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2-Propanol treatment of *Candida rugosa* lipase and its hydrolytic activity

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Abstract

We studied the effects of the 2-propanol treatment of *Candida rugosa* lipase (CRL) by the addition of 2-propanol in the range of 0–35 ml against 25 ml of the solution extracted from 5 g of CRL on its hydrolytic activity, stereoselectivity, and recovered protein. Consequently, the following four results were revealed: (1) the highest hydrolytic specific activity against *p*-nitrophenyl acetate (PNPA) and the *p*-nitrophenyl esters of a series of straight-chain carboxylic acids was shown by CRL treated with 20 ml 2-propanol; (2) the total activity for PNPA monotonously increased up to 15 ml of 2-propanol addition while over 15 ml of 2-propanol addition resulted in a drastic decrease in total activity; (3) stereoselectivity for methyl 2-phenylpropanoate was improved in accordance with the increase in the 2-propanol added, and 20 ml of 2-propanol addition produced an *E* value over 500; and (4) recovered protein decreased in accordance with the volume of 2-propanol added and significantly decreased, especially at 5 ml and over 15 ml of 2-propanol addition.

Keywords: *Candida rugosa*; 2-Propanol treatment; Specific activity; Recovered protein; Enantioselective hydrolysis

1. Introduction

Lipases are usually used for the hydrolysis of lipids on an industrial scale and for the kinetic resolution of racemic carboxylic acids and alcohols in the laboratory [1–4]. The interests of organic chemists are usually directed to attain excellent resolution with commercially available enzymes and they strive to increase the enantioselectivity rather than to increase the activity or specific activity of the lipase. In 1995, Kazlauskas and coworkers reported a new method for increasing both the hydrolytic activity and enantioselectivity toward esters of the 2-aryl-propanoic acid and 2-(aryloxy)propanoic acids [5]. This new method was to treat natural *Candida rugosa* lipase (CRL) with 50 vol.% 2-propanol. They suggested that the 2-propanol treatment converted the closed form of the conformation of CRL to its open form [6–9]. However, they did not examine and explain the details of 2-propanol treatment of CRL. In this paper, results about the changes in the recovered protein, hydrolytic activity, and enantioselectivity for the CRL treated with several volumes of 2-propanol are discussed [10].

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2. Materials and methods

2.1. Materials

CRL (lipase OF) was obtained from Meito Sangyo Co. (Japan). All chemicals were of reagent grade and were purchased from Wako Pure Chemical Industries Ltd. (Japan).

2.2. 2-Propanol treatment of CRL

Commercial CRL (5 g) was dissolved in a 2-(*N*-morpholino)ethanesulfonic acid buffer solution (50 mM, pH 6.0, 25 ml), stirred at 4 °C for 24 h and the suspension was then centrifuged at 15,000 rpm for 30 min at 0 °C. After adjusting to 25 ml with the addition of distilled water to the obtained protein solutions, 5, 10, 15, 20, 25, 30 and 35 ml of 2-propanol were added dropwise over 40 min and the resultant mixture were stirred for 42 h at 4 °C. After removal of the precipitate by centrifugation under the same conditions, the supernatant was dialyzed against deionized distilled water (3× 1 l) every 24th hour. The resultant solutions were used for the following analyses.

2.3. Recovered protein

The concentration of the recovered protein was measured by Lowry's method using bovine serum albumin as the protein standard [11]. The weights of the recovered protein from 5 g of commercial CRL after the 2-propanol treatments are shown in Fig. 2.

2.4. Hydrolytic activity for p-nitrophenyl esters

The hydrolytic activity was measured at pH 7.5 and 25 °C using *p*-nitrophenyl acetate (PNPA, **1** ($n = 2$)), *p*-nitrophenyl esters of a series of straight-chain carboxylic acids (**1** ($n = 3$–12)) and (*R*)-*p*-nitrophenyl 2-phenylpropanoate and (*S*)-*p*-nitrophenyl 2-phenylpropanoate (**2**) as the substrates. An aliquot (10 ml) of the above enzyme solution was dissolved in phosphate buffer (10 mM, pH 7.5, 2 ml). To this was added an aliquot of a *p*-nitrophenyl ester solution of acetonitrile (50 mM, 10 ml) at 25 °C. The initial rate of formation of the *p*-nitrophenoxy ion, generated through the reaction as shown in Fig. 1, was monitored at 404 nm for 2 min (6 s interval). An extinction coefficient of 11,600 M^{-1} cm^{-1}, which accounts for the incomplete ionization of the *p*-nitrophenoxy ion at pH 7.5, was used to calculate the activity [12]. The total activity of CRL (5 g) for PNPA (**1** ($n = 2$)) and specific activity are shown in Figs. 2–5.

