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Activation of subtilisin Carlsberg in organic solvents by methyl-β-cyclodextrin: Lyoprotection versus substrate and product-complex effect

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Abstract

Enzyme catalysis in organic solvents is a valuable tool for the synthesis of a number of important chiral compounds. However, their usually low activity in these applications is a major drawback which hampers their potential. To overcome this, research groups around the world have successfully employed a variety of additives to protect enzyme catalysts from the detrimental effects of dehydration (mostly by freeze-drying, a required step prior to introducing the catalyst to organic solvents) and subsequent exposure to organic media. Modified cyclodextrins are among the most successful additives employed for this purpose. However, to maximize their constructive effect it is necessary to determine how these cyclic macromolecules enhance enzyme catalytic activity as well as the enantioselectivity. Our data suggests that MβCD activates the serine protease subtilisin C. predominantly by preserving the enzyme structure. Supporting data include the facts that: (a) MβCD must be co-dissolved with the enzyme in buffer prior to lyophilization; (b) formation of inclusion complexes is excluded from contributing to the activation because no increase in enantioselectivity or in activity was observed when MβCD was added directly to the enzyme–organic solvent mixture; (c) MβCD works at an optimum concentration, after which a decrease in enzyme activity is observed; (d) removal of the additive by washing steps only causes minor activity decreases; (e) co-lyophilization of a lipase was also found to increase its enantioselectivity towards the opposite enantiomer than subtilisin. These data are irreconcilable with a mechanism of activation that involves the formation of inclusion complexes between the additive, the substrates and the products, which has been proposed to contribute to the activation of a lipase.

Keywords: Subtilisin Carlsberg; Enzyme activation in organic solvents; Methyl-β-cyclodextrin; Co-lyophilization

1. Introduction

The usefulness of enzymatic catalysis in organic solvents for introducing chirality to key biologically relevant compounds is well recognized [1–8]. However, there are still drawbacks to this approach, in particular, the activity of enzymes in organic solvents is much lower than that observed in aqueous solutions [9] which, substantially limits the maturity of enzyme catalysis in organic solvents to its full potential. Lyophilization, the most common method of enzyme preparation, and other dehydration methods, cause protein structural perturbations [10,11]. Although they are frequently reversible upon dissolving the protein in water, they are mostly not reversible when using the protein powder in non-aqueous enzymology [12,13]. In the latter case, the lyophilized enzyme catalysts are generally suspended as amorphous powders in an organic solvent with the enzyme locked in a non-native conformation, resulting in a less active biocatalyst [13].

There have been a number of studies focusing on improving the activity of enzymes suspended in organic solvents [14–18]. One of the most popular ones is co-lyophilization of the enzyme with excipients. Successful excipients include polyethylene glycol [19], crown ethers [20], sugars [21], salts [22], and imprinting agents [23,24]. Generally, it is believed they improve the

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activity of enzymes in organic solvents either by affording a structurally more native enzyme (globally or at the active site), or by enhancing enzyme dynamics or a combination of both [25]. A common feature among these additives is that they have to be added to the enzyme-buffer solution prior to lyophilization to afford a substantial activation effect. Furthermore; the magnitude of the activation depends on the ratio of the additive-to-enzyme, and it displays an optimum additive-concentration effect. It has been shown that removal of some of these additives after co-lyophilization does not reduce the high enzyme activity significantly [19]. Actually, to obtain any substantial increase in activity using imprinting agents or sugars, this washing step has been shown to be necessary; otherwise, these additives tend to block the enzyme's active site.

During the past few years there have been numerous studies of enzyme activation by cyclodextrins [26,27]. We have recently reported that methyl-β-cyclodextrin (MβCD) dramatically activates the serine protease subtilisin Carlsberg in tetrahydrofuran (THF) and 1,4-dioxane [28,29]. Our results suggested that MβCD activates this enzyme by two mechanisms: it acts as a molecular lubricant enhancing enzyme dynamics and it reduced protein structural perturbations during lyophilization [30]. However, it has recently been speculated that in addition to a lyoprotectant effect, the activation of a lipase by a similar cyclodextrin (peracetylated-β-cyclodextrin) involves primarily a substrate and product complexation within the cyclodextrin-hydrophobic cavity [31]. The study also suggests that activation is the result of reduced inhibition of the lipase by the product, and the enhanced enantioselectivity observed was interpreted as being the result of a sequestering effect of the least-reactive substrate by the cyclodextrin.

