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Lipase-catalyzed chemoselective aminolysis of various aminoalcohols with fatty acids

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ABSTRACT

Candida antarctica lipase is well known to convert amines and alcohols into amides and esters. This report describes the development of a solvent-free enzymatic process for the production of fatty alkanolamides. The aminolysis of linoleyl ethyl ester with several aminoalcohols from C2 to C6 (linear or branched compounds), and the very high selectivity of amide compounds have been observed.

Our investigation leads us to develop an original biotechnological process for the chemoselective synthesis of new active molecules for cutaneous application.

1. Introduction

Biocatalysis offers a clean and ecological way to perform chemical processes, in mild reaction conditions and with high degree of selectivity. The use of lipases for the synthesis of chiral drugs, cosmetic or nutritional compounds is well established since years ago.

Lipid modifications strategies for industry include processes such as fractionation, hydrogenation, and interesterification. While each of the two first processes has specific uses and advantages, interesterification reaction offers the greatest potential application.[1] It includes three approaches: acidolysis, alcoholysis and transesterification. Chemically, it can be induced by the use of alkali catalysts in a reaction which lacks specificity and offers little or no control over the positional distribution of fatty acids in the final product [1,2].

To overcome such difficulties, enzymatic approach is used. Applied biocatalysts are microbial lipases and are based mainly on their specificity. They are divided in two main groups: random lipases, which cleave fatty acids at all position on the glycerol molecule (e.g. lipases from *Candida antarctica*, *Candida rugosa*, *Corynebacterium acnes* and *Staphylococcus aureus*), and *sn*-1, *sn*-3-specific lipases, which act preferentially at the *sn*-1 and *sn*-3 positions of the glycerol molecule (e.g. lipases from *Rhizomucor miehei*, *Rhizopus oryzae*, *Aspergillus niger*, ...) [3]. The second group of lipases show specificity for particular fatty acids. An example is the lipase from *Geotrichum candidum*, which has a marked specificity for long-chain fatty acids that contain *cis*-9 unsaturation [4].

Lipases (triacyl glycerol hydrolase, EC 3.1.1.3) are stable and rugged enzymes that act as well on a wide variety on nonnatural reactants. The studies of their characteristic features have become an essential part of the synthetic repertoire [5]. De Zoete et al. [6] reported that ammonia acts as an unnatural acyl acceptor (ammonolysis). Carboxylic esters are converted to the corresponding carboxylic amides in essentially quantitative yield in an exothermic and, hence, irreversible reaction. The lipase from *C. antarctica* (B type, Novozym 435) emerged from the investigations as the catalyst of choice.

Several other studies demonstrate the possibility of utilizing lipases to catalyze aminolysis reactions using a wide range of amines [7]. More specifically lipases have been tested for their ability to catalyze the synthesis of aminoalcohol compounds [8]. An important issue was chemo-selectivity, since alkanolamines are susceptible for acylation both at the amine and alcoholic group. It was found that most lipases catalyze both the aminolysis and esterification of alkanolamines.

The impact of various reaction parameters in the lipase-catalyzed production of alkanolamides has been studied, using Novozym 435 (immobilized lipase B from *C. antarctica*) in various organic solvents, using free carboxylic acids. It was found that the kinetics for the aminolysis using free acids was controlled by the

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solubility of the ion-pair formed by the reactants. Moreover, it was found that the selectivity of the reaction depended on the solubility of the product in the solvent used and that the choice of solvent was critical in obtaining an efficient process.

Even though organic solvents provide several advantages in enzyme catalysis, such as the shift in the equilibrium in hydrolytic reactions and modification of reactivity as mentioned above, their use in industrial processes is undesirable for various reasons. Organic solvents are a source of volatile organic compounds, and their use also requires costly post-treatment in the form of solvent evaporation and recycling. Therefore, a solvent-free process would be beneficial both from environmental as well as from an economical perspective.

This report describes the development of a solvent-free enzymatic process for the production of fatty alkanolamides. The aim of the investigation was to develop an environmentally benign process suite for industrial production of high quality fatty alkanolamides from fatty acid ethyl esters. The aminolysis of linoleyl ethyl ester with several aminoalcohols from C2 to C6 (linear or branched compounds), and the selectivity of amide compounds have been observed.

Our investigation leads us to develop an original biotechnological process for the chemoselective synthesis of new active molecules.

