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Enantioselectivity of *Candida rugosa* lipase in the hydrolysis of 2-chloropropionic acid methyl ester

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Abstract

The enantioselectivity of lipase from *Candida rugosa* in the hydrolysis of methyl 2-chloropropionate was measured at several substrate concentrations. Initial reaction rates for the pure enantiomers differ by a factor of 2.1 (low substrate concentration) and 1.6 (high substrate concentration), respectively. Determination of the enantiomeric ratio, *E*-value, in (1) kinetic resolution experiments, and (2) by the method of initial reaction rates on mixed enantiomers, however, showed $E = 1$ (no enantioselectivity) for the full range of substrate concentrations.

Keywords: *Candida rugosa* lipase; Enantioselectivity; Kinetic resolution; Enantiomeric ratio; 2-Chloropropionic acid methyl ester

1. Introduction

Enzymes are potentially useful catalysts for the production of enantiomerically pure compounds. The importance of the enantiomeric ratio, *E*-value, as a key process parameter in kinetic resolutions, has been well recognized [1]. Although, by definition, $E = (k^{R}_{cat}/k^{S}_{cat}) \times (K^{S}_{M}/K^{R}_{M})$ represents the product of the ratio of catalytic constants and the (reciprocal) ratio of the Michaelis constants for the pure enantiomers, it is not uncommon to use either the ratio of catalytic constants, k^{R}_{cat}/k^{S}_{cat}, or the ratio of maximum rates, V^{R}_{max}/V^{S}_{max}, or the ratio of reaction rates under saturating conditions, r^{R}_{sat}/r^{S}_{sat}, as a first-order indication of the enantioselectivity of an enzyme/substrate couple. So far, disregarding the properties of racemazes, where thermodynamic constraints demand complete reciprocity of the contributions of k^{R}_{cat}/k^{S}_{cat} and K^{R}_{M}/K^{S}_{M}, violations of this 'rule of thumb' have been reported in a few cases only (e.g. [2]). As part of an investigation into the influence of the enzyme conformation on lipase enantioselectivity [3], we measured the enantioselectivity of *Candida rugosa* lipase in the hydrolysis of various chiral esters at 25 °C. While different reaction rates were found for pure (*R*)- and (*S*)-2-chloropropionic acid methyl esters, kinetic resolution of the racemic mixture showed the enzyme to be completely non-specific. Since this appears to be the first example of a (non-racemizing) enzyme for which the combination of different microscopic kinetic constants leads to complete cancellation of enantioselectivity, we decided to investigate this phenomenon more closely.

2. Materials and methods

C. rugosa lipase, CrL, purified as described [4] was a generous gift from Altus Biologics (Cambridge, USA). 2-Chloropropionic acid methyl esters,

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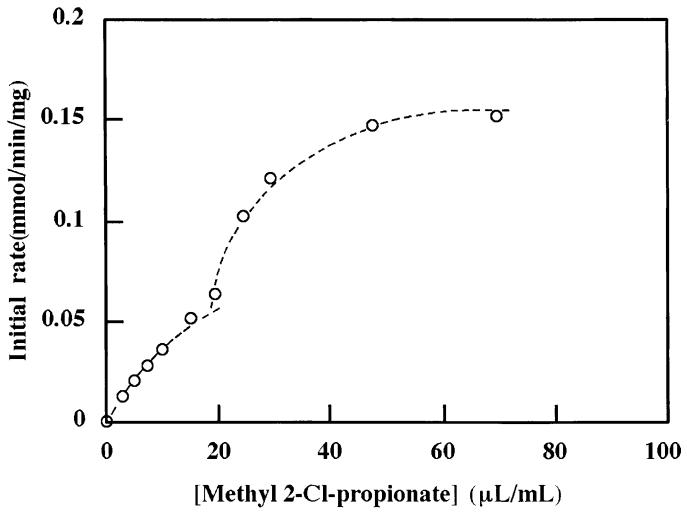

Fig. 1. Initial reaction rates for the *C. rugosa* lipase-catalyzed hydrolysis of methyl 2-chloropropionate at varying substrate concentrations.

