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Immobilized lipase-mediated acidolysis of butteroil with conjugated linoleic acid: batch reactor and packed bed reactor studies

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Abstract

Six commercial lipases, in either free or immobilized forms, were screened for their ability to catalyze acyl exchange between the triacylglycerols of butteroil (milkfat) and conjugated linoleic acid (CLA) in an organic solvent-free medium. Immobilized lipase preparations from *Candida antarctica* and *Mucor miehei* demonstrated the ability to increase the CLA content of the milk fat acylglycerols from the native value of 0.6 g/100 g fat to values which were at least an order of magnitude higher. Comparable increases were also obtained with a free enzyme from *Candida rugosa*.

In addition to the screening studies, the effects of the weight ratio of milkfat to CLA on the product distribution and of the water content on the kinetics and maximum extent of this acidolysis reaction were systematically investigated in a batch reactor: The fatty acids liberated from the butteroil triacylglycerols were primarily short chain fatty acids, especially butyric and caproic acids.

Modified butteroils were also produced via acidolysis of butteroil with CLA in a packed bed reactor containing an immobilized lipase preparation from *C. antarctica.* Significant enrichment of the butteroil in CLA residues was accomplished at reactor space times (fluid residence times) of 2–4 h at 40–60°C. Under these conditions, approximately 80–90% of the free CLA fed to the reactor is (inter)esterified. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Conjugated linoleic acid; Butteroil; Milkfat; Lipase; Acidolysis; Interesterification

1. Introduction

Conjugated linoleic acid (CLA) has aroused considerable interest on the part of food manufacturers because of its potential for use in the formulation of nutraceuticals. Nutraceuticals are foods (or parts of foods) which provide therapeutic or preventative medicinal values in addition to nutritional benefits. The market for such foods reflects increasing consumer interest in foods that offer significant health benefits in terms of prevention of particular disease conditions, e.g., certain forms of cancer and cardiovascular disease. CLA is a generic term that refers to

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a mixture of geometric and positional isomers of straight chain C18 fatty acids containing two conjugated double bonds. Commercially available CLA preparations are prepared by chemical isomerization and contain at least 12 different isomers of CLA, as determined by silver ion HPLC [1].

The physiological benefits of CLA consumption have been documented in a wide variety of animal models, and extensive toxicological and epidemiological studies are currently in progress. The first volume in a new series edited by Yurawecz et al. [2] provides an excellent summary of the various studies documenting the benefits of incorporating CLA in the diets of several species. These benefits include anticancer and antiatherogenic effects, enhancement of immunological functions, and alteration of metabolic processes to reduce production of fatty tissue and increase muscle mass. In this context, CLA is an extremely promising substance for use in the formulation of nutraceuticals.

One mechanism by which CLA could be readily incorporated in human diets is by enrichment of naturally occurring fats and oils in CLA residues. Dairy manufacturers are particularly interested in the market potential of dairy products fortified in CLA by partial or complete replacement of the fatty acid residues present in native milkfat by CLA residues, thereby producing a nutraceutical with the desired prophylactic/therapeutic effect. Among the wide variety of dairy products containing milkfat that could be fortified in this manner are dairy spreads, frozen desserts, and fluid products. Because of the wide variety of other processed foods that also incorporate milkfat in their production, the dairy industry constitutes a logical entry point for the development of technology by which CLA may be incorporated in triacylglycerols. To this end, we have investigated the kinetics and the extent of incorporation of CLA in the triacylglycerols that constitute milkfat as a consequence of interesterification (acidolysis) reactions. The work described below represents an extension of earlier batch reactor studies involving both the interesterification of butteroil with CLA [3,4] and the polyesterification reactions of CLA and glycerol [5,6]. Results obtained in a preliminary study of the feasibility of using a packed bed reactor to effect the continuous interesterification of butteroil with CLA have also been reported elsewhere [7].

2. Experimental procedures

2.1. Materials

Enzymes: F-AP16 (referred to as F) from *Rhizopus oryzae*, PS (referred to as P) from *Pseudomonas*, and lipase AY-30 (referred to as Y) from *Candida rugosa* (all free) were obtained from Amano (Lombard, IL). Chirazyme L-2 from *Candida antarctica* fraction B, Chirazyme L-5 from *C. antartica* fraction A, and Chirazyme L-9 from *Mucor miehei* (all carrier fixed) were obtained from Boëhringer-Mannheim (Indianapolis, IN).

A butteroil fraction that remains liquid at 20°C was provided by Grassland Dairy Products (Greenwood, WI). CLA (98% purity) was kindly provided by Dr. Michael Pariza of the Food Research Institute of the University of Wisconsin-Madison.

