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Subtilisin Catalysis of Substrate Anchored in Cyclodextrin

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ABSTRACT

The N-benzoyl-L-tyrosine ethyl ester (BTEE) anchored in cyclodextrin (β and β-methyl CD) serves as an excellent substrate for subtilisin Carlsberg catalysis. The rate of hydrolysis was found to be approximately twofold higher than that of the methanolic substrate. The K_{mapp} and V_{max} values for the CD anchored substrates were significantly higher than the methanol-solubilized BTEE.

Index Entries: Anchored substrate; cyclodextrin; subtilisin; catalysis.

INTRODUCTION

Enzymes are currently being extensively used in the food, beverage, and pharmaceutical industries, because both their catalytic efficiency and environment-friendly nature favor their employment for these purposes *(1)*. However, in addition to constraints imposed by their narrow substrate specificity *(2)* and thermal instability *(3)*, enzymatic catalysis of hydrophobic compounds is rather poor because of the insoluble nature of the substrates in aqueous environment. In order to overcome the substrate solubility problem, organic solvents and detergents are often used *(5)*. Though enzymes have been shown to be catalytically active in nonaqueous environments, their low activity, arising from the removal of the essential water from the surface of enzymes, is a serious disadvantage *(4)*. Although the enzymes exhibit higher activity toward detergent-solubilized substrates *(5)*, higher concentrations of detergents also inhibit the enzyme activity *(6)*. Further, at higher temperatures, detergent micelles are unsta-

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ble, and, as a result, the substrate solubility rapidly decreases, causing turbidity that interferes with the enzyme assay *(7)*. In order to mitigate these problems and render the hydrophobic substrates soluble in an aqueous environment, cyclodextrins have been used with some success in a few instances *(8–11,22)*. We report here our observations on the subtilisin catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) anchored in cyclodextrin.

MATERIALS AND METHODS

BTEE, β-cyclodextrin (β-CD), calcium chloride, and subtilisin Carlsberg (EC.3.4.21.14) were purchased from Sigma (St. Louis, MO). β-Methyl cyclodextrin (MCD), degree of substitution = 1.8, was from Wacker-Chemie GmbH, (Munich, Germany). All other reagents and chemicals used in this investigation were of analytical grade. Subtilisin Carlsberg was used as such without any further purification, since no contaminating proteins were detected apart from the 25 kDa band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue dye.

The BTEE substrate was checked for any contaminants by thin layer chromatography on a precast silica gel plate (0.2 mm), developed with chloroform:acetone (85:15). BTEE migrated as a single fluorescent spot (R_f = 0.75). High performance liquid chromatography (HPLC) (Waters, Milford, MA) analysis was carried out using a Novapack® C_{18} column (3.9 × 150mm) with 30% acetonitrile (v/v) at a flow rate of 1 mL/min. The peaks were detected at 254 nm. BTEE eluted as a single peak (retention time = 7.81 min).

The solubilization protocol of BTEE in cyclodextrin involved the addition of 100 µL of methanolic solution of BTEE (10.6 mg) to a 2.5 molar equivalent of either β-CD or MCD in a total volume of 10 mL of 0.05*M* Tris-Cl buffer, pH 8.0. Though the complete solubilization required 2 mol of cyclodextrin per mol of BTEE, 2.5 mol of CD was used to avoid any turbidity arising out of the dissociation of the guest (BTEE). The methanol–BTEE solution (control) was prepared by dissolving 15.6 mg of BTEE in 5.0 mL methanol (50%) in a volumetric flask. The maximum concentration of methanol used in the regular assay was 2% (v/v).

All the kinetic and pH-activity data were fitted to a nonlinear regression program (r = 0.96–0.99) of Sigma Plot software (Jandel Scientific). EZ-FIT Version 1.1 software developed by F. W. Perrella, E. I. DuPont de Nemours, Glenolden, PA, was also used for the calculation of the kinetic parameters.

The protein was estimated by the method of Lowry et al. *(12)*, using bovine serum albumin as standard. Both substrate and enzyme solutions were prepared fresh on the day of use.

