Enzymatic Synthesis of N-ε-Acyllysines

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Chemical synthesis of N-ε-acyllysines on an industrial scale corresponds to new requirements in the surfactant field; only one chemical synthesis process is currently known for this type of molecule. A number of organic synthesis conducted in the laboratory so far have not made it possible to consider industrial development. However, biotechnologically, it has been shown that this molecule is easily accessible using a commercially available fixed lipase. Synthesis is carried out by causing a triglyceride to react with the lysine either with or without a solvent. The latter method gives the best results at the current stage of research. Given the availability of at least one fixed lipase on the market today, and the possibility of directly applying the reaction using oils, this reaction could be scaled up rapidly for industrial development.

KEY WORDS: Acyl amide, enzymatic synthesis, fatty acids, lysine.

Industrial synthesis of N-ε-acyllysines corresponds to new requirements in the surfactant field. Only one chemical synthesis process is currently known for this type of molecule (1). The difficulty lies in the fact that the two substrates of the reaction (namely, a fatty acid with chain length exceeding eight carbon and lysine) are molecules with no common solvent; in particular, lysine is only soluble in media containing high proportions of water. In addition, when a molecule contains two amine functions, such as lysine, acylation of one of them alone is almost impossible without previously protecting the other using a very fastidious protection/deprotection process.

Most work already carried out in the laboratory has shown that it is possible to synthesize the amide bond; particularly worth noting is the work published by Servat *et al.* (2,3) on the synthesis and titration of hydroxamic acids, and work by Montet *et al.* on the synthesis of N-lauryloleylamide (4) and the synthesis of acylated aminopropanols (5). These works have been described in the patent by Graille *et al.* (6). The method proposed here describes the enzymatic synthesis of N-oleyllysine under different conditions; in addition, a few spectroscopic characteristics obtained on the purified product are given.

MATERIALS AND METHODS

Solvents. The solvents used for synthesis were all pure, purchased from Aldrich Chemical Co. (Milwaukee, WI).

Reagents. The enzyme is a fixed lipase bonded to an anionic macroporous resin, provided by Novo Industri (Bagsvaerd, Denmark) as under the trade mark LIPOZYME. Thin-layer chromatography (TLC) plates were silicagel 60 G plates ready for use from Merck (West Germany).

Synthesis with a solvent. The reactions were carried out in 25-mL flasks and stirred on a vibrator in a temperature controlled oven at different temperatures. The composition of the medium was as follows: free lysine, 292 mg; soybean oil, 282 mg; Lipozyme, 50 mg; and solvent, 3 mL.

Synthesis without a solvent. The quantities of substrates and enzyme were as follows: soybean oil, 2.82 g; free lysine, 1.46 g; and Lipozyme, 250 mg. These products were brought into contact in a 50-mL flask stirred on a rotary evaporator which was used as a reactor at atmospheric pressure, at reduced pressure or under nitrogen at 1 atmosphere.

Analysis of reactions. Due to the heterogeneity of the reaction medium, it was totally taken up by 100 mL of a butanol/acetic acid/water mixture (40:10:10, v/v/v), a homogeneous solvent solubilizing oleyllysine, lysine and the different glycerides. Different dilutions then were made to appropriately titrate the oleyllysine by (TLC).

RESULTS AND DISCUSSION

Firstly, we planned to synthesize N-ε-oleyllysine by Lipozyme action on lysine and oleic acid. The tests conducted in numerous solvents at various temperatures (30-100°C) gave very low yields (< 1%); nevertheless, they suggested that synthesis of this molecule was possible.

The first satisfactory reaction in terms of yield was carried out in a closed reactor in ethyl ether at a temperature of approximately 65°C (3 atmospheres), using triglycerides as a fatty acid source. The composition of the medium is generally as follows: free lysine, 292 mg; soybean oil, 282 mg; Lipozyme, 50 mg; solvent, 3 mL (duration, 24 hr).

A systematic study was made to determine the parameters that make the reaction possible. We initially varied the carbon chain of the ether by conducting the reaction in ethers for which a few characteristics are given; two temperatures were also tested 25 and 65°C (Table 1).