2.2. Apparatus

2.2.1. Batch reactor

The batch reactor experiments were conducted in 15×100 mm screw cap vials. Identical quantities of the stock mixture of reactants, combined with identical quantities of the enzyme of interest, were placed in the vials, which were then immersed in an orbital shaker operating at ca. 200 rpm and the temperature of interest.

2.2.2. Packed bed reactor

The packed bed reactor consisted of a Delrin tube which was 10 cm long and 1.13 cm in internal diameter. The end fittings were also constructed of Delrin. To obtain temperature control, the reactor was contained inside a jacket connected to a flow loop containing a water circulator and a constant temperature bath. The internal volume of the tube was ca. 10 ml. When the tube was packed with 3.26 g of the immobilized lipase preparation Chirazyme L-2, the void volume was 7.34 ml.

The substrate mixture was fed by a syringe pump at rates that produced space times ranging from 10 min to 12 h. (For a liquid phase system, the reactor space time is equal to the residence time of the fluid in the reactor [8].)

2.3. Experimental procedures

2.3.1. Batch reactor

A mixture of butterfat and CLA in the desired ratio was prepared and stored under nitrogen. One-gram portions of the mixture were placed in 15 × 100 mm screw-cap vials, the enzyme was added, and the tubes were closed under nitrogen. The vials were placed in an orbital shaker operating at ca. 200 rpm and the temperature of interest. Vials were removed from the shaker at periodic intervals for subsequent analysis. The enzymes were removed by filtration using a 0.45-μm membrane filter. Samples were refrigerated at −20°C until they could be analyzed.

2.3.2. Packed bed reactor

The reactor and its contents were first allowed to come to thermal equilibrium with the water circulating in the surrounding jacket. Reactants were then fed to the packed bed reactor at the desired flow rate for each reactant. After start-up of the reactor, a time on the clock equal to at least 1.5 reactor space times (fluid residence times) was allowed to elapse before effluent samples were taken for use in the analysis of the reaction kinetics. Thus, the experimental points correspond to quasi steady-state values of the effluent composition. Samples of the effluent stream were taken at periodic intervals and refrigerated at −20°C until they could be analyzed.

2.4. Analytical procedures

2.4.1. Gas chromatography

Esterified and total fatty acids in product mixtures were analyzed by gas chromatography using the method of Chin et al. [9], as modified by Williams et al. [10], which involves parallel selective methylation of duplicate samples, one with NaOH–methanol and the other with HCl–methanol, respectively. Sample handling procedures were based on an analytical protocol described previously [3].

Triacylglycerol profiles of the interesterified product mixtures were also examined by gas chromatography. The various fractions emerging from the HPLC column were collected using a splitter valve. Portions containing ca. 30 mg TAG were diluted with isooctane to a concentration of ca. 1 mg/ml. Then, 1 μl of the resulting solution was injected to a Hewlett-Packard 5890 Series II-Plus gas chromatograph equipped with an on-column injector, FID and an Alltech Heliflex AT-1 30 m by 0.25 mm i.d. column. The TAG were separated using a temperature programmed ramp from 220°C to 327°C at 3°C/min followed by holding the column at 327°C for an additional 36 min.

2.4.2. HPLC

Acylglycerols (1{3}-mono, 2-mono, 1,2-di, 1,3-di, and tri- as well as free fatty acids) in the reaction mixtures were quantified by HPLC following the procedure described by Liu et al. [11]. Calibration curves for each component were prepared using high purity standards obtained from Sigma. The acylglycerols were subjected to analysis in a system containing an evaporative light-scattering detector.

2.4.3. Positional analysis

Analysis of the positions occupied by the various fatty acid residues in the triacylglycerols was conducted using a modified version of the method of Williams et al. [10].

3. Results and discussion

A particularly appealing vehicle for addition of CLA to human diets is milkfat (perhaps in the form of butteroil) because of the wide variety of foods in which this material can easily be incorporated. A butteroil fraction that remains liquid at room temperature was selected as the form of milkfat to be reacted with CLA in order to facilitate handling of the feedstock. Use of this material has the additional advantage that it permits one to employ a wider range of temperatures in the studies of the reaction kinetics than would otherwise be possible. This material has previously been used by Garcia et al. [4,7] in preliminary studies of the acidolysis of milkfat with CLA.

3.1. Batch reactor studies

The results of preliminary process feasibility studies in a batch reactor are reported elsewhere [3,4].