Since most of the evidence provided in that study regarding the formation of product and substrate complexes with cyclodextrins was compelling, and it is well known that cyclodextrins form inclusion complexes with a variety of host compounds, we decided to investigate to which extent, if at all, complexation contributes to the activity increase and enhanced enantioselectivity we observe with our co-lyophilized subtilisin-MβCD powder. The results obtained from this work corroborate our previous findings that the main mechanism by which MβCD activates and enhances the enantioselectivity of subtilisin C. is the prevention of structural perturbation during the initial lyophilization process. Additional smaller contributions to increased activity may stem from increased enzyme dynamics by perhaps a "lubricant" effect, and by diminishing mass-transfer limitations.

2. Materials and Methods

2.1. Enzyme and reagents

Subtilisin Carlsberg (alkaline protease from *Bacillus licheniformis*, EC 3.4.21.14) and Lipase from *Candida rugosa*. EC 3.1.1.3 were purchased as lyophilized powders from Sigma. The solvents were purchased in the anhydrous form (Aldrich Sure/Seal bottles, water content below 0.005%). Vinyl butyrate (1) was purchased from TCI America Co. MβCD, the crown ethers, sec-phenethyl alcohol (2), butyril chloride (4), *N*-acetyl-L-phenylalanine ethyl ester, and 1-propanol were purchased from Aldrich.

2.2. Enzyme preparation

Subtilisin C. and Lipase from *Candida rugosa* powders were used as received from the supplier, and activated by lyophilization from 20 mM potassium phosphate buffer for 24 h at a concentration of 5 mg/ml at pH 7.8 and 7.0, respectively. Co-lyophilization of the enzymes with MβCD and the crown ethers was done the same way, except that the additive was added to the enzyme-buffer solution prior to lyophilization (at a protein:additive weight ratio of 1:6, for subtilisin and lipase from *Candida rugosa*, and 1:4 for subtilisin C. co-lyophilized with the crown ethers).

2.3. Kinetic measurements

The synthesis of the vinyl ester derivative presented in Tables 2 and 5 (using *Candida rugosa*—Table 5) were performed at a constant water activity of 0.11 obtained by gas-phase equilibrium in a reactor containing a saturated aqueous solution of LiCl. All the reactants, solvent (THF), MβCD and enzyme were pre-equilibrated in separate vials in the reactor for 24 h before the reaction. The reactions were then performed as follows:

Lyophilised enzyme: racemic *sec*-phenethyl alcohol (70 mM) was dissolved in 1.0 ml of THF containing 5 μl of dodecane as internal standard. To this solution, 6.0 mg of lyophilized enzyme (10.0 mg of powder) was added. After 5 min, *n*-butyric acid vinyl ester (200 mM) was added and the reaction was allowed to proceed with constant stirring at the defined water activity at room temperature. The reactions were performed in triplicate.

Co-lyophilized enzyme with MβCD: the procedure is the same as the one described above for lyophilized enzyme except for the amount of enzyme used: 0.26 mg of co-lyophilized enzyme (2.0 mg of powder) instead of 6.0 mg of lyophilized enzyme (10.0 mg of powder). The experiments using neat THF (Table 1) were performed in a similar manner as those described above, except that the temperature was 45 °C, water activity was not equilibrated, and therefore the incubation steps were avoided. The activity data for subtilisin presented in Table 3 were determined by following the product formation of the transesterification reaction *N*-acetyl-L-phenyl ethyl ester with 1-propanol in THF by HPLC, as previously described [25,28]. In brief, the reaction was performed at 25 °C and 250 rpm on an orbital shaker and contained 100 mM substrate, 1 M 1-propanol, and 1 mg of enzyme per ml of THF. All other activity data were obtained by following the product formation by gas chromatography (GC). The GC instruments (HP 6850, and HP 6890, fitted with Chirasil CB chiral columns, FID detectors, and He as carrier gas) were calibrated with the chiral esters (3)—synthesized as reported [28].

