Synthesis of Fatty Hydroxamic Acids Catalyzed by the Lipase of *Mucor Miehei*

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Biotechnological synthesis of a new class of amphiphilic molecules—fatty hydroxamic acids—was carried out using the lipase of *Mucor miehei* by reacting hydroxyl amine with the fatty acids in their free or methyl ester form. Concurrently with enzymatic synthesis, chemical synthesis of hydroxamic fatty acids has also been developed by adapting methods that already existed for water-soluble acids. Different parameters were studied to determine the optimum operating conditions: temperature, molar ratio of reagents, quantity of biocatalyst and length of reaction. A general method, whatever the type of fatty acids used, is described.

KEY WORDS: Amide, fatty acid, fatty hydroxamic acids, lipase, synthesis, triglyceride.

Hydroxamic acids, whose general formula RCONHOH enables their assimilation with N-substituted amides, are chelating agents which chelate transition metals in particular. This property is mainly used in analytical chemistry, therapeutics and agronomy. They are also used as heavy metal conveyors in liquid-liquid extraction (aqueous phase/organic solvent) in the nuclear industry.

So far, low carbon condensation hydroxamic acids have mostly been obtained chemically (1). It should be noted, however, that Thiery *et al.* (2) succeeded in obtaining the corresponding hydroxamic acids from water-soluble acids in a buffered medium by using the amidase of *Brevibacterium* as a catalyst; in particular, they synthesized acetylhydroxamic acid.

Based on the work of these authors, we attempted to extrapolate the reaction to fatty chains in an organic medium in the presence of a lipase. Lipases are acyltransferases like amidases and offer, in addition, the advantage of being available on the specialized market.

This new biotechnological synthetic pathway would appear to be completely original since, as far as we know, the literature contains no mention of this type of synthesis for aliphatic hydroxamic acids.

The use of lipases, now well known for problem-free catalysis of the ester bond formation (3,4), may appear surprising for the creation of the C-N bond; the transfer of acyls to a nitrogen atom, catalyzed by a lipase, has nonetheless been reported by Inada *et al.* (5), who studied the reaction of laurylamine on laurylstearate to obtain N-laurylstearamide, and also by Zaks and Klibanov (6), who described an ester aminolysis reaction with pancreatic lipase. Gancet *et al.* (7) suggested the possibility of creating an amide bond using the lipase of *Rhizopus arrhizus* in a nonaqueous medium. Later, Graille *et al.* (8) confirmed the ability of lipases to catalyze the formation of the amide bond in the fatty series. Then Montet *et al.* (9,10) successfully achieved N-lauryloleylamide synthesis and N-acylation of aminopropanols using the fixed lipase of *Mucor miehei*, marketed by Novo Industri under the trade name Lipozyme.

These positive results led us on the synthesis of new amphiphilic molecules to use this biocatalyst for hydroxylamine substitution. These new molecules could be the precursors of surfactants with a wide range of properties enabling them to be used in numerous specialized fields, such as household cleaning products, cosmetics, pharmacology, etc.

Synthesis was undertaken in a heterogeneous medium in the presence of Lipozyme, reacting hydroxylamine and fatty acids or their methyl esters from C_8 to C_{22}. In addition, we synthesized fatty hydroxamic acids chemically by adapting the methods that already exist for water-soluble acids (11,12) to obtain model molecules.

MATERIAL AND METHODS

The commercial lipase used was Novo Lipozyme® (lipase of *Mucor miehei* fixed on a macroporous anion exchange resin). The acids, caprylic (Aldrich Chemical Co., Milwaukee, WI), capric (Prolabo, Paris, France), palmitic (Stéarinerie Dubois, Scoury, France), stearic (Hyfran, Compiègne, France), oleic (Prolabo), erucic (Fluka AG, Buchs, Switzerland), linoleic (Aldrich) and HCl-hydroxylamine (Aldrich), were used without purification. Soybean methyl esters were obtained by methanolysis of commercial soybean oil, by the IUPAC method, using sodium methylate and methanolic HCl (13).

Chemical synthesis. A solution of 7 g of hyroxylamine hydrochloride (137.3 mmoles) in 100 mL of methanol was added to a solution of 5 g of sodium (217.4 mmoles) in 150 mL of methanol. After elimination of the sodium chloride by filtration, 29.3 g of soybean methyl ester with a mean molecular weight of 293 (100 mmoles) was added.

