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Combination of modern plant breeding and enzyme technology to obtain highly enriched erucic acid from Crambe oil

Natalia Volkova[1], Xueyuan Li[2], Li-Hua Zhu[2] and Patrick Adlercreutz[1*]

Abstract

Background: Fatty acids from vegetable oils are useful building blocks for industrial materials. The purpose of this work was to prepare erucic acid with high purity from a vegetable oil. High purity erucic acid is used for the production of erucamide with applications in plastics manufacturing. A newly developed transgenic Crambe line produces seed oil with 68 % erucic acid compared to 53 % in the wild type oil.

Results: Further enrichment of erucic acid from Crambe (wild type and transgenic) oil was achieved by selective enzymatic hydrolysis. Using *Candida rugosa* lipase as catalyst, other fatty acids were preferentially hydrolysed from the triacylglycerols and erucic acid was enriched in the acylglycerol fraction. The highest content of erucic acid achieved in that fraction was 95 %.

Conclusions: The combination of modern plant breeding and enzyme technology is a promising approach for preparation of fatty acids of high purity.

Keywords: Crambe, Erucic acid, Lipase, Enzymatic enrichment

Background

Vegetable oils constitute a renewable raw material with great potential not only for production of food and feed but also in the production of industrial materials and fuels. Transesterification of vegetable oils to methyl esters to be used as biodiesel is carried out industrially [1] and fatty acids are used as building blocks in many products. In a typical example, a fatty acid is esterified with a fatty alcohol to produce a wax ester [2], or the wax ester is formed in the alcoholysis of a vegetable oil with a fatty alcohol [3]. Wax esters can be used for various applications, such as coatings for wood [4] or as ingredients for cosmetics [5]. Furthermore, fatty acids can be used as the hydrophobic building blocks of surfactants such as carbohydrate esters [6, 7]. Still another application is the use of erucic acid and its derivatives, such as erucamide, in manufacturing plastics, nylon13-13 and high temperature lubricants [8].

Sometimes the natural mixture of fatty acids from a vegetable oil can be used in the final product, but in most cases there is a need for purification of fatty acids. In the case of production of erucamide, it is important to have erucic acid with high enough purity. Modern plant breeding techniques enable us to develop new crop varieties in a more precise and efficient way. Through genetic transformation, we have previously obtained transgenic Crambe, a dedicated industry crop species, with significantly increased level of erucic acid in the seed oil through expressing the *LdLPAAT*, *BnFAE1* and *CaFAD2*-RNAi genes [9, 10]. However, the erucic acid level still needs to be enriched further for erucamide production.

One way to enrich fatty acids from natural oils is to use fractionation, by distillation or crystallization or to utilize the fatty acid selectivity of lipases. Lipases often accept a wide variety of carboxylic acids as substrates and the catalytic activity depends on how well the particular acid fits in the active site of the lipase. The ideal lipase would

*Correspondence: Patrick.Adlercreutz@biotek.lu.se
[1] Department of Biotechnology, Lund University, P.O. Box 124, 221 00 Lund, Sweden
Full list of author information is available at the end of the article

convert the desired fatty acid(s) much slower or faster than the other fatty acids in the original mixture. After the lipase-catalyzed conversion reacted and un-reacted fatty acids are separated by extraction, distillation, etc. A particularly successful example concerns enrichment of long-chain omega-3 fatty acids [11–13], but enrichment of erucic acid [14] and gamma-linolenic acid [15] has also been reported.

The aim of the present study was to study if the erucic acid content of acylglycerols from wildtype and transgenic Crambe could be further increased by selective lipase-catalyzed hydrolysis of the oils.

Results and discussion

The fatty acid compositions of the wild type oil and the high erucic oil are shown in Table 1. The high erucic oil contained substantially larger amounts of erucic acid and C20:1 than the wild type oil. On the other hand, the contents of palmitic and stearic acids were lower, and those of linoleic and linolenic acid were substantially lower in the high erucic oil compared to the wild type oil.

In order to enrich erucic acid further, selective enzymatic hydrolysis was attempted. The method is based on the fatty acid selectivity of lipases. A lipase should thus selectively hydrolyse off other fatty acids from the triacylglycerols. After separation of the acylglycerol and free fatty acid fractions (by extraction, distillation, etc.) a product further enriched in erucic acid can be obtained. Previous model studies using equimolar mixtures of erucic acids and other relevant fatty acids showed that the lipases from *Candida rugosa, Thermomyces lanuginosus* and *Pseudomonas cepacia* express the desired fatty acid selectivity [16], although the selectivity is not quite as strong as in cases involving long-chain omega-3 fatty acids, such as EPA and DHA [17, 18].

