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# INTERESTERIFICATION AND SYNTHESIS BY CANDIDA cylindracea LIPASE IN MICROEMULSIONS

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**SUMMARY:** Unusual reactions of interesterification and synthesis catalyzed by Candida cylindracea lipase have been tested in reverse microemulsions . The microemulsions used are made of fatty acids or triglycerides, the enzyme dissolved in a very low water quantity, Brij 35 used as surfactant and an alcoholic cosurfactant . In such a system, fats and alcohols are both the substrates of the enzyme and the microemulsion components .
Incidentally, non specific Candida cylindracea lipase does not catalyze interesterification of short chain triglycerides, revealing a specificity for the chain length . Interesterification reactions tested in the presence of a given water quantity but with varying water activities show that it is the water activity and not the water quantity which is a fundamental parameter of the system. The effect of the surfactant (Brij 35) on the interesterification reaction is studied.
Heptyl-oleate synthesis catalyzed by non-specific lipase is obtained in microemulsions at a 98 % yield. Synthesis of glycerol esters is also tested in monophasic medium and mono and diglycerides are obtained. 

The possibility of including enzymes in emulsions or microemulsions allows the study of the transformation of aqueous insoluble substrates and even, the modification of the aqueous content to obtain synthesis or group transferring reactions (1 - 9) .
Microemulsions are monophasic, transparent, isotropic and stable from the kinetic and thermodynamic point of view . In a microemulsion the exchanges are very rapid (10 - 13).

The industrial potential of such systems include : tertiary oil recovery, octane improvement, pollution abatement, lubricants, pesticides and chemical processing . Microemulsions are part of many cosmetics (14) . The use of microemulsions in enzymology has had very little attention in the literature (15) . We have chosen to study the behavior of lipases in microemulsions . It is well-known that these enzymes act at the interface between the aqueous and organic phases (16). In a microemulsion there is no "physical" interface, but the contact surface between aqueous and organic phases is greater than with an emulsion. By modifying the quantities of the different components, it is possible to have direct microemulsions (oil in water) or reverse microemulsions (water in oil) (10, 14)

The microemulsions used are composed of four constituents : triglycerides or fatty acids constitute the organic phase, the enzyme in water the aqueous one, the surfactant is Brij 35 (polyoxyethylen-23-lauryl ether) and the cosurfactant, a primary or a tertiary alcohol .
In our case, the system permits not only the solubilization of substrates and products but also demonstrates unconventional reactions such as interesterifications and synthesis .

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The reactions studied are as follows :

- for interesterification $R_1 X + R_2 Y \text{------} > R_1 Y + R_2 X$

- for synthesis **alcohol + fatty acid(s) ---------- > fatty acid ester(s) + $H_2O$**

One of the fundamental parameters influencing hydrolysis or formation of new fatty acid esters is the water activity . According to the mass action law, a high yield of water will bring on the hydrolysis of esters, whereas a low yield of water will favor synthesis or group transfers .

## MATERIALS AND METHODS

The microbial lipase from Candida cylindracea (non specific) is purchased from Sigma . Its hydrolytic activity tested in an emulsion made of olive oil and $10^{-2}$M, pH 6.8 phosphate buffer by a chemostat method is 15 mmoles of fatty acids/mg of protein x min .
The organic phase is commercial olive oil, triglycerides (trriolein, trinonanoin, triheptanoin, tricaproin or tributyrin from Fluka) or oleic acid (Fluka) . The surfactant is Brij 35 (Sigma) and the cosurfactant heptanol (Fluka), 2-methyl-2-hexanol (Aldrich) or 2-methyl-2-butanol (Aldrich).

- **Phase diagram**

Microemulsion areas are determined by phase diagram construction .
All the experiments are made with reverse microemulsions . In all cases, the microemulsion composition is given in mass yields .