The mixture was refluxed for 30 min, then cooled to ambient temperature, acidified by the addition of 500 mL of 20% aqueous HCl to give a mixture containing a 1:2 ratio by volume of methanol/water. This solution was extracted with approximately 300 mL of hexane. The hexane solution was washed until neutral and dried over anhydrous sodium sulphate. The soybean hydroxamic acids obtained in this way were then purified using the $FeCl_3$ chelating method, which will be described later.

Enzymatic synthesis. The reaction took place in 25-mL glass flasks hermetically closed with a teflon seal; the flasks were mechanically stirred with a vibrator in a temperature controlled oven. Initially, basing

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ourselves on the conditions described by Thiery *et al.* (2) for the synthesis of water-soluble hydroxamic acids, enzymatic synthesis was conducted as indicated in the following paragraph.

One hundred fifteen mg of soybean methyl esters (mean MW 293) (i.e., 0.4 mM) and 140 mg of hydroxylamine hydrochloride (MW 70) neutralized with 4N sodium hydroxide (i.e., 20 mM), were reacted in the presence of 100 mg of Lipozyme in 3 mL of phosphate buffer (50 mM, Ph7) for 24 hr at 40°C. With oleic acid, enzymatic synthesis was conducted in the presence of 3 mL of phosphate buffer, 50 mM, pH 7, or 3 mL of hexane and with the same quantities of reagents, i.e., 113 mg of oleic acid, 140 mg of hydroxylamine and 100 mg of Lipozyme. Different temperatures, between 25°C and 70°C, were tested during the 24 hr period. To study the effect of the hydroxylamine hydrochloride/oleic acid molar ratio, the following conditions were chosen: The length of the reaction was fixed at 24 hr; the medium was buffered and the hydroxylamine hydrochloride/oleic acid molar ratio varied from 0.4 to 40 on the basis of a constant quantity of oleic acid (282 mg, or 1 mM). The reaction took place at 37°C in the presence of 100 mg of Lipozyme.

Yields according to the amount of Lipozyme were determined with the other parameters fixed at their optimum values. We varied the amount of Lipozyme from 3–200 mg, i.e., a variation of the biocatalyst/fatty acid ponderal ratio (P/P) from 0.01 to 0.70.

Thin-layer chromatography (TLC). Products were analyzed by TLC on Merck 0.25 mm thick, ready-to-use silica gel plates (60 G), develo ed by a hexane/ethyl ether/acetic acid mixture (20/80/1 v/v/v). Plates were heated at 180°C for 4 min after eing sprayed with a solution containing a mixture of aturated copper acetate in water and 85% phospho ic acid (50/50, v/v). Under these conditions, methyl sters migrate to the front—fatty acids and hydroxami acids have an Rf of 0.7 and 0.3, respectively. The ethod was accurate enough to monitor synthesis evol ition, for more accurate determinations a colorimet ic method (14) was used.

Quantification of hydroxamic acids. The fatty hydroxamic acids with carbon cha ns greater than 10 were colorimetrically quantitated i hexane as described by Servat *et al.* (14). Then 0.5 m of $FeCl_3$ $6H_2O$ (6% P/V in HCl 2N) were added to 3 ml of fatty hydroxamic acid solution appropriately dilute with hexane. After stirring, the colorimetric values ere read at 450 nm on the hexane phase, in comparisc with a control. The fatty hydroxamic acids with carb on chain lengths under 10 were quantitated using the Brammar and Clarke method (15).

Purification and identification The hydroxamic acids were purified, either by crys allization in hexane at 14°C, or by chelation with F Cl_3, summarized as follows: Actual chelation, salific ation of fatty acids with 0.1 N sodium hydroxide an l destruction of the chelate in an acid medium (HCl 2N). After purification, the degree of purity as cor firmed by elemental analysis and by IR spectrometr (Philips SP3-200S), by ^1H NMR (Brucker AC-250) an l by fast atom bombardment (FAB) at 70eV in nitrol enzyl alcohol (JEOL JNM-DX 300).

RESULTS AND DISCUSSION

Monitoring of the reaction over time using TLC revealed the gradual reduction of the soybean methyl esters and the appearance of hydroxamic acids accompanied by free fatty acids in the reaction medium; these free fatty acids are probably intermediates in the synthesis process.