2.4.1. Enantioselective enzymatic resolution

The (*R, S*)-methyl 2-phenylpropanoate (**3**) (100 mg) dissolved in isooctane (5 ml) was added to the 2-PrOH-treated CRL solution (10 ml, 100 U of activity by PNPA (**1** ($n = 2$) assay) mixed with 1 M phosphate buffer (1 ml) at 25 °C and the mixture was thoroughly stirred for several hours at this temperature. The progress of the kinetic resolution was monitored by HPLC as described below. The reaction was stopped when the ee of the remaining ester reached about 80%. The remaining ester and the hydrolyzed acid were separated in the usual manner.

2.4.2. Analytical methods for enantioselective enzymatic resolution

The enantiomers of the ester (**3**) in the organic phase were separated by HPLC (LC-6A system, Shimadzu Co., Japan) with a Daicel Chiralcel OJ column (Daicel Chemical Co., Japan) under the following conditions: mobile phase, *n*-hexane/2-propanol

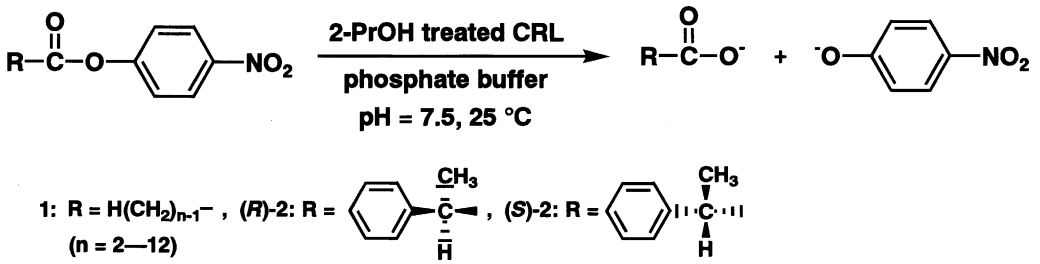


Fig. 1. Determination of hydrolytic activity.

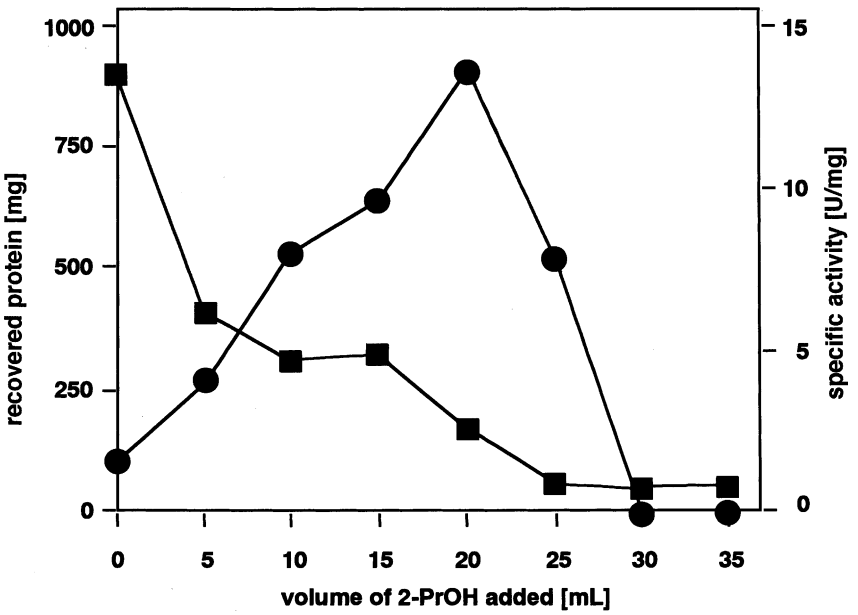


Fig. 2. Effect of 2-propanol treatment on specific activity of CRL against PNPA (**1** ($n = 2$)) and on recovered protein: (●) specific activity; (■) recovered protein.