- **Water activity measurements ($A_w$)**

Water activity of the reaction medium is measured at 30°C by using a NOVASINA hygrometric sensor calibrated with 3 salts having known $A_w$ (LiCl, $H_2O$, $A_w$=0.113 ; $Mg(NO_3)_2$, $6H_2O$, $A_w$=0.514 ; $KNO_3$, $A_w$=0.914)

- **Hydrolytic activity**

The hydrolytic activity of Candida cylindracea lipase is determined with an emulsion of 22.66 µM tributyrin in a $10^{-2}$ M pH 6.8 phosphate buffer. The emulsion is realized by using a Branson ultrasonic apparatus. Free fatty acids are determined by 0.1 M NaOH neutralization. The specific hydrolytic activity of Candida cylindracea lipase is 15 units (µmoles of product/min x mg of enzyme).

- **Interesterification and synthesis activities**

The reactions are followed by gas chromatography or thin layer chromatography

- Phase gas chromatography :

The separation is made according to the molecular weight and polarity of the lipids .
A Girdel 3000 chromatograph equiped with a 10 meters capillary OV1 column is used with temperature programme from 60°C to 340°C at 10°C/min. A 1 µl aliquot reaction medium is introduced in a tube containing 1 ml of $CH_2Cl_2$ and 0.1 ml of trilaurin in isooctane (0.34 mg/ml) as internal standard.
The gas flows are as follows : 20 ml/min. for hydrogen, 300 ml/min for air and 10 ml/min for vector gas. The leakage of nitrogen or helium vector gas is 30 ml/min and its pressure 0.4 bar . The injector and detector (FID) temperature is 375°C.

-Thin layer chromatography

Reaction products obtained during glycerol ester synthesis are separated by thin layer chromatography using silica gel plates 60 $F_{254}$ (Merck). In this experiment, the samples are developped by using hexane/diethyl ether/acetic acid, 80/20/1, (V/V/V). The spots of products are visualized by spraying dichloro-2',7'-flurescein and compared with standards.

## RESULTS AND DISCUSSION

- **Interesterification :**

Interesterifications are tried with triglycerides having varying fatty acid chain lengths (from $C_4$ to $C_9$) .
Microemulsions are composed of 33 % triolein, 1 % water containing 60 units/ml of Candida cylindracea lipase 30 % 2-methyl-2-hexanol and 0.1 % Brij 35. 37 % tributyrin ($C_4$), 37 % tricaproin ($C_6$), 37 % triheptanoin ($C_7$) or 37 % trinonanoin ($C_9$) are added to the microemulsions .

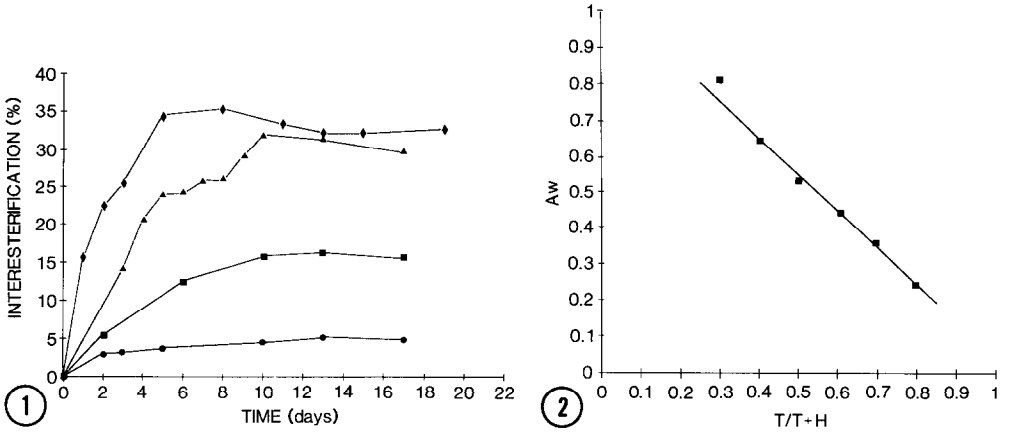


FIGURE 1 . Interesterification yield for triolein-tributyrin (-●-), triolein-tricaproin (-■-), triolein-triheptanoin (-▲-) and triolein-trinonanoin (-◆-) as a function of time for Candida cylindracea lipase in microemulsions made of 33% triolein, 36 % tributyrin, tricaproin, triheptanoin or trinonanoin, 30 % 2-methyl-2-hexanol, 1% water containing 60 units/ml of enzyme and 0.1 % Brij 35.

