0273-2289/91/2702—0185$2.00

Use of Cyclodextrins in Enzymology to Enhance the Solubility of Hydrophobic Compounds in Water

CRISTINA OTERO,* CARMEN CRUZADO, AND ANTONIO BALLESTEROS

Instituto de Catálisis, C.S.I.C., 28006 Madrid, Spain

Received and Accepted April 3, 1990

ABSTRACT

In enzyme catalysis, hydrophobic compounds are usually made soluble in water by the addition of low amounts of a water-miscible organic solvent. In the present study, taking advantage of the solubilizing properties of inclusion compounds (cyclodextrins), a new methodology is proposed. The hydrolysis of *p*-nitrophenyl esters catalyzed by several hydrolases (lipase from *C. cylindracea*, porcine pancreatic lipase, *R. arrhizus* lipase and cholesterol esterase) in the presence of cyclodextrins (CD), has been investigated. This procedure presents many advantages:

1. The enzyme parameters are not affected substantially (the catalytic constant may even increase);
2. The reproducibility is higher than with previous methods;
3. CDs enhance the solubility of substrates; and
4. CDs do not destabilize enzyme structure. In some cases a thermal stabilization is observed.

Index Entries: Lipases; hydrolases; cyclodextrins; solubilizing agents, cyclodextrins as; insoluble substrates, cyclodextrins as solubilizers of.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Enzymes are traditionally used in aqueous media, where most of their substrates and products are soluble. In the case of hydrophobic compounds, they are made soluble in water by adding low amounts of a water-miscible organic solvent to the reaction mixture. Acetonitrile at 1–4% (v/v) has been usually employed (*1*). The value of the enzyme kinetic constants were thus obtained in the presence of the solvent, that may alter the intrinsic kinetic constants of the enzyme in water. Indeed, it has been shown that in organic media the specificity of enzymes is reversed (*2*). On the other hand, another approach to facilitate the solubility of organic molecules in water could be, instead of changing the properties of the solvent (water) by the addition of another solvent, the use of inclusion compounds having hydrophobic cavities able to include the insoluble substrates and products.

Cyclodextrins (cycloamyloses) are cyclic oligosaccharides enzymatically produced from starch. The main cyclodextrins (α-, β- and γ-CD) are composed of 6, 7 or 8 α (1→4)-linked D-glucose units, respectively. Each of these cyclic molecules is a torus (doughnut-shaped), with a hydrophilic shell and a hydrophobic cavity. The most important consequence of this structure is the ability of the CDs to form inclusion complexes, in which a wide variety of suitable sized "guest" molecules, (such as fatty acids, vitamins, flavor compounds, etc), can be enclosed within the cavity (*3,4*). CDs and their derivatives enhance the solubility of complexed substrates in aqueous media. As inclusion represents a molecular encapsulation, this phenomenon contributes to the stabilization of sensitive substances improving their handling and dosage, and to the sequestration of compounds toxic to cells. Encapsulation by CDs makes possible a very broad range of industrial applications, mainly in the food, cosmetic, pesticide, pharmaceutical and sewage purification areas (*5–7*).

The basic concept of using CDs in enzyme catalysis has been forwarded by Szejtli (*7,8*). The hydrolysis of olive oil or triolein by lipases was 2–3 times faster in the presence of dimethyl-β CD than in the presence of a detergent (*9*). In addition, CDs have been used recently in many processes involving cells: stimulation of the production of antibiotics (*7,10*), intensification of microbial steroid conversion (*7,11,12*), enhancement of vaccine production (*7*), and others (*13*).

Many hydrolytic enzymes have been studied using not their natural substrates but simple synthetic ones (i.e., *p*-nitrophenyl acetate) whose hydrolysis can be followed easily in the laboratory. In the case of lipases and esterases, *p*-nitrophenyl butyrate (PNPB) (and other *p*-nitrophenyl esters of longer hydrocarbon chain) is generally employed to determine, in these standard and reference conditions, the activity and behavior of the enzymes. The low solubility in water of PNPB (and higher esters) is

usually increased using surfactants, low concentrations of acetonitrile, ultrasonic disintegration, and so on, factors that may affect the intrinsic properties of the enzyme. In this paper, we propose a modification of the method to determine the enzymatic activity of enzymes with *p*-nitrophenyl esters in aqueous media, using the ability of CDs to form water soluble inclusion complexes.