The results reported here focus first on additional batch reactor screening studies designed to further identify appropriate enzymes for use in mediating the acidolysis reaction, characterize the distribution of product triacylglycerols, and ascertain the effects of moisture level on both rate and yield. Once quasi-optimum conditions for acidolysis of butteroil with CLA were identified, experiments were conducted in a packed bed reactor.

3.1.1. Enzyme screening

In an effort to identify lipases with activity for acidolysis of milkfat with CLA, screening experiments were conducted. The extent to which each enzyme is able to effect the incorporation of CLA in milkfat triacylglycerols in 24 h of reaction is depicted in Fig. 1. Because manufacturers do not provide information concerning the precise amount of lipase contained in the proprietary formulations, the screening experiments were based on the use of a large amount of enzyme to ensure that the yields obtained with each of the various enzymes would not be limited by the reaction kinetics.

Examination of Fig. 1 indicates that for acidolysis under these conditions, the free lipases P and Y from Amano, and the immobilized lipases L-2, L-5, and L-9 from Boëhringer-Mannheim, all exhibit comparable catalytic activity for this reaction. Hence, five of these preparations could, in principle, be employed to catalyze the acidolysis of milkfat with CLA. Hence, further experiments were conducted with each of these preparations to further characterize the properties of these biocatalysts. Because Lipase F was not an effective catalyst in the present context, no further studies of this preparation were conducted.

Although lipases P and Y demonstrated their ability to effect a higher percentage of incorporation of CLA in milkfat triacylglycerols, there are a number of advantages that make immobilized enzymes more attractive for industrial applications of this reaction. For example, immobilized preparations usually exhibit higher stability, are more easily recovered for subsequent reuse, and offer economic benefits in terms of being able to produce a greater quantity of product per unit of enzyme consumed. Hence, potential manufacturers of nutraceuticals are more interested in employing an immobilized enzyme for this application than soluble enzymes. In this context, the properties of the three immobilized lipases from Boëhringer-Mannheim, L-2, L-5, and L-9, indicated that these enzymes merited further investigation.

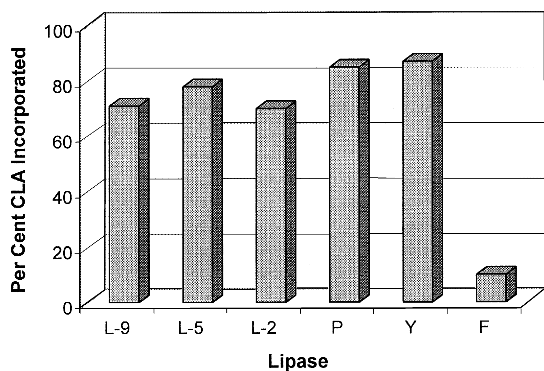

Fig. 1. Screening of commercial lipases for their ability to catalyze the acidolysis reaction of CLA and a low melting fraction of butteroil at 50°C after 24 h. The feedstock consisted of 1 g of a 1:10 (v/v) mixture of CLA and butteroil, together with 150 mg of one of the following immobilized enzymes: Chirazyme L-9, Chirazyme L-2, and Chirazyme L-5; or 250 mg of one of the following free enzymes: Amano P, Amano Y, and Amano F.

3.1.2. Effect of the weight ratio of CLA to milk fat on product distribution

3.1.2.1. Effect on total number of carbon atoms present in fatty acid residues. In order to ascertain the nature of the products resulting from the acidolysis reaction, gas chromatography was employed to determine triacylglycerol profiles of the interesterified product mixtures. Examination of Fig. 2 indicates that acidolysis of milkfat mediated by Chirazyme L-2 leads to significant decreases in triacylglycerols with total residue carbon numbers from C34 to C42 and to significant increases in triacylglycerols with total residue carbon numbers from C46 to C54. These results reflect the substitution of CLA, a long chain fatty acid (C18:2) for medium and short chain fatty acid residues whose corresponding triacylglycerols in native butteroil are normally located in the C34 to C42 region (primarily C4:0,C14:0,C16; C4:0,C14:0,C18; C4:0,C16:0,C16; C6:0,C14:0,C18; and C6:0,C16:0,C16). This result is consistent with the decreases observed in the per-