FIGURE 2 . Water activity ($A_w$) as a function of T/T+H in microemulsions made of triolein, triheptanoin, Brij 35, 2-methyl-2-butanol (Brij 35/2-methyl-2-butanol = 5 $10^{-3}$), 1.5% water and 60 units/ml of lipase.

The results obtained as a function of time are presented on figure 1. In all cases the interesterification happens.

Interesterification yields are defined as :

$$\% \text{ Interesterification} = \frac{\text{Interesterified triglycerides + mixed diglycerides}}{\text{Initial triglycerides}} \times 100$$

40 % interesterification are obtained for triolein-triheptanoin and triolein-Trinonanoin systems but only 17% and 6% interesterification are seen with triolein-tricaproin and triolein-tributyrin respectively. It seems that this non specific lipase in particular conditions such as reverse microemulsions is able to acquire a kind of specificity and cannot accept short chain triglycerides as the substrate for interesterification .

In these systems, one of the fundamental parameters influencing the reaction is the water activity. In numerous papers, authors speak about water quantities and not about water activities. In microemulsions it is possible with always the same water quantity to obtain variations of water activity by changing the mass fraction of T (T/H+T) with T as surfactant/cosurfactant and H as lipidic phase. The interesterification reactions between triolein and triheptanoin are tested with a 1.5 % water quantity but with water activities ranging from 0.24 to 0.8 by varying the ratio between T (Brij35/2-methyl-2-butanol = 1/199 (weight/weight)) and T + H. As Brij 35 and 2-methyl-2-butanol are all the two hydrophiles, when T increases the water is less available.

By varying T/T+H from 0.3 to 0.8 the water activity varies from 0.24 to 0.8 and $A_w$ is a linear function with $A_w = -106.6\ T/T+H + 109.1$ as represented on figure 2.

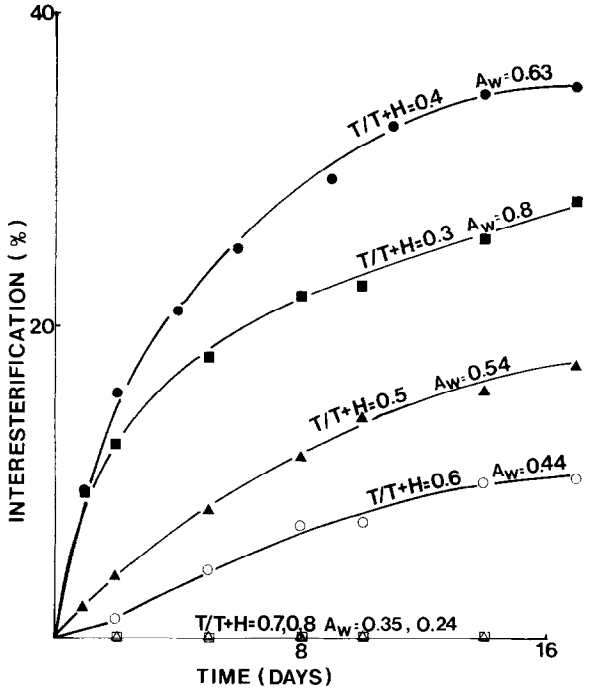


FIGURE 3 . Interesterification yield for triolein-triheptanoin system as a function of time tested with 1.5 % water quantity but with water activities varying from 0.25 to 0.8 by varying the ratio between T (surfactant/cosurfactant = 5 $10^{-3}$) and T+H. Microemulsions are made of lipidic phase (triolein and triheptanoin), Brij 35/ 2-methyl-2-butanol = 5 $10^{-3}$ and 1.5 % water containing 60 units/ml of Candida cylindracea lipase.

