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ORIGINAL PAPER

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Biocatalyzed acidolysis of olive oil triacylglycerols with 9*c*,11*t* and 10*t*,12*c* isomers of conjugated linoleic acid

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Abstract Lipase catalyzed acidolysis of triacylglycerols (TAG) of olive oil with 9*c*,11*t* and 10*t*,12*c* isomers of conjugated linoleic acid (CLA) in an organic solvent was studied. The CLA isomers were first obtained in good yield starting from sunflower oil. The acidolysis reactions were carried out with two immobilized lipase, an *sn*-1,3-regiospecific one from *Rhizomucor miehei* and a nonregiospecific one from *Candida antartica*, in order to valuate not only the total incorporation of CLA isomers in olive TAG but also the distribution of the cited isomers in the three *sn* positions of TAG molecules. The effect of reaction time was also investigated; in fact two series of reactions, with the two lipases, were carried out for 24, 48 and 72 h. The TAG fractions relative to the starting olive oil TAG (OOTAG) and to the samples obtained from the acidolysis reactions were analyzed to obtain the total and positional fatty acid percentage compositions. The results show that after 24 h of reaction, high levels of CLA isomers were incorporated in OOTAG using Lipozyme IM and that slightly higher values were obtained by increasing the reaction time; Novozym 435 was less effective in catalyzing the incorporation of CLA isomers and CLA isomers were incorporated in OOTAG to a lesser degree. The results of stereospecific analysis of TAG fractions showed that, at every reaction time, the CLA isomers were incorporated mainly in *sn*-1 and *sn*-3 positions, as expected on the basis of the enzyme regiospecificity. As regards the TAG *sn*-2 position, the incorporation of CLA isomers was not observed after 24 h, but after 48 and 72 h; this occurrence was probably due to isomerization phenomena or regiospecificity loss after extended reaction times.

Keywords Olive oil triacylglycerols · Conjugated linoleic acid · Lipase catalyzed acidolysis

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Introduction

In recent years, the understanding of the nutritional values of lipids, in particular the effects associated with consumption of triacylglycerols (TAG) containing specific fatty acid (FA) residues in specific positions of the glycerol backbone, has led to the development of novel technologies based on the modification of fats and oils to enhance the health benefits resulting from ingestion of these substances [1]. Research [2] has focused on the use of lipases in organic solvents to modify TAG; the positional and acylic specificity may result in the formation of structured lipids with specific FA at specific sites within the TAG molecules [3].

Conjugated linoleic acids (CLA) represent a family of conjugated polyunsaturated FA with different positional and geometric double bonds.

The main dietary sources of CLA are animal products from ruminants (3–4 mg/g in beef, 5–7 mg/g in milk and butter), but the presence of CLA was also verified in seed oils (0.01–0.12% of total FA) [4, 5].

CLA exert a variety of effects in experimental animals, including anticancer and antiatherogenic actions [6–8]. In addition to the diverse positive influence of CLA, many authors found some beneficial effects of dietary CLA on lipid metabolism, such as body fat reduction with enhancement of lean body mass [9, 10]. Sakono et al. [11] found that CLA influenced FA metabolism not only in the liver but also in extra-hepatic tissues.

The 9*c*,11*t*- and the 10*t*,12*c*- isomers of CLA are the isomers that are believed to be primarily responsible for the beneficial physiological effects of this substance.

Much research has been carried out in order to produce TAG containing CLA residues: in particular, enzymatic reactions mediated by lipases have been carried out in order to "place" CLA isomers in TAG structures such as butter oil [12, 13], tricaprylin [14] or in acylglycerols of eicosapentaenoic acid and docohexaenoic acid [15]. The present study was undertaken in order to evaluate the insertion of the 9*c*,11*t*- and 10*t*,12*c*- isomers of CLA in the TAG fraction of olive oil (OOTAG); in the first step

the CLA isomers were prepared starting from a vegetable oil, rich in linoleic acid (LA), such as sunflower oil, then the acidolysis reactions of OOTAG with the CLA isomers were carried out. The objectives were to verify the CLA incorporation using two different lipases, Lipozyme IM, an *sn*-1- and *sn*-3- regiospecific one, and the nonregiospecific Novozym 435. Moreover, the acidolysis reactions were protracted for different times, in order to verify both the extent of incorporation of CLA isomers and then the percentage distribution of these isomers in the three *sn* positions of TAG; in fact the percentage total and positional FA composition of TAG were determined for all the samples considered.