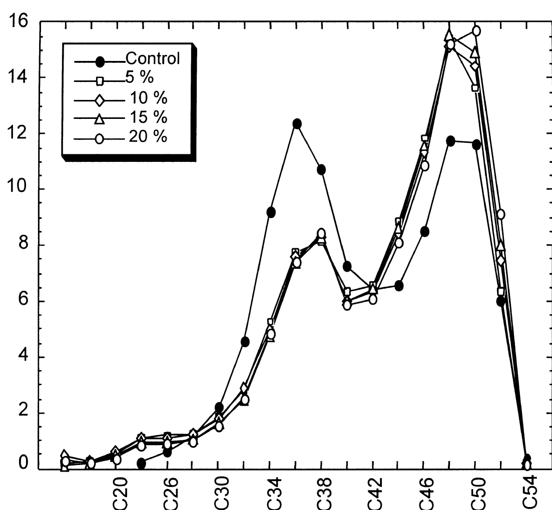

Fig. 2. Distribution of triacylglycerols by total number of residue carbon atoms as effected by interesterification in the presence of Chirazyme L-2 with different proportions of CLA in the initial reaction mixture. The control is the original low melting fraction of butteroil.

centages of short chain fatty acid residues reported in Tables 1 and 2. Triacylglycerols containing 32 to 50 carbon atoms do not exhibit significant shifts in their relative proportions for products formed by acidolysis of butteroil with weight percentages of CLA ranging from 5% to 20%. However, larger amounts of triacylglycerols with total residue carbon numbers of 52 and 54 were obtained when the percentage of CLA in the original mixture was increased to higher levels. The small increase in the number of triacylglycerols with residue carbon numbers totaling C50 and C52, which is observed when the ratio of CLA to milk fat is increased, could also be a consequence of substitution of CLA for palmitic acid (see Tables 1 and 2).

3.1.2.2. Effect on the composition of the fatty acid residues in modified milkfat triacylglycerols. The influence of the ratio of CLA to milk fat on the percentage of CLA incorporated as residues in milkfat triacylglycerols and the distribution of fatty acid residues in these triacylglycerols was studied for three different ratios (w/w) of CLA to the low melting fraction of butteroil. Distributions of fatty acid residues in milkfat triacylglycerols both prior to and subsequent to the acidolysis reaction are indicated in Tables 1 and 2 for immobilized preparations

Table 1
Effect of the initial weight ratio of CLA to butteroil on the weight percentages of various fatty acid residues in triacylglycerols produced in the acidolysis of butteroil when catalyzed by three different immobilized lipases

Fatty acid	Initial milk fat g residue/100 g fat	Enzyme								
		Lipase L-2			Lipase L-5			Lipase L-9		
		CLA/milk fat (w/w)								
		1/10	1/7	1/4	1/10	1/7	1/4	1/10	1/7	1/4
		g residue/100 g fat			g residue/100 g fat			g residue/100 g fat		
C4	3.9	2.0	1.6	0.9	1.8	1.1	1.1	2.0	1.5	0.7
C6	2.4	0.7	0.9	0.6	1.0	1.5	0.9	1.5	1.0	0.5
C8	1.4	0.6	1.0	0.6	0.5	1.1	0.5	1.0	1.2	0.4
C10	3.1	1.6	2.3	1.4	1.7	2.3	1.5	2.0	2.3	1.2
C12	3.5	2.2	2.6	1.9	1.8	2.7	2.1	2.0	2.5	1.8
C14	12.8	10.5	10.8	9.4	10.0	11.0	10.2	10.3	11.3	9.2
C16:1	1.7	1.3	1.5	1.5	1.4	1.4	1.4	0.6	1.7	1.4
C16	25.5	25.6	22.6	23.5	26.0	22.7	21.0	25.4	21.3	21.6
CLA	0.7	7.1	12.2	13.0	7.8	13.0	16.9	7.1	13.7	17.3
C18:2	3.7	3.3	3.1	3.1	2.7	3.1	2.9	2.6	3.4	2.8
C18:1	27.3	28.0	26.1	29.0	27.0	24.8	28.1	28.3	25.5	28.9
C18	9.1	11.1	8.6	10.6	11.0	8.7	8.2	10.0	7.8	9.0
Others	5.1	6.0	6.5	4.7	7.3	6.4	5.2	7.2	6.9	5.2

Conditions: 1 g of butteroil, 0.1 g of CLA, 150 mg of lipase. The orbital shaker was operated at 50°C and 200 rpm.