The interesterification yield as a function of time is presented on the figure 3. The best interesterification is obtained for T/T+H of 0.4 and a water activity of 0.63. For a smaller mass fraction of T and a higher water activity, the hydrolytic reaction is favored. For mass fraction equal or higher than 0.7 and water activities smaller than 0.35, interesterification cannot occur. In these cases, if by adding water, the water activity becomes 0.63, hydrolysis immediately occurs but it is impossible to obtain interesterification.

Another important parameter in reverse microemulsions is the ratio between surfactant and cosurfactant. This factor is studied for the triolein-triheptanoin interesterification. The results are presented on the figure 4. In these experiments, water quantity is 1.5 %, triolein and triheptanoin are 40 and 20 % respectively, water activity is 0.63 in all the cases and the ratio Brij 35/2-methyl-2-butanol is from 0 to 5 $10^{-2}$ (corresponding to Brij 35 amounts from 0 to 2 %). For comparison, experiments are tested with emulsions made of 1 % water and 99 % lipidic phase with a water activity of 0.63. The results in emulsion present an important variability due to the instability of the emulsion.

The lower the Brij 35 amount the faster the interesterification reachs its maximum but after the maximum the interesterified triglycerides decrease.

When the Brij 35 amount increases, the maximum interesterification is obtained latter but is higher. In media without surfactant or with a low amount of surfactant, a small variation of the water percentage

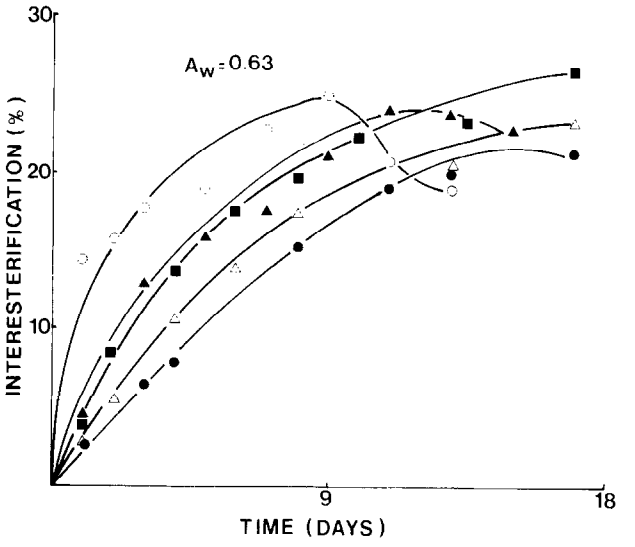


FIGURE 4 . Triolein-triheptanoin interesterification yield as a function of time in emulsion made of 1 % water and 99 % lipidic phase (-○-), in microemulsions made of 20 % triheptanoin, 40 % triolein, 1.5 % water and a ratio Brij 35/2-methyl-2-butanol of 0 (0 % Brij 35) (-▲-), 5 $10^{-3}$ (0.2 % brij 35) (-●-), 2 $10^{-2}$ (0.8 % Brij 35) (-■-) and 5 $10^{-2}$ (2 % Brij 35) (-△-).
In all the cases $A_W$ = 0.63.

induced a great variation of the water activity. This phenomenon is not observed in presence of high Brij 35 amounts.