MATERIALS

Candida cylindracea (now named *C. rugosa*) Type VII lipase and porcine pancreas Tipe II lipase (containing, respectively, 700 and 160 U/mg powder using olive oil as substrate) were purchased from Sigma; *Rhizopus arrhizus* lipase (14000 U/mg protein, using olive oil) and cholesterol esterase (48 U/mg powder) were from Boehringer Mannheim; and *p*-nitrophenyl butyrate (PNPB), *p*-nitrophenyl caprylate (PNPC), α-CD, β-CD, γ-CD, and maltoheptaose were also from Sigma.

METHODS

Preparation of Inclusion Complexes of PNPB

Thirty mg of β-CD (26 μmol) were dissolved in 20 mL of standard buffer (0.1*M* potassium phosphate buffer, pH=7.26, 0.1*M* NaCl). 3 μL of PNPB (3.6 μg, 1.7 10^{-2} μmol) were added and dissolved by stirring for 20 min. This solution was stored at 4°C. Unless otherwise specified, the molar ratio between β-CD and PNPB was kept constant (1.5:1) in all the experiments. Owing to precipitation of the inclusion complex, it was not possible to work with PNPB concentrations higher than 1 m*M*.

Preparation of PNPB Solution Using Acetonitrile

A typical PNPB solution (2.9 10^{-4} m*M*) in standard buffer, with 3% (v/v) of acetonitrile was prepared by submitting the mixture to disruption in a Branson sonifier for 2×5 min. This solution was kept at 4°C.

Preparation of Enzyme Solutions

Appropriate quantities of the enzymes were stirred in standard buffer during 30 min. at room temperature. The final concentration of the enzymes was the following: 2–50 mg/mL, for *Candida* lipase; 50 mg/mL, for PPL; and 2 mg/mL, for cholesterol esterase. (Commercial suspension of *Rhizopus* lipase was used as such in the reaction).

Hydrolysis of PNPB

The kinetics of hydrolysis of PNPB has been followed spectrophotometrically at 400 nm and 30°C in a Varian Cary spectrophotometer provided with magnetic stirring. Three mL of the PNPB solution in standard buffer were deposited in a cuvet in the spectrophotometer, left 5 min., and the reaction started with an appropriate amount of enzyme. The value found of the molar absorption coefficient (ϵ) at 400 nm of *p*-nitrophenolate in these conditions was 13400 $M^{-1}cm^{-1}$.

The activation energy, with and without CD, was determined in the same way but changing the temperature in the range between 20 and 50°C.

Thermal Stability

Samples of lipase (10 mg/mL) in standard buffer containing, respectively, maltoheptaose, α-CD, β-CD, γ-CD (1.32 m*M* for all), or acetonitrile (3% v/v), were incubated at 30 or 50°C. At different times, aliquots were drawn and the residual enzyme activity for the hydrolysis of PNPB measured as above (at 30°C, in standard buffer containing 3% (v/v) of acetonitrile). The stabilization factor was calculated as the ratio between the half-life of the enzyme in the presence and in the absence of CD.

Solubility of PNPB and PNPC

Substrate (PNPB and PNPC) solubility in standard buffer with or without 1.5 equivalents of β-CD have been determined spectrophotometrically at 400 nm and 30°C. In order to know which fraction of the substrate was already hydrolyzed in the Sigma reagents, the absorbance at pH=7.26 was measured (A_0) immediately after preparing the solutions. Then, total alkaline hydrolysis was effected by raising the pH to 11.5, and —after lowering the pH to 7.26— the absorbance was again determined (A). *p*-nitrophenyl esters solubility was calculated according to Eq. (1), where ϵ is the molar absorption coefficient of *p*-nitrophenolate ion at pH=7.26 and 30°C.

$$s = (A - A_0) / \epsilon \quad (1)$$

A_0 was always smaller than 5% of A.

RESULTS

We have studied the variation of the initial rate of PNPB hydrolysis, catalyzed by the nonspecific *Candida c.* lipase, in the presence of acetonitrile (3% v/v) or with β-CD. The results obtained at different substrate and enzyme concentrations are plotted in Fig. 1 and 2, respectively. This study has been extended to other hydrolases like porcine pancreatic lipase (PPL), *R. arrhizus* lipase (both of them 1,3-specific), and colesterol esterase (not shown).