= 90/10; flow rate, 0.5 ml/min; column temperature, 30 °C; detection, 254 nm. Retention times of (*R*)-**3** and (*S*)-**3** were 13.98 and 16.08 min, respectively. On the other hand, the enantiomers of the acid (**4**) in the aqueous phase were separated by HPLC (CCPD System, Tosoh Co., Japan) with a Daicel Chiralcel OJ-R: mobile phase, 0.2 M phosphate buffer (pH2.0)/acetonitrile = 70/30. Retention times of

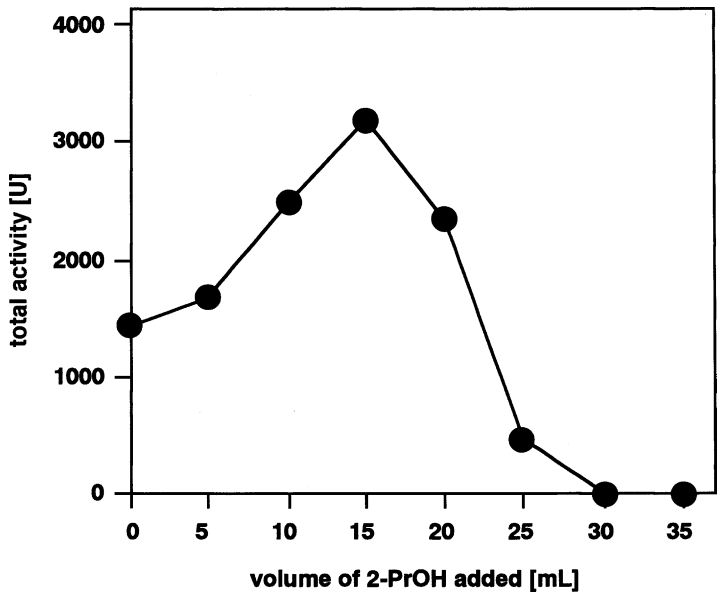


Fig. 3. Effect of 2-propanol treatment on total activity of CRL against PNPA (**1** ($n = 2$)).

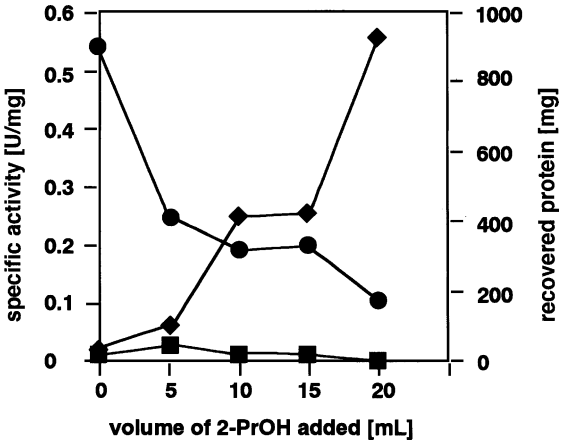


Fig. 4. Effect of 2-propanol treatment on specific activity of CRL against (*R*)-*p*-nitrophenyl 2-phenylpropanoate and (*S*)-*p*-nitrophenyl 2-phenylpropanoate: (■) specific activity against (*R*)-*p*-nitrophenyl 2-phenylpropanoate; (◆) specific activity against (*S*)-*p*-nitrophenyl 2-phenylpropanoate; (●) recovered protein.

(*R*)-**4** and (*S*)-**4** were 19.46 and 20.86 min, respectively. The enantiomeric ratio, the *E* value, was calculated using $E = \ln[1 - c(1 + \mathrm{ee_P})]/\ln[1 - c(1 - \mathrm{ee_P})]$ and $c = \mathrm{ee_S}/(\mathrm{ee_S} + \mathrm{ee_P})$ where $\mathrm{ee_S}$ represents the ee of the remaining ester and $\mathrm{ee_P}$ represents the ee of the hydrolyzed acid [13]. The obtained results are listed in Table 1.

3. Results and discussion

3.1. Effect of 2-propanol treatment on the recovered protein and hydrolytic activity for PNPA

As plotted in Fig. 2, the recovered protein decreased with an increase in the 2-propanol added and a dramatic decrease was observed with the addition of 5 ml of 2-propanol. Interestingly, the recovered protein did not very change in the range of 5–15 ml 2-propanol treatments and the addition of over 15 ml of 2-propanol significantly reduced the recovered protein.