2. Material and methods

2.1. Enzyme and chemicals

Novozym 435 (immobilized *C. antarctica* lipase B), and Lipozyme RM IM (immobilized *R. miehei* lipase), were kindly provided by Novozymes A/S, Bagsvaerd, Denmark. Linoleyl ethyl ester (70%, for synthesis) was purchased from Stearinerie Dubois (Ciron, France), while aminoalcohols (>97%) were from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

2.2. Enzymatic reactions

The aminoalcohol was added to the acyl donor (linoleyl ethyl ester concentration was adjusted to have a aminoalcohol/linoleyl ethyl ester molar ratio equals 1) in a glass reactor. Then, the temperature mixture was increased at 65 °C (except for ethanolamine compound −40 °C- and 2-amino-1-butanol −50 °C) under stirring (about 300 rpm, using an overhead stirrer IKA RW 16 Basic, Staufen, Germany, equipped with a plastic propeller). After 5 min under vacuum (50 mbar) to remove the gazes of the system, the lipase Novozym 435 (5 g/mol of substrate) was added to the mixture. The reaction was then run under vacuum.

For all experiments, the reaction was stopped after 20 h. The mixture was recovered by filtering off the enzyme preparation. Disappearance of linoleyl ethyl ester and formation of products were monitored using HPLC, to determine the rate of conversion of the reaction.

Products obtained were analysed by ^{1}H and ^{13}C NMR to confirm their structure.

2.3. Analytical methods

2.3.1. High-performance liquid chromatography (HPLC analysis)

The aminolysis reaction was monitored by HPLC analysis carried out in a system (Alliance-Waters) composed of a column (Xterra MS C18 5 μM, 150 mm × 2.1 mm), a column oven (temperature 40 °C), an autoinjector, a UV/vis detector (PDA, W2996, λ = 210 nm).

Table 1
Gradient elution for HPLC analysis

Time (min)	Flow rate (ml/min)	% A	% B
0	0.27	80	20
5	0.27	80	20
25	0.27	100	0
50	0.27	100	0
55	0.27	80	20

The compounds were eluted by gradient elution with an eluent system of methanol (A) and water (B), both containing 0.1% of trifluoroacetic acid (see Table 1).

2.3.2. Nuclear magnetic resonance (NMR) analysis

The chemical structures of the synthesised products were determined by ^{1}H NMR and ^{13}C NMR in $CDCl_3$ using a Brucker AM 400 spectrometer at 400 MHz.

3. Results and discussion

3.1. The reaction parameters

In order to run enzymatic reactions under solvent-free conditions, at least one of the reactants has to be kept in the liquid state. The reaction temperature is therefore a crucial parameter that has to be chosen by taking both the melting points and the miscibility of the reactants into account. Moreover, the formation of products leads to viscous mixture; high viscosity leads to a low mass transfer rate and therefore, the temperature has to be high enough to shift the equilibrium in the right sense.

In our case, the equilibrium is as well shifted in the synthesis way (not the hydrolysis way) due to the evaporation of ethanol formed during the reaction; thanks to vacuum, the possible reverse reaction (hydrolysis) is limited.

3.2. Choice of lipase

Novozym 435 has been chosen for the assays. Novozym 435 proved to be very active for aminolysis, regarded to other industrial lipases. For example, the reactions between linoleyl ethyl ester with 1-aminopropan-3-ol or 1-amino-propan-2,3-diol in the presence of Lipozyme RM IM with the enzymatic method described above (65 °C, 50 mbar, 20 h) does not lead to any aminolysis.

3.3. Rate of aminolysis and esterification

The lipase preparations were tested for their ability to catalyze aminolysis and/or esterification. The reactions were carried out with linoleyl ethyl ester as acyl donor and several aminoalcohols, under solvent-free conditions (Fig. 1):

Table 2 shows the aminoalcohols used.

Table 3 shows that only ramified aminoalcohols provides very good yield (in term of disappearance of linoleyl ethyl ester), with very high selectivity for aminolysis, regarded to esterification (the results are observed with HPLC).

