, 66 mM.

standard. The method had a coefficient of variation of 10% and single determinations were made.

Results

In a relatively low-water system, the lipase Palatase catalysed the synthesis of ethyl butanoate from ethanol and tributyrin, being linear for the first 10 min, but not from ethanol and butanoic acid (Fig. 1). There was no formation of ethyl butanoate in the control to which no lipase was added. Likewise, we found that a number of other commercial fungal lipases (not mammalian lipases) were able to synthesize ethyl butanoate from tributyrin and ethanol; none of these lipases could produce ethyl butanoate from butanoic acid and ethanol under similar conditions (data not shown).

Figure 2 shows the impact of a_w and NaCl on ethyl butanoate synthesis by Palatase from ethanol and tributyrin. Lowering the a_w from, 0·99 to 0·83 moderately increased the initial rate of ethyl butanoate synthesis (10–30%) and the yield of ethyl butanoate (30–40%) (Fig. 2a). NaCl at concentrations up to 6 g/100 ml caused a moderate increase (10–30%) in the initial rate of ethyl butanoate synthesis and a slight increase (<10%) in the yield (Fig. 2b).

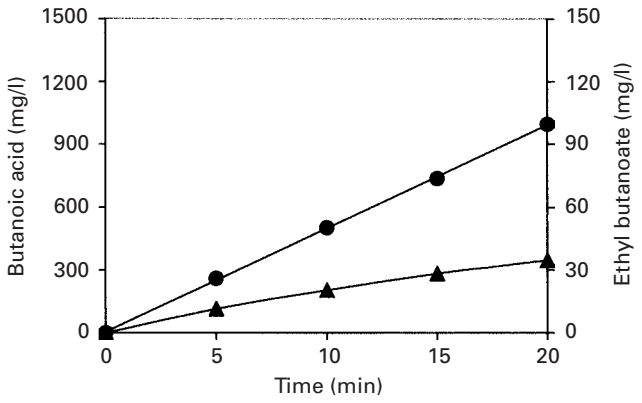

Fig. 4. Formation of butanoic acid during synthesis of ethyl butanoate by Palatase at a_w 0·83 (PEG 200 as the humectant) from 33 mM-tributyrin and 100 mM-ethanol in phosphate buffer. ●, butanoic acid; ▲, ethyl butanoate.

Ethyl butanoate synthesis was not affected by the pH conditions tested (from pH 5·2 to pH 8·0) (data not shown).

Figure 3 shows the influence of ethanol and tributyrin concentrations on ethyl butanoate synthesis by Palatase. Both the initial rate of ester synthesis and the yield of ethyl butanoate were directly proportional to the ethanol concentration up to 100 mM (Fig. 3a). The K_m value for ethanol was estimated to be 200 mM based on the Lineweaver–Burk reciprocal plot (plot not shown). The initial rate of ester synthesis and the yield of ethyl butanoate were also directly proportional to the tributyrin concentration, but only up to 8·3 mM (Fig. 3b). The K_m value for tributyrin was approximately 4 mM. A concentration of 16·5 mM-tributyrin was saturating for the lipase, as indicated in Fig. 3b.

Figure 4 shows the formation of butanoic acid during synthesis of ethyl butanoate by Palatase from 33 mM-tributyrin and 100 mM-ethanol in the same reaction mixture. The rate of butanoic acid release (hydrolysis) and the yield of butanoic acid released were much higher than those of ethyl butanoate synthesis (alcoholysis). Similar results were obtained with 33 mM-tributyrin and various ethanol concentrations tested, as indicated in Fig. 3a.

Figure 5 shows the production of ethyl butanoate by Palatase in a cheese-based medium. There was consistent formation of this ester when the medium was spiked with ethanol. On the contrary, no ester was detected when either ethanol or Palatase was not added to the medium. In addition to ethyl butanoate, other esters such as ethyl hexanoate were detected but not quantifiable in the medium supplemented with ethanol (data not shown).

