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TEMPERATURE AS AN ENANTIOSELECTIVE PARAMETER IN ENZYMATIC RESOLUTIONS OF RACEMIC MIXTURES

Erland Holmberg and Karl Hult*

Department of Biochemistry and Biotechnology,
Royal Institute of Technology,
S-100 44 Stockholm, SWEDEN

SUMMARY

In the field of enhancement of the enantiomeric excess, the effect of temperature on the enantiomeric ratio, E, was investigated in lipase catalysed hydrolysis and transesterification. It was found that the equation $(E_1)^{T_1}=(E_2)^{T_2}$ correlated well with the experimental data obtained.

INTRODUCTION

The use of enzymes (e.g.lipases) has opened an attractive route for the production of homochiral compounds from mixtures of isomers or from prochiral compounds. If not always a high enantioselectivity, then at least a moderate one can be found using commercial available enzymes. It is then possible to enhance the enantiomeric excess by several methods (Chen and Sih, 1989; Otto, 1990). To extend this field with additional tools we studied the effect of temperature on the enantioselectivity using transesterification and hydrolysis of 1-phenylethyl butyrate catalysed by lipase from *Candida cylindracea (Candida rugosa)*.

MATERIALS AND METHODS

Enzyme
Commercial extracellular lipase from *Candida cylindracea* was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The preparation had a specific activity of 665 units/mg solid and 4750 units/mg protein. One unit will hydrolyse 1.0 microequivalent of fatty acid from a triglyceride (olive oil) in one hour at pH 7.7 at 37 °C (incubation time: 30 min).

Substrate
1-Phenylethyl butyrate was synthesized from the butyryl chloride and 1-phenylethanol in dichloroethane.

Enzymatic Hydrolysis
The incubations contained enzyme (20 mg) (2.5 mg/ml) and substrate (0.2 M). The substrate was emulsified by sonication in a buffered solution of potassium phosphate (0.25 M, pH 8.0) and gum arabic (5%, w/v) in a total volume of 8 ml in test tubes. The mixtures were incubated at 6 °C or at 37 °C in an end-over-end incubator (22 rpm) until a racemic mixture of the alcohol was obtained. At the end of the reaction the reaction rate was very slow and an additional amount of enzyme was added (20 mg).

Enzymatic Transesterification
The incubations contained enzyme (2 g), ester (0.1 M), 1-heptanol (2.0 M) and cyclohexane to a total volume of 8 ml in test tubes. The mixtures were incubated at 6 °C or at 37 °C in an end-over-end incubator (22 rpm). No water was added.

Determination of Conversion
Samples from on-going hydrolytic experiments were taken in duplicates after sonication (20 s). The remaining ester and the produced alcohol were immediately extracted by addition of diethyl ether (1 ml) and using a vortex mixer. Samples from the transesterification experiments were taken in duplicates after centrifugation. The conversion was determined by gas chromatography using a Perkin Elmer 5000 equipped with an FFAP-CB column (CHROMPACK; 25 m, 0.32 μm, 0.32 mm). Nitrogen was used as a carrier gas. Hot on-column technique was used and a temperature program (135-195 °C) was applied. The detection was made with a FID, which was set at 350 °C. A relative response factor for the ester to the alcohol was determined and was used for calculation of the conversion.

Determination of the Enantiomeric Excess
Samples (0.10-0.25 ml) were withdrawn after sonication and were extracted with cyclohexane (1-3 ml) on a vortex mixer (1 min). The enantiomeric excess of the liberated alcohol was analyzed by gas chromatography (König, Francke and Benecke, 1982) using a Carlo Erba Strumentazione equipped with a chiral column (XE-60-S-Valine-S-α-phenylethyl amide; 50 m, 0.12 μm, 0.25 mm). Helium was used as a carrier gas and the oven temperature was set at 154 °C. The temperature of the split injector was set at 275 °C and detection was accomplished with a FID set at 300 °C.

RESULTS AND DISCUSSION

It has been observed that the temperature can affect the enantioselectivity. However both an increase (Lam et al., 1986) and a decrease (Boutelje et al., 1988) of the selectivity with the lowering of the temperature have been observed. It has also been reported that the temperature had no effect on the enantioselectivity (Barton et al., 1990). From their result however a slight increase in enantioselectivity at lower temperature can be seen. To investigate the effect of temperature on the enantioselectivity, traditional enzyme kinetic equations were set up. It can be shown that (Fersht, 1985):

$$(k_{cat}/K_M)_R/(k_{cat}/K_M)_S=\exp(-\Delta\Delta G^{\#}_T/RT) \quad (1)$$

Where $\Delta\Delta G^{\#}_T$ is the difference in activation energies for the two enantiomers and k_{cat}/K_M are the specificity constants for the *R*- and the *S*-enantiomer respectively.