Under all conditions, the kinetic experiments were terminated before 10% of the product had been formed. The enzyme enantioselectivity was determined from the initial rates of ester product formation. The retention time of the "*R*" and "*S*" ester

Table 1
Effect of MβCD on subtilisin activity and enantioselectivity in neat THF[a]

Entry	Preparation	MβCD added[b]	Washed[c]	Enantioselectivity[d]	V_S[e]	Water[f] (%)
1	Lyophilized	No	No	30	0.023 ± 0.003	0.29
2	Lyophilized	Yes	No	21	0.021 ± 0.003	0.38
3	Co-lyophilized[g]	No	No	42	1.2 ± 0.15	–
4	Co-lyophilized	No	Yes	42	0.62 ± 0.07	–

[a] Reaction conditions: transesterification between sec-phenethyl alcohol and vinyl butyrate, at 45 °C.
[b] MβCD was added directly to the organic solvent–enzyme mixture.
[c] MβCD was removed from the co-lyophilizate by washing it 3 times with THF, see Section 2 for details.
[d] Enantioselectivity was determined from the initial rates for the formation of the enantiomeric products, see Section 2 for details.
[e] Initial velocity for the formation of the *S* enantiomer (μmol min^{-1} mg^{-1}).
[f] The water content (%) was determined by Karl Fisher titration.
[g] Subtilisin was co-lyophilized with MβCD at a 1:6 weight ratio of enzyme-to-cyclodextrin.

products was determined by analyzing samples of the pure enantiomers synthesized from the corresponding alcohol enantiomers. The enzyme enantioselectivity for either substrate is equal to the ratio: $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S = V_R[S]/V_S[R]$ [32]. Note that this relationship is valid only when a racemic mixture of the substrates is being studied (as in our experiments) so that both chiral substrates are competing for the binding site simultaneously.

2.4. FTIR measurements

FTIR studies were carried out using a Nicolet Magna-IR 560 optical bench [25,28,33]. Samples were measured as suspension in Perkin Elmer cells equipped with CaF_2 windows or as solid powder pressed into KBr pellets (1 mg of protein per 200 mg of KBr) as described [25]. Each sample was measured at least five times and the spectra obtained corrected for the solvent and water vapor contributions [25,28]. Every spectrum was analyzed by calculation of the second derivative spectrum for the component composition of the amide I band (1615–1700 cm^{-1}). Second derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}). The secondary structure composition (percentage of α-helix and β-sheet secondary structure) of subtilisin was determined by Gaussian curve fitting of the resolution-enhanced amide I spectra as described [28,30]. Spectral correlation coefficients (SCC) were used as measure for overall perturbations of subtilisin secondary structure upon lyophilization and suspension in organic solvents. Amide I second derivative spectra of subtilisin after exposure to the conditions to be tested were compared to that of subtilisin in aqueous buffer [11,34,35]. A SCC value of 1 shows spectral and thus structural identity, a value of less than 1 is a quantitative description of overall structural differences.

2.5. Calculation of the molar ratio of additive-to-protein

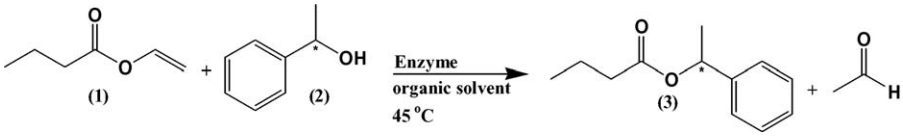
FTIR spectroscopy was used to monitor the removal of the additives from the formulations after repeated washing steps with THF (see above). First, calibration curves were obtained by rationing the area of characteristic IR absorption bands of the additives versus the area of the amide I protein band. The additives 18-crown-6 and MβCD were co-dissolved with subtilisin at certain molar ratios followed by subsequent lyophilization. The powders were then pressed into KBr pellets and the areas of the protein amide I band (1700–1600 cm^{-1}), a crown ether band (1955–2000 cm^{-1}) or a MβCD band (1210–1180 cm^{-1}) determined from the FTIR spectra. The band areas corresponding to the additives were obtained by subtracting the spectrum of the lyophilized powder from that of the co-lyophilized enzyme. The ratios of the band areas of additive/protein were plotted versus the known molar ratio to produce calibration curves. The moles of additive remaining in the enzyme powder after each washing step were calculated using the calibration curves.