For example, when linear aminopropanol is employed, the conversion rate is 32.9%, and there is no selectivity observed at 20 h. On the contrary, when branched aminopropanediol (3-amino-1,2-propanediol, or 2-amino-1,3-propanediol) is used, the yield is >98% only in favor of linoleylamide. In this C3 family, the use of 1,3-diamino-2-propanol leads to 88.6% yield, with only aminolysis (a mixture of monoamide 70/diamide 30 is obtained in this case). At the same time, we tried the substrate glycerol (propanetriol) and the yield obtained was quite low (84.5%, calculated with disap-

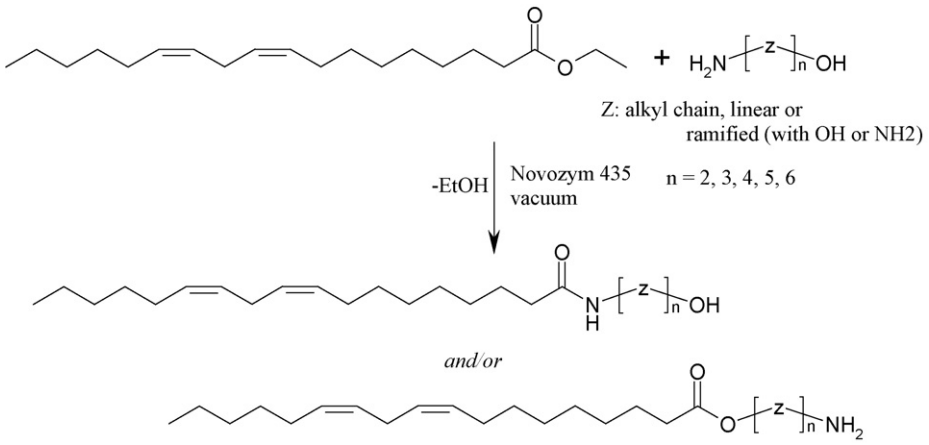


Fig. 1.

Table 2

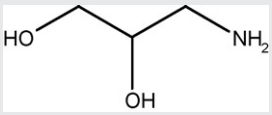
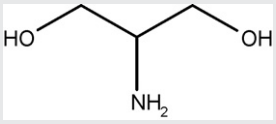
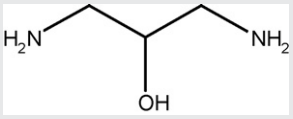
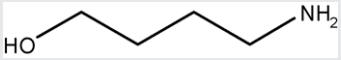
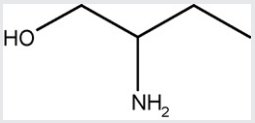
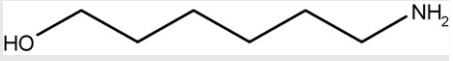
Aminoalcohol	Structure
Ethanolamine	
Aminopropanol	
3-Amino-1,2-propanediol	
2-Amino-1,3-propanediol	
1,3-Diamino-2-propanol	
4-Amino-1-butanol	
2-Amino-1-butanol	
5-Amino-1-pentanol	
6-Amino-1-hexanol	

Table 3

Aminoalcohol	% Linoleyl ethyl ester t_0[a]	% Linoleyl ethyl ester $t_{20\,h}$[a]	Yield[b]	Amide (A) and/or ester (E) formation[c]
Ethanolamine	83.5	63.2	24.3	A + E
Aminopropanol	77.2	51.8	32.9	A + E
3-Amino-1,2-propanediol	77.2	0.0	100.0	A
2-Amino-1,3-propanediol	77.2	1.5	98.1	A
1,3-Diamino-2-propanol	77.4	8.8	88.6	A
4-Amino-1-butanol	77.6	45.9	40.9	A + E
2-Amino-1-butanol	77.6	3.1	96.0	A
5-Amino-1-pentanol	74.9	43.8	28.2	A + E
6-Amino-1-hexanol	72.0	40.9	43.2	A + E

[a] Determined by HPLC.
[b] Calculated with disappearance of linoleyl ethyl ester.
[c] Visualized with ^{1}H and ^{13}C NMR.

pearance of linoleyl ethyl ester), with a mixture of mono-, di- and tri-transesterification.

Results are the same with any other length of chain: with linear aminoalcohols chain, the conversion rate is very low, and no selectivity for aminolysis has been observed. On the contrary, with branched aminoalcohols chain, the conversion rate is very high and very high selectivity for aminolysis has been observed.

Thanks to the results, especially the very high selectivity for aminolysis while using branched aminoalcohols, an original biotechnological process for the chemoselective synthesis of new active derivatives has been developed in our laboratories, for cosmetic applications.