Material and methods

Materials

The starting sunflower oil and extra-virgin olive oil were from a known and certified origin. The lipases, Lipozyme IM and Novozym 435, were kindly donated by Novo Nordisk (Bagsvaerd, Denmark); the first enzyme, derived from *Mucor miehei*, was immobilized on a macroporous anion-exchange resin and has 1,3-positional specificity, while Novozym 435 is a lipase from *Candida antarctica* immobilized on a macroporous acrylic resin. All chemicals and solvents were of ACS purity. The TAG fraction of oils, before and after the acidolysis reactions, were analyzed using the steps in the next subsection.

Experimental procedures

All the experimental steps were carried out at least twice and the results reported represent the average values.

Preparation of the CLA isomers

Sunflower oil (5 g) was reacted with 1.15 g potassium hydroxide, 2.2 ml water, 13.2 ml 95% ethanol; 1 mg butylated hydroxytoluene was also added. The reaction was carried out for 1 h at 70 °C, then the mixture was cooled and, after the addition of water, 10 ml, it was washed with hexane, 2×20 ml. HCl (3N) was added to the aqueous phase to reach pH 1, then it was extracted with hexane, 2×10 ml, to isolate the free FA. The organic layer was dried over anhydrous sodium sulfate, then the FA obtained were subjected to the following step of urea purification. Urea (6 g) was dissolved in 12 ml methanol warmed to 60–70 °C under magnetic stirring to obtain a clear solution; then the FA were added, in small portions, to the cooled urea solution, always maintaining magnetic stirring. The mixture was then cooled to 5 °C, maintained overnight at this temperature and then subjected to filtration under vacuum; water, 10 ml, was added to the solution and the pH was adjusted to pH<2 with 6N HCl. The FA fraction enriched in LA was extracted with hexane, 2×5 ml, washed with three portions of 30% methanol in water, then the solvent was evaporated. The residue was subjected to the following isomerization reaction to obtain the CLA isomers from LA. In brief, to the FA fraction, enriched in LA, 5 ml 1-butanol and 1 g KOH were added and the reaction was heated to reflux for 12 h to 120 °C under a gentle stream of nitrogen. After cooling, 5 ml water was added to the reaction mixture, the mixture was acidified to pH 1 with 6N HCl and extracted with hexane, 2×10 ml; the organic extracts collected were washed with 5% NaCl, 2×5 ml, and with water to reach neutrality, then they were dried over anhydrous sodium sulfate and evaporated under vacuum.

The FA percentage compositions of the products, obtained from (1) alcaline hydrolysis of sunflower oil, (2) LA purification with urea and (3) alcaline isomerization of a fraction enriched in LA to obtain the CLA isomers, were determined after the following methylation reaction and the high-resolution gas chromatography (HRGC) analysis of FA methyl esters (FAME) were carried out.

Acid methylation of FA

An aliquot of FA, about 10 mg, was added to 1 ml 1% H_2SO_4 in methanol and maintained at 70 °C for 1 h; then 5% NaCl aqueous solution, 5 ml, was added and the FAME were extracted with hexane, 2×5 ml. The organic extracts were then washed with 4% $KHCO_3$ aqueous solution, 4 ml.

HRGC analysis of FAME

A PerkinElmer Autosystem (Norwalk, CT) gas chromatograph equipped with a split/splitless injector and a flame ionization detector was used for the FAME analysis; the separation was carried out on a CP-Sil 88 column (50-m×0.25-mm i.d., d.f.=0.25 μm) (Varian, Middelburg, The Netherlands). The data acquisition and integration were performed by the PE Turbochrom Navigator 4.1 software. The chromatographic conditions were the following: injector and detector temperature 250 °C; initial oven temperature 180 °C, maintained for 6 min then raised by 1 °C/min to 225 °C and maintained for 10 min. The carrier gas (He) flow was 1 ml/min, the detector gas flows were air 250 ml/min, H_2 25 ml/min; make up (N_2) 25 ml/min.