Table 2
Effect of the initial weight ratio of CLA to butteroil on the weight percentages of various fatty acid residues in triacylglycerols produced in the acidolysis of butteroil when catalyzed by two different free lipases

Fatty acid	Initial milkfat g residue/100 g fat	Enzyme					
		Lipase P			Lipase Y		
		CLA/milk fat (w/w)					
		1/10	1/7	1/4	1/10	1/7	1/4
		g residue/100 g fat			g residue/100 g fat		
C4	3.9	2.0	1.5	1.6	2.4	2.2	2.0
C6	2.4	1.8	1.0	0.9	1.6	1.5	1.3
C8	1.4	0.3	0.7	0.6	0.9	1.0	0.8
C10	3.1	1.1	1.6	1.4	2.1	2.1	1.7
C12	3.5	2.0	2.0	1.8	2.5	2.5	2.1
C14	12.8	9.9	9.8	9.3	11.2	10.2	9.6
C16:1	1.7	1.6	1.4	1.3	0.7	1.4	1.4
C16	25.5	23.5	24.9	24.1	24.7	21.2	20.5
CLA	0.7	8.5	10.1	14.7	8.7	12.7	17.2
C18:2	3.7	3.3	3.0	2.6	3.3	3.0	2.7
C18:1	27.3	32.0	28.8	25.5	26.0	26.0	26.8
C18	9.1	9.5	10.4	10.9	10.7	9.1	8.7
Others	5.1	4.4	4.8	5.3	5.2	7.1	5.3

Conditions: 1 g of butteroil, 0.1 g of CLA, 250 mg of lipase. The orbital shaker was operated at 50°C and 200 rpm.

and free enzymes, respectively. For weight ratios of CLA to milkfat of 1/10 and 1/7, all three immobilized enzyme preparations and soluble lipases P and Y were able to bring about incorporation of between 70% and 90% of the free CLA employed as a reagent.

With the exception of the lipase from *Pseudomonas* sp. (lipase P), all of the lipases investigated achieve a maximum in the percentage of CLA incorporated as a triacylglycerol residue when the weight ratio of CLA to milkfat is 1/7. Further studies will be required to further pinpoint the exact ratio at which this percentage is maximized.

Examination of the entries in Tables 1 and 2 indicates that incorporation of CLA residues in milkfat triacylglycerols is accompanied by a significant decrease in percentages of the short chain fatty acid residues, specifically the C4, C6, and C8 fatty acids that are primarily located at the sn-1 and sn-3 positions of the glycerol backbone. It is important to recognize that because of the low molecular weights of these fatty acids, a sample that contains 4% by weight of butyric acid (C4) may correspond to a composition of as much 10 mol%. Moreover, it must be recognized that these species play important roles in sensory phenomena associated with flavor generation. Consequently, further studies will be required to ascertain the dependence of the physicochemical and organoleptic properties of milkfats enriched in CLA on their CLA content. In nutraceutical applications, it will be important to retain the functional properties of the CLA modified milkfats so that dairy-type flavors will be retained, and the properties of the dairy products in which these modified milk fats are employed will retain the sensory and textural attributes that make them attractive to consumers.

Small decreases in the percentage of C14 residues were also observed for all lipases and weight ratios of CLA to milkfat. When the initial weight ratio of CLA to butteroil is 1/4, as much as 20% of the palmitic acid residues present in the native milkfat can be released during acidolysis in the presence of all of the lipases investigated, except for lipase P. The percentages of C18:1 and C18 residues in the milkfat triacylglycerols are not changed significantly by the acidolysis reaction. The minor increments observed in the percentages of these fatty acids in some of the experiments could be the result of the analytical error or a consequence of impurities in the CLA mixture utilized.

It is also interesting to note that the experiments conducted with the lipase from *C. rugosa* (lipase Y) reveal that this enzyme has a quite different selectivity than the other soluble lipase studied (lipase P). During acidolysis, lipase Y releases a much greater percentage of C16 and fewer residues with chain lengths of 12 or less.

3.1.2.3. Characterization of the positional distribution of fatty acid residues in product triacylglycerols. Since the fatty acid residues located at the sn-2 position of triacylglycerols are those that are absorbed to the greatest extent during the digestion process, it would be desirable to exchange CLA for the fatty acid present at this position in the triacylglycerols of milk fat. Preliminary studies related to the positional distribution of fatty acid residues in the modified milkfat triacylglycerols indicate that for the various enzyme preparations employed in the present studies, CLA is primarily incorporated at the sn-1 and sn-3 positions of the glycerol backbone. The CLA residues primarily replace short chain fatty acids (namely, C4, C6, and C8). The preliminary results also indicate that the amount of CLA esterified at the sn-2 position increased as the initial ratio of fatty acid to glycerol increased. The selectivity of the various lipases for exchange with the fatty acid originally located at the sn-2 position of the triacylglycerol decreases in the order L-5 > L-2 > Y > L-9 > P.