3.4. Application to the chemoselective synthesis of ceramide analogues

Numerous studies have confirmed the interest of ceramides. These play a key role in the skin, particularly in maintaining the water balance [9,10]. It is relatively difficult to obtain these molecules in their natural form. These compounds can also be synthesised, but this is still, nevertheless, tricky. Linoleic acid (Omega 6) is an essential fatty acid in the skin. It is very unstable (especially face to oxidation) and cannot be synthesised by the body but are fundamental to the skin. They intervene at both a structural (by the double bonds) and biochemical level [11].

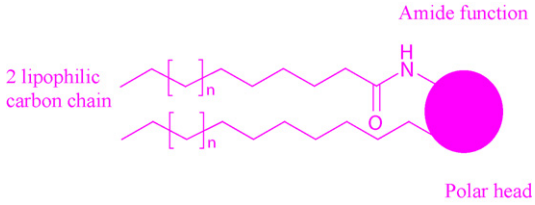


Fig. 2.

A modeling system realized on the ceramide revealed the presence of 3 key points in the molecule: a polar head and 2 lipophilic carbon chains, one of which is linked by amide function to the polar section of the molecule (Fig. 2).

Previous works realized in the laboratory indicate that PUFAs (polyunsaturated fatty acids) can be stabilized on an amide form. With this idea, we expected that Omega 6 could be stabilized on the upon lipophilic carbon chain, and to mime ceramide structure, palmitic acid can be integrated on the above lipophilic chain.

For this synthesis, we suggested an original biotechnological process (Fig. 3) [12].

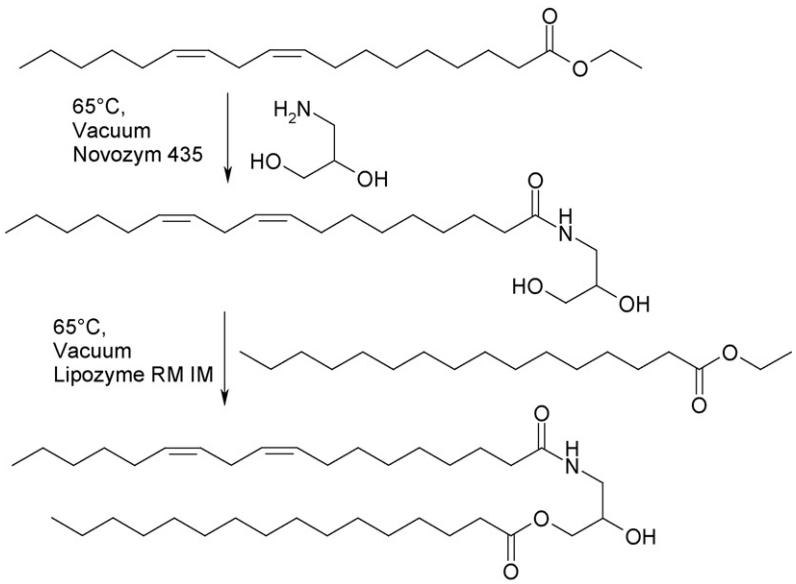


Fig. 3.

Starting from linoleyl ethyl ester (from an oil rich in Omega 6), the first step consists in a chemoselective aminolysis using the lipase Novozym 435.

The intermediate is engaged in the second step for transesterification with the lipase Lipozyme RM IM.

The 2 steps process is realized under vacuum, without any solvent, at 65 °C.

The reduced pressure lead to the removing of alcohol released during the condensation, and thus significantly accelerates the kinetic of the reaction.

Moreover, the reduced pressure and the low temperature of the reaction limits the degradation of the oxidizable fatty acid.

The use of immobilized enzymes (absorbed on a macroporous resin) allows them to be easily removed from the reaction medium and to be then recycled (minimum ten times).

Conversion rate is greater than 99% with perfect chemoselectivity (over 99%).

This molecule revealed to be very active in the biological point view.

4. Conclusion

This report clearly shows that enzymatic synthesis provides an attractive and environmentally more benign alternative to the conventional chemical approaches used for the production of high quality alkanolamides. The selective aminolysis of various aminoalcohols with Novozym 435 (immobilized *C. antarctica* lipase B) has been demonstrated and such very high selectivity for aminolysis of branched aminoalcohols has been used for the synthesis of new active ingredients, used for cosmetic application.

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