Enzymatic Enrichment of Conjugated Linoleic Acid Isomers and Incorporation into Triglycerides.

Gerald P. McNeill[a,*], Chris Rawlins[b], and Anne C. Peilow[b]

[a]Loders Croklaan NA, Channahon, Illinois 60410-5249,
and [b]Unilever Research Colworth, Sharnbrook, Bedford MK44 1LQ, England

ABSTRACT: A method was developed for the enrichment of either the *cis*9,*trans*11 or the *trans*10,*cis*12 isomer of conjugated linoleic acid (CLA) from a synthetic CLA mixture consisting predominantly of these isomers in equal amounts. Lipases were screened for their ability to selectively esterify one isomer at a significantly greater rate than the other isomer. An immobilized lipase from *Rhizomucor miehei* was nonselective, but a lipase from *Geotrichum candidum* esterified the *cis*9,*trans*11 isomer more rapidly than the *trans*10,*cis*12 isomer. This selectivity was exploited at the kilogram scale to prepare an ester fraction with a content of 91% *cis*9,*trans*11 CLA and an unreacted free fatty acid fraction consisting of 82% *trans*10,*cis*12 CLA, based on total CLA content. The components of the reaction mixture were separated by molecular distillation. Each enriched fraction was then incorporated into palm oil triglycerides by interesterification with the non-selective lipase from *R. miehei.* Two triglyceride fats resulted, which were enriched in either *cis*9,*trans*11 CLA (26.5% *cis*9,*trans*11 and 1.7% *trans*10,*cis*12) or *trans*10,*cis*12 CLA (3.5% *cis*9,*trans*11 and 22.9% *trans*10,*cis*12).

Paper no. J9356 in *JAOCS 76,* 1265–1268 (November 1999).

KEY WORDS: CLA, *Geotrichum candidum,* lipase, purification, *Rhizomucor miehei.*

Conjugated linoleic acid (CLA) is a group of C18 fatty acids containing a pair of conjugated double bonds in either *cis* or *trans* configuration, located at various positions on the carbon chain. Naturally occurring CLA is composed mainly of the *cis*9,*trans*11 isomer, believed to be generated by rumen bacteria during the digestive process and subsequently incorporated into the body fat and milk fat of ruminant animals (1). An anticancer activity found in beef was attributed to the CLA component (2), and subsequent to that discovery, many studies have confirmed anticancer activity in a variety of models (3). Other beneficial bioactivities were also revealed, in particular the inhibition of body fat deposition during development of certain animal species, beneficial effects on risk of heart disease and improved immune function in farm animals (4–6). Unfortunately, all of the latter studies were performed with synthetic CLA containing between 2 and 12 CLA isomers; in some cases the isomer composition was not reported. It was therefore not known if one or more than one of the CLA isomers was responsible for the observed bioactivities.

The main objective of this study was to develop a method to enrich either the *cis*9,*trans*11 isomer or the *trans*10,*cis*12 isomer from a synthetic mixture. The synthetic mixture was carefully produced to be composed primarily of these two isomers with minimal contamination from other isomers. Owing to the similar chemical and physical properties of these isomers, separation by traditional techniques is difficult. Previous work by this group and others showed that lipase technology can be a powerful tool for the enrichment of fatty acids that are otherwise difficult to purify (7,8). It was therefore decided to investigate selective lipase esterification to facilitate enrichment of each of the CLA isomers. A second objective of the study was to convert the CLA fatty acids into triglyceride form, suitable for bioactivity studies in animal models.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Geotrichum candidum* (GC-4) was obtained from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Safflower oil and palm oil were provided by Unilever Research (Vlaardingen, The Netherlands). 1-Dodec-anol (lauryl alcohol, 98%) was purchased from Aldrich Chemicals (Milwaukee, WI), and 1-octanol (octyl alcohol, 98%) was purchased from Sigma Chemicals (St. Louis, MO).

Preparation of CLA. Safflower oil was isomerized according to the method of Ip *et al.* (9) by reacting the oil with an excess of sodium hydroxide in propylene glycol at elevated temperature.

Screening of lipases. For screening of lipases, a small-scale model esterification system was developed. 1-Octanol and fatty acids were mixed in a mole ratio of 1:1 to give a total weight of approximately 9 g. In the case of lipase from *G. candidum*, 1% powder (based on the weight of fatty acid) was dissolved in 18 mL distilled water and added to the octanol/fatty acid mixture. For immobilized lipase, 2% based on the weight of fatty acids was used. The reaction was carried out by agitating the reaction mixture at 25°C using magnetic stirring. The oil phase was sampled periodically and the

*To whom correspondence should be addressed at Loders Croklan NA, 24708 W. Durkee Rd., Channahon, IL 60410-5249.
E-mail: gerald.mcneill@unilever.com

progress of the reaction followed by measuring the free fatty acid (FFA) concentration in the samples, as determined by titration with dilute HCl. Conversion is defined as the decrease in FFA divided by the FFA at the start of the reaction, expressed as a percentage.