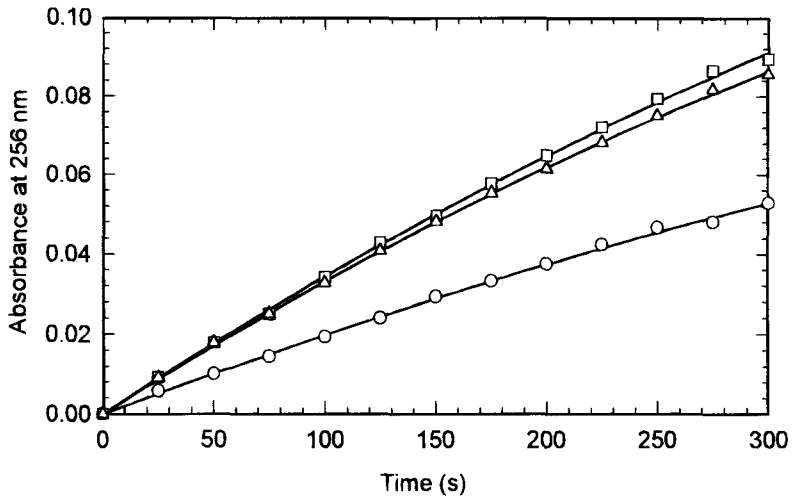

Fig. 1. Time-course of subtilisin reaction. BTEE (0.4 m*M*) in methanol (O) or anchored in β-CD (□) or MCD (△) was used. The concentration of β-CD/MCD was 2.5 times that of BTEE. Reactions were performed in 0.05 m*M* Tris-Cl buffer, pH 8.0, at 25°C containing $CaCl_2$ with 0.9 μg of enzyme per assay.

Enzyme Assay

The assay for subtilisin was carried out in a total volume of 1 mL, containing 0.05 *M* Tris-Cl buffer, pH 8.0, 0.01 *M* calcium chloride, and 0.4 m*M* BTEE. The enzyme activity was calculated from the initial phase (0–10 s) of the increase in absorbance at 256 nm, after enzyme addition to the assay mixture maintained at 25°C in a thermostatically controlled Shimadzu UV2100 double-beam, UV-visible spectrophotometer fitted with a CPS-260 cell temperature controller. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μmol of BTEE per minute under the given conditions. The activity was calculated based on the molar absorption coefficient of 810 M^{-1} cm^{-1} for the N-benzoyl-L-tyrosine at 256 nm *(13)*.

RESULTS

Time-Course of Hydrolysis of BTEE Anchored in Cyclodextrin

In the present investigation, it was observed that the BTEE anchored in cyclodextrin served as an excellent substrate for subtilisin. The progress curve for the hydrolysis of BTEE anchored in cyclodextrin, and for that solubilized in methanol, was scanned for a period of 5 min. As seen from Fig. 1, the rates of hydrolyses of all the three substrates were linear up to a period of 60–75 s, and thereafter, deviated from linearity. The rates for the β-CD- and MCD-anchored BTEE were almost twofold higher than that for

