INVESTIGACION

# Specificity of *Mucor miehei* lipase on methyl ester substrates

By **G. Aggelis** (1), **M. Komaitis** * (2), **M. Pina** (3) and **J. Graille** (3)
1. Department of Agricultural Biology and Biotechnology.
2. Department of Food Sciences and Technology. Agricultural University of Athens. Iera Odos 75, 118.55 Athens-Greece.
3. Division Chimie des Corps Gras. CIRAD IRHO. BP 5035, 340.32 Montpellier - France.
* To whom all correspondence should be addressed.

**RESUMEN**

**Especificidad de lipasa *Mucor miehei* en sustrato de ésteres metílicos.**

Los ésteres metílicos de ácidos grasos constituyen un buen sustrato para la caracterización de tipos de especificidad de lipasa. En el presente trabajo, se estudió la acción hidrolítica de lipasa de *Mucor miehei.* Se demostró que esta lipasa cataliza preferencialmente la hidrólisis de ésteres metílicos de ácidos grasos con número pequeño de dobles enlaces. Se encontró también que esta lipasa muestra una especificidad en la hidrólisis de ésteres metílicos de ácidos grasos con cadena alifática corta.

*PALABRAS-CLAVE: Especificidad - Ester metílico - Lipasa (Mucor miehei) - Sustrato.*

**SUMMARY**

**Specificity of *Mucor miehei* lipase on methyl ester substrates.**

Fatty acid methyl esters constitute a good substrate for the characterization of lipase typospecificity. In the present work, the hydrolytic action of lipase from *Mucor miehei* was studied. It was demonstrated that this lipase preferentially catalyses the hydrolysis of fatty acid methyl esters with small number of double bonds. It was also found that this lipase shows a specificity in the hydrolysis of fatty acid methyl esters with short aliphatic chain.

*KEY-WORDS: Lipase (Mucor miehei) - Methyl ester - Specificity - Substrate.*

## 1. INTRODUCTION

Lipase typospecificity as well as the stereospecificity for the sn-1,3 or sn-2 positions is of great theoretical and practical interest (Posorske, 1984; Galzy et al., 1986; Schuch and Mukherjee, 1987; Chang et al., 1990; Ghoshray and Bhattacharya, 1992). They affect the composition of the lipid hydrolysis products (in hydrophilic reaction media) or the composition of new triglycerides and other molecules (in hydrophobic reaction media) (Macrae, 1983; Rattray, 1984; Muderhwa et al., 1988 a;b;c; Hills et al., 1990; Yadwad et al., 1991; Tanaka et al., 1992).

Most extracellular microbial lipases exhibit little fatty acid specificity when incubated with natural fats and oils (Macrae, 1983). The *Geotrichum candidum* lipase was found to possess a fatty acid specificity in the hydrolysis of esters of a particular type of long-chain fatty acid (Jensen, 1974). The lipase characterization using a triglyceride substrate is very difficult because the composition of the hydrolysis product is influenced not only by the fatty acid specificity but also by the stereospecificity which is, usually, the dominant factor (Muderhwa et al., 1987; Sonnet and Antonian, 1988).

In this study, mixtures of fatty acid methyl esters were used as substrate for the characterization of *Mucor miehei* lipase. By using this mixtures first we have an ester bond similar to that found in the sn-1,3-positions of the triglyceride molecules and second there is no possibility for stereospecific action of lipase because the fatty acid are not esterified to a glycerol molecule. Thus, the difference in the composition of hydrolysis product would be the result of the specific action of the enzyme on the aliphatic chain-length and the number of double bonds.

## 2. MATERIAL AND METHODS

Lipase of *Mucor miehei* immobilized on anion exchange resin was a product of Novo Industrie GmbH Danemark. Methyl esters of polyunsaturated fatty acids were prepared from Borago and cod-liver oil. Borago oil (*Borago officinalis L.*) with high content of unsaturated fatty acids was prepared from natural Borago oil in the laboratories of DCCG, IRHO/CIRAD (Montpellier-France). The methyl esters were prepared according to the method of AFNOR (AFNOR 1984). The hydrolysis of oils was carried out in a closed conical flask containing 50 ml R113 ($CCl_2$ $FCClF_2$) : $H_2O$ (95:5) and at 37±1°C. The ratio enzyme-substrate was 1g of immobilized enzyme - 0.5 g methyl esters. The products of hydrolysis and the non- hydrolysed esters were analysed as described below. The two fractions were separated by TLC on silicic acid plates (Muderhwa, 1989). The two fractions (methyl esters and fatty acids) were scraped off from the TLC plates and were analysed by GLC (Aggelis et al.,

1987). The gas chromatographic separation was performed on a Carlo Erba 4160 apparatus equipped with a flame ionization detector and a 30m x 3mm DB Wax 30W (J et W) column. The operation parameters were as follows: carrier gas helium 3 ml/min, column temperature 190 °C, detector temperature 275 °C, injector temperature 250 °C. The identification was carried out by comparison with standard solutions of fatty acid methyl esters.