Acidolysis reactions

Two series of reactions were carried out using the two different lipases, Lipozyme IM and Novozym 435, for different times, 24, 48 and 72 h; in Table 1, the samples considered are reported. In general, extra-virgin olive oil TAG -OOTAG- (150 mg) was mixed with CLA isomers at a molar ratio of 2.3:1 in hexane (2 ml). The enzymatic preparation was first dried under vacuum for 60 min and then 7 mg (4% w/w) was added to the reaction mixture. At the end of the enzymatic reaction, the lipase was separated by filtration and the TAG fraction was isolated by thin layer chromatography (TLC), as described in the nest subsection.

Analysis of TAG FA percentage composition

The TAG fraction was purified by TLC (silica gel precoated plates, 20×20 cm, 250 μm) using hexane/diethyl ether/formic acid (80:20:2, v/v/v) as the developing solvent [16]. The TAG fraction ($R_f \cong 0.6$ - 0.7) was extracted from silica with diethyl ether (3×2 ml) and the organic extracts were pooled.

An aliquot of TAG was dissolved with 2 ml hexane and then 0.4 ml 2 M KOH in anhydrous methanol was added [17]; after 3 min, 3 ml water was added. The organic layer, separated by centrifugation, was dried over anhydrous sodium sulfate, then concentrated with a N_2 stream to around 0.5 ml for HRGC analysis of FAME (as described earlier).

Table 1 Olive oil triacylglycerol (*OOTAG*) modified samples.

Sample	Reaction time, h	Enzyme
L24	24	Lipozyme IM
L48	48	Lipozyme IM
L72	72	Lipozyme IM
N24	24	Novozym 435
N48	48	Novozym 435
N72	72	Novozym 435

Analysis of FA percentage composition of the TAG sn-2 position

The TAG fraction was subjected to pancreatic lipase hydrolysis [18] to determine the FA percentage composition of the TAG *sn*-2 position; the determination of the FA percentage composition of the *sn*-2-monoacylglycerols was carried out with a procedure analogous to that described for the TAG fraction in the previous subsection.

Stereospecific analysis of TAG by the sn-1,2-diacylglycerolkinase method

The FA percentage positional compositions of TAG were obtained using the *sn*-1,2-diacylglycerolkinase procedure, as reported in a previous paper [16]. In brief, methyl magnesium bromide was added to the TAG fraction in dry diethyl ether and then the hydrolysis mixture was applied to TLC plates to isolate the *sn*-1,2(2,3)-diacylglycerol fraction. This fraction was extracted with diethyl ether, then the solvent was evaporated and 0.1 ml methanolic cardiolipin solution (5 mg/ml, Fluka, Bucks, Switzerland) was added; after ultrasonication for 1–2 min, the solvent was removed under a N_2 stream. To the residue were added 20 μl *sn*-1,2-diacylglycerol kinase buffered solution (from *E. coli*, 1 mg/ml in 10 mM phosphate buffer pH 7.0, 20% glycerol, 2 mM β-mercaptoethanol, with defined specific activity—this specimen 10.6 U/mg, Calbiochem Co, La Jolla, CA), 1 ml tris(hydroxymethyl)aminomethane buffer (pH 6.6) and 100 μl Na_2ATP aqueous solution (8 mM). The enzymatic reaction was carried out at 40 °C for 90 min under constant stirring, then the required products were extracted with chloroform/methanol (1:1, v/v); the *sn*-1,2-phosphatidic acids were then isolated by TLC. The phosphatidic acids were transesterified and the FAME obtained were analyzed by HRGC (as described earlier).

The results were then used to obtain the *sn*-1-, *sn*-2- and *sn*-3-FA percentage compositions according to the method in Ref. [16].