Given this observation, it appeared logical to conduct the reaction directly with free fatty acids. That reaction conducted under the same conditions with the same molar proportion of soybean free fatty acids gave rise to the same product with the same efficiency. Further, the chromatographic behavior of the synthesized product was similar to that of the fatty hydroxamic acids obtained chemically from soybean methyl esters.

Chemical synthesis carried out without optimization of the operating conditions gave a yield of 30%, whereas the first enzymatic synthesis trials gave a yield of 54%. Given the obvious economic advantages offered by direct utilization of free fatty acids, that biotechnological pathway seemed to be the best way of producing fatty hydroxamic acids. We then studied and optimized different parameters governing the reaction with oleic acid as the substrate.

Definition of the reaction medium. Bringing oleic acid into contact with hydroxylamine hydrochloride neutralized with 4 N sodium hydroxide produces a viscous reaction medium; stirring is therefore impossible and dilution is necessary. Two ways were possible—either diluting the aqueous phase containing the hydroxylamine as per Thiery *et al.* (2), or diluting the organic phase constituted by the fatty acids. In the first case, dilution of the medium by a buffered solution at pH 7 gave a two-phase system consisting of a solid phase (the Lipozyme) and a translucent liquid phase (the aqueous phase containing the hydroxylamine, the buffer and the hydroxylammonium oleate, this soap being responsible for the translucence of the aqueous phase). As time goes by, a low density product was seen to appear on the surface of the reaction medium.

In the second case, hexane, a good fatty acid solvent, was introduced into the medium to give a three-phase system. The liquid part consisted of two phases, an organic phase containing fatty acids in solution and an aqueous phase containing hydroxylamine; the Lipozyme makes up the solid phase. During the reaction, the two liquid phases remained transparent.

Effect of temperature. The two dilutents, phosphate buffer (50 mM, pH 7) and hexane, were evaluated over a temperature range of 25 to 70°C. A control reaction was undertaken with no catalyst for each set of experiments. The buffered reactions yielded from 42% to 8% conversion over the temperature range with maximum values (53% to 55%) from 30–45°C. Hexane diluted reactions ranged from 5% to none with maxima at about 10% from 37–50°C. Synthesis of oleylhydroxamic acid was more substantial in all cases in a buffered medium than in a hexane medium. It is well known that lipases have a greater tendency to catalyze hydrolysis reactions in an aqueous medium and synthesis reactions in an organic medium in the presence of a limited aqueous phase. It is likely that increasing the volume of the organic phase with hex-

TABLE 1

Effect of the Molar Ratio of the Reagents on the Oleylhydroxamic Yield

NH_2OH, HCl (mg)	28	70	175	350	700	1050	1400	2100	2800
Oleic acid (mg)	282	282	282	282	282	282	282	282	282
Molar ratio	0.4	1	2.5	5	10	15	20	30	40
Yield (%)	10	16	30	48	60	65	70	76	75

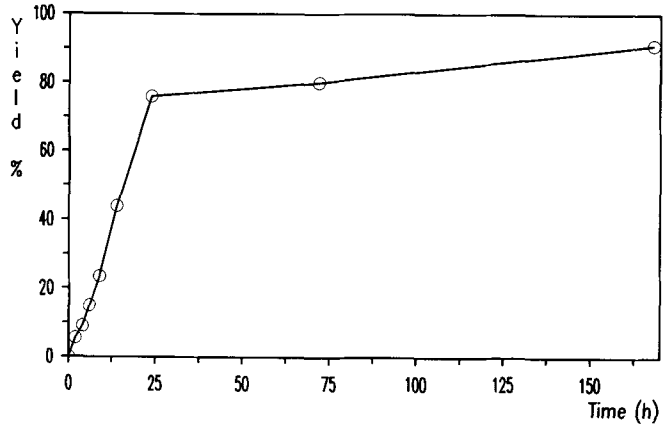

FIG. 1. Evolution of oleylhydroxamic acid synthesis over time.

ane lowers the quantity of fatty acids available at the interface, which slows down the reaction.

In a buffered aqueous medium at pH 7, the hydroxylammonium soaps probably arrange themselves in mixed surfactant/free fatty acid micelles, in the manner of sodium soaps. The arrangement of free fatty acids in this type of macrostructure certainly enables them to present their carboxylic part to the surface of the micelles, i.e., to the aqueous phase. This organization would facilitate the formation of acylenzyme, an intermediate subsequently evolving into a hydroxamic acid by reaction with a hydroxylamine molecule, a species which is even more available when the hydroxylamine concentration increases.