Lipase selectivity was determined by measuring the content of CLA isomers in either the unreacted fatty acid fraction or the octyl esters. Esters were separated from unreacted fatty acids by thin-layer chromatography (TLC), converted to methyl esters, and analyzed by gas chromatography (GC) as described below.

Pilot scale experiments. Lipase reactions were performed in a 20-L glass vessel with overhead stirring, temperature control, and nitrogen blanketing. The reaction temperature was 25°C, and a 1:1 mole ratio of lauryl alcohol/fatty acids was used. A volume of distilled water equal to that of the alcohol + fatty acid was added to the reaction vessel. The lipase from *G. candidum* was used at 1% of the weight of the fatty acids and dissolved in the water before addition to the reaction vessel. Separation of the products was carried out using short-path molecular distillation on a Fischer KD500/S evaporator (Loughborough, United Kingdom).

Interesterification reactions were carried out using 5% immobilized *R. miehei* lipase at 60°C under vacuum. In the case of interesterification between CLA fatty acids and palm oil triglycerides, enough glycerol was added to convert all FFA to triglyceride, to ensure that the interesterified product was composed predominantly of glyceride.

TLC. One drop of the oil phase of the reaction mixture was applied to a TLC plate (20 cm × 20 cm × 0.5 mm, silica gel G). The developing solvent was 60:40:1 diethylether/hexane/formic acid. Bands were visualized by spraying the dried plate with a 1% solution of 2,7-dichlorofluorescein in methanol followed by viewing under an ultraviolet lamp at a wavelength of 254 nm. The bands were scraped from the plates, and the esters and FFA were converted into methyl esters without further extraction.

GC. Fatty acid compositions were determined by GC of the methyl ester derivatives. The methylating reagent for FFA was 3 mL of 5% HCl in methanol, heated at 70°C for 2 min (to minimize artifact formation). The esters were converted using 1 mL of 0.5% sodium methoxide in methanol, with heating at 70°C for 5 min. Methyl esters were extracted into isooctane and washed with deionized water.

Methyl esters were analyzed on a 50 m, 0.25 mm i.d., 0.2 μm film, Chrompack CP-Sil 88 capillary GC column (Middelburg, The Netherlands) using helium as the carrier gas. The column oven was held at 120°C for 1 min, then raised to 160°C at 20°C/min, 160–180°C at 0.5°C/min, and finally 180–230°C at 3°C/min, giving a total run time of 60 min. A Perkin Elmer (Norwalk, CT) 8420 GC system fitted with programmable temperature vaporizing injection (PTV) in split injection mode and a flame-ionization detector (260°C) was used.

RESULTS AND DISCUSSION

Synthesis of CLA. Preparation of CLA from vegetable oils using basic catalysis in ethylene glycol was previously reported to give a CLA component comprising predominantly *cis*9,*trans*11 and *trans*10,*cis*12 isomers in roughly equal amounts (9). Some commercially available materials were recently shown to contain significant quantities of other positional and geometric isomers (10,11); however the method of synthesis was not reported. In this study the method described by Ip and coworkers (9) was used to provide a starting material with the lowest possible levels of undesirable CLA isomers. Moreover, a high-resolution GC analysis method was employed (see Experimental Procedures section) to adequately resolve the isomers, as low-resolution GC was reported to be inadequate for the separation of several contaminating CLA isomers (12). The composition of the principal fatty acids in the safflower substrate and in the CLA-containing product is shown in Table 1. The majority (96%) of the safflower linoleic acid was converted into CLA. The sum of unreacted linoleic acid plus all CLA isomers in the product equals the linoleic acid content in the safflower, indicating that products other than CLA isomers were not generated. Of the CLA in the product, 94.6% was *cis*9,*trans*11 and *trans*10,*cis*12 isomers in about equal amounts, the remainder being other positional and geometric isomers.