Although additional experiments are necessary to obtain a more accurate quantitative picture of the positional distribution of fatty acid residues subsequent to the acidolysis reaction, the preliminary analysis reveals that when a weight ratio of CLA to milkfat of 1/10 and Chirazyme L-5 are employed, up to 50% of the CLA is esterified at the 2-position, Rogalska et al. [14] have previously reported good regioselectivity of this lipase for the hydrolysis of the sn-2 ester group in triolein.

Since the goal of the present research effort is to develop a continuous procedure for incorporating CLA into milk fat triacylglycerols in a packed bed reactor with a view towards eventual commercialization of this technology, Chirazyme L-2 was selected for further use in subsequent experiments. This biocatalyst is an immobilized form of *C. antartica* fraction B, what facilitates its use in a packed bed reactor. It permits one to obtain up to 85% incorporation of the original CLA when a weight ratio of CLA to milkfat of 1/7 is employed, has significant selectivity for reaction at the sn-2 position, and is available at reasonable cost. However, the immobilized lipases Chirazyme L-5 and L-9 or an immobilized form of lipase Y could also be considered for this application, provided that future developments of the commercial potential of these enzymes lead to approval for food use by regulatory agencies and to improved process economics by virtue of reduced cost of production or to demonstration of superior stability under process conditions.

3.1.3. Effect of water content

To study the effect of water content on the rate and extent of incorporation of CLA residues in milkfat triacylglycerols, additional amounts of water were added to the enzyme preparation to adjust the water content of the corresponding initial mixtures to levels ranging from 0.5% to 2.0%. In this study, the initial reaction mixture consisted of 1 g of butteroil, 0.1 g of CLA and 100 mg of the *C. antarctica* lipase preparation Chirazyme L-2. For reaction at 50°C, the data presented in Fig. 3 indicate that the extent of incorporation of CLA residues was always greatest for the situation in which no water was added to the original system. The rate of incorporation of CLA

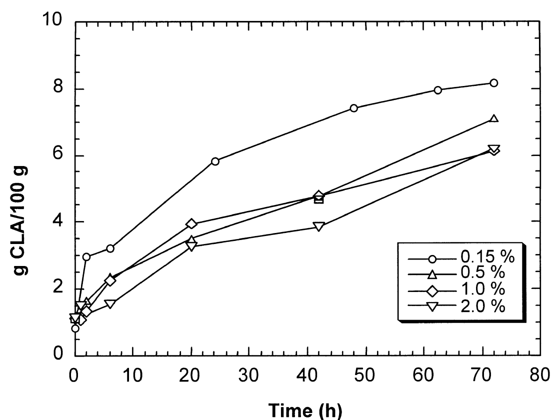

Fig. 3. Effect of water concentration on the interesterification of CLA and a low melting fraction of butteroil at 50°C. The initial reaction mixture consisted of 1 g of butteroil, 0.1 g of CLA, and 100 mg of the immobilized *C. antarctica* lipase preparation Chirazyme L-2 (from Boëhringer-Mannheim).

was fastest for the system to which no water was added. The effect of the initial moisture content of the system on the composition of the system resulting from 42 h of reaction at 50°C is shown in Table 3. This time is not sufficient for chemical reaction equilibrium to be approached, but it is sufficiently long to achieve very significant incorporation of CLA residues in the milkfat.

The presence of water in the reaction mixture is expected to have two primary effects. Because the first step in interesterification (acidolysis) of a triacylglycerol is hydrolysis of an ester bond to form a lower acylglycerol and release a fatty acid, it is reasonable to expect that the overall rate of the acidolysis reaction will be affected by the concentration of water in this system. Because the position of thermodynamic equilibrium for esterification/hydrolysis reactions is influenced by the water content of the system, the equilibrium extent of incorporation of CLA in milkfat triacylglycerols will also be influenced by this variable.

Analyses for moisture content by Karl Fischer titration revealed that the water contents of the initial CLA concentrate, the butteroil, and the enzyme preparation were 1.58% (w/w) ($a_w = 0.476$), 0.058%, and 0.303%, respectively. The weighted average value of the moisture content of the initial system was thus 0.15%. Examination of the plots in Fig. 3 and the entries in Table 3 indicates that the reaction occurs most rapidly when no water is added to the initial mixture, but that the differences between the composition of the product mixture obtained when no water is added, and that obtained from a system initially containing 0.5% moisture were relatively small. The system to which no water was added contained a very large proportion of TAG, an appreciable level of free fatty acids, and minimal amounts of monoacylglycerols (MAG) and diacylglycerols (DAG). However, as the moisture content of the initial system was increased beyond 0.5% there was relatively little effect of the rate of incorporation of CLA, but there was a marked shift in the composition of the product mixture. The concentrations of free fatty acids and lower acylglycerols (MAG and DAG) in the product mixture all increased with increasing water content of the initial mixture. The free fatty acid concentrations differed by a factor of ca. 2 between the systems containing 0.15% and 2.0% moisture. Moreover, the MAG and DAG in these systems differed by an even larger factor (> 5). For the acyl exchange reaction of butteroil and oleic acid in isooctane solution, Oba and Witholt [12] observed no interesterification activity at water contents of 0.1% or lower, but this activity was optimum for systems containing between 0.2% and 0.3% moisture. In our experiments, the optimum is located near the intrinsic moisture content of the starting materials to which no water has been added.