The specific activity of CRL toward PNPA (**1** ($n = 2$)) increased linearly from 1.6 to 13.6 U/mg with an increase in the 2-propanol from 0 to 20 ml and then, a drastic drop was observed when adding over 20 ml of 2-propanol (Fig. 2). Though in the range of 5–15 ml of 2-propanol, the recovered protein was not significantly reduced (411–312 mg), the specific activity was enhanced, according to the increase in the 2-propanol added, to 2.6, 5.0, and 6.0 times greater than the non-treated one, respectively. The highest specific activity was obtained by treatment with 20 ml of 2-propanol and this volume was the second decreasing point of the recovered protein. Treatment with over 20 ml of 2-propanol led to a significant decrease in the specific activity (7.8 U/mg with 25 ml of 2-propanol) and both protein solutions treated with 30 ml and with 35 ml of 2-propanol had no hydrolytic activity. We also checked the hydrolytic specific activity of the precipitate after the 2-propanol treatment. It possessed no or negligible activity.

The changes in total activity of the CRL for PNPA (**1** ($n = 2$)) through treatment with eight different volumes of 2-propanol (0–35 ml) toward 5 g of commercial CRL are plotted in Fig. 3. Treatment with 5 ml of 2-propanol seemed not very effective on the increase in the total activity (only 1.1 times enhancement), but the addition of 15 ml of 2-propanol caused the total activity to double compared with the non-treated one. Treatment with over 15 ml of 2-propanol lead to a large decrease in the total activity while the 30 ml treatment completely deactivated the CRL.

We conceived that the 2-propanol treatment had three types of effects on the commercial CRL, that is, the removal effect of proteins other than CRL, which were not hydrolases, a conformation change effect on the CRL from a closed form to an open form, and the deactivation effect on the active CRL.

From the changes in the three kinds of results, the recovered protein, specific activity, and total activity, it was postulated that the proteins in the solution in accordance with the volume of the 2-propanol added varied in four stages: first stage, region of 0–5 ml of 2-propanol; second stage, region of 5–15 ml of 2-propanol; third stage, region of 15–20 ml of 2-propanol; and fourth stage, region of over 20 ml of 2-propanol. The changes in three different variables in each four stage could be comprehended by considering the following. (1) Five milliliters of 2-propanol reduced the recovered protein to half and this reduction resulted in making doubling the specific activity, but the total activity did not change. Thus, in the first stage, 2-propanol mainly behaved as a remover of proteins other than CRL. (2) The second stage was the crucial stage and we saw that the conformational change of CRL from the “closed form” to “open

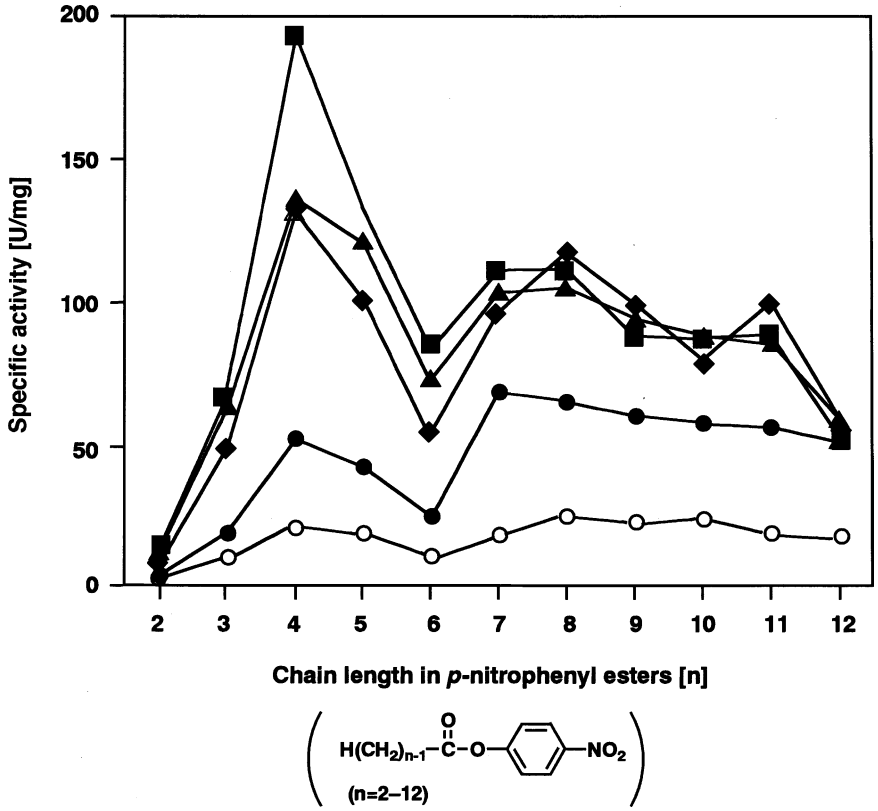
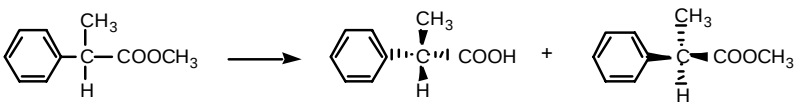