For example, for a water amount of 1.5 %, the water activity is 0.633, 0.632 and 0.633 for Brij 35 amounts of 0.2, 0.8 and 2.0 % respectively, when, for a water amount of 2 %, the water activity is 0.753, 0.735 and 0.706 for 0.2, 0.8 and 2.0 % Brij 35 respectively. Furthermore, if interesterification after 12 days with 1.5 % water is given as 100% for all the Brij 35 amounts, after 12 days with 2.0 % water amounts it becomes 73.6 %, 94 % and 98,8 % for 0.2, 0.8 and 2.0 % Brij 35 respectively.
A possible hypothesis is that Brij 35 in high amount is able to combine with excess water so variation in water quantity does not induce important variation in water activity or in interesterification yield. So, it is possible that for interesterification, in the range of Brij 35 amounts studied, the surfactant insures for the lipase a good catalytic efficiency in the time by a regulation of the available water.

-Synthesis :

The lipase is used for the synthesis of heptyl oleate . As described before heptanol is the substrate of the enzyme and the cosurfactant of the microemulsion . Ester synthesis as a function of the heptanol/oleic acid molar ratio is studied for microemulsions containing 1 % water with 60 units/ml of lipase, 0.1 % Brij 35, and heptanol and oleic acid in a molar ratio varying from 1 to 5 . The results are presented on figure 5 .
Synthesis yields are defined as :

$$\% \text{ synthesis} = \frac{\text{ester}}{\text{ester} + \text{acid}} \times 100$$

The reaction is considerably more rapid and the ester synthesis much more important when the

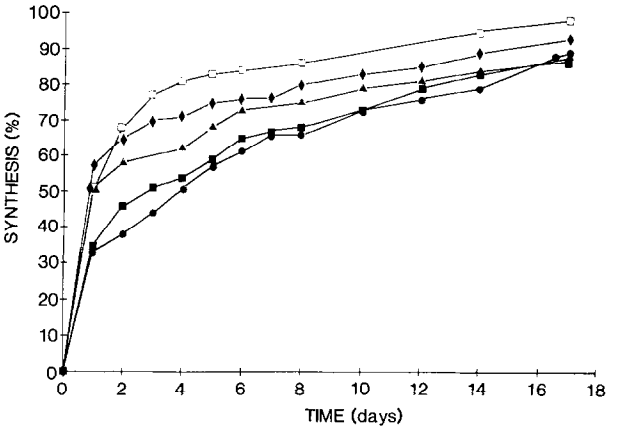


FIGURE 5 . Heptyl-oleate synthesis as a function of time with Candida cylindracea lipase for a molar ratio heptanol/oleic acid of 1 (-●-), 2 (-■-), 3 (-▲-), 4 (-◆-), 5 (-□-) in microemulsions made of heptanol and oleic acid in a molar ratio from 1 to 5, 1 % water containing 60 units/ml of enzyme and 0.1% Brij 35.

heptanol/oleic acid ratio is high . For a molar ratio of 5, the synthesis yield is 80 % after 4 days and around 100 % after 17 days of the reaction . For example, with a primary alcohol which is as reactive as possible, it is impossible to obtain more than 67 % ester synthesis by chemical processing .

The preparation of microemulsions containing glycerol is quite difficult because of the viscosity of glycerol ; so, as an example, the synthesis of esters of glycerol and oleic acid is studied in a monophasic reaction medium made of 2-methyl-2-propanol (63 %), oleic acid (27 %), glycerol (9 %), water (1 %) and 90 units/ml of lipase. After 72 hours of reaction at 25 °C, reaction medium is analyzed by thin layer chromatography. The results are presented on the figure 6. Oleic acid, mono and