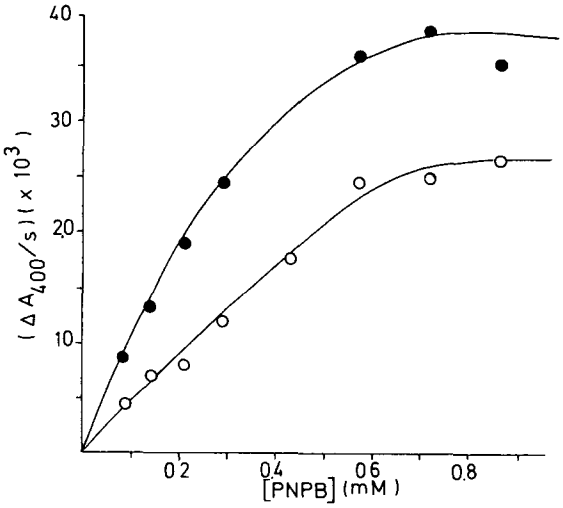

Fig. 1. Initial rate of hydrolysis by Candida lipase as a function of PNPB concentration, in the presence of: ○, CH_3CH; ●, β-CD. The enzyme concentration was 0.05 mg/mL.

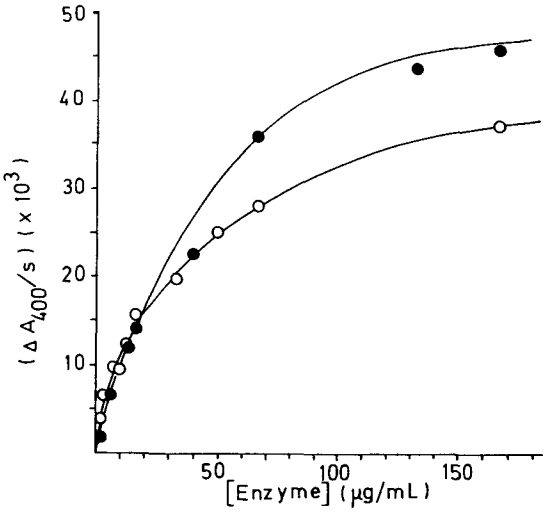

Fig. 2. Initial rate of PNPB hydrolysis by Candida lipase as a function of enzyme concentration, in the presence of: ○, CH_3CN; ●, β-CD. The substrate concentration was 0.57 m*M*.

As can be seen in Figs. 1 and 2 as well as in the experiments not shown, this kind of kinetics corresponds to a Michaelis-Menten mechanism. The values of the Michaelis-Menten constant (K_m) and the maximal reaction rate (V_{max}) were obtained from a double-reciprocal plot (see Table 1). From the initial slope of the curves, the value of the catalytic constant, k_{cat}, has been calculated. Taking into account the mol wt of the *C. cylindracea* lipase and the purity of the commercial preparation obtained from Sigma ((*14*), and M. L. Rúa and A. Ballesteros, unpublished experiments), it has been possible to express k_{cat} in reciprocal time. The values obtained are 501

Table 1
K_m and V_{max} Values for the Hydrolysis of PNPB in the Presence of CH_3CN or β-CD, Catalyzed by Different Hydrolases

	Candida c.[a]		PPL[b]	Rhizopus[c]	esterase[d]
	CH_3CN	β-CD	β-CD	β-CD	β-CD
K_m (mM)	0.35	0.27	0.25	16.0	23.0
V_{max} ($M\ s^{-1}$) x 10^6	2.2	3.2	0.74	33.5	66.0

The amount of the enzyme used (per mL of reaction mixture) were: [a]0.05 mg; [b]1 mg; [c]1 μL; [d]2.07 mg.

Table 2
Initial Rate of PNPB (0.096 m*M*) Hydrolysis by Candida Lipase (0.05 mg/mL) in the Presence of β-CD (0.33 mg/mL) vs Product Concentration

Product (μM)	0	29	58	87	116
A_{400}/s (x 10^3)[a]	7.77	6.67	5.92	5.83	5.67
% Inhibition	0	14	24	25	27

[a]Error: ±3%.

and 395 s^{-1}, for the lipase in the presence of either acetonitrile or β-CD, respectively.

