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Enzymic Enhancement of ω3 Polyunsaturated Fatty Acids Content in Brazilian Sardine Oil

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SUMMARY. In an attempt to concentrate the content of ω3 polyunsaturated fatty acids (ω3 PUFA) in the partially hydrolyzed glycerides, Brazilian sardine oil was hydrolyzed with four kinds of microbial lipases. The enzyme from *Candida cylindracea* was the most effective for the production of oil with high concentration of docosahexaenoic acid (DHA). Sardine oil was treated at 35 °C with this lipase for 16 h reaction and 60.0% hydrolysis resulted in an increase in the DHA content from 10.2% in the original oil to 22.5% (2.20-fold enrichment) in the unhydrolysed acylglycerol. None the four lipases tested could raise the EPA content significantly.

RESUMEN. "Aumento enzimático del contenido de los ácidos grasos polinsaturados ω3 en aceite de sardina brasileño". Se ensayaron cuatro lipasas microbianas con la intención de aumentar el contenido de los ácidos grasos poliinsaturados de la serie ω3 (AGPI ω3) en la fracción de glicéridos no hidrolizados después de la hidrólisis del aceite de sardina brasileño. La lipasa de *Candida cylindracea* fue la más eficiente entre las enzimas ensayadas, aumentando el contenido de ácido decosahexaenoico (DHA) del 10,2% en el aceite original al 22,5% en relación a los ácidos grasos totales después de 16 h de reacción a 35 °C, lo que equivale a un incremento del 120% en relación a la cantidad presente antes de la hidrólisis. Las cuatro lipasas probadas no fueron eficientes para aumentar el contenido del ácido eicosapentenoico (EPA) en el aceite de sardina brasileño en las condiciones del ensayo.

INTRODUCTION

An intense interest in the health benefits of fish oils was stimulated in the early 1970's by the research of Dyerberg and co-workers [1,2]. The ω3 polyunsaturated fatty acids (ω3 PUFA), cis-5,8,11,14,17-eicosapentaenoic acid (EPA 20:5) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA 22:6) are the active components of such oils. Both EPA and DHA have several benefits on cardiovascular disorders [3,4], autoimune and inflammatory diseases [5,6] and cancer [7]. The beneficial effects of ω3 PUFA are attributed to eicosanoid synthesis such as prostaglandins, thromboxanes and leukotrienes. The importance of these fatty acids to infant nutrition is particularly relevant because DHA is important for fetal and term-infant neural development [8]. Although PUFA can be synthesized in the body by elongation and desaturation of α-linolenic acid, ingestion of the preformed molecules usually is more effective, especially for the very young or the elderly [9]. Consequently, there is interest in methods for production of more purified ω3 PUFA concentrates and, indeed, for bulk quantities of EPA and DHA.

An alternative approach for enrichment of PUFA in fish oil is based on enzymatic techniques. Lipases display two different specificities, namely fatty acid specificity and positional specificity. The regiospecificity is one of the major advantages of using lipase technology for the modification of oils and fats to produce high-value added products. Lipases are known to have little reactivity on PUFA (e.g., gamma-linolenic acid, arachidonic acid, EPA and DHA), and these acids can be enriched by selective hydrolysis [10-12], direct esterification of glycerol with EPA and DHA [13-15], and interesterification [16-18].

In this paper, we report how DHA and EPA can be effectively concentrated in Brazilian sardine oil by selective hydrolysis reaction catalyzed by lipase of *Candida cylindracea*.

KEY WORDS: Docosahexaenoic acid, Hydrolysis, Lipase, Sardine Oil.
PALABRAS CLAVE: Aceite de Sardina, Ácido decosahexaenóico, Hidrólisis, Lipasa.

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MATERIAL AND METHODS

Lipases

Four types of lipases were obtained from following sources: *Candida cylindracea* lipase (85 U/mg, Sigma Chemical Co.), *Rhizopus delemar* lipase (6.5 U/mg, Amano Pharmaceutical Manufacturing Co.), *Aspergillus oryzae* lipase (8.5 U/mg Novo Industry) and *Chromobacterium viscosum* lipase (4.5 U/mg Novo Industry). The specific lipase activity was measured by titration of fatty acids liberated from fish oil with 0.05N KOH. One unit of lipase activity was defined as the amount of enzyme that liberated 1 (mol of fatty acids per min. The lipases from *R. delemar* and *A. oryzae* are *sn*-1, *sn*-3 stereo-specific, and those from *C. cylindracea* and *C. viscosum* are nonpositional specific.

Fish oil

Refined sardine (Sardinella braziliensis) oil was obtained from Alcyon Co. (São Paulo - Brazil) with the following properties: acid value, 0.89, saponification value, 182.6, triglyceride content, 98.4%, ω3 PUFA content, 25.5% (15.3% EPA and 10.2% DHA).