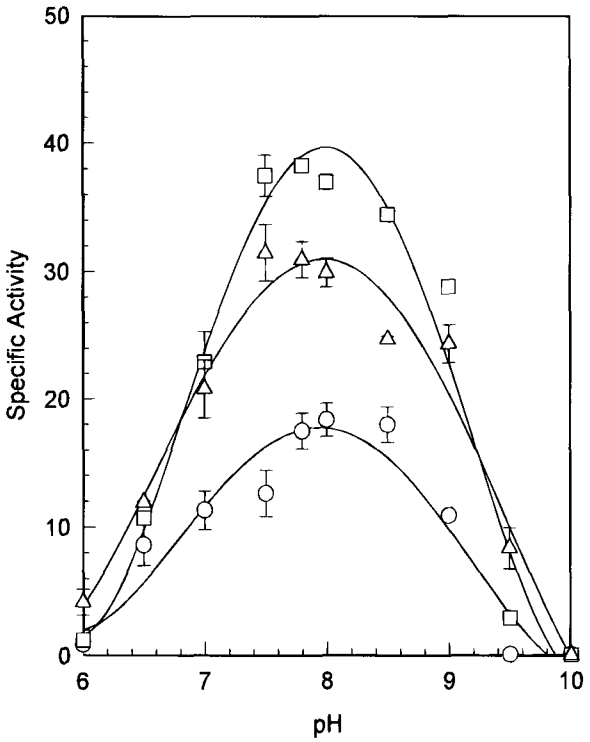

Fig. 2. pH-activity profile of the hydrolysis of BTEE. The following buffers (0.05 *M*; containing 0.01 *M* $CaCl_2$) were used: cacodylate buffer, pH 6.0–7.0; Tris-Cl buffer, pH 7.0–8.0; and glycine-NaOH buffer, pH 9.0–10.0. Respective buffer blanks were used. All other reaction conditions were same as mentioned in Fig. 1. BTEE in methanol (O) or anchored in β-CD (□) or MCD (△).

the methanol-solubilized substrate. However, the rates with both the cyclodextrin-anchored substrates were only marginally different.

The possibilities of the formation of N-benzoyl-L-tyrosine methyl ester (BT-OMe), a transesterification reaction product, was checked in order to rule out the participation of methanol in the reaction. HPLC analysis of the reaction mixture showed that the transesterifcation product is not formed (data not shown). Also, the use of Tris in the reaction did not lead to products other than the expected N-benzoyl-L-tyrosine.

Effect of pH on the Hydrolysis of Anchored Substrate

The pH-activity profile of the subtilisin catalyzed hydrolysis of BTEE anchored in cyclodextrin showed an optimum around 8.0 (Fig. 2). The pH optimum for the hydrolysis of methanol-solubilized BTEE was also around 8.0. It was observed that at pH values in the range of 6.0–9.5, there was a significant increase in the reaction rate with cyclodextrin(s)-anchored BTEE, compared to that observed with the methanolic substrate. The fact

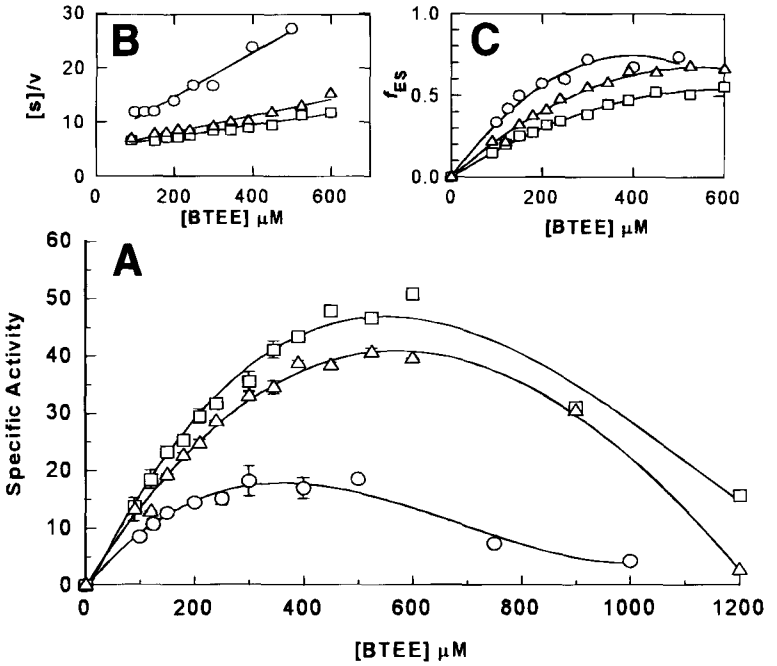

Fig. 3. **(A)** Plot of v vs S. BTEE in methanol (O) or anchored in β-CD (□) or MCD (△) was varied from 0.1–1.0 m*M* (for methanolic substrate) and 0.09–1.2 m*M* (for cyclodextrin substrate). The reactions were started with appropriate amounts of enzyme. All other reaction conditions were the same as mentioned in Fig. 1. **(B)** Analysis of the data by Hanes-Woolf plot. **(C)** Plot of the fraction of active sites (f_{ES}) of subtilisin Carlsberg filled by BTEE. Data from Fig. 3A was fitted to equation (1) to obtain the f_{ES} values; Substrate in methanol (O) or anchored in β-CD (□) or MCD (△).

that the pH profile was unaltered in the presence of cyclodextrins suggests that the ionization at the active site was unaffected by their presence.