Screening of lipases. The fatty acid mixture containing CLA was subjected to esterification with 1-octanol, catalyzed by lipases from either *G. candidum* or *R. miehei*. The CLA isomer composition in both the ester phase and the unreacted fatty acid phase was monitored during the course of the reaction as described in the Experimental Procedures section, and the results are shown in Figure 1. To simplify interpretation of results, only the *cis*9,*trans*11 and *trans*10,*cis*12 isomers are reported. In the case of *R. miehei* lipase, the concentration of the *cis*9,*trans*11 isomer in the ester phase rose slightly from 50% in the starting material to 61%, at 64% conversion. This indicates a weak preference by this lipase for the *cis*9,*trans*11 isomer, but for practical purposes it is nonselective.

The lipase from *G. candidum* exhibited a completely dif-

TABLE 1
Composition of the Principal Fatty Acids in Safflower Oil Before and After Alkali-Catalyzed Isomerisation[a]

	Fatty acid (wt%)						
					CLA		
	C16:0	C18:0	C18:1	C18:2	*c*9,*t*11	*t*10,*c*12	Other
Safflower oil	7.1	2.5	13.3	75.7	0	0	0
Conjugated product	7.2	2.4	13.3	3.0	34.2	34.6	3.9

[a]CLA, conjugated linoleic acid.

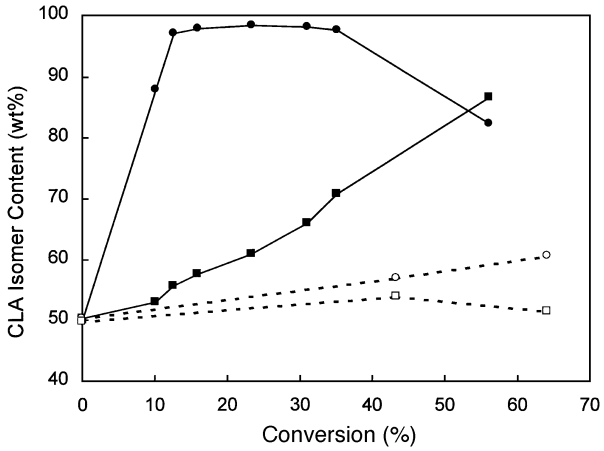
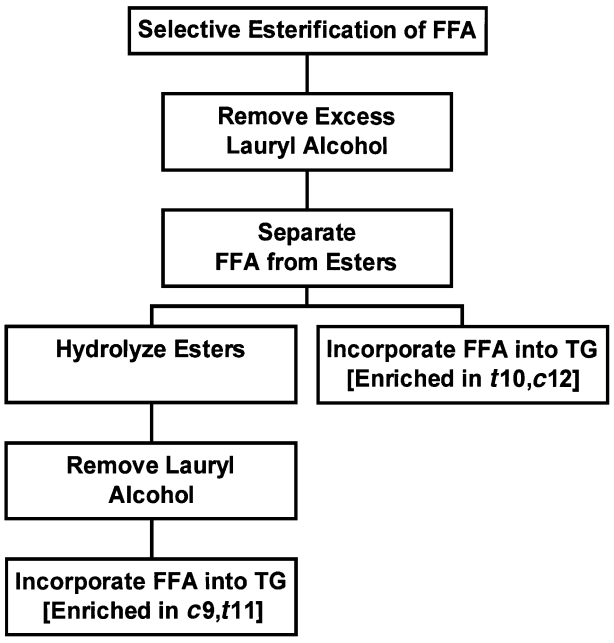


FIG. 1. Time course for the lipase-catalyzed esterification of safflower oil conjugated linoleic acid (CLA) with octanol. Solid lines: *Geotrichum candidum* lipase, (●) *cis*9,*trans*11 content in the ester fraction, and (■) *trans*10,*cis*12 content in the free fatty acid (FFA) fraction. Broken lines: immobilized *Rhizomucor miehei* lipase, (○) *cis*9,*trans*11 content in the ester fraction and (□) *trans*10,*cis*12 content in the FFA fraction. Concentration of CLA is normalized on the *cis*9,*trans*11 and the *trans*10,*cis*12 isomers.



SCHEME 1

ferent time course from that of *R. miehei* (Fig. 1). At the earlier stages of the reaction, the ester fraction was highly enriched in the *cis*9,*trans*11 isomer, reaching a maximum of 97–98% between 13–35% conversion. In the FFA fraction, the *trans*10,*cis*12 isomer was only slightly enriched at the early part of the reaction but became highly enriched after 40% conversion, reaching a maximum of 87% at 56% conversion. Clearly, lipase from *G. candidum* catalyzes the esterification of the *cis*9,*trans*11 isomer at a much higher rate than for the *trans*10,*cis*12 isomer, thus forming the basis for an efficient enrichment process for these isomers.