Hydrolysis reaction

The reaction mixture containing 500 mg sardine oil and 150 U lipase powder in 3,5 ml potassium phosphate buffer (pH 7.0, 0.08 M) was placed in a 50 ml Erlenmeyer flask. Air in the flask was replaced by nitrogen, and the suspension formed was agitated on an orbital shaker at 200 rpm at 35 °C for various periods. The reaction was terminated by the addition of 2 ml CH_2Cl_2/MeOH (1:1). All the data presented here represent the means of two or three determinations.

Analytical procedures

To separate fatty acids from acylglycerols, the products of lipolysis were dissolved in 20 mL *n*- hexane and extracted with 10 mL de 0.5 N ethanolic NaOH. The aqueous extracts were separated and acidified with 2 mL 6 N HCl, and the fatty acids were extracted with diethyl ether. Both acylglycerols and fatty acids were converted to methyl esters by treatment with methanol-BF_3 [19] and analyzed by gas chromatography in a Chrompack CG instrument equipped with flame ionization detectors (FID). The separations were carried out on a fused silica WCOT CP-Sil 88 capillary column (Chrompack, Holland) using a programmed temperature of 180-220 °C, 5 °C/min. Hydrogen was used as carrier gas. The composition of fatty acids was identified by comparing retention times with authentic standards (Sigma Chemical Co.) and determined by relative percentage.

RESULTS

Table 1 shows the results of enrichment of EPA and DHA in unhydrolysed acylglycerol by treatment with the lipases tested in this article. Selective hydrolysis of sardine oil with *Candida cylindracea* (nonspecific) lipase led to an increase in the total ω3 PUFA (EPA + DHA) content from 25.5% in the original oil to 40.4% in the unhydrolysed acylglycerol. Most of the enrichment was due to DHA, which increased twofold, whereas the EPA content remained essentially unchanged compared to the original oil after 8 h reaction. Hydrolysis with *Rhizopus delemar* (1,3-positional specific) and *Chromobacterium viscosum* (nonspecific) lipases also enriched DHA in the unhydrolysed acylglycerol; however, these lipases were not as effective as *Candida cylindracea* lipase. On the contrary, *Aspergillus oryzae* (1,3-positional specific) lipase did not result in a significant increase in the DHA contents in the unhydrolysed acylglycerol. The extent of hydrolysis by the last three lipases was almost the same (approximately 30% hydrolysis). None the four lipases tested could raise the EPA content significantly.

Lipases	Components	Amount in total products (%)	PUFA content of fatty acid (%)			Enrichment	
			EPA	DHA	EPA + DHA	EPA	DHA
None	Acylglycerol	~100	15.3	10.2	25.5	1.00	1.00
Candida cylindraceaa	Acylglycerol Fatty acid	51.10 48.90	19.7	20.7	40.4	1.29	2.02
Rhizopus delemar [b]	Acylglycerol Fatty acid	67.40 32.60	15.9	15.5	31.4	1.03	1.52
Chromobacterium viscosum [a]	Acylglycerol Fatty acid	73.10 28.90	15.7	15.2	30.9	1.02	1.49
Aspergillus oryzae [b]	Acylglycerol Fatty acid	73.80 26.20	14.8	13.2	28.0	-	1.29

Table 1. Enrichment of ω3 PUFA in unhydrolysed acylglycerol by selective hydrolysis of sardine oil for 8 h using using various lipases. [a] Nonpositional specific lipase, [b] 1,3-positional specific lipases; [c] Data shows the average of two or three determinations. Relative standard deviation (RSD) ≤ 0.37%.

Reaction time	Components	Amount in total products (%)	PUFA content of fatty acid (%)		Enrichment	
			EPA	DHA	EPA	DHA
0	Acylglycerol	~100	15.3	10.2	1.00	1.00
2	Acylglycerol	73.60	17.9	12.3	1.17	1.20
	Fatty acid	26.40	10.6	7.4		
4	Acylglycerol	60.00	19.1	16.1	1.24	1.58
	Fatty acid	40.00	6.3	3.2		
8	Acylglycerol	53.10	19.7	20.7	1.29	2.02
	Fatty acid	46.90	8.6	3.0		
16	Acylglycerol	40.00	20.7	22.5	1.35	2.20
	Fatty acid	60.00	4.0	2.8		
24	Acylglycerol	37.20	16.9	17.2	1.10	1.68
	Fatty acid	62.80	7.9	5.8		

Table 2. Enrichment of ω3 PUFA in unhydrolysed acylglycerol by selective hydrolysis of sardine oil using lipase from *Candida cylindracea*. [a] Data shows the average of two or three determinations. Relative standard deviation (RSD) ≤ 0.34%.

For this reason only *Candida cylindracea* lipase was used for enrichment of PUFA in sardine oil by selective hydrolysis in the following experiments. Table 2 shows the changes in EPA and DHA contents of unhydrolyzed acylglycerols and fatty acids in the reaction products obtained upon partial hydrolysis of the sardine oil by *Candida cylindracea* lipase for various periods. The course of hydrolysis shows an increase in the level of EPA and DHA in the unhydrolyzed acylglycerols, with a concomitant decrease in the PUFA content of the liberated fatty acids. Thus, after 16 h reaction, leading to 60% hydrolysis of the oil, the level of EPA and DHA in the acylglycerols was increased to 20.7 (1.35-fold enrichment) and 22.5% (2.20-fold enrichment) respectively. Further increase in reaction time leads to a decrease in the extent of enrichment of PUFA.