## 3. RESULTS AND DISCUSSION

The enzyme specificity was quantitatively characterized by the hydrolysis coefficient K(FA).

$$K(FA) = \frac{[FA]p}{[FE]s}$$

where [FA]p the concentration % of fatty acid in the hydrolysis product and [FE]s the concentration % of the fatty acid methyl ester substrate. The coefficient of hydrolysis is a measure of the lipase specificity. When the fatty acid esters are hydrolysed with a rate less than the mean rate of hydrolysis, K[FA] < 1. When an ester is preferentially hydrolysed, K[FA] > 1.

### 3.1. Substrate from methyl esters of Borago oil

The fatty acid esters produced from Borago oil is a mixture of oleic, linoleic and gamma linolenic esters. Consequently, it is suitable for the study of lipase specificity on the number and position of double bonds because the aliphatic chains have the same number of carbon atoms.

Table I
**Composition of substrate and hydrolysis products during the hydrolytic action of the lipase from *Mucor miehie* on a borago oil substrate**

| t(hr) | methyl esters | | | fatty acids | | |
|---|---|---|---|---|---|---|
| | C18:1 | C18:2 | C18:3 | C18:1 | C18:2 | C18:3 |
| 0h00 | 13.0 | 45.4 | 39.1 | 0.0 | 0.0 | 0.0 |
| 0h45 | 11.3 | 39.5 | 46.5 | 25.4 | 61.7 | 6.0 |
| 1h45 | 10.3 | 36.6 | 50.0 | 25.3 | 60.0 | 8.5 |
| 2h15 | 10.1 | 35.9 | 51.9 | 24.0 | 61.4 | 9.4 |
| 3h45 | 10.2 | 35.8 | 51.2 | 24.2 | 56.7 | 10.9 |
| 4h30 | 11.6 | 34.3 | 49.6 | 22.2 | 60.2 | 10.9 |
| 5h00 | 11.0 | 35.2 | 50.1 | 21.5 | 60.5 | 11.2 |

The changes in composition of the substrate and the hydrolysis products are shown in Table I. As can be seen the hydrolysis product was enriched in oleic and linoleic acid but not in gamma linolenic acid. During the hydrolysis process the concentration of oleic acid ester varies between 10.1-13.0%, while in the product the concentration of oleic acid is more than double. The same was observed for the concentration of linoleic acid ester. Contrary to these findings the concentration of gamma linolenic ester in the substrate varied between 39.1-51.9% while in the hydrolysis product it varied between 6.0-11.2%. These results suggest that the lipase shows specificity on the various aliphatic chains.

The changes in the hydrolysis coefficients for the three different fatty acid methyl esters and at three different time periods are shown in Figure 1. It was found that the K[FA] values for oleic and linoleic acid were higher than those for gamma linolenic acid (K[18:1] > K[18:2] > 1 > K[18:3]). A decrease in the value of coefficient K[18:1] and an increase in the values of coefficients K[18:2] and K[18:3] was also observed. A possible explanation of this phenomenon is that aloss or some kind of modification of the lipase specificity might have occured.

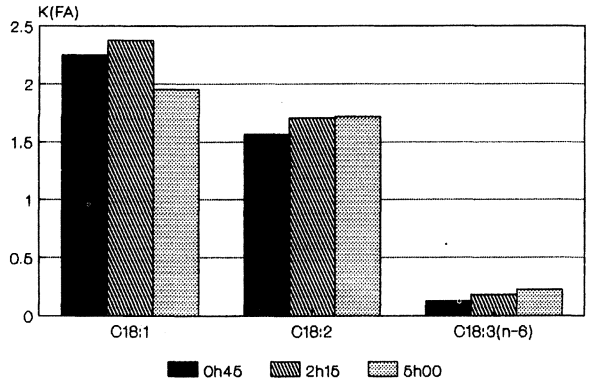


Fig. 1
Changes in the hydrolysis coefficients of fatty acid methyl esters produced from Borago oil.