At 25°C, traces of nonquantifiable synthesized products are observed in diethylether, diisopropylether and methyltertiobutylether, whereas this rises to 1.5% and 1% in dibutylether and dipentylether. At 65°C, the yield of the

TABLE 1

Synthesis Yields in Different Ethers at 25 and 65°C

Ethers	Boiling point (°C)	Water content (%)	Yield (%) 25°C	Yield (%) 65°C
Diethylether	34.6	0.06	0	16.5[a]
Diisopropylether	68	0.08	0	12.6
Methyltertiobutylether	70	0.12	0	14.2
Dibutylether	142	0.07	1.5	12.0
Dipentylether	187	0.09	1	5.5
Tetrahydrofuran	67	0.06	0	0
Dioxane	100	0.16	0	0

[a]At 3 atm.

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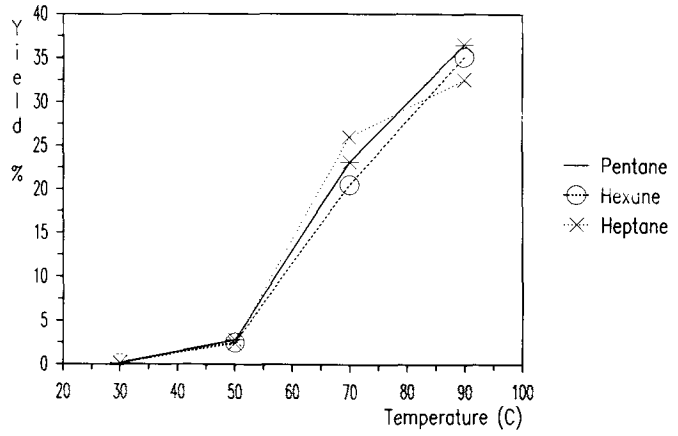

FIG. 1. Yield vs type of solvent.

reaction seems to increase when the CHO ratio of the ether used decreases, or when the oxygen content of the ether increases. This observation could mean that the smaller the ether's substituents, the more the polar zone of the amino acid tends to combine with the ether's oxygen atom, thereby making the NH_2 group at the end of the chain more accessible, and hence, more available for acylation.

As the water contents of these ethers were not significantly different, it can be considered that yield was not affected by this parameter, as long as water is only present as traces.

No synthesis is seen in the presence of two cyclic ethers tested (dioxane, THF), and, so far, we have no explanation for this.

In order to sidestep the effect of oxygen, we conducted the acylation reaction in hydrocarbons (pentane, hexane, heptane) whose water contents are around 0.01%. The study was carried out at different temperatures (30, 50, 70, and 90°C); the yield, depending on temperature (Fig. 1), gives similar diagrams for the three solvents. In addition, in all cases yields increase in line with temperature and no effect is noted for the chain length of the three hydrocarbons. The maximum yield obtained after 24 hr of reaction was approximately 35% at 90°C in the three sets of experiments.

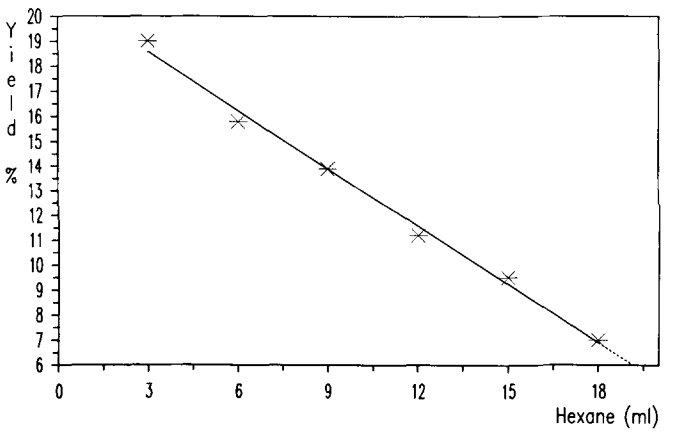

FIG. 2. Effect of the volume of solvent on reaction yield.