Results

The results relative to the first step of this work, that is the preparation of the CLA isomers to use in the acidolysis reactions, are reported in Table 2; the FA percentage compositions of the starting sunflower oil TAG show that LA is the most abundant FA and that satisfactory enrichment of LA was obtained by means of the urea treatment. Finally, good results were obtained with reference to the CLA isomers obtained after the alcaline isomerization of the FA fraction enriched in LA; in fact the total conversion of LA to CLA was obtained with satisfactory percentage contents of the two CLA isomers and a slightly higher percentage content of the 10*t*, 12*c*-CLA isomer compared with the 9*c*,11*t* one.

Table 2 Fatty acid (*FA*) percentage compositions. Linoleic acid (*LA*), conjugated LA (*CLA*)

	Sunflower TAG	LA-enriched FA fraction	CLA
C16:0	6.5	0.1	0.1
C16:1 (*n*-9+*n*-7)	0.1	–[a]	–
C18:0	3.8	–	–
C18:1 (*n*-9+*n*-7)	31.7	8.1	9.7
C18:2 *n*-6	57.4	91.8	–
C18:3 *n*-3	0.2	–	–
9*c*,11*t*- CLA	–	–	40.0
10*t*,12*c*- CLA	–	–	45.6
t,*t* CLA	–	–	1.7
Other CLA isomers	–	–	2.9

[a] Lower than 0.1%

Table 3 Total and positional FA percentage compositions of OOTAG.

OOTAG				
FA	TAG	*sn*-1	*sn*-2	*sn*-3
C 16:0	11.9	14.5	0.5	20.7
C 16:1 (*n*-9–*n*-7)	0.9	2.9	0.7	–[a]
C 18:0	1.5	2.0	0.1	2.6
C 18:1 (*n*-9–*n*-7)	78.7	71.2	89.5	75.5
C 18:2 (*n*-6)	6.4	9.0	8.7	1.6
C 18:3 (*n*-3)	0.5	0.5	0.6	0.4

[a] Lower than 0.1%

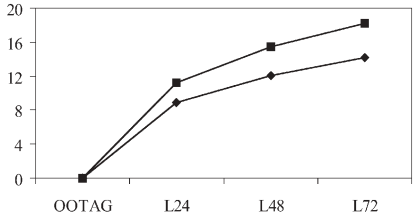

Fig. 1 Trends of percentage contents of 9*c*,11*t*-conjugated linoleic acid (*CLA*) (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in olive oil triacylglycerols (*OOTAG*) and in TAG of the samples treated with Lipozyme IM for different times.

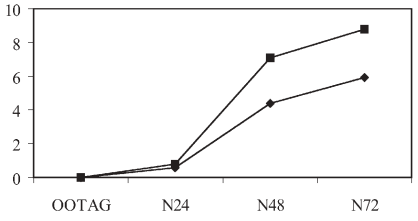

Fig. 2 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in OOTAG and in TAG of the samples treated with Novozym 435 for different times.

The results relative to the total and positional FA percentage compositions of the starting OOTAG, reported in Table 3, show that the most abundant FA, C18:1 oleic acid, is greatly represented in the *sn*-2 position as well as the C18:2 (LA), while the saturated FA are esterified mainly in the *sn*-1 and *sn*-3 positions of TAG; these results are in agreement with some previously reported results [16].

With reference to the modified OOTAG, obtained after the enzymatic acidolysis reactions, the results are reported in Figs. 1–8; the trends of the CLA isomers incorporated in total TAG for OOTAG and the samples treated for different times (24, 48 and 72 h) with Lipozyme IM and Novozym 435 are shown, respectively, in Figs. 1 and 2. It can be observed that with Lipozyme IM high levels of 9*c*,11*t*- and 10*t*,12*c*-CLA were incorporated in TAG as early as after 24 h and that a gradual increase was ob-

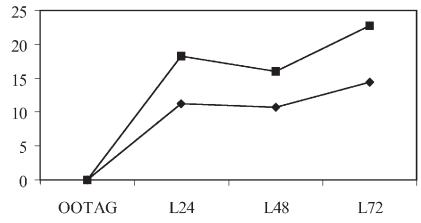

Fig. 3 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-1 position of OOTAG and of the samples treated with Lipozyme IM for different times.