Fig. 5. Effect of 2-propanol treatment on specific activity for straight-chain carboxylic acid *p*-nitrophenylesters: (○) with non-treated CRL; (●) with CRL treated with 5 ml of 2-propanol; (◆) with CRL treated with 10 ml of 2-propanol; (▲) with CRL treated with 15 ml of 2-propanol; (■) with CRL treated with 20 ml of 2-propanol.

Table 1
Effect of 2-propanol treatment on enantioselectivity of CRL



(*R*, *S*)-**3** (*S*)-**4** (*R*)-**3**

2-Propanol (ml)	ee_S (%)	ee_P (%)	Conversion (%)	*E*
0	50	68	42	8.5
5	89	71	56	17.2
10	62	97	39	124
15	69	98	41	205
20	81.6	>99	45	>500[a]
25	76.2	>99	43	>500[a]

[a] *E* value was calculated as $ee_S = 99.99$.

form" occurred since the specific activity and total activity were enhanced though the recovered protein did not so change. (3) In the third stage, in comparison with the results of the 15 ml of 2-propanol added, the recovered protein decreased to half and the specific activity was improved to 1.5-fold with the addition of 20 ml of 2-propanol. However, the total activity fell to 74% in comparison with the 15 ml addition of 2-propanol. These results meant that improvement in the specific activity was responsible for the reduction in the "closed-form CRL" by further progress in the conformational change, and the decrease in the recovered protein was attributed to the removal of both the deactivated "open-form CRL" and proteins other than CRL. (4) In the fourth stage, the values of all three items significantly decreased. Both the remarkable decrease in the recovered protein and total activity were explained as the removal of the "open-form CRL" by the predominant deactivation effect. Reduction of the specific activity could be understood to consider both the decrease in the active CRL by the deactivation effect and the existence of a certain protein which strongly resists the 2-propanol treatment.

3.1.1. Effect of 2-propanol treatment on enantioselectivity

We next examined the effect of the 2-propanol treatment on the enantioselectivity of CRL toward the methyl (*R*, *S*)-2-phenylpropanoate ((*R*, *S*)-**3**). CRL was treated using several different 2-propanol volumes as described in the Section 2 and each 100 U of the pre-treated CRL was used for the hydrolysis of 100 mg of (*R*, *S*)-**3**. As shown in Table 1, *E* value dramatically changed with the addition of more than 10 ml of 2-propanol. The best result was obtained with the 20 ml 2-propanol-treated CRL having more than a 58-fold improvement in the enantioselectivity ($E > 500$; $ee_S = 82\%$ and $ee_P > 99\%$ at 45% conversion). Large-scale application of this enzymatic resolution and purification of each enantiomer will be described in the near future.

3.1.2. Effect of 2-propanol treatment on hydrolytic activity for each enantiomer ester

In order to investigate in detail, the changes in the enantioselectivity for methyl 2-phenylpropanoate the specific activity for the *p*-nitrophenyl esters of the optically active 2-phenylpropanoic acid, as (*R*)-**2** and (*S*)-**2** was measured with the 2-propanol-treated CRL. The addition of 2-propanol in the range of 0–15 ml hardly improved the specific activity for (*R*)-**2** at all and the treatment with 20 ml of 2-propanol made CRL inactive, whereas the (*S*)-**2** specific activity increased from 0.022 U/mg (0 ml) to 0.558 U/mg (20 ml) in accordance with the increase in the 2-propanol. The relationship between the volume of 2-propanol and both the specific activity for (*R*, *S*)-**2** and recovered protein is shown in Fig. 4.