The enantiomeric ratio E, is defined as (Chen et al., 1982):

$$E = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S \quad (2)$$

Combination of (1) and (2) at two different temperatures T_1 and T_2 (R is the gas constant):

$$RT_1 \ln E_1=-\Delta\Delta G^{\#}_{T_1} \quad (3)$$

$$RT_2 \ln E_2=-\Delta\Delta G^{\#}_{T_2} \quad (4)$$

Assuming that there is no major difference in conformation at the two different temperatures, then:

$$\Delta\Delta G^{\#}_{T_1}=\Delta\Delta G^{\#}_{T_2} \quad (5)$$

Combination of (3), (4) and (5):

$$RT_1 \ln E_1=RT_2 \ln E_2 \quad (6)$$

And thus:

$$(E_1)^{T_1}=(E_2)^{T_2} \quad (7)$$

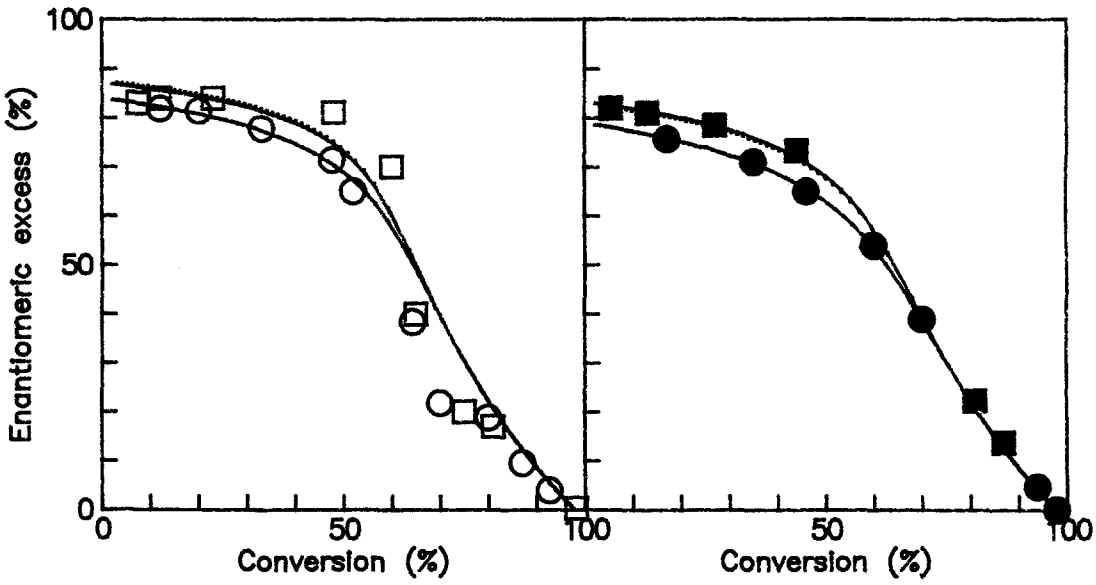

Figure 1 The enantiomeric excess of produced 1-phenylethanol during hydrolysis of 1-phenylethyl butyrate catalysed by lipase from *Candida cylindracea*. Incubations at 37 °C (○) and at 6 °C (□). The two continuous curves represents E-values of 8.5 (37 °C) and 11.0 (6 °C) and an equilibrium conversion of 97.8%. The dotted curve represents the curve obtained when equation (6) was used to calculate the theoretical value E=10.8 at 6 °C from the experimental data at 37 °C.

Figure 2 The enantiomeric excess of produced 1-phenylethanol during transesterification of 1-phenylethyl butyrate with 1-heptanol catalysed by lipase from *Candida cylindracea*. Incubations at 37 °C (●) and at 6 °C (■). The two continuous curves represents E-values of 11.5 (37 °C) and 14.6 (6 °C) and an equilibrium conversion of 97.8%. The dotted curve represents the curve obtained when equation (6) was used to calculate the theoretical value E=15.1 at 6 °C from the experimental data at 37 °C.

A similar equation, without accompanying experimental proof, was recently reported by Otto (1990). We can now confirm these equations by experimental data. Figure 1 shows the hydrolysis and Figure 2 the transesterification of 1-phenylethyl butyrate, at 37 °C and 6 °C. The computed curves in each figure at the two different temperatures represents the E-values obtained, 8.5 and 11.0 at 37 °C and 6 °C, respectively, for hydrolysis and 11.5 and 14.6 at 37 °C and 6 °C, respectively, for transesterification. When equation (6) was used to calculate the E-values at 6 °C from the values experimentally determined at 37 °C, E=10.8 was obtained for hydrolysis and E=15.1 was obtained for transesterification. Thus the experimentally determined data are in good agreement with theoretical calculation.

As can be seen from equation (6) the temperature is proportional to the logarithm of the E-value or as in equation (7) the E-value is an exponential function of the temperature. With an E-value of 10, a decrease of the temperature from 37 °C to 6 °C increases the E-value to 13 (a factor 1.3). With the same temperatures and an E-value of 100 it will increase to 167 (a factor 1.67). That is; when decreasing the temperature the increase in E-value will increase with the E-value. However, the E-value is a logarithmic function of the enantiomeric excess (Chen et al., 1987), a raised E-value starting

from a high E-value will not enhance the enantiomeric excess as much as when starting from a lower E-value (equation 8, K is the equilibrium constant for the reaction, ee_S is the enantiomeric excess for the substrate, c is the conversion of the substrate).

$$E = \frac{\ln\{1-[1+K]\cdot[c+ee_S\cdot(1-c)]\}}{\ln\{1-[1+K]\cdot[c+ee_S\,(1-c)]\}} \qquad (8)$$

At 37 °C with an E-value=100 and a conversion of 45%, the product will be obtained with an enantiomeric excess of 95%. A decrease of the temperature to 6 °C increases the E-value to 167 and the enantiomeric excess will be 96.7 at 45% conversion. For optical active compounds which are to be used as drugs or pesticides, every percentage of enantiomeric excess may be very important. Even though only a limited increase in enantiomeric ratio can be expected from a decrease in temperature, that is possible in practical use, we propose that the effect of temperature can be added as a tool for enhancement of the enantiomeric excess in cases where a decrease in reaction rate can be accepted.

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