This mechanism seems to be corroborated by the fact that the reaction yield increases with the hydroxylamine concentration (Table 1). However, because the product is insoluble in the medium, the balance of the reaction is naturally displaced towards synthesis by virtue of the application of the law of mass action.

Effect of the hydroxylamine hydrochloride/oleic acid molar ratio. Table 1 shows that synthesis is maximized when the reagents are brought together at a molar ratio of 30, i.e., when there is a large surplus of hydroxylamine. Montet *et al.* (10) also show that when a fatty acid is made to react with water-soluble molecules, such as aminopropanols, synthesis yield increases when the quantity of the low molecular weight molecule is substantially increased.

Yield according to the amount of Lipozyme. As could be expected, yield increased when the amount of Lipozyme increased. The best compromise between yield and the quantity of biocatalyst was reached with 21 mg of Lipozyme, when a 75% yield was obtained for a relatively low biocatalyst/oleic acid ratio (0.07).

Synthesis evolution over time. With optimum reaction conditions, the reaction proceeded rapidly and virtually linearly for 24 hr (Fig. 1), during which time the yield reached 75%. After a day's reaction, production slowed down and increased asymptotically; thus, after seven days, yield reached 91% and it is possible, by extrapolation, to forecast that the reaction would be almost complete at the end of a fortnight. This slowing down of the reaction could be explained by the fact that the fatty hydroxamic acids rise to the surface of the reaction medium as they form and agglomerate and trap some of the fatty acids. This phenomenon has been verified by TLC, because the fatty hydroxamic acids recovered and dissolved in hexane contained the remainder of the fatty acids.

If this agglomeration did not occur, the reaction would likely continue to be linear beyond 24 hr and would then go to completion much more rapidly.

Identification and purity of the oleylhydroxamic acid. The IR spectra of the oleylhydroxamic acids synthesized chemically and by enzymatic catalysis were identical and both show the typical bands of amides:

$$\upsilon_{C=O}: 1650\ cm^{-1},\ \upsilon_{NH\text{-}OH}: 3420, 3250\ cm^{-1}$$

TABLE 2

Signals of ^{1}HNMR Spectrum in $CDCl_3$

(18) (11) (10) (9) (8) (3) (2) (1)
$CH_3(CH_2)_6\text{-}CH_2\text{-}CH = CH\text{-}CH_2\text{-}(CH_2)_4\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}NHOH$

δ (ppm)	Signal	Integration	J Hz	Attribution
0.88	triplet	(3H)	6.5	CH_3 terminal (18)
1.27	multiplet	(20H)		CH_2 of the chain
1.62	quintuplet	(2H)		CH_2 (3)
2.00	multiplet	(4H)		CH_2 (8) and (11)
2.14	triplet	(2H)	7.5	CH_2 (2)
5.34	multiplet	(2H)		CH 9 and 10

The two protons α to the carbonyl group are clearly more shielded in the hydroxamic acid as compared to the methyl oleate; the signals are at 2.30 ppm for methyl oleate and 2.14 ppm in the oleylhydroxamic acid. This difference is obviously due to the difference in the inductive effects of the nitrogen and oxygen

atoms. All the other signals are identical to those of methyl oleate, except for the absence of the methyl ester signal. Under our measurement conditions we did not observe the N-H and O-H protons. As expected, FAB mass spectrometry gave the protonated molecular ion at m/e = 298. Ions were also seen at m/e = 282 (loss of terminal methyl group), m/e = 265 (loss of NH_2OH) and m/e = 154 (allylic splitting, loss of C_6H_{11}-CO-NHOH).

Elemental analysis was C: 72,68; H: 12,00; N: 4,56; O: 10,74; $C_{18}H_{35}NO_2$ requires C: 72,73; H: 11,78; N: 4,71; O: 10,77.

In order to check whether this synthesis could be applied and extended to other fatty acids, a series of experiments was carried out under the optimized operating conditions using caprylic, capric, palmitic, stearic, oleic, erucic and linoleic acids. In all cases, the yield was similar to that obtained with oleic acid. This method therefore appears to be applicable to most fatty acids, whether saturated mono-, or polyunsaturated, over a carbon chain length range of 8–22.

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