A recent study on the use of lipases for the enrichment of CLA isomers reported that lipase from *G. candidum* had poor selectivity (13). This is in contrast to the results obtained here, where *G. candidum* was found to be highly selective for the *cis*9,*trans*11 isomer.

Pilot-scale enrichment of CLA isomers. Based on the results of the screening procedure above, lipase from *G. candidum* was used to selectively esterify approximately 6 kg of fatty acids with lauryl alcohol (see Experimental Procedures for details). As the intended use of the enriched CLA isomer products was in animal studies, 1-octanol was substituted by lauryl alcohol as the latter has a considerably lower toxicity.

An outline of the enrichment procedure is shown in Scheme 1 (where TG = triglycerides). After selective esterification with *G. candidum* to approximately 50% conversion, the CLA composition of the ester and FFA fractions was determined by TLC/GC analysis. In the lauryl ester fraction, 94% of the CLA was the *cis*9,*trans*11 isomer, and in the FFA fraction, the *trans*10,*cis*12 isomer constituted 82% of the CLA. The level of enrichment of *cis*9,*trans*11 isomer in the ester fraction and the *trans*10,*cis*12 in the FFA fraction was similar to that obtained in the model system using octanol. Unreacted lauryl alcohol was removed from the reaction mixture by molecular distillation at 130°C. The FFA and lauryl esters were separated by distillation at 170°C. Residual FFA in the lauryl ester fraction was removed by conversion of the FFA to soap using dilute KOH solution, followed by water washing. Removal of residual lauryl ester in the FFA fraction proved to be more difficult and required several redistillations at 170°C.

The purified lauryl ester fraction was hydrolyzed using ethanolic KOH to release the CLA as FFA and to generate lauryl alcohol. The lauryl alcohol was then removed by distillation at 130°C.

At this stage both the *cis*9,*trans*11- and the *trans*10,*cis*12-enriched fractions were contaminated by several percent of lauryl alcohol (products 1a and 2a in Table 2). Further distillation did not result in removal of this contaminant. The lauryl alcohol was therefore reconverted *in situ* to the lauryl ester by esterification with immobilized *R. miehei* lipase. This product was distilled at 170°C to evaporate the FFA from the lauryl esters. By using this method, the lauryl alcohol content was reduced more than 20-fold and the lauryl ester content reduced by about 50% (products 1b and 2b in Table 2).

Products 1b and 2b were considered the final products of the enrichment procedure. The enrichment in either the *cis*9,*trans*11 (92.4%) or *trans*10,*cis*12 (81.0%) CLA isomers was very close to that detected by TLC analysis immediately after the lipase reaction. The slightly lower enrichment in the final product was presumably due to the inability to completely separate the ester from the FFA fraction.

Incorporation of enriched CLA isomers into palm oil. The ultimate use of the CLA isomers enriched as described above is evaluation of biological activity in animal models through dietary supplementation. As dietary fatty acids are normally ingested in the form of glycerides, we decided to incorporate

TABLE 2
Composition of the Enriched CLA Fractions Before (product 1a and 2a) and After (product 1b and 2b) Removal of Lauryl Alcohol by Enzymatic Esterification and Molecular Distillation[a]

	Composition (wt%)					
			CLA			
	Lauryl alcohol	Lauryl ester	*c*9,*t*11	*t*10,*c*12	Other	FFA
Product 1a	2.3	4.8	12.6	81.1	6.3	92.9
Product 1b	0.08	2.8	12.4	81.0	6.6	97.2
Product 2a	3.2	9.1	89.4	5.6	5.0	87.7
Product 2b	0.04	4.0	92.4	5.8	1.8	95.9

[a]CLA normalized to 100%. FFA, free fatty acids. For other abbreviations see Table 1.

TABLE 3
Composition of the Palm Oil-Based Glycerides Enriched in Either the *cis*9,*trans*11 CLA Isomer or the *trans*10,*cis*12 CLA Isomer[a]

	Composition (wt%)									
							CLA			
	Lauryl alcohol	Lauryl ester	C16:0	C18:0	C18:1	C18:2	*c*9,*t*11	*t*10,*c*12	Other	FFA
Enriched *c*9,*t*11	0.002	1.9	27.5	2.6	32.7	7.9	26.5	1.7	0.3	0.7
Enriched *t*10,*c*12	0.02	1.4	31.7	4.6	27.1	6.8	3.5	22.9	1.9	1.2

[a]For abbreviations see Tables 1 and 2.

the enriched CLA fatty acids into palm oil, a typical fat used in feed formulation for animal studies.