Kinetics of the Hydrolysis of BTEE Anchored in Cyclodextrin

The plot of reaction rate as a function of increasing concentration of BTEE either as methanol-solubilized, or as CD-anchored substrate, showed similar profiles for all of the three substrate preparations (Fig. 3A). The Hanes-Woolf plot was used to derive the kinetic parameters (Table 1). The data was also fitted to the EZ-FIT software, and the values so obtained were in total agreement with those calculated from the Hanes-Woolf plot. Inhibition at higher concentration of BTEE (>400 μ*M* with methanolic BTEE, and >600 μ*M* with both β- and β-methyl-CD) was observed (Fig. 3A), and such data points were not included in the Hanes-Woolf plot (Fig. 3B). There was a noticeable difference in the rates of hydrolyses of BTEE anchored in β-CD and MCD.

Table 1
Kinetic Parameters of Subtilisin Carlsberg with CD-Anchored BTEE and Effect of α-D-Glucose and D-Sorbitol on Hydrolysis of Methanol-Solubilized BTEE

BTEE in	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Methanol	156.2 ± 2	25.0 ± 1	6.0 ± 0.4	3.8×10^4
β-CD	454.6 ± 29	91.9 ± 5	18.8 ± 1	4.1×10^4
MCD	352.9 ± 27	59.7 ± 7	12.9 ± 0.8	3.6×10^4
Methanolic BTEE +				
a. α-D-Glucose	165.8	21.2	5.8	3.5×10^4
b. D-Sorbitol	146.3	21.7	5.9	4.1×10^4

Reaction conditions in case of CD-anchored BTEE hydrolysis were as mentioned in Fig. 3. For the sugar-control experiments, BTEE in methanol was varied from 0.1 to 0.5 m*M*, with either α-D-glucose and D-sorbitol added at a concentration of 0.1 *M* to the assay mixture. The reaction was started with appropriate amounts of the enzyme. Experiments were done in triplicates, and averages computed. All other reaction conditions were identical to those mentioned in Fig. 1.

It was found that the K_{mapp} for the β-CD- and MCD-anchored BTEE were in the range of 363 and 455 μ*M* respectively; in the case of methanol-solubilized substrate, the K_{mapp} was 155 μ*M*. The V_{max} values for the cyclodextrin-anchored substrate was 2–4 fold higher than that for the methanolic substrate (Table 1). Experiments carried out at pH 7.0 also showed an increase in both K_m and V_{max} values for the CD-anchored substrates (data not shown).

In order to check whether the change in kinetic parameters was caused by polyol effect, assays of methanolic substrate were carried out in the presence of 0.1 *M* each of α-D-glucose and D-sorbitol. The results suggest that the sugar moieties *per se* have no effect on the kinetic parameters (Table 1).

Although the V_{max} values derived from various plots were higher for the BTEE in cyclodextrins, the maximum activity measured was only about one-half of these values. Since this difference appeared considerably larger than expected, we analyzed the fraction of active sites filled (f_{ES}) according to the equation (1) *(14)*:

$$f_{ES} = v/V_{max} \quad (1)$$

where v is the rate. The plot of the fraction of active sites filled vs substrate concentration (Fig. 3C) showed that the methanol-solubilized substrate could saturate 80% of the active site; the β-CD- and MCD-anchored

BTEE brought about only 54 and 65% saturation, respectively. In view of the increase in both the kinetic parameters, we also tested the effect of free β-CD (3 m*M*) either in the assay system containing methanolic substrate or in the preincubation mixture containing only the enzyme. The results suggest that free β-CD has no effect on enzyme activity (data not shown).