Therefore, the values of the specificity constant, k_{cat}/K_m, for the hydrolysis of PNPB by *C. cylindracea* lipase in the presence of either CH_3CN or β-CD are, respectively, 1.43 10^6 and 1.46 10^6 $s^{-1}M^{-1}$.

The activation energy (E_A) for the PNPB hydrolysis reaction catalyzed by Candida lipase in the presence of either acetonitrile (3%) or β-CD has been determined over the temperature range between 20 and 50°C. An Arrhenius plot of these data (not shown) yielded E_A values of 4.5 and 5.2 kcal/mol, respectively.

The values obtained for the solubility of PNPB and PNPC in water in the presence of 1.5 parts of β-CD were 1050 and 24.9 μ*M*, respectively, which are 15.4 and 11 times higher than in the presence of 3% acetonitrile (68.2 and 2.26 μM, respectively).

Next, the possible inhibition of the substrate inclusion complex by the reaction product (*p*-nitrophenolate) was investigated. For it, the initial rate of PNPB hydrolysis was measured at fixed concentrations of PNPB, lipase and β-CD, and variable product concentrations (different amounts of a concentrated product solution were added to a cuvet containing fixed volumes of substrate and enzyme solutions. To reach a final volume of 3 mL for the mixture, the necessary amount of the standard buffer solution was added). Table 2 summarizes the results obtained, which show that the product inclusion complex produces an inhibition effect that increases

Table 3
Initial Rate of PNPB (0.29 mM) Hydrolysis
by Candida Lipase (0.05 mg/mL) vs β-CD Concentration

β-CD (mM)	0.44	0.80	1.14	1.30	1.76	2.60	4.40
A_{400}/s (x 10^3)[a]	18.1	15.7	15.0	14.0	13.5	14.2	14.3
% Inhibition	0	13	17	22	25	21	21

[a]Error: ±3%.

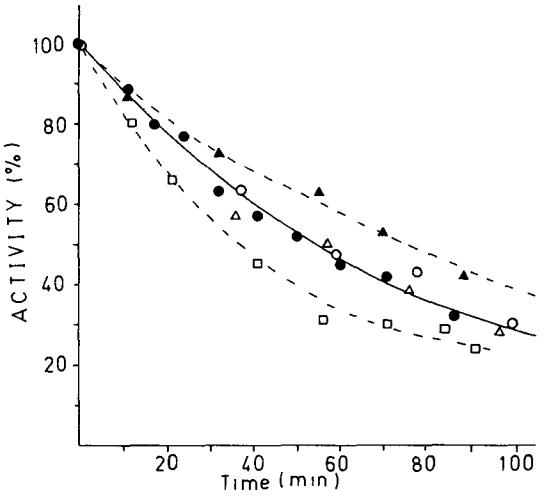

Fig. 3. Storage stability of Candida lipase in standard buffer at 50°C and in the presence of: ●, none; □, α-CD; ▲, β-CD; △, γ-CD; ○, maltoheptaose.

slowly reaching about 25% for product concentrations of 58–116 μ*M* (product/substrate ratio 1:2 or 1:1). We can see that the inhibition is important when *p*-nitrophenolate concentration is ≥ 50% of substrate concentration.

Table 3 presents the effect of β-CD concentration in the PNPB hydrolysis rate, measured at fixed concentrations of substrate (0.29 m*M*) and enzyme (0.05 mg/mL). From these results, it appears that β-CD produces an inhibition effect that increases with CD concentration reaching a plateau at concentrations over 1.2 m*M*, concentrations at which the ratio β-CD/PNPB is >4. The fact that the inhibition is not very large and reach a plateau is advantageous when, to prepare very high concentrations of substrate in solution, large amounts of CDs are required.

A similar inhibition effect has been found in experiments (not shown) carried out with 6 μg/mL of enzyme and a PNPB concentration (57 μM) lower than its solubility in water (68 μ*M*). In this case a smaller inhibition effect is seen, which reaches a plateau when CD is several times in excess over PNPB.

The effect of the acetonitrile (3%), α-CD, β-CD, γ-CD or maltoheptaose (1.32 m*M* for all) on the thermal storage stability of Candida lipase at 30° and 50°C has been studied. Figure 3 shows the results obtained at 50°C.