MCPs, were purchased from Fluka, Buchs. All other chemicals were of analytical grade, obtained from commercial suppliers, and used as provided. Rate measurements were carried out in a pH-stat system from Metrohm (Dosimat model 655, Impulsomat model 614, and pH-meter model 632) in a well-stirred thermostated reaction vessel at 25 °C. After equilibration, substrate esters were added to 10 ml of buffer (10 mM phosphate, pH 7.0). The pH of the reaction mixture was kept constant at 7 by the addition of 10 or 100 mM KOH solution. The progress of the reaction was expressed as millimoles of alkali consumed per minute per milligram of pure protein. Kinetic resolution experiments were carried out in the pH-stat system starting with a well-stirred mixture of 1 ml of racemic MCP in 50 ml of 10 mM phosphate buffer,

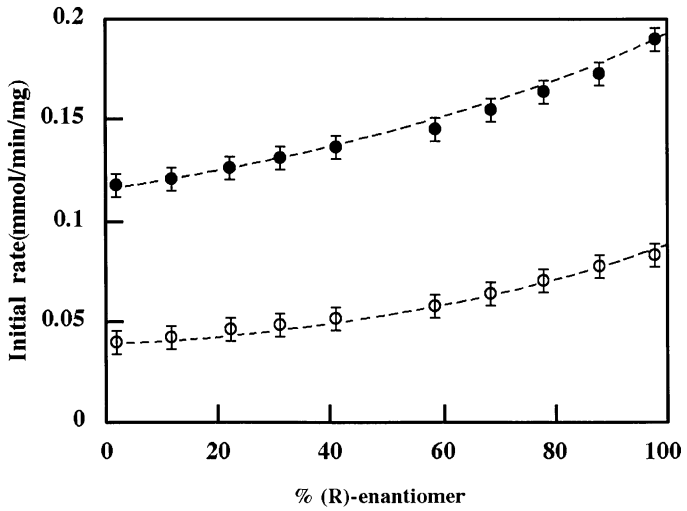

Fig. 2. Initial reaction rates for the *C. rugosa* lipase-catalyzed hydrolysis of methyl 2-chloropropionate in aqueous solution at various ratios of (*S*)- and (*R*)-enantiomers. Total substrate concentration: (○) 10 μl/ml buffer; (●) 100 μl/ml buffer.

pH 7, at 25 °C. Samples were prepared by periodically withdrawing 20 μl of the reaction mixture, extraction of the ester with 200 μl dichloromethane, separation of the organic layer and drying over anhydrous $MgSO_4$. Chiral analyses were carried out by GC (Chrompack CP9002, with flame ionization detector and autosampler) on a β-cyclodextrin column (CP cyclodex B 236 M, Chrompack, The Netherlands).

3. Results and discussion

The limiting solubility of methyl 2-chloropropionate, MCP, in water is approximately 20 μl/ml. At higher substrate concentrations the organic compound forms a second phase. As shown in Fig. 1, CrL displays 'interfacial activation' with racemic MCP, as has been observed for other esters [4]. Initial reaction rates on pure (*R*)- and (*S*)-MCP at a concentration of 10 μl/ml showed (*R*)-MCP to be converted approximately twice as fast as (*S*)-MCP. Kinetic resolution experiments using racemic MCP were carried out as outlined above. Chiral analysis of the remaining substrate showed identical concentrations of (*R*)- and (*S*)-MCP throughout the reaction (up to 90% conversion). Considering the possibility that phase changes during the conversion might lead to enantioselectivity differences, as has been observed for the PPL-catalyzed resolution of glycidyl butyrate [5], we measured the initial reaction rates on mixtures of MCP enantiomers at concentrations below (10 μl/ml) and above (100 μl/ml) the solubility limit. The results are shown in Fig. 2. Analysis of the data using the appropriate relation [6] showed $E = 1.00 \pm 0.03$ for both low and high substrate concentrations.

Clearly, the properties of CrL in the hydrolysis of MCP can be attributed to the fact that the 'racemic temperature' [7] of this system happens to be located at 25 °C. In this respect, it will be interesting to establish the temperature dependence of CrL enantioselectivity in this reaction. Experiments are in progress.

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