TABLE 2

Test in Trichlorotrifluoroethane (CF_2ClCCl_2F)

Temperature (°C)	Yield (%)
30	0
65	15.5

Tests in trifluotrichloroethane (CF_2ClCCl_2F), a less polar solvent than hydrocarbons, gave yields equivalent to the latter at the same temperatures (Table 2).

For practical reasons, hexane was chosen as solvent for studying the effect of solvent volume on yield. Figure 2 shows, without ambiguity, that reducing the volume of solvent very distinctly favors yield, since it decreases from 19% for 3 mL to 7% for 18 mL. It then appeared obvious to us that we should work with no solvent at all, despite all the technical difficulties that could be envisaged, i.e., particularly that the two substrates are not absolutely mutually miscible, which suggested that difficulties would be encountered for bringing the reagents into contact with each other and with the biocatalyst.

To try and solve this problem, we used a rotary evaporator as the reactor; indeed, such an apparatus seems to be excellent for phase contact and also offers the advantage of being able to operate at reduced pressure, if necessary. Table 3 gives the results of the different experiments carried out with this type of apparatus, with or without pressure reduction.

The quantities of substrates and enzymes used were as follows: soybean oil, 2.82 g; free lysine, 1.46 g; and Lipozyme, 250 mg.

Table 3 leads to several conclusions: in one experiment only, the oil/lysine molar ratio was doubled, so as to try and make the medium more fluid; as a matter of fact, for the ratio 1:1, the medium has a viscous consistency which does not favor exchanges between phases. The results show that this strategy does not favor synthesis; the reaction without a solvent gives better yields in general than with a solvent, and reducing the pressure has a somewhat unfavorable effect on synthesis, contrary to what might have been expected. In fact, it is probable that not only the synthesis water is eliminated, but also the water making up the Lipozyme (which makes the latter inoperative), and although peroxides are reputed to be

TABLE 3

Yields (%) Obtained Without Solvent

Conditions	70°C	90°C
TG/lysine = 2	6.0	7.0
Without catalyst	0	0
Resin only	0	0
Under reduced pressure	18.0	5.0
1 atm. with N_2	25.6	36.6
1 atm. without N_2	24.0	29.2
2 Days	39.4	not titrated
4 Days	39.6	60.7
7 Days	not titrated	73.0
adding 90 μL of water	not titrated	16.0

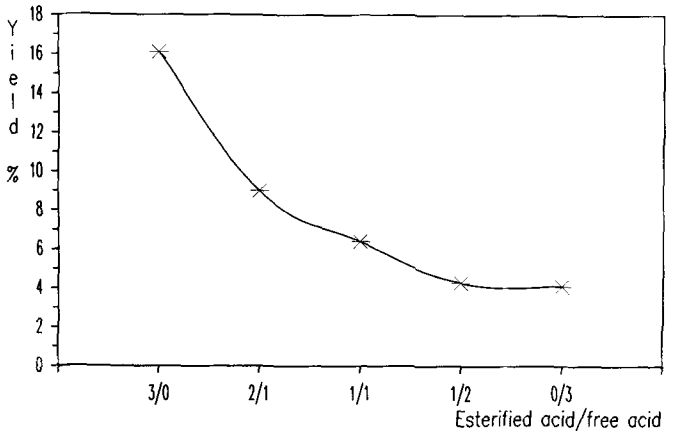

FIG. 3. Evolution of synthesis vs the FA/TG ratio.

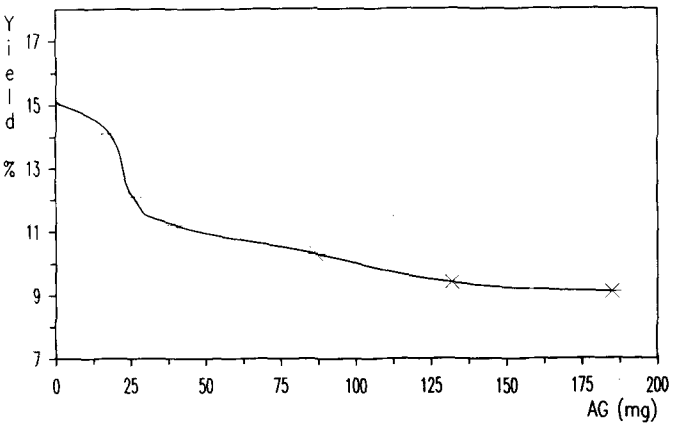

FIG. 4. Inhibition of synthesis by oleic acid with a constant TG/Lysine ratio.