All three lipases catalyzed the selective hydrolysis of other fatty acids, leading to enrichment of erucic acid in the acylglycerol fraction (Table 2). Using *T. lanuginosus* lipase only modest increases in erucic acid content were achieved for both oils. Higher erucic acid contents were achieved with *P. cepacia* lipase, and the best results were obtained with *C. rugosa* lipase. The time course of the enrichment using *C. rugosa* lipase is shown in Fig. 1.

During the initial phase of the reactions, the content of erucic acid in the acylglycerol fraction gradually increased because of hydrolysis of other fatty acids, but in the end of the reaction, the erucic acid content started to decrease again because the lipase can remove even erucic acid and this happens to an appreciable extent when erucic acid is the dominating fatty acid remaining in the acylglycerols. As in most other cases of enzymatic fatty acid enrichment it is thus important to stop the reaction at a suitable reaction time. The maximum was reached in a shorter reaction time with the high erucic oil, maybe because it contained a lower amount of the fatty acids to be removed. In addition to the level of erucic acid achieved in the acylglycerol fraction, the yield of erucic acid is of importance. The erucic acid which has been hydrolyzed from the acylglycerols can be considered as lost (unless additional process steps are added). The recovery of erucic acid can be expressed as the ratio of erucic present in the acyl glycerol fraction and the initial amount of erucic acid in the starting material.

Table 1 Fatty acid composition (in mol%) of the Crambe oils used in this study (mean ± standard deviation, n = 3)

Fatty acids	Wild type oil	High erucic oil
C 16:0	5.1 ± 0.2	3.9 ± 0.2
C 18:0	1.7 ± 0.8	1.4 ± 0.6
C 18:1	30.0 ± 0.8	17.8 ± 0.5
C 18:2	4.8 ± 0.2	1.3 ± 0.2
C 18:3	2.0 ± 0.2	0.7 ± 0.1
C 20:1	2.7 ± 0.4	6.3 ± 0.6
C 22:1	52.8 ± 0.5	67.9 ± 0.7

Table 2 Maximal content of erucic acid in the acylglycerol fraction during lipase-catalyzed hydrolysis of Crambe oils

Treatment	Mol% C 22:1	
	Wild type oil	High erucic oil
Original oil	52.8	67.9
Product; *Thermomyces lanuginosus*	56	73
Product; *Pseudomonas cepacia*	62	80
Product; *Candida rugosa*	82	95

Total reaction time 40 h. Only the maximal values are shown

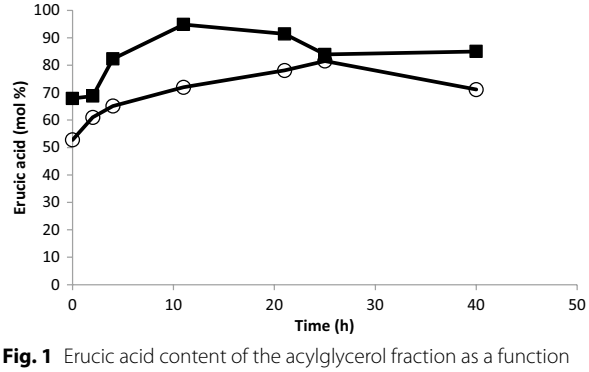

Fig. 1 Erucic acid content of the acylglycerol fraction as a function of time of hydrolysis of Crambe oils by *C. rugosa* lipase. *Filled squares* high erucic oil; *open circles* wild type oil

The recovery of erucic acid decreased with increasing reaction time, especially for the wild type oil, which decreased to very low values after 40 h reaction, indicating close to total hydrolysis of the oil (Fig. 2). The recoveries at the maximal percentage of erucic acid in the acylglycerol fraction were about 60 % for the high erucic oil and about 10 % for the wild type oil. The main other fatty acid remaining in the acylglycerol fraction was oleic acid (Table 3).