γ-CD, acetonitrile and maltoheptaose do not change greatly the stability of the enzyme. Whereas β-CD produces a slight stabilization effect (1.5 fold), α-CD acts in the opposite way. Similar results have been found in the experiments at 30°C.

DISCUSSION

In the present work, the kinetic constants and the activation energy of the hydrolysis of PNPB catalyzed by *C. cylindracea* lipase in the presence of CH_3CN (3%) or β-CD (1.5-fold concentration over substrate) have been compared. The values obtained are not very different. The affinity of the enzyme for the substrate is higher in the presence of β-CD ($K_m = 0.27$ m*M*) than in the presence of 3% acetonitrile (0.35 m*M*); this relaxation of enzyme affinity in the presence of organic solvents has also been observed before (*15*). On the other hand, the k_{cat} value is 27% higher (501 s^{-1}) with acetonitrile than with CD (395 s^{-1}). These figures yield the same value for the specificity constant in both cases (1.43 10^6 and 1.46 10^6 s^{-1} M^{-1}).

CDs are being increasingly utilized in chemical analysis (*16*) and in downstream processing (*17*), therefore their properties are very well known. The microencapsulation of the guest is usually instantaneous in solution. The complexes are stable even at elevated temperatures for long periods of time. Commonly, poor water solubility of the guest favors the complexation. CDs catalyze many chemical reactions, and because of this have been considered as enzyme mimics (*4,18*). They hydrolyze phenyl ester very efficiently at pH≥11 (*19*), the catalytic activity varying with the ester chain length: it decreases, then increases. This effect could be apparently related to the mode of insertion of the chain, since it has been postulated that up to hexanoate only the *p*-nitrophenyl moiety is included in the CD cavity, whereas, for longer esters, the hydrocarbon chain might also be inserted into it. In the conditions of the present investigation, pH around 7, hydrolytic activity by the CD added was insignificant compared with the enzymic activity.

The use of CDs allows the preparation of *p*-nitrophenyl ester solutions many fold more concentrated. With 3% acetonitrile—usually employed to raise the solubility of hydrophobic compounds in water—the solubility of PNPB is 68 μ*M*. However, with β-CD a solubility value of 1050 μ*M* is attained. At this concentration the inclusion complex with β-CD (PNPB/CD ratio 1:1.5) precipitates. While the aqueous solubilities of α- and γ-CD at 25°C are 121 m*M* (145 g/L) and 168 m*M* (232 g/L), respectively, that of β-CD is roughly a factor of 10 less, i.e., 16.3 m*M* (18.5 g/L) (*20,16*). Using γ-CD we have not been able to obtain solutions of PNPB of concentrations higher than 1 m*M*, which indicates that although this CD is much more soluble, its inclusion complexes are not more soluble than those of β-CD.

The use of β-CD derivatives more soluble in water (hydroxyethyl-, hydroxypropyl-, maltosyl-β-CD or β-CD polymer) (*21*) may help to overcome these problems in preparing substrate solutions of higher concentrations.

Stabilization of enzymes is one of the most important parameters for their utilization. Generally, enzymes increase in thermostability in the presence of substrates, products and inhibitors (*22*). In the present work, β-CD increases slightly (about 1.5 fold) the stability of Candida lipase at 50°C*; however, a noncylic heptasaccharide (maltoheptaose) does not exert any effect on stabilization. On the other hand, α-CD slightly destabilize this lipase. The effect of CDs on the stability of many substances has been reported (*24,25*). Ezure et al. (*26*), studying glucoamylase, tested the influence of CDs as substrate analogs not hydrolyzable by the enzyme: only α-CD had a good stabilizing effect at 60°C.

CONCLUSIONS

In the present report we have proposed the use of CDs in enzyme (hydrolytic) reaction mixtures in order to enhance the solubility of hydrophobic compounds (i.e., esters of *p*-nitrophenol). As compared to previous methodologies (the addition of organic solvents or surfactants, ultrasonic desintegration), our procedure presents many advantages:

1. The enzyme parameters are not affected substantially when compared with their values in 3% acetonitrile.
2. Much more concentrated solutions of substrates can be prepared.
3. The reproducibility is higher, owing to the perfect solution obtained for the substrate.
4. CDs are cheap (because of their production in industrial quantities).
5. The use of a ultrasonic desintegrator—although a simple apparatus—is not necessary.
6. The addition of a hydrophilic compound, CD, to the reaction mixture does not change the properties of the solvent (water). Contrariwise, the addition of acetonitrile or other water-miscible solvent will alter (*27*) water properties (dielectric constant, partition coefficient, hydrogen-bond acidity and basicity).