Discussion

The mechanism of ester synthesis in raw milk and cheeses is not well understood and has been a subject of hypothesis for many years. Esterification of free fatty acids and ethanol

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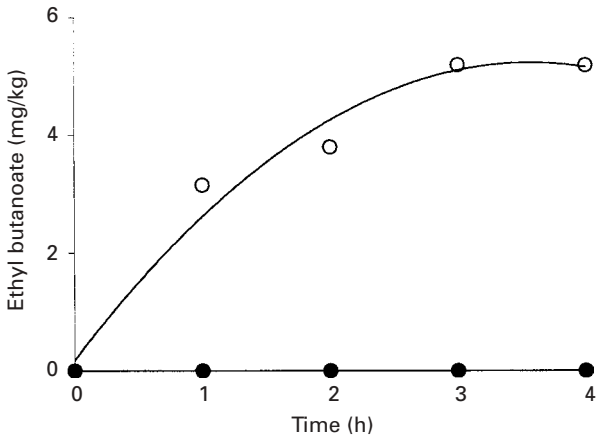

Fig. 5. Synthesis of ethyl butanoate by Palatase in a cheese-based medium spiked with ethanol (○). The control (●) had no ethanol added.

by esterases from lactic acid bacteria and pseudomonads is generally recognized as the mechanism of ester formation in raw milk and cheeses (Hosono et al. 1974; Morgan, 1976). However, in this study, the fungal lipase Palatase could not use butanoic acid; rather tributyrin was used in the synthesis of ethyl butanoate from ethanol (Fig. 1). This was not an esterification reaction, but a transferase reaction (alcoholysis) in which the butyryl group from the tributyrin was transferred to ethanol. In the synthesis of ethyl butanoate in the cheese-based medium spiked with ethanol (Fig. 5), presumably the butyryl group was derived from glycerides in milkfat and transferred to ethanol. Alcoholysis is the transfer of fatty acyl groups from acylglycerols to alcohols without direct involvement of water (Briand et al. 1995). Therefore, the fungal lipase Palatase is essentially an acyltransferase and the acyl acceptor may be either water (hydrolysis) or alcohol (alcoholysis) (Fig. 4). Ester synthesis through alcoholysis has been reported for lipases from other sources (Briand et al. 1994, 1995; Lecointe et al. 1996). Therefore, ester synthesis via alcoholysis may be a common feature of lipases in an aqueous environment such as cheese.

It should be pointed out that the fungal lipase Palatase used here also catalysed the hydrolysis of tributyrin and milkfat (Fig. 4 and unpublished data). The hydrolytic activity of this lipase appeared to dominate over its transferase activity (ester synthesis via alcoholysis) (Fig. 4). Milkfat hydrolysis is the primary function of lipases in the manufacture of natural cheeses and EMC (El Soda, 1997; Fox & Wallace, 1997; Kilcawley et al. 1998). The results from our study suggest that lipases may contribute to both lipolytic and estery/fruity flavours of natural cheeses and EMC, provided a sufficient amount of alcohol (ethanol) is available. It should also be stressed that certain lipases can synthesize esters in an aqueous medium by reversing hydrolysis (esterification) and that numerous lipases are capable of esterification in organic media containing little water (Malcata et al. 1992; Yahya et al. 1998).

The fungal lipase Palatase had a relatively high K_m for ethanol (200 mM) and a relatively low K_m for tributyrin (4 mM) (Fig. 3) and the cheese conditions either had no adverse impact on (low pH) or were conducive to (reduced a_w or addition of NaCl) (Fig. 2) ester synthesis. These findings suggest that, when Palatase is used in cheese systems, the ethanol concentration rather than the cheese environment is the limiting factor in determining the rate of ester synthesis and the yield of esters. The ethanol concentrations in natural cheeses vary with cheese type, manufacturing conditions and practices, ranging from 36 to 320 ppm in non-fruity cheese and from 400 to 2040 ppm in fruity cheese (Sandine et al. 1972). Ethanol in cheeses arises mainly from sugar fermentation (glycolysis) by starter cultures and adventitious microorganisms. Therefore, the selection of starter cultures and control of adventitious microorganisms become important as to the development (or lack) of fruity flavour in fermented dairy products.

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