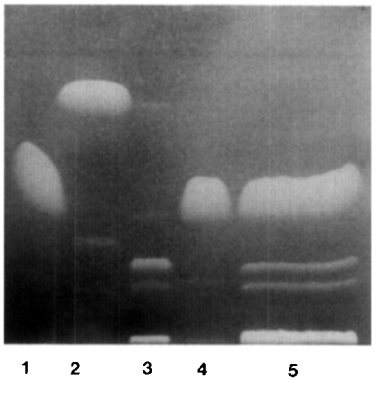


FIGURE 6 . Thin layer chromatography of the products of the glycerol ester synthesis after 72 hours of reaction.
1 : oleic acid
2 : triolein
3 : mono and diglycerides (standards)
4 : blank test : reaction medium without enzyme
5 : reaction medium with enzyme showing production of monoolein, 1-2 and 1-3 diolein

diglycerides and triolein are used as standards. As a blank test, the same experiment without enzyme is also tested. The analysis of the results shows a production of monoolein, 1-2 diolein and 1-3 diolein. These results showing the feasibility of the synthesis of glycerol esters in monophasic sytems are preliminary and are now in optimization.

The obtention of enzymatic reactions and especially group transfer or synthesis reactions with microemulsion systems constitutes a new approach .
In the case of the lipases, lipids and alcohols are the enzymatic substrates and microemulsion components .

We have shown that it is possible to make microemulsions using liquid fats, a surfactant, an alcoholic cosurfactant and a very few water quantity .
The lipase addition into the aqueous phase does not break the microemulsion . Under these conditions, lipase has an hydrolytic activity and for adapted water activities, interesterification and synthesis capacities. The influence of the surfactant is studied and seems to have a stabilizing effect on the lipase. The use of Candida cylindracea lipase in reverse microemulsions reveals that this non specific enzyme cannot accept all the triglycerides as substrates when acting in particular conditions .

The obtained results show that lipases do not need a "physical" interface to be functional . Microemulsions, by their structure and their stability open important application fields for enzyme catalysis . The non conventional lipase reactions (interesterification and ester synthesis) observed in microemulsions are an example .

## REFERENCES


1. Hoq M.M., Yamane T., Shimizu S., Funada T. and S. Ishida (1985) J. Am. Oil Chem. Soc., 62, 6, 1016-1021
2. Macrae A.R. (1983) J. Am. Oil Chem. Soc., 60, 2, 291-294
3. Marlot C., Langrand G., Triantaphylides C. and J. Baratti (1985) Biotechnol. Lett., 7, 9, 647-650
4. Morita S., Narita H., Matoba T. and M. Kito (1984) J. Am. Oil Chem. Soc., 61, 10, 1571-1574
5. Sreenivasan B. (1978) J. Am. Oil Chem. Soc., 55, 796-805
6. Takahashi K., Kodera Y., Yoshimoto T., Ajima A., Matsushima A. and Y Inada (1985) Biochem. Biophys. Res. Commun., 131, 2, 532-53
7. Tanaka T., Ono E., Ishihara M., Yamanaka S. and K. Takinami (1981) Agric. Biol. Chem., 45, 10, 2387-2389
8. Yokozeki K., Yamanaka S., Takinami K., Hirose Y., Tanaka A., Sonomoto K. and S. Fukui (1982) Eur. J. Appl. Microbiol. Biotechnol., 14, 1-5
9. Zaks A. and A.M. Klibanov (1985) Proc. Natl. Acad. Sci. USA, 82, 3192-3196
10. Clausse M., Zradba A. and L. Nicolas-Morgantini (1985) Coll. and Polym. Sci., 263, 9, 767-770
11. Luisi P.L. (1985) Angew. Chem. Int. Ed. Engl., 24, 439-450
12. Martinek K., Levashov A.V., Klyachko N.L., Pantin V.I. and I.V. Berezin (1981) Biochim. Biophys. Acta, 657, 277-294
13. Mittal K.L. and E.J. Fendler (1982) Solution behavior of surfactants, vol. 1, Plenum Press, New York,
14. Langevin D., Meunier J. et A.M. Cazabat (1985) La Recherche, 167, 16, 720-728
15. Fletcher P.D.I., Rees G.D., Robinson B.H. and R.B. Freedman (1985) Biochim. Biophys. Acta , 832, 204-214
16. Entressangles B. and P. Desnuelle (1968) Biochim. Biophys. Acta, 159, 285-295