The procedure is simple, does not require any instrumentation, and many different types of CDs are commercially available.

* In a previous study in our laboratory, this enzyme was stabilized 140 times by immobilization on Sepharose (*23*).

ACKNOWLEDGMENTS

We thank Dr. V. M. Fernández and Dr. M. Martin-Lomas and Dr. S. Penadés for help and advice. This work was supported by grants from the EEC (BAP.0402.E) and the Spanish CICYT (BIO88-0241).

REFERENCES

1. Benohr, H. C. and Krisch, K. (1967), *Z. Physiol. Chem.* **348,** 1102.
2. Zaks, A. and Klibanov, A. M. (1986), *J. Am. Chem. Soc.* **108,** 2767.
3. Bender, M. L. and Komiyama, M. (1978), *"Cyclodextrin Chemistry"*, Springer-Verlag, New York.
4. Saenger, W. (1980), *Angew. Chem. Int. Ed.* **19,** 344.
5. Konno, A., Misaki, M., Toda, J., Wada, T., and Yasumatsu, K. (1982), *Agric. Biol. Chem.* **46,** 2203.
6. Ito, K., Kikuchi, K., Okazaki, N., and Kobayashi, S. (1988), *Agric. Biol. Chem.* **52,** 2763.
7. Szejtli, J. (1986), *Starch* **38,** 388.
8. Szejtli, J. (1988), *Kontakte* **1,** 31.
9. Szejtli, J., Szente, L., Kálói, K., Marton, J., and Gerlóczy, A. (1985), Hungarian Patent Appl., 75/85.
10. Sawada, H., Suzuki, T., Akiyama, S., and Nakao, Y. (1987), *Appl. Microbiol. Biotechnol.* **26,** 522.
11. Hesselink, P. G. M., de Vries, H., and Witholt, B. (1987), In *Proc 4th European Congress on Biotechnology,* Vol. 2; Neijssel, O. M., Van der Meer, R. R., and Luyben, K. Ch. A. M., Ed.; Elsevier, pp. 229.
12. Hesselink, P. G. M., van Vliet, S., de Vries, H., and Witholt, B. (1989), *Enzyme, Mic. Technol.* **11,** 398.
13. Bar, R. (1989), *Trends Biotechnol.* **7,** 2.
14. Kawase, M. and Tanaka, A. (1989), *Enzyme Microb. Technol.* **11,** 44.
15. Nasri, M. and Thomas, D. (1986), *Nucl. Acid. Res.* **14,** 811.
16. Pharr, D. Y., Fu, Z. S., Smith, T. K., and Hinze, W. L. (1989), *Anal. Chem.* **61,** 275.
17. Szejtli, J. (1989), *Trends Biotechnol.* **7,** 170.
18. Rideout, D. C. and Breslow, R. (1980), *J. Am. Chem. Soc.* **102,** 7816.
19. Ueno, A., Suzuki, I., Hino, Y., Suzuki, A., and Osa, Tetsuo (1985), *Chem. Lett.* 159.
20. French, D., Levine, M. L., Pazur, J. H., and Norverg, E. (1949), *J. Am. Chem. Soc.* **71,** 353.
21. Pitha, J., Irie, T., Sklar, P. B. and Nye, J. S. (1988), *Life Sciences,* **43,** 493.
22. Citry, N. (1973), *Adv. Enzymol.* **37,** 397.
23. Otero, C., Ballesteros, A. and Guisan, J. M. (1988), *Appl. Biochem. Biotechnol.* **19,** 163.
24. Uekama, K. (1979), *J. Pharm. Bull* **68,** 1059.
25. Schlenk, H. (1955), *J. Am. Chem. Soc.* **77,** 3587.
26. Ezure, Y., Maruo, S., Kojima, M., Yamashita, H., and Sugiyama, M. (1988), *Agric. Biol. Chem.* **52,** 1073.
27. Alcantara, A. R., Garcia-Blanco, F., Heras, A. M., Sinisterra, J. V., and Ballesteros, A. (1989), *J. Mol. Catal.* **52,** 323.