Studies with partially purified subtilisin BPN', an extracellular serine protease from *B. amyloliquefaciens*, showed that the activities with either cyclodextrin-anchored or methanol-solubilized BTEE were sevenfold lower than the Carlsberg enzyme. A similar diminished reactivity of subtilisin BPN' toward peptide substrates was also reported earlier *(20)*. Although the reactivity toward the synthetic substrate is lower for subtilisin BPN', compared to subtilisin Carlsberg, the increase in *Km* and *Vmax* values observed with the cyclodextrin-anchored substrate for the former were essentially similar to that of the latter (Prabhu, unpublished results).

Effect of Temperature on the Hydrolysis of BTEE Anchored in Cyclodextrin

The effect of increasing temperature on the hydrolysis of BTEE anchored in CD and of that solubilized in methanol was checked. The maximum temperature for the BTEE hydrolysis, in all three cases, was found to be around 50°C. At higher temperatures (above 60°C), the activity was reduced in all preparations. It was observed that at temperatures in the range of 20–50°C, the increase in the reaction rate with both β-CD- and MCD-anchored BTEE was significantly higher than with the methanolic substrate. Arrhenius plot was used to calculate the energy of activation (*Ea*). The *Ea* values of both β-CD- and MCD-anchored substrates were lower than the methanol-solubilised BTEE (Table 2), indicating a possible higher degree of stabilization of the transition state.

DISCUSSION

We have compared the subtilisin catalyzed hydrolysis of BTEE in β-cyclodextrin (or its methyl derivative) with that of BTEE solubilized in methanol. The results show that subtilisin can access BTEE solubilized in CD. The *Vmax* values for CD-anchored substrates were higher than the methanolic BTEE. The increase in rate can be attributed to a better productive encounter of the substrate–CD complex with the enzyme. It should, however, be noted that the observed maximum rate (V_{max}) is only one-half the value derived from the graphical plot. Evaluation of the fraction of active sites filled in the enzyme indicates only 55–65% occupancy of the available sites (Fig. 3C). Because, at higher substrate concentration, there is inhibition of activity, it is not surprising to find that under actual assay conditions, there is a larger apparent decrease in V_{max} value.

Table 2
Activation Energy Parameters for the Hydrolysis of Cyclodextrin-Anchored BTEE

Substrate in	*Ea* (k_{cal}/mol)
Methanol	5.47
β-CD	4.04
MCD	3.36

Reaction mixture contained 0.02 *M* HEPES buffer, pH 8.0, with 0.01 *M* $CaCl_2$. The temperature was varied from 25°C to 50°C. The buffered substrate solution was preincubated for 30 min in order to ensure correct temperature. All other conditions were the same as mentioned in Fig. 1. Experiments were done in duplicates and averages computed.

It is known that some enzymes exhibit one-half of the site reactivity *(26)*. Although the presence of two active sites in serine proteases has been speculated *(25)*, X-ray crystallographic studies of α-chymotrypsin *(17)* and subtilisin *(21)* do not provide any evidence for the presence of two distinct active sites for consideration of one-half of the site reactivity. However, the existence of subsites in serine proteases *(18,19,24)*, and a second inhibitor site in subtilisin, is well-recognized *(23)*. Therefore, at higher concentrations, the synthetic substrate (either as CD-anchored or methanol-solubilized) may bind to one of these subsites and reduce the activity.

In addition to V_{max}, K_m is also considerably increased with CD-anchored substrates (Table 1). If the CD–substrate complex were to bind to the enzyme *per se*, then the observed change in K_m could indicate distortion of the active site. It may be relevant to mention that the active site hydrophobic pocket present in subtilisin is wider and more exposed than in α-chymotrypsin *(15–17,23)* and could accommodate the CD-complex with some slight structural adjustments. Instead of the complex, if free BTEE were to be the actual substrate, then the concentration of the free species will be much lower