The lipase-catalyzed enrichment caused a considerable increase in erucic acid content, but the recovery of erucic acid probably needs to be higher than that achieved in this study to be economically feasible on a large scale. A main factor which determines the outcome of the enzymatic enrichment reactions is the intrinsic specificity of the lipase for erucic acid in comparison with the specificity for the other fatty acids present in the substrate mixture. This is best quantified in terms of competitive factors [19], which are the ratios of k_{cat}/K_m of the two substrates to be compared. Competitive factors of lipases for erucic acid and other fatty acids $[(k_{cat}/K_m)_{fatty\ acid}/(k_{cat}/K_m)_{erucic\ acid}]$ have been measured in the hydrolysis of fatty acid methyl esters and in the esterification of free fatty acids and were reported to be in the range 1.4–6.0 [16]. On the other hand, the corresponding competitive factors of up to over 100 have been reported for lipase-catalyzed enrichment of the long-chain omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid [17]. The situation is further complicated if the fatty acids are part of triacylglycerol molecules, since all acylglycerols are potential substrates and other fatty acids than the one hydrolyzed off can be expected to have an influence. In the case of long-chain omega-3 fatty acids it has been shown that enrichment from triacylglycerols is less efficient than from simple esters [18]. There is a clear need for lipases with even higher selectivity in the enrichment of erucic acid. This could be achieved by screening among natural lipases or protein engineering of known lipases [20]. The commercial preparation of *C. rugosa* lipase, which was the best enzyme in the present study, contains isoenzymes with slightly different properties [21]. Starting with one of these isoenzymes and mutating it, Pleiss and co-workers managed to block the active site so that fatty acids longer than a critical length (C10 or C14) were strongly discriminated against [22]. This demonstrates that there are good possibilities to create new lipases with improved fatty acid selectivity and a similar methodology could be useful to prepare a lipase well suited for enrichment of erucic acid.

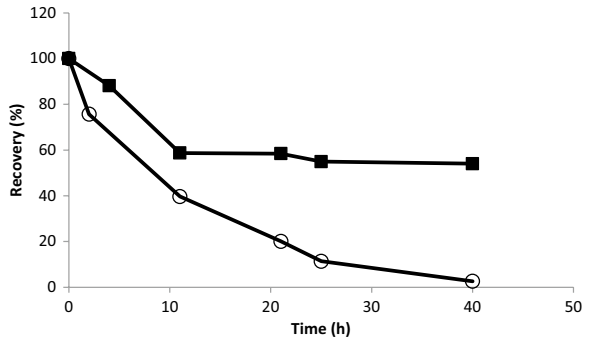

Fig. 2 Recovery of erucic acid in the acylglycerol fraction as a function of time of hydrolysis of Crambe oils by *C. rugosa* lipase. *Filled squares* high erucic oil; *open circles* wild type oil

Table 3 Fatty acid composition (in mol%) of the acylglycerol fractions after enzymatic enrichment for optimal reaction time (mentioned in parentheses)

Fatty acids	Wild type oil (25 h)	High erucic oil (11 h)
C 16:0	2	1
C 18:0	–	–
C 18:1	13	4
C 18:2	–	–
C 18:3	–	–
C 20:1	3	–
C 22:1	82	95

Experimental

Materials

Solvents: heptane, methanol, petroleum ether, diethyl ether, cyclohexane of analytical grade were obtained from Merck and used without further purification. Concentrated H_2SO_4 and 30 % solution of sodium methylate were from Merck. Inorganic salts were purchased from Sigma Aldrich.

A transgenic line with high erucic acid and the non-transgenic wild type of *Crambe abyssinica* Hochst. cv Galactica were used in this study. The plants were grown in greenhouse with 16 h photoperiod, 21/13 °C temperature (day/night) and standard management. The harvested seeds were kept at 4 °C before the oil composition analysis.

Extraction of seed oil

Crambe seeds, both wild type and high erucic, were grinded in a small food processor to obtain a powder. The powder (25 g) was mixed with 150 ml heptane in Erlenmeyer flask and stirred by magnetic stirrer (600 rpm) at room temperature for about 20 h. The mixture was vacuum filtered by use of Schott filter (No.3). The filter cake was washed with pure heptane and dry Na_2SO_4 was added to the combined heptane phases to remove

possible traces of water. The salt was removed by filtration using cotton as a filter in a regular funnel. The salt was washed with pure heptane and filtrated again. All heptane fractions were collected. Heptane was evaporated by use of a vacuum rotary evaporator (Heidolph, W60) at room temperature, the cooling water temperature was kept at 5 °C. The obtained yields were 26 % for wild type Crambe oil and 29 % for high erucic Crambe oil.