Improvement of the *E* value by the 2-propanol treatment was explained by considering that though CRL was mostly pure hydrolase, it was composed of a "main hydrolytic protein" which had strict (*S*)-**2** preference, partly the "other hydrolytic protein" whose stereoselectivity was not as strict as the main one and proteins other than CRL which were not hydrolases. This main hydrolytic protein adopted an open or closed conformation and the former had hydrolytic activity while the latter had the possibility to be altered to the active open form by having the treatment of 5–15 ml of 2-propanol. In this range of added 2-propanol the recovered protein did not significantly changed. Since the active main hydrolytic protein would have strict stereoselectivity for (*S*)-**2**, an increase in the open-form CRL resulted in improving the *E* value. The large increase in the specific activity for (*S*)-**2** by treatment with 20 ml of 2-propanol was responsible for the increase in the ratio of the active main hydrolytic protein obtained by a conformational change to "open-form CRL" in spite of the decrease in the recovered protein. In spite of the increase in the ratio of the active main hydrolytic protein, the fact that the specific activity for (*R*)-**2** did not change very much supported the hypothesis that the stereoselectivity of the active main hydrolytic protein was strictly preferential for (*S*)-**2** and implied the existence of "another hydrolytic protein" whose stereoselectivity was not very good. The 1.75-fold increase in the specific activity for (*R*)-**2** with the addition of 5 ml of 2-propanol compared with the case without 2-propanol treatment was mainly attributed to the removal of proteins other than CRL which was not a hydrolase. The "other hydrolytic protein" suffered a slight deactivation from treatment with 10–15 ml of 2-propanol and treatment with 20 ml of 2-propanol made the "other hydrolytic protein" perfectly deactivated. From the results of the specific activity for (*R*)-**2** and (*S*)-**2** and the recovered

protein, we believe that the 2-propanol treatment contributed to both the activation and purification of the "main hydrolytic protein" in CRL.

3.2. Effect of 2-propanol treatment on the hydrolytic activity for p-nitrophenyl esters of straight-chain carboxylic acids

The specific activities for a series of straight-chain carboxylic acid *p*-nitrophenyl esters (**1** ($n = 2$–12)) were obtained by the reactions with CRL solutions treated with 0–20 ml of 2-propanol [14]. All the results are plotted in Fig. 5. Two striking characteristics are shown in the graph: (1) the specific activity for each ester increased according to the increase in the volume of the 2-propanol added; and (2) in a series of alterations in specific activity depending on the alkyl chain length, two maximum points were observed for the ester **1** ($n = 4$) and **1** ($n = 8$).

An X-ray structure of the CRL-inhibitor complex [9] demonstrates that in the active site, CRL has a tunnel as a pocket for accommodating the straight-chain moiety in the ester and this tunnel was long, narrow, hydrophobic, and bent toward the center of the CRL. This unique bent form of the tunnel in the active site seemed to be related with the specific activity of CRL showing two maximum points for **1** ($n = 4$) and **1** ($n = 8$).

4. Conclusion

We investigated the effects of 2-propanol treatment on the activity, enantioselectivity and recovered protein of CRL by means of changing the volumes of 2-propanol added in the range of 0–35 ml. CRL treated with 20 ml of 2-propanol afforded both the highest specific activity for PNPA and enantioselectivity for the methyl 2-phenyl propanoate and the highest total activity was obtained by treatment with 15 ml of 2-propanol. From the obtained results, it seemed that the added 2-propanol played important roles, first by removing proteins other than hydrolases, resulting in changes in the recovered protein, and secondly, to activate the CRL by causing a conformational change from the "closed-form CRL" to the "open-form one" leading to the increase in the total activity and improvement of specific activity, and the increase in the active "open-form CRL", whose enantioselectivity might be highly preferential, resulted in improvement of the *E* value for the enantioselective hydrolysis of methyl 2-phenylpropanoate. Needless to say, the addition of a large excess of 2-propanol deactivated the active CRL and reduced both the activity and recovered protein. However, concerning the reason for the improvement in enantioselectivity, the possibility for a favorable conformational change in the active site of CRL by the 2-propanol treatment could not be exactly contradicted.

Acknowledgements

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