Method for the measurement of lipase activity in milk

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Lipolysis has many effects in dairy technology. Undesirable consequences include deleterious effects on the organoleptic properties of milk and some cheeses; however, it is desirable in some other cheeses.

The usual methods for detecting lipolysis in milk measure the amounts of free fatty acids liberated from triacylglycerols (International Dairy Federation, 1991). Lipase activity is detected by incubation of samples with substrates such as tributyrin (Castberg *et al.* 1975) or triolein (Nilsson-Ehle & Schotz, 1976; Shirai *et al.* 1982). A few reports describe methods using synthetic chromogenic substrates, e.g. *p*-nitrophenyl butyrate (Shirai & Jackson, 1982), *β*-naphthyl caprylate (McKellar, 1986; McKellar & Cholette, 1986) or 4-methylumbelliferyl oleate (Stead, 1983). Tsakalidou *et al.* (1992) have developed the detection of esterase activities on electrophoresis gels.

Clarifying Reagent®, a patented product (Linden *et al.* 1987), is a mixture of organic solvents and detergents with an apparent pH > 13 and a very low absorbance between 340 and 800 nm. It is already used in many routine analyses of milk such as the measurement of amino and sulphydryl groups (Humbert *et al.* 1990; Guingamp *et al.* 1993) and *N*-acetyl-*β*-glucosaminidase activity (Humbert *et al.* 1995). The measurement of lipase activity in milk appears to be another potential application of this reagent since milk is a turbid fluid that interferes with colorimetric procedures. Clarifying Reagent renders casein micelles and fat globules soluble and allows direct spectrophotometric measurements to be made without the preliminary separation and extraction steps needed in many protocols.

The purpose of the present study was to test a method for measuring lipase activity in milk samples using Clarifying Reagent to eliminate turbidity and to compare this method with that of Castberg *et al.* (1975). Measurements on cheeses were also possible. The requirement was that when the Clarifying Reagent was added at the end of the incubation it should not hydrolyse the excess substrate, destroy any reaction product or interfere with the spectrophotometric measurement. It was an added advantage if it contributed to stopping the enzymic reaction by raising the pH.

MATERIALS AND METHODS

Materials

Individual and bulk fresh bovine and caprine milk samples were collected from a local experimental farm. Cheeses were bought at a local market.

Chemical reagents, phenylmethanesulphonyl fluoride (PMSF), dimethyl-

formamide (DMF), EDTA and tributyrin were from Merck (D-64293 Darmstadt 1, Germany). Tributyrin was emulsified at 100 g/l in gum arabic (100 g/l) and the emulsified mixture ultrasonicated at 5 °C for 5 min. Clarifying Reagent was obtained from Prolabo (F-5011 Paris, France). The substrate *p*-nitrophenyl butyrate was from Sigma Chemical Co. (St Louis, MO 63178, USA). It was dissolved in acetonitrile and stored in aliquots at −20 °C.

Lipase measurements

The lipase activity in the milk samples was measured both by our proposed method incorporating the use of Clarifying Reagent and by a standard procedure.

The principle of our method was similar to that reported by Humbert *et al.* (1995) for *N*-acetyl-*β*-glucosaminidase activity measurements. A synthetic substrate (Shirai & Jackson, 1982) in buffered solution was added to the milk sample. After incubation, a portion of Clarifying Reagent was mixed with the reaction mixture to clarify it and stop the enzymic reaction.

The protocol given here was adopted after tests to determine optimum conditions (results not reported). In a test tube, milk sample (0·5 ml) was mixed with 2 ml 0·05 M-barbital buffer, pH 7·6. Then 0·4 ml inhibiting mixture (three volumes 0·06 M-EDTA adjusted to pH 7·6 with 2 M-NaOH plus one volume 0·06 M-PMSF in DMF) was added only to the blank tube. These mixtures were incubated at 37 °C for 15 min. Then substrate (0·05 ml 50 mM-*p*-nitrophenyl butyrate in acetonitrile) was added and the reaction mixture was shaken and incubated at 37 °C for 10 min. Then inhibiting mixture (0·4 ml) was added only to the sample tube and mixed. Finally 2 ml Clarifying Reagent was added to all the tubes. After shaking again they were incubated at 37 °C for 3–5 min. These mixtures were transferred into cuvettes and the absorbance at 420 nm was read within 15 min. Values for absorbance were converted to *μ*mol *p*-nitrophenol using a standard curve obtained by using 0–150 *μ*mol per assay. Blanks were prepared using the same conditions as assays except that lipase activity inhibitors were added to the milk sample before adding the substrate solution.

This method was compared with the titration procedure of Castberg *et al.* (1975) which measures the amount of butyric acid liberated from emulsified tributyrin. A milk sample (0·5 ml) was mixed with 2 ml reaction mixture (distilled water–1 M-Tris–1 M-NaCl buffer, pH 8·5–emulsified tributyrin in gum arabic (100 g/l), 2 : 1 : 1 by vol.). The mixture was incubated at 37 °C for 30 min, then butyric acid was extracted with 2 ml 0·1 M-H_2SO_4–1 M-NaCl and 6 ml hexane–diethyl ether (1 : 2·75, v/v). After shaking and decanting, the acidity of 3 ml of the organic layer was determined by titration with 0·01 M-NaOH in the presence of 1 ml thymol blue (0·2 g/l in isopropanol).

Absorbances were measured with a Shimadzu UV2100 spectrophotometer (Roucaire, F-78143 Velizy-Villacoublay, France).