2.6. Removal of additives

Additives were removed employing a tri-sequential procedure by washing with anhydrous THF (which was also used as the organic solvent for the kinetic studies). Briefly, THF was added to the co-lyophilized enzyme to achieve 1 mg/ml powder concentration, the resulting suspension was sonicated for about 20 s, agitated for 10 min on an orbital shaker at 25 °C and 250 rpm, and the enzyme recovered by centrifugation for 20 min at 6000 rpm. The supernatant and the powder were analyzed by ^1H NMR (between 3–4 and 3.5 ppm for MβCD and crown ether, respectively, 400 scans using a 400 MHz Bruker NMR) and FTIR to monitor the additive removal. The remaining powder was dried with nitrogen gas for FTIR and kinetic experiments used to determine the residual additive content or used without drying for kinetic experiments and FTIR structural studies of the enzyme suspended in THF. Note that drying of the enzyme powder prior to experiments causes loss in activity [19].

3. Results and discussions

The ability of cyclodextrins to form inclusion complexes with a variety of compounds and to catalyze a number of reactions is well documented. Therefore, in an enzyme-catalyzed reaction, it is feasible to propose a mechanism in which a cyclodextrin additive could have different binding constants for each enantiomer of a racemic mixture of substrates and their corresponding products, resulting in an increased enzyme enantioselectivity and activity (if the reaction is product-inhibited as previously suggested [31]).



Scheme 1. Transesterification reaction between sec-phenethyl alcohol and vinyl butyrate used to measure enzyme activity and enantioselectivity.

We previously reported that co-lyophilization of subtilisin Carlsberg with MβCD improves its enantioselectivity and activity [28–30]. Thus far, substrate or product complexation had not been excluded from being partially responsible for some of the reported effects. To address this experimentally, first we added MβCD to the enzyme lyophilized in the absence of the excipient in the reaction mixture using neat THF. Data presented in Table 1 show that the activity did not increase, for the reaction depicted in Scheme 1, when MβCD was added directly to the solvent-enzyme system. Next, we co-lyophilized subtilisin with MβCD and removed the additive by washing it with THF from the preparation. Even though washing reduced the activity by 50%, the residual enzyme activity was still much higher than that of the enzyme lyophilized without the additive (27 times). Furthermore, the washing process had no effect on the enzyme enantioselectivity (Table 1). To ensure that possible changes in water activity did not influence the experimental outcome, the above experiments were also performed at a constant water activity of 0.11 at 25 °C (Table 2). These results are consistent with the previous ones: adding MβCD to the lyophilized powder did not change the enzyme activity or enantioselectivity. Washing the additive from the co-lyophilized preparation did not alter the enzyme enantioselectivity, but as previously observed, even though some activity was lost during the washing step the residual activity was still 26 times higher than that of the lyophilized powder (Table 2).

A possible explanation for the activity loss observed as the result of the washing procedure could be that this step leads to some degree of enzyme denaturation. Fourier transform infrared (FTIR) spectroscopy experiments were conducted to address these questions. The spectra obtained for the different preparations were analyzed by calculating the spectral correlation coefficient (SCC) between the second derivative amide I spectrum of subtilisin in aqueous solution and lyophilized subtilisin suspended in THF. Identical spectra produce a SCC value of 1 and values lower than 1 reveal protein structural perturbations. The SCC calculated for the co-lyophilized powder at a water activity of 0.11 was 0.78 ± 0.05 and that calculated for the washed enzyme was 0.73 ± 0.03. For the powder lyophilized without additives, subsequent washing steps to some extent improved the secondary structure resulting in SCC values closer to 1 (Table 3). The enzyme activity (determined from the model transesterification reaction between *N*-acetyl-L-phenylalanine ethyl ester and 1-propanol) was not influenced by these washing steps. No structural changes were noted when washing the powder containing MβCD (Table 3) even though activity was influenced by the washing procedure. These data exclude global structural changes being responsible for the observed activity drop (by a factor of 7 in neat THF, and by a factor of 5 in THF at a a_w of 0.11). In this context we also studied the behavior of the enzyme co-lyophilized with other macrocycles known to activate subtilisin, namely crown ethers [25]. The findings were similar to those obtained with MβCD: washing did not cause marked structural changes and the activity was reduced to some extent. However, under all circumstances and even after the washing steps, the powder was much more active than the powder lyophilized without these additives.