The observed differences in the hydrolysis coefficients indicate that the *Mucor miehei* lipase discriminates against fatty acids/acyl moieties having one, two of three double bonds. These findings are in agreement with the findings of the other workers (Hills et al., 1990). They reported that lipase preparations from seedling of rape and *Mucor miehei* in non-aqueous media discriminate against 18:1, 18:2 and 18:3 fatty acids. Utilising this property they increased the content of gamma linolenic acid of evening primrose oil to nine -fold. Similarly, docosahexaenoic acid of cod liver oil has been enriched to five - fold by selective esterification of fatty acids (other than docosahexaenoic acid) with butanol.

A similar approach of selective esterification of fatty acids with methanol was also used to concentrate docosahexaenoic acid from a marine oil (Langholz et al., 1989).

### 3.2. Substrate from methyl esters of cod-liver oil

This substrate is more complicated and includes fatty esters of palmitic, palmitoleic, oleic, 9-eicosenoic, eicosapentaenoic and docosahexaenoic acids. Table II shows the

Table II
**Composition of substrate and hydrolysis products during the hydrolytic action of the lipase from *Mucor miehei* on a cod-liver oil substrate**

| t(hr) | methyl esters | | | | | | fatty acids | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C16:0 | C16:1 | C18:1 | C20:1 | C20:5 | C22:6 | C16:0 | C16:1 | C18:1 | C20:1 | C20:5 | C22:6 |
| 0h00 | 11.4 | 10.2 | 23.0 | 11.6 | 8.9 | 9.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 1h00 | 11.6 | 10.6 | 23.2 | 10.4 | 10.1 | 11.5 | 18.5 | 29.8 | 27.4 | 3.4 | tr | tr |
| 3h00 | 10.3 | 9.4 | 21.1 | 9.0 | 10.4 | 14.2 | 14.3 | 18.0 | 25.2 | 8.0 | 4.2 | 4.2 |
| 5h30 | 11.2 | 10.0 | 22.3 | 11.2 | 8.5 | 13.2 | 13.1 | 18.4 | 23.2 | 8.8 | 8.2 | 4.6 |

composition of substrate and that of hydrolysis products during the reaction. It was found that the hydrolysis products were enriched in palmitic, palmitoleic and oleic but not in 9-eicosaenoic, eicosapentaenoic and docosahexaenoic acids. These results mean that *Mucor miehei* lipase has acyl chain specificity. The latter was evaluated in term of hydrolysis coefficients. Figure 2 shows the changes of K(FA) for the various fatty acids. The order of hydrolysis is C16 > C18 > C20 > C22. The values of K[16:0], K[16:1], K[18:1] were higher than one while the values of K[20:1], K[20:5], K[22:6] never exceeded one. It was also found that K[20:1] > K[20:5] and K[16:1] > K[16:0]. During the reaction a decrease in the values of K[16:0], K[16:1] and K[18:1] and an increase in the values of K[20:1], K[20:5] and K[22:6] were observed. This fact strengthens the supposition that some changes of the lipase specificity might have taken place.

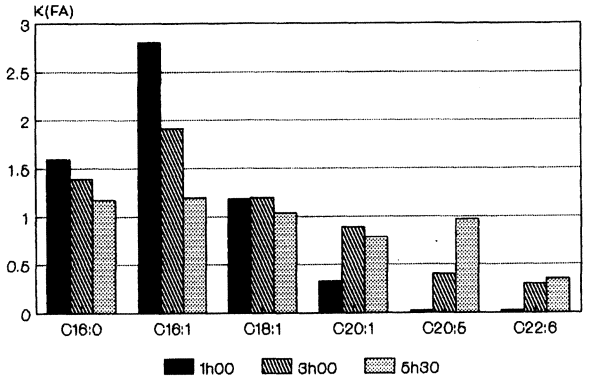


Fig. 2
Changes in the hydrolysis coefficients of fatty acid methyl esters produced from cod-liver oil.

## 4. CONCLUSION

The aim of this work was to investigate the hydrolytic action of the lipase of *Mucor miehei* on various methyl ester substrates. The results of the present work demonstrate that this lipase preferentially hydrolyses the fatty acid esters with small number of double bonds and short aliphatic chain. A possible explanation of this phenomenon is that long aliphatic and polyunsaturated chains show a stereochemical hindrance and consequently fatty acid esters with small number of double bonds and/or short aliphatic chain are hydrolysed more quickly.

The double bond and chain specificity of lipase is a property which has been very little studied compared with that of stereospecificity. This might be particularly interesting in the synthesis of new fats of high nutritional value.