In this case, it was necessary to use a lipase that could easily and nonselectively incorporate both the *cis*9,*trans*11 CLA isomer and the *trans*10,*cis*12 CLA isomer into the triglycerides. Immobilized lipase from *R. miehei* was found in this study to be virtually nonselective (Fig. 1) and was chosen to incorporate the CLA isomers into palm oil (for details see the Experimental Procedures section).

A ratio of CLA and palm oil that would result in approximately 30% total CLA in the product was chosen. To ensure complete conversion of FFA to glycerides, an amount of glycerol that was calculated to convert all the FFA to triglycerides was added to the palm/CLA reaction mixture. The reaction thus consisted of a simultaneous esterification and interesterification with the CLA fatty acids randomly distributed throughout the palm triglycerides.

As shown in Table 3, the *R. miehei* lipase effectively incorporated both the *cis*9,*trans*11 and the *trans*10,*cis*12 CLA isomers into palm oil with a low residual FFA content. The CLA content was approximately 30%, and the degree of enrichment of each isomer was identical to that of the unesterified CLA. Only traces of lauryl alcohol remained while lauryl ester content was reduced to below 2%.

Both the *cis*9,*trans*11 and *trans*10,*cis*12 enriched isomer fats were used in animal model studies designed to determine which CLA isomers posses biological activity and the results will be reported elsewhere (14).

REFERENCES

1. Kepler, C.R., K.P. Hirons, J.J. McNeill, and S.B. Tove, Intermediates and Products of the Biohydrogenation of Linoleic Acid by *Butyrovibrio fibrisolvens, J. Biol. Chem. 241*:1350–1354 (1966).
2. Pariza, M..W., and W.A. Hargraves, A Beef-Derived Mutagenesis Modulator Inhibits Initiation of Mouse Epidermal Tumors by 7,12-Dimethylbenz[a]anthracene, *Carcinogenesis 6*:591–593 (1985).
3. Belury, M.A., Conjugated Dienoic Linoleate: a Polyunsaturated Fatty Acid with Unique Chemoprotective Properties, *Nutr. Rev. 53*:83–89 (1995).
4. Park, Y., K.J. Albright, W. Liu, J.M. Storkson, M.E. Cook, and M.W. Pariza, Effect of Conjugated Linoleic Acid on Body Composition in Mice, *Lipids 32*:853–858 (1997).
5. Nicolosi, R.J., E.J. Rogers, D. Kritchevsky, J.A. Scimeca, and P.J. Huth, Dietary Conjugated Linoleic Acid Reduces Plasma Lipoproteins and Early Aortic Atherosclerosis in Hypercholesterolemic Hamsters, *Artery 22*:266–277 (1997).
6. Miller, C.C., Y. Park, M.W. Pariza, and M.E. Cook, Feeding Conjugated Linoleic Acid to Animals Partially Overcomes Catabolic Responses Due to Endotoxin Injection, *Biochem. Biophys. Res. Commun. 198*:1107–1112 (1994).
7. McNeill, G.P., R.G. Ackman, and S.R. Moore, Lipase-Catalyzed Enrichment of Long-Chain Polyunsaturated Fatty Acids, *J. Am. Oil Chem. Soc. 73*:1403–1407 (1996).
8. McNeill, G.P., and P.E. Sonnet, Isolation of Erucic Acid from Rapeseed Oil by Lipase-Catalyzed Hydrolysis, *Ibid.. 72*:213–218 (1995).
9. Ip, C., S.F. Chin, J.A. Scimeca, and M.W. Pariza, Mammary Cancer Prevention by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 51*:6118–6124 (1991).
10. Christie, W.W., G. Dobson, and F.D. Gunstone, Isomers in Commercial Samples of Conjugated Linoleic Acid, *Lipids 17*:197–203 (1997).
11. Davis, A.L., G.P. McNeill, and D.C. Caswell, Analysis of Conjugated Linoleic Acid Isomers by ^{13}C NMR Spectroscopy, *Chem. Phys. Lipids 97*:155–165 (1999).
12. Kramer, J.K.C., V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoba, and M.P. Yurawecz, Evaluating Acid and Base Catalysts in the Methylation of Milk and Rumen Fatty Acids with Special Emphasis on Conjugated Dienes and Total *Trans* Fatty Acids, *Lipids 32*:1219–1228 (1997).
13. Chen, C.A., and C.J. Sih, Chemoenzymatic Synthesis of Conjugated Linoleic Acid, *J. Org. Chem. 63*:9620–9621 (1998).
14. DeDeckere, E.A.M., J.M.M. VanAmelsvoort, G.P. McNeill, and P. Jones, Effects of Conjugated Linoleic Acid (CLA) Isomers on