To ensure that no additive was left after the washing steps NMR and FTIR studies were conducted (see Section 2 for details). In the case of MβCD it was found that no additive was left after two washing steps. In the case of 18-crown-6, 10 washing steps were necessary to completely remove the additive. When comparing the activity data to the residual additive content, it is evident that the first washing step is largely responsible for reducing the enzyme activity (Table 3). Further washing resulted in complete additive removal and did not cause any substantial decrease in activity.

We are presently interpreting these data as follows: the larger fraction of the enzyme activation observed is probably due to a lyoprotection effect. It is evident from our results that the protein has to be co-lyophilized with the additive to achieve this activation effect. After complete removal of the additives

Table 2
Effect of MβCD on subtilisin activity and enantioselectivity in THF, at a constant water activity[a]

Entry	Preparation	MβCD added[b]	Washed[c]	Enantioselectivity ($a_w = 0.11$)[d]	V_S ($a_w = 0.11$)[e]
1	Lyophilized	No	No	15.1 ± 2.4	0.0034 ± 0.00059
2	Lyophilized	Yes	No	18.2 ± 1.6	0.0021 ± 0.00011
3	Co-lyophilized[f]	No	No	31.5 ± 3.16	0.290 ± 0.017
4	Co-lyophilized	No	Yes	28.5 ± 3.8	0.062 ± 0.0071

[a] Reaction conditions: transesterification between sec-phenethyl alcohol and vinyl butyrate at 25 °C.
[b] MβCD was added directly to the organic solvent–enzyme mixture.
[c] MβCD was removed from the co-lyophilizate by washing with THF three times, see Section 2 for details.
[d] Enantioselectivity was determined from the initial rates for the formation of the enantiomeric products, see Section 2 for details.
[e] Initial velocity for the formation of the *S* enantiomer (μmol min^{-1} mg^{-1}).
[f] Subtilisin was co-lyophilized with MβCD at a 1:6 weight ratio of enzyme-to-cyclodextrin.

Table 3
Effect of the removal of additives by successive washing on the structure and activity of subtilisin in THF

Additive	Wash no.	Mole of additive bound per mole of protein[a]	SCC[b]	V_0[c]
Dried powder[d]	0	n.a.	0.65 ± 0.10	7 ± 1[g]
	1	n.a.	0.85 ± 0.00	6 ± 1
	2	n.a.	0.78 ± 0.01	7 ± 0
	3	n.a.	0.86 ± 0.01	8 ± 1
	10	n.a.	n.d.	6 ± 1
MβCD[d,e]	0	41	0.81 ± 0.01	1367 ± 34
	1	2 ± 0	0.81 ± 0.01	214 ± 14
	2	∼0	0.88 ± 0.02	213 ± 22
	3	∼0	0.83 ± 0.01	180 ± 24
	10	0	n.d.	190 ± 15
12-c-4[d,f]	0	n.d.	0.67 ± 0.01	729 ± 49[g]
	1	n.d.	0.65 ± 0.02	314 ± 44
	3	n.d.	0.76 ± 0.01	244 ± 15
	10	n.d.	n.d.	250 ± 12
15-c-5[d,f]	0	n.d	0.76 ± 0.04	482 ± 80[g]
	1	n.d.	0.70 ± 0.04	218 ± 9
	3	n.d.	0.78 ± 0.01	251 ± 66
	10	n.d.	n.d.	310 ± 30
18-c-6[d,f]	0	409	0.79 ± 0.01	538 ± 96[g]
	1	12 ± 1	0.77 ± 0.01	159 ± 50
	3	6 ± 1	0.80 ± 0.02	244 ± 26
	10	0	n.d.	235 ± 12