Results and discussion

Under the assay conditions used, the enzymic reaction was linear for 12 min. Under the same conditions of pH, temperature and substrate, with lipoprotein lipase, Shirai & Jackson (1982) reported linearity over 13 min.

The specificities of psychrotrophic lipases and milk lipoprotein lipase in relation to triacylglycerol length and characteristics were discussed by Lawrence (1967), and by Jensen & Pitas (1976) and Morley & Kuksis (1977) respectively. These enzymes also

hydrolyse synthetic substrates if they are dissolved in water-miscible organic solvents (Entressangles & Desnuelle, 1974). Shirai *et al.* (1982) have used *p*-nitrophenyl butyrate as the substrate for investigating the catalytic characteristics of lipoprotein lipase. For these reasons we have chosen this substrate and used it dissolved in acetonitrile.

Under our conditions, the value of K_m determined by a Lineweaver–Burk plot ($y = 0{\cdot}835x + 1{\cdot}6477$) was 0·51 mM, and was similar to that reported by Shirai & Jackson (1982). The low stability of the substrate prevented our using a higher substrate concentration.

Preliminary studies have shown that different buffers such as carbonate, triethanolamine, borate, Tris and Tris–maleate under the same buffering conditions induced appreciable hydrolysis of *p*-nitrophenyl butyrate. Barbital was the only buffer with a pK_a value compatible with optimal enzymic reaction, at the same time causing no significant chemical degradation of this substrate.

Some inhibiting reagents previously used are EDTA (Laboureur & Labrousse, 1966), PMSF (Quinn *et al.* 1982) and ethylene glycol (Touraine & Drapron, 1987). In the present study it was impossible to inactivate enzymic activity by using these inhibitors separately, even after 30 min incubation. Total inactivation was obtained by mixing EDTA, PMSF and DMF, after 15 min incubation at 37 °C.

The use of 2 ml Clarifying Reagent produced a clear solution when the assay was carried out as reported in Materials and Methods. Because of its chemical composition, Clarifying Reagent denatures the enzyme molecules and so stops enzyme activity. EDTA promotes the action of Clarifying Reagent by complexing Ca^{2+} and avoids the pH rising above 8·8, which could damage the substrate. The absorbance of the resulting clear solution remained stable at room temperature for at least 15 min, allowing sufficient time to read a series of samples.

The repeatability of our method was satisfactory: after 10 min incubation a series of 20 measurements on a single sample of raw milk produced a lipase activity (mean ± SD) of 0·102 ± 0·003 μmol/min per ml milk with a CV of 3·25%. In a study carried out on 29 individual milk samples, there was a good correlation (Fig. 1) between results obtained with our proposed method and those using the titration method of Castberg *et al.* (1975). The regression equation was $y = 1{\cdot}44 + 863{\cdot}55x$ ($r = 0{\cdot}89$). However, some milk samples gave different results with the two methods. All the samples involved were individual milk samples and so the specificity of their lipases could be different towards the two kinds of substrate, soluble and insoluble. Alternatively, they could be doubtful experimental results.

The proposed procedure was applied over time to individual bovine milk samples, and mean values were 0·06 and 0·12 μmol/min per ml for five winter and five summer milk samples respectively. The mean value for spring caprine milk was 0·11 μmol/min per ml. It was also tested on varieties of cheese (10 g/l aqueous suspensions), and mean lipase activities for Brie-type and blue-type cheeses were 1·9 and 2·4 μmol/min per g respectively. These activities were higher than in milk samples because cheeses contain lipolytic enzymes from psychrotrophic bacteria of milk, starters and ripening flora.

In conclusion, the present study has shown that our proposed technique gave satisfactory results for lipase activity measurement and correlated well with a standard titration method. The major advantage of our method is the elimination of the extraction and centrifugation steps used to separate reaction products in the procedure of Castberg *et al.* (1975) and in other fatty acid titration methods. Our method is sensitive, rapid and simple, requiring only a colorimeter and, for example,

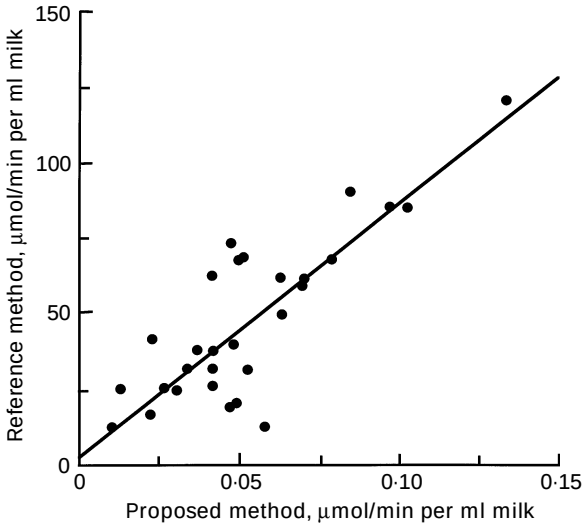

Fig. 1. Correlation between method proposed in this paper for measuring lipase activity (x axis) and the reference method of Castberg *et al.* (1975) (y axis) for 29 bovine milk samples. Enzyme activities are expressed in μmol p-nitrophenol released/min per ml milk for the proposed method and μmol acidity/min per ml milk for reference method.

no pH-stat titrator or gas chromatograph as in the method of Deeth *et al.* (1983) or even a spectrofluorimeter as in the method of Stead (1983). Because of these advantages it could easily be automated and used for routine analysis.

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