TABLE 4

Reaction Mixture Composition

Free lysine (mg)	292	292	292	292	292
Esterified fatty acids[a](mg)	282	188	141	94	0
Oleic acid (mg)	0	94	141	188	282
Lipozyme (mg)	50	50	50	50	50
Hexane (mL)	3	3	3	3	3

[a]In soybean oil form.

lipase inhibitors, adding nitrogen to prevent any oxidation of lipids has few beneficial effects; adding water, corresponding to the quantity required for obtaining 50% hydrolysis of soybean oil, adversely affects the reaction; this result shows that, for the reaction to occur properly, the concentration of fatty acids must remain low. In fact, triglycerides play the role of a feeding tank delivering fatty acids as and when required by the reaction, and despite the viscosity of the reaction medium, after a few hours the evolution over time is important, since at 90°C it increases from 29% in 24 hr to 60% in 4 days.

As the temperature of 90°C is not usual for a biological catalyst, we initially suspected that the macroporous anion exchange resin could have had a catalytic effect due to its structure. Tests conducted at both 70 and 90°C with no catalyst or with virgin resin do not give rise to synthesis. Therefore, it appears that the catalytic effect can only come from the combination of the lipase of *Mucor miehei* and the resin. In other words, the resin/enzyme bond is characterized by exceptional activity which the two elements are incapable of individually. Grafting this lipase onto this type of resin provides a remarkable and exceptional thermal stability factor.

As synthesis of the molecule required is effective from triglycerides (TG), it was logical to examine the fact that yields are low in the presence of oleic acid and high in the presence of triglycerides. Our way of approaching this phenomenon was to conduct tests using oleic acid and triglyceride mixtures in two different ways—varying the fatty acid (FA)/TG ratio; and keeping a constant TG/lysine ratio while varying fatty acid concentration.

Study of varying the FA/TG ratio. Experiments were conducted using hexane as the solvent. The quantity of lysine and the total FA + TG remained constant, and are given in Table 4.

If yield is expressed as a function of the esterified acid/free acid ratio (Fig. 3), strong inhibition of the reaction by free fatty acids is seen. A yield of 4% was obtained in the presence of oleic acid alone. The fatty acid is therefore not a strict inhibitor. Perhaps its inhibiting action is simply due to ionic fixation of the fatty acid onto the Lipozyme's macroporous anion exchange resin, thereby creating a lipophilic macrostructure preventing the lysine from gaining access to the active site of the lipase.

Study of the effect of fatty acids keeping a constant TG/lysine ratio. The quantities of lysine and soybean oil were fixed at 292 mg and 282 mg, respectively. The quantities of fatty acids were varied from 0 to 181 mg. Figure 4 reveals a considerable inhibiting effect of the oleic acid on the reaction, though it did not stop it. Yields dropped from 15.1% in the best case, to 8.9% in the most unfavorable case, i.e., in a reaction medium containing 181 mg of oleic acid. These results enable us to categorically conclude that free fatty acids have an inhibiting effect.

When using fatty acids in TG form, it is possible to add the latter to the medium as and when they are hydrolyzed, thereby avoiding their accumulation. It is also possible that, under these conditions, the fatty acids are found in an active enzyme-substrate compound form, favoring transacylation, by preventing the straight release of fatty acids into the medium.