[a] Number of additive moles remained after different pretreatment steps determined by extrapolating the ratio of areas of additive to protein on the calibration curves.
[b] Spectral correlation coefficients were determined by using second derivative amide I spectra of subtilisin in aqueous buffer vs. the sample to be compared.
[c] Initial rates (nmol mg^{-1} min^{-1}) determined for the transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester (100 mM) with 1-propanol (1 M) in anhydrous THF at 25 °C, 250 rpm; enzyme concentration: 1 mg/ml.
[d] Lyophilized from 10 mM potassium phosphate buffer solution at pH 7.8.
[e] Co-lyophilized at a 1:2 weight ratio of protein-to-additive.
[f] Co-lyophilized at 1:4 weight ratio of protein-to-additive.
[g] Values taken from Santos et al. [25].

the enzyme activities for the formulations are still larger than for the lyophilized powder. The large activity increase is likely entirely related to preservation of the active site structure by the additives. A smaller portion of the activity increase which disappears after removal of the additives is likely related to other factors known to influence enzyme activity in organic solvents, specifically enzyme dynamics [33,36,37].

In agreement with the data presented here, previous studies have also suggested that the nature of the enzyme activation, induced by cyclodextrins, must be one of structural preservation

Table 4
Subtilisin structure (% of α-helix), activity and enantioselectivity in various solvents

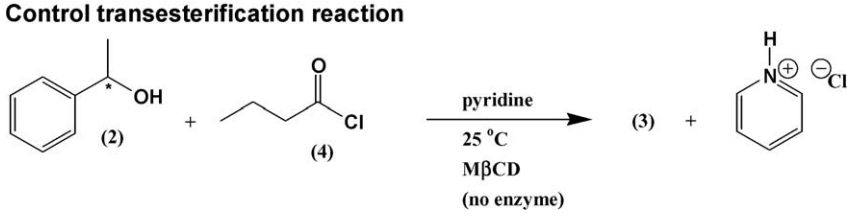
Solvents	% of native α-helix retained after suspension	Activating and enantioselectivity enhancement factor (co-lyophilized with MβCD/lyophilized powder)	
		V_S[a]	Enantioselectivity[a]
THF	94	164	1.8
1,4-Dioxane	88	112	1.4
CH_2Cl_2	76	19	1.0
Acetonitrile	65	17	1.2
Toluene	76	11	0.6
Octane	85	4	0.5

[a] Data taken from Griebenow et al. [28].

of the enzyme, and dismissed an inclusion-complex mechanism [38]. In fact, this has already been confirmed by us using FTIR spectroscopy [28]. It was found that co-lyophilization of subtilisin with MβCD reduced structural perturbations caused by the lyophilization process. The less-perturbed structure was maintained upon suspension in solvents such as THF and 1,4-dioxane (in which the enzyme was most active), while other solvents caused some structural perturbations (e.g., acetonitrile and dichloromethane), suggesting an alternate activating mechanism besides lyoprotection, perhaps, reduction of possible mass-transfer limitations (by helping to reduce the particle size [39]), or by increasing the enzyme's dynamics (Table 4).

Although the results presented so far consistently suggest that MβCD is primarily activating subtilisin by preventing structural perturbations and not by interacting with the substrates or products, we had to exclude that MβCD could in some way indeed assist in the formation of the enantiomeric product of our model reaction. A control transesterification between a racemic mixture of sec-phenethyl alcohol (2) and butyric chloride (4) in pyridine was performed in the presence of the same quantity of MβCD that was present in the enzyme-catalyzed reactions (Scheme 2). After 24 h of reaction, a racemic product was obtained indicative that for our model reaction MβCD does not promote the formation of any one enantiomer in particular.

Another argument against substrate and product complexation effects is that activation by additives such as MβCD is observed at a certain enzyme:additive ratio in co-lyophilization experiments. Below or above this optimum ratio (of MβCD-to-enzyme) the enzyme activity decreases, while its enantioselectivity remains unchanged (Fig. 1). These data are hard to reconcile with substrate and product complexation events because activity and enantioselectivity should be favored at high additive concentrations. Similarly, when enzymes such as lipases



Scheme 2. Control transesterification reaction.