Figure 1 shows the time course of hydrolysis and the accompanying concentration ratios of the main fatty acids of unhydrolysed acylglycerols after the hydrolysis of sardine oil with *Candida cylindracea* lipase. It is also evident that the course of PUFA enrichment was accompanied by a moderate decrease in the levels of palmitic (16:0) palmitoleic (16:1) and oleic acid (18:1) in the unhydrolysed acylglycerols.

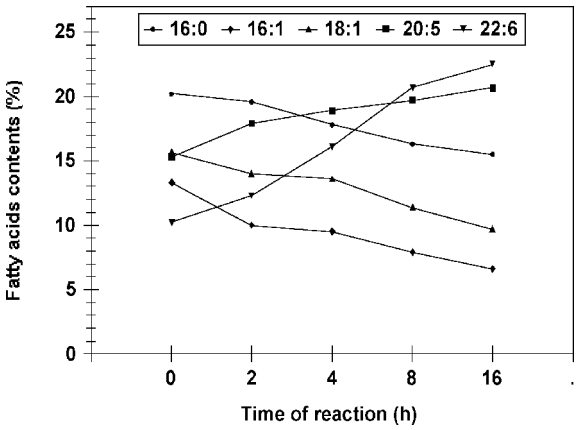

Figure 1. Time course of the main fatty acids contents of unhydrolysed acylglycerols after the hydrolysis of *Candida cylindracea* lipase. The fatty acids contents in glycerides were expressed relative to those in the original oil. **Fatty acid components of original sardine oil:** Palmitic acid (16:0): 20.2%, Palmitoleic acid (16:1): 13.3%, Oleic acid (18:1): 15.7%, Eicosapentaenoic acid (20:5) 15.3%, Docosahexaenoic acid (22:6): 10.2%. Data shows the average of two or three determinations. Relative standard deviation (RSD) ≤ 0.48%

DISCUSSION

It has been reported that because ω3 PUFA is located in the 2-position of triglyceride, hydrolysis of sardine oil with 1,3-specific lipase should produce PUFA rich 2- monoglyceride and 1,2 (2,3)-diglyceride [12,20]. However, in this report, both nonpositional specific and 1,3-specific lipases were tested, and nonspecific *Candida cylindracea* lipase was more effective for the enrichment of DHA in unhydrolysed acylglycerol of sardine oil. The partial hydrolysis of the sardine oil by *Candida cylindracea* lipase indicates strong discrimination by the lipase against DHA and only moderate discrimination against EPA. This implies that the concept of positional specificity alone cannot explain the observed diversity in the hydrolysis of the sardine oil with *Candida cylindracea* lipase. This could be partly due to fatty acid chain length selectivity, showing higher activity with C18 or shorter fatty acids than with C20 and C22 fatty

acids [21]. Bottino *et al.* [20] postulated that in the resistant acid the location of the double bonds in the terminal methyl groups are close to carboxyl group, producing a steric hindrance effect, which protects against hydrolysis. According to Tanaka *et al.* [10] this enzyme has "fatty acid acyl chain specificity" and appears to be unique in its resistance to DHA. The other lipases tested (regiospecific and nonspecific) hydrolyzed PUFA to the same extent as saturated and monoenoic acids because they have less acyl chain specificity, and PUFA can to be transfer from the 2- to the 1,3-position of triglycerides [10].

A lipase from *Candida cylindracea* immobilized on microporus polypropylene fibers was used to selectively hydrolyze the saturated and monounsaturated fatty acid residues of menhaden oil in a hollow-fiber reactor [22]. Since *Candida cylindracea* lipase shows preferential selectivity for or against individual ester bonds of DHA, it was expected that saturated and monoenoic acids in triglycerides are hydrolyzed preferentially, regardless of the presence of DHA in unhydrolysed acylglycerols.

These results confirms those obtained by Tanaka *et al.* [10, 23] showing no clear correlation between the positional specificity and the ease of the fish oil hydrolysis for enrichment of PUFA. After 70% hydrolysis of tuna oil with *Candida cylindracea* lipase, the glyceride mixtures contained two times more DHA than the original oil [10]. As DHA-rich triglyceride was obtained and DHA-rich monoglyceride was absent, the results indicated that the lipase recognizes the whole triglyceride structure. They defined this difference of activity to triglyceride species as "triglyceride specificity" [23].

Although the hypotheses seem difficult to explain in terms of enzymatic specificity, for enrichment of ω3 PUFA in the Brazilian sardine oil, approximately 60% hydrolysis with a moderate level of lipase from *Candida cylindracea* is adequate over a 16 h reaction time at 35 °C.

In Brazil, where there is a substantial sardine industry; it is expected that the present study, along with others of similar nature, may provide valuable information for the preparation of PUFA concentrates, which could be recommended for nutritional or medical purposes.

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