## REFERENCES

1. Aggelis, G., Pina, M., Ratomahenina, R., Arnaund, A., Graille, J., Galzy, P., Martín-Privat, P. and Perraud, J.P. (1987).–«Production d'huiles riches en acide gamma linolenique par diverses souches de Phycomycetes».–Oleagineux **42**, 379-386.
2. AFNOR (1984).–«Recueil de Normes Francaises des corps gras, graines oleagineux et produites derives».–3ª Ed. NF T 60-233, P. 95.
3. Chang, M-K., Abraham, G. and John V.T. (1990).–«Production of cocoa butter-like fat from interesterification of vegetable oils».–J. Am. Oil Chemists'Soc. **67,** 832-834.
4. Galzy, P., Ratomahenina, R., Graille, J. and Pina, M. (1986).–«Microbiologie appliquee a l'industrie des lipides».–Rev. Fr. Corps Gras **33,** 307-309.
5. Jensen, R.G. (1974).–«Microbial lipolytic enzymes».–Lipids **9**, 149-152.
6. Ghoshray, S. and Bhattacharya, D.K. (1992).–«Enzymatic preparation of ricinoleic acid esters of long-chain monohydric alcohols and properties of the esters».–J. Am. Oil Chemists'Soc. **69,** 85-88.
7. Hills, M., Klewitt, I. and Mukherjee, K. (1990).–«Enrichment of γ-linolenic acid and docosahexaenoic acid by selective esterification catalyzed by lipases».–J. Am. Oil Chemists'Soc. **67,** 561-564.
8. Langholz, P., Andersen, P., Forskov, T. and Schmidtsdorff, W. (1989).–«Application of a specificity of *Mucor miehei* lipase to concentrate docosahexaenoic acid (DHA)».–J. Am. Oil Chemists'Soc. **66,** 1120-1123.
9. Macrae, A.R. (1983).–«Lipase-catalyzed interesterification of oils and fats».–J. Am. Oil Chemists'Soc. **60,** 291-294.
10. Muderhwa, J.M., Pina, M., and Graille, J. (1987).–«Probleme du dosage des acides gras en positions internes et externes des triglycerides des huiles lauriques; approche de la specificite des lipases vis-a-vis de la nature des acides gras.»–Rev. Fr. Corps Gras **34,** 533-542.
11. Muderhwa, J., Pina, M. and Graille, J. (1988a).–«Aptitude a la transesterification de quelques lipases regioselectives 1-3. I. Determination de l'activite specifique de transesterification».–Oleagineux **43,** 385-392.
12. Muderhwa, J., Pina, M. and Graille, J. (1988b).–«Aptitude a la transesterification de quelques lipases regio-selectives 1-3. II. Taux de conversion et glycerides partiels en foction de l'activite de l'eau des biocatalyseurs».–Oleagineux **43,** 427-433.

13. Muderhwa, J., Pina, M. and Graille, J. (1988c).–«Aptitude a la transesterification de quelques lipases regio-selectives 1-3. III. Stabilite de la regio-selectivite 1,3 des biocatalyseurs».–Oleagineux **43**, 465-470.
14. Muderwa, J., Pina, M. and Graille, J. (1989).–«Interesterification catalysee par les lipases regio-selectives 1,3 en milieu fondu: applications a l'huile de palme et a sa fraction concrete».–Rev. Fr. Corps Gras **36,** 11-19.
15. Posorske, L.H. (1984).–«Industrial –scale application of enzymes to the fats and oil industry».–J. Am. Oil Chemists'Soc. **61,** 1758-1760.
16. Rattray, J. (1984).–«Biotechnology and the fats and oils industry-an overview».–J. Am. Oil Chemists'Soc. **61,** 1701-1711.
17. Schuch, R. and Mukherjee, D. (1987).–«Interesterification of lipids using an immobilized sn-1,3 specific triacylglycerol lipase».–J. Agric. Food Chem. **35,** 1005-1008.
18. Sonnet, P. and Antonian, E. (1988).–«Synthesis and evaluation of pseudolipids to characterize lipase selectivities».–J. Agric. Food Chem. **36,** 856-862.
19. Tanaka, Y., Hirano, J. and Funada, T. (1992).–«Concentration of docosahexaenoic acid in glyceride by hydrolysis of fish oil with *Candida cylindracea* lipase».–J. Am. Oil Chemists'Soc. **69,** 1210-1214.
20. Yadwad, V.A., Ward, O.P. and Noronha, L.C. (1991).–«Application of lipase to concentrate the Docosahexaenoic acid (DHA) fraction of fish oil».–Biotechnol. Bioengineering **38,** 956-959.

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