Enzymatic hydrolysis of Crambe oil

Oil samples, obtained by the procedure described above, were hydrolysed by three different lipases: *Candida rugosa* (Sigma), *Thermomyces lanuginosus* (Sigma) and *Pseudomonas cepacia* (Amano Enzymes). The reactions were carried out in 4-ml glass vials (inner diameter 12 mm) with screw caps and Teflon lined septa at 25 °C in thermo mixer at 600 rpm (MKR 13, HLC BioTech, Bovenden, Germany) with free enzymes. Shaking was rotary horizontal with a throw distance of about 3 mm. The oil samples of 0.3 g were mixed with 300 µl 0.2 M phosphate buffer (pH 7.0) and 0.02 g of free lipase was added to start the reaction. Samples were taken for analysis during the reaction.

Thin-layer chromatography (TLC)

The samples (10 µl) taken from reaction mixture at different time were mixed with 40 µl hexane and separated on 16 cm long TLC silica gel 60 F_{254} aluminium sheets (Merck). The separation was performed in a glass chamber by use of petroleum ether-diethyl ether-acetic acid (80:20:1) as mobile phase. Bands were visualised in an iodine vapour chamber. The marked bands were collected by scraping of the silica to glass tubes for further treatment. As shown by their position on the TLC plates they contained: free fatty acids (FFA), monoglycerides (MAG), diglycerides (DAG) and triglycerides (TAG), which were treated by two different procedures (basic and acidic), described in the next section.

Sample preparation for gas chromatography

Two ml of freshly prepared 0.5 M sodium methoxide solution in dry methanol, 475 µl dry cyclohexane and 25 µl internal standard solution (5 mM C 13:0 methyl esters dissolved in dry methanol) were added to silica scrapes containing MAG, DAG or TAG. Samples were mixed and incubated at 52 °C for 30 min in a thermo mixer (500 rpm). After that 4 ml of saturated NaCl solution was added to the mixtures, the samples were vortexed (3 × 5 s) and centrifuged at 1800 or 4400 rpm (WIFUG, England) to achieve efficient phase separation. 100 µl of the upper organic phase was used for gas chromatography analysis.

FFA silica scrapes were first wetted with 475 µl dry cyclohexane and 25 µl internal standard solution (see above), then 1.25 ml of freshly prepared 1.5 % sulphuric acid in dry methanol was added. Tubes were incubated at 52 °C for 2 h in thermo mixer (500 rpm) and after that 3 ml of saturated NaCl solution was added to the mixtures. The samples were vortexed and then mixed with 4 ml of 2 % sodium carbonate solution for neutralization, vortexed (3 × 5 s) again and centrifuged at 1800 or 4400 rpm, and 100 µl of the upper organic phase was used for gas chromatography analysis.

Gas chromatography

Gas chromatography (GC) analyses were performed by use of Varian 430-GC instrument with a split/splitless injector, FID detector and Varian CP-8400 autosampler (Agilent Technologies Inc., USA). Supelcowax 10 fused silica capillary column (53 m × 0.32 mm × 0.25 µm film thickness) was used for determinations. Isobaric conditions (24psi) with helium as a carrier gas were used. The initial column temperature was 100 °C and after 1 min it was increased to 220 °C with 50 °C/min and kept for 10 min. The second step to reach the temperature 240 °C was done with a heating rate 15 °C/min. This final temperature (240 °C) was kept for 15 min. The instrument was calibrated with one-point calibration, as described previously [23]. The injection volume was 1 µl in all the experiments. Galaxie Chromatography Data System (vers.1.9) from Agilent Technologies was used for the data treatment.

Conclusions

Plant breeding can be used to increase the content of a specific fatty acid in the seed oil to high levels, but further enrichment is often needed to achieve above 90 % purity. Selective lipase-catalyzed hydrolysis was shown useful for increasing the content of erucic acid in transgenic Crambe seed oil from 68 to 95 % with a recovery of 60 %. To achieve even more efficient lipase-catalyzed enrichment, lipases with even higher selectivity are needed.

Authors' contributions

NV carried out the practical work on the extraction of oil from the seeds and the enzymatic processing of the oil. XL and LHZ carried out all work with Crambe plants and supplied the seeds. PA initiated the project and coordinated the work on enzymatic processing and writing of the manuscript. All authors gave input to the manuscript. All authors read and approved the final manuscript.

Author details

[1] Department of Biotechnology, Lund University, P.O. Box 124, 221 00 Lund, Sweden. [2] Department of Plant Breeding, Swedish University of Agricultural Sciences, P.O. Box 101, 230 53 Alnarp, Sweden.

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Competing interests
The authors declare that they have no competing interests.

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