Table 5
Activation of the lipase *Candida rugosa* with cyclodextrins

Enzyme	Preparation	Enantioselectivity	Initial velocity, "*R*" enantiomer (μmol min^{-1} mg^{-1})
Lipase from *Candida rugosa*	Co-lyophilized with MβCD	23.1 ± 2	0.079 ± 0.009[a]
	Lyophilized powder (no additive)	13.1 ± 0.8	0.0069 ± 0.0011[a]

[a] Determined from the initial rates of the formation of the enantiomeric products (V_R/V_S) in dichloromethane. Reaction: sec-phenethyl alcohol and vinyl butyrate, constant a_w (0.11), 25 °C.

and α-chymotrypsin are co-lyophilized with different rations of known lyoprotectants such as crown ethers and polyethylene glycol, they too display optimum activity at a certain additive-to-enzyme ratio [33], and it has been demonstrated that at high ratios of 18-crown-6 the enzyme loses its native structure [33] which might offer an explanation for the drop in activity at high additive concentrations.

Furthermore, if cyclodextrin–substrate complexation would contribute to the observed enantioselectivity increase to any degree, one would expect similar results regardless of the enzyme catalyzing the reaction. To test this, we decided to use a lipase, which actually prefer the opposite enantiomer than subtilisin. Lipase from *Candida rugosa* was activated and its enantioselectivity enhanced by co-lyophilization with MβCD in dichloromethane (one of the solvents in which this enzyme was most active, and it could be activated by MβCD). These data (Table 5) clearly demonstrates that cyclodextrins must enhance this lipase enantioselectivity by increasing its selectivity towards the "*R*" enantiomer of sec-phenethyl alcohol, the opposite enantiomer for which subtilisin is most selective. These results are in agreement with a lyoprotection mechanism rather than a cyclodextrin–substrate complex effect. Similar results have been previously obtained with the same lipase co-lyophilized with MβCD, in which the enantioselectivity towards the "*R*" enantiomer of *N*-acetyl phenylalanine was increased, while subtilisin prefers the opposite enantiomer [28]. In addition, lipase LP from Amano, co-lyophilizated with two types of cyclodextrins (β-and methyl-β-cyclodextrin) showed the same effect in the transesterification between sulcatol and vinyl acetate (data not published).

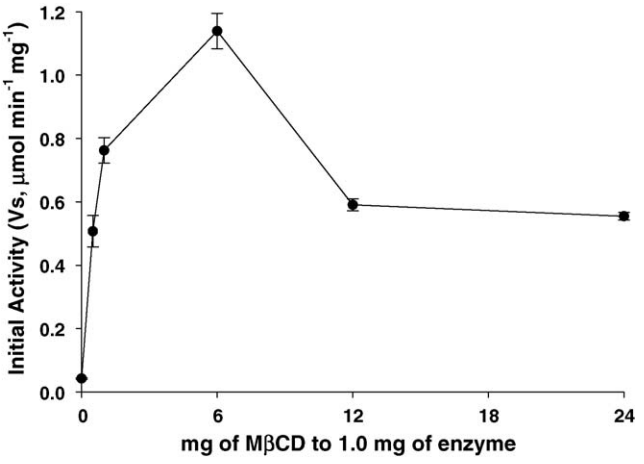


Fig. 1. Initial activity (V_S) vs. the MβCD concentration. Subtilisin C. in THF, co-lyophilized with different concentrations of MβCD. Reaction: transesterification between sec-phenethyl alcohol and vinyl butyrate.

4. Conclusions

The results presented and those previously reported on the activation and the enantioselectivity enhancement observed by co-lyophilizing an enzyme with the additive MβCD, provide ample evidence in support of a mechanism based on preservation of active site structure. Minor contributions to increased activity likely stem from other factors, such as, reduction of possible mass transport limitations and changes in enzyme dynamics. For the model reactions employed we clearly excluded any contribution to enhanced activity and enantioselectivity caused by formation of cyclodextrin–substrate or -product complexes.

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