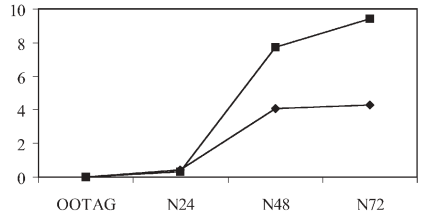

Fig. 4 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds)* and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-1 position of OOTAG and of the samples treated with Novozym 435 for different times.

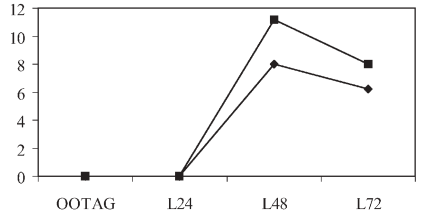

Fig. 5 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-2 position of OOTAG and of the samples treated with Lipozyme IM for different times.

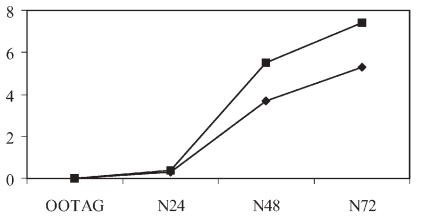

Fig. 6 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-2 position of OOTAG and of the samples treated with Novozym 435 for different times.

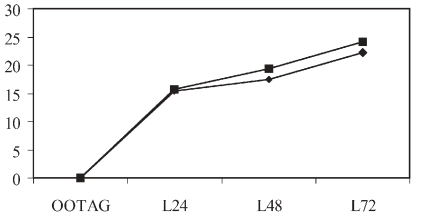

Fig. 7 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-3 position of OOTAG and of the samples treated with Lipozyme IM for different times.

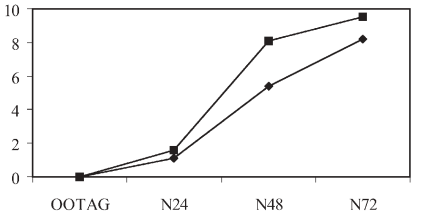

Fig. 8 Trends of percentage contents of 9*c*,11*t*-CLA (*diamonds*) and 10*t*,12*c*-CLA (*squares*) in the TAG *sn*-3 position of OOTAG and of the samples treated with Novozym 435 for different times.

served after 48 and 72 h; this increase was also observed in the samples treated with Novozym 435 but with much lower percentage contents of the CLA isomers.

The trends of the CLA isomers incorporated in the three *sn* positions of TAG for OOTAG and the samples treated for different times (24, 48 and 72 h) with Lipozyme IM are reported in Figs. 3, 5 and 7, respectively, for *sn*-1, *sn*-2 and *sn*-3 positions. The results show that the CLA isomers have been incorporated mainly in *sn*-1 and *sn*-3 positions, with a little preference for the last, at all reaction times. With reference to the *sn*-2 position, after 24 h no CLA isomer incorporation has been observed as a consequence of the Lipozyme IM regiospecificity; however, after 48 and 72 h the resulting CLA isomers were also incorporated in this position, probably because of isomerization phenomena or enzyme regiospecificity loss.

The results of FA positional analysis in OOTAG and in samples treated for different times (24, 48 and 72 h) with Novozym 435 are reported in Figs. 4, 6 and 8, respectively, for *sn*-1, *sn*-2 and *sn*-3 positions. As already observed for the total TAG FA composition, lower levels of CLA isomer incorporation were observed compared with the results obtained with Lipozyme IM; moreover comparable values of CLA isomers were obtained in the three positions of TAG, as expected considering the enzyme nonpositional specificity.

It can be concluded that the acidolysis reactions carried out with Lipozyme IM under the experimental conditions described are able to produce TAG with good levels of CLA isomers and, even if the enzyme is *sn*-1 and *sn*-3 regiospecific, the CLA incorporation is also in the *sn*-2 position under selected experimental conditions; this last occurrence confers a positive feature from a nutritional point of view to these structured lipids because, as reported by several researchers on the basis of physiological studies, the long-chain polyunsaturated FA esterified at the TAG *sn*-2 position are characterized by better intestinal absorption [19, 20].

Further analyses are under way to investigate the influence of some parameters of the acidolysis reactions on the incorporation of CLA isomers in OOTAG as well as in other TAG matrices.

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