TABLE 5

Fragmentation Obtained with Mass Spectrometry

Mass	Fragments
41	$CH{=}CHCH_2 + H^+$
55	$CH_2CH{=}CHCH_2 + H^+$
84	$(CH_2)_4CH$
154	$CH_3(CH_2)_7CH{=}CHCH_2 + H^+$
166	$CH_3(CH_2)_7CH{=}CH(CH_2)_2$
244	$(CH_2)_5\underset{\underset{OH}{\vert}}{C}N(CH_2)_4\underset{\underset{COOH}{\vert}}{C}HNH_2$
307	$M - COOH - CH_3(CH_2)_3 + H^+$
368	$M - CH_3(CH_2)_2 + H^+$
411	Mass peak + H^+

TABLE 6

Interpretation of the NMR Spectrum

ppm	5.32	4.85	3.2	2.2	2.02	1.55	1.30	0.88
Intensity	1.100	55.283	1.749	1.104	2.421	18.212		1.784
Type of signal	Sing.	Mas.	Mult.	Tripl.	Mas.	Mas.	Mas.	Tripl.
Number of protons	2	—	3	2	4	8	20	3
Attribution	CH=CH	Solv.	CH-COOH NH_2 CH_2NC HO	CH_2C O	CH_2	CH_2	CH_2	CH_3
Carbon no.[a]	16;17	—	2;6	9	15;18	3;10 14;19	4-5 20 to 24 11 to 13	25

[a]The molecule is numbered starting from the carbon of the lysine's carboxyl function. Sing., singlet; mas., Massif; mult., multiplet; trip., triplet; and solv., solvent.

TABLE 7

Oleyllysine Microanalysis

Element	Theoretical %	Measured %
C	70.24	68.28
H	11.22	11.09
O	11.71	12.22
N	6.83	6.50

Confirmation of N-ε-oleyllysine structure. In order to check whether the product obtained was in fact N-ε-oleyllysine, we produced the mass, proton nuclear magnetic resonance and microanalysis spectra for it. The mass spectrum was obtained under Fast Atom Bombardment with a p-nitrobenzoic alcohol matrix. The characteristic fragments identified are given in Table 5. The mass peak obtained confirms the authenticity of the product. The proton spectrum was obtained on a Bruker AC 250 apparatus (Brucker Instruments, Inc., Karlsruhe, Germany), and Table 6 gives its interpretation. This spectrum confirms that the lysine and the oleic acid are linked by an amide bond only concerning the amine function at the end of the chain. Table 7 gives the microanalytical results obtained per measurement, as compared to the theoretical results and confirms the atomic structure of the molecule. The melting points measured for the control N-ε-lauryllysine and the N-ε-oleyllysine obtained enzymatically are 263°C and 229°C, respectively. It is seen that the difference between the melting points of lauric and oleic acids is reflected in the corresponding N-ε-acyllysines.

Therefore, there is hope of mastering the rheological properties of these types of compounds according to formula requirements.

The success of this synthesis shows that it is possible to obtain these molecules biotechnologically, whereas conventional chemical methods have not enabled us to achieve our objectives so far, for different reasons.

The essential difficulty stems from the presence of two amine functions, whereas we are only interested in acylating the one occupying the end of the lysine chain, without prior protection of the amine function located in α position of the carboxyl group.

These results can be added to those already published elsewhere (2-6) and confirm that acylation of an amine function by a fatty acid, whose carbon chain length is > 8, is possible.

REFERENCES

1. Takizawa, K., and R. Yoshida, U.S. Patent No. 3, 897, 466 (1975).
2. Servat, F., D. Montet, M. Pina, J. Graille and L. Marcou, *Rev. Fr. Corps Gras 36*:217 (1989).
3. Servat, F., D. Montet, M. Pina, A. Arnaud, P. Galzy, M. Ledon, L. Marcou and J. Graille, *J. Am. Oil Chem. Soc.*, accepted for publication.
4. Montet, D., M. Pina, J. Graille, G. Renard and J. Grimaud, *Fett Wissenschaft Technologie 91*:14 (1989).
5. Montet, D., J. Graille, F. Servat, L. Marcou and G. Renard, *Rev. Fr. Corps Gras 36*:79 (1989).
6. Graille, J., D. Montet, F. Servat, J. Grimaud, G. Renard, P. Galzy, A. Arnaud and L. Marcou, European Patent No. 0,298,796,A1 (1988).

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