Table 3
Effect of moisture content of the initial reaction mixture on the composition of the product (in weight percentage) obtained after 42 h of reaction

	Moisture wt.%				
	0.15	0.50	1.0	1.5	2.0
	Component wt.%				
Triacylglycerols	91.25	91.86	87.90	81.86	79.51
Free fatty acids	8.36	7.60	10.18	13.90	16.53
1,3-DAG	0.29	0.33	1.15	2.34	1.66
1,2-DAG	0.11	0.21	0.54	1.16	0.98
MAG	0.00	0.00	0.23	0.74	1.32

Conditions: 1 g of butteroil, 0.1 g of CLA, 100 mg of L-2 lipase with moisture adjusted by direct addition of water to the enzyme preparation. The orbital shaker was operated at 50°C and 200 rpm.

3.2. Packed bed reactor studies

In the packed bed reactor studies involving Chirazyme L-2, the composition of the effluent was determined as a function of the space time at several temperatures and at several different ratios of reactants. (For a liquid phase system the reactor space time is equal to the residence time of the fluid in the reactor [8].)

3.2.1. Effect of temperature

Inspection of the plots in Fig. 4 indicates that as was the case for a batch reactor study [4], operation of the packed bed reactor at 50°C is preferable to operation at higher or lower temperatures. This optimum reflects the interactions of the effects of temperature on both the rate constants for the elementary reactions constituting the enzyme-mediated reaction

mechanism and the rate constant for deactivation of the enzyme. Higher temperatures increase the rates of the intrinsic reaction of interest, but also lead to the loss of enzyme activity via a variety of processes, e.g., denaturation of the enzyme.

Relatively short space times (0.5–2 h) are sufficient to obtain an effluent in which the triacylglycerols contain very significant amounts of CLA residues. As the length of time the mixture of substrates is in contact with the immobilized enzyme increases, the extents of incorporation appear to approach asymptotic limits that are temperature-dependent. The differences in the asymptotes may reflect shifts in the positions for the several equilibria associated with the interesterification reactions.

3.2.2. Effect of ratio of reactants

Data characterizing the dependence of the extent of interesterification on the reactor space time and the ratio of substrates are shown in Fig. 5 for reaction at 50°C. Inspection of these data indicates that for the space times of greatest industrial interest, namely, space times less than 3 h, very significant extents of interesterification can be obtained at 50°C.

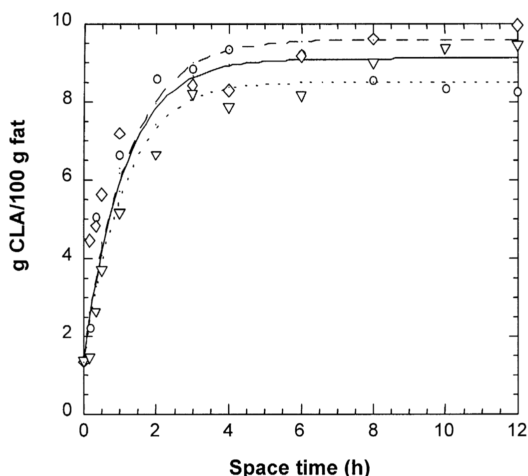

Fig. 4. Effect of temperature on the effluent extent of interesterification of CLA with the triacylglycerides of butteroil for a weight ratio of CLA to butteroil of 1:10. Operating temperatures: 40°C (inverted triangles, dotted line), 50°C (open diamonds, long dashes), and 60°C (filled circles, solid line). The smooth curves correspond to the best fits of the data to the rate expression proposed by Reyes and Hill [13].

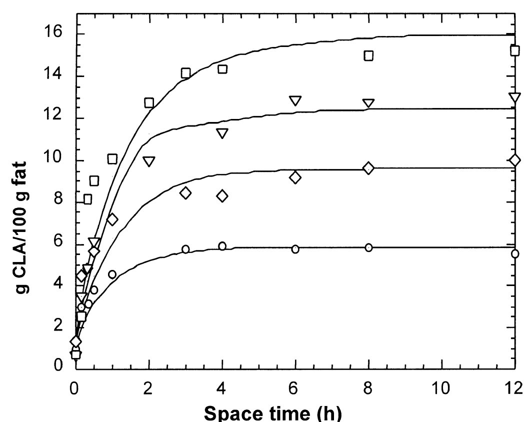

Fig. 5. Extent of interesterification of CLA and butteroil at 50°C as a function of the weight ratio of CLA to butteroil and the reactor space time. Ratios (w/w): 1:5 (squares), 3:20 (triangles), 1:10 (diamonds) and 1:20 (circles). The smooth curves correspond to the best fits of the data to the rate expression proposed by Reyes and Hill [13].

This inspection also reveals that for all ratios of the substrates, equilibrium is approached at space times of less than 6 h. In all cases, the asymptotic limits correspond to conversion of ca. 80–90% of the free CLA originally fed to the reactor to its esterified form. The smooth curves in Fig. 5 again correspond to the best fit of the mathematical form of the rate expression suggested by Reyes and Hill [13] to the data. This rate expression provides a reasonable fit of the experimental data for different ratios of CLA to butteroil. The model can also be employed to predict the rate at which CLA will be incorporated at different temperatures; however, for very short space times (less than 1 h), the model tends to underestimate the extent of interesterification.

4. Conclusions

The results presented in this paper suggest that enrichment of butteroil with CLA residues can be accomplished in a solvent-free system at reasonable rates using a continuous flow packed-bed reactor or a batch reactor containing Chirazyme L-2 from *C. antartica* fraction B. The immobilized lipases Chirazyme L-9 from *M. miehei* and Chirazyme L-5

from *C. antartica* fraction A also merit further investigation in this context.

CLA is mainly incorporated in the glycerol backbone by releasing short chain fatty acid residues bound to the sn-1 and sn-3 positions. The level of incorporation can be controlled by varying the ratio of CLA to butteroil fed to the reactor, the temperature, and the time of contact with the enzyme. A rate expression of the general Michaelis–Menten form provides a reasonable fit of the experimental data at different temperatures and various ratios of CLA to butteroil.

Further engineering studies will be necessary to evaluate process economics, as well as the desirability of incorporating various purification steps to remove the free fatty acids (and perhaps byproduct MAG and DAG) from the CLA-enriched butteroil product. In addition, thorough assessments of the physical and organoleptic properties of the products are needed to determine their functional properties and their suitability for use in formulating nutraceutical forms of manufactured dairy products.

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References

[1] N. Sehat, M.P. Yurawecz, J.A.G. Roach, M.M. Mossoba, J.K.G. Kramer, Y. Ku, Lipids 33 (1998) 217.
[2] M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza, G. Nelson (Eds.), Advances in Conjugated Linoleic Acid Research, vol. I, American Oil Chemists' Society Press, Champaign, IL, 1999.
[3] H.S. Garcia, J.M. Storkson, M.W. Pariza, C.G. Hill Jr., Biotechnol. Lett. 20 (1988) 393.
[4] H.S. Garcia, K.J. Keough, J.A. Arcos, C.G. Hill Jr., J. Dairy Sci. 83 (2000) 371.
[5] J.A. Arcos, C. Otero, C.G. Hill Jr., Biotechnol. Lett. 20 (1998) 617.
[6] J.A. Arcos, H.S. Garcia, C.G. Hill Jr., Biotechnol. Bioeng. (in press).
[7] H.S. Garcia, K.J. Keough, J.A. Arcos, C.G. Hill Jr., Biotechnol. Tech. 13 (1999) 369.
[8] C.G. Hill Jr., An Introduction to Chemical Engineering Kinetics and Reactor Design, Wiley, New York, 1977, pp. 255–256.
[9] S.F. Chin, W. Liu, J.M. Storkson, Y.L. Ha, M.W. Pariza, J. Food Compos. Anal. 5 (1992) 185.
[10] P.J. Williams, M.U. Khan, D. Wong, J. Lipid Res. 36 (1995) 1407.
[11] J. Liu, T. Lee, E. Bobik Jr., M. Guzman-Harty, C. Hastilow, J. Am. Oil Chem. Soc. 70 (1993) 343.
[12] T. Oba, B. Witholt, J. Dairy Sci. 77 (1994) 1790.
[13] H.R. Reyes, C.G. Hill Jr., Biotechnol. Bioeng. 43 (1994) 171.
[14] E. Rogalska, C. Cudrey, F. Ferrato, R. Verger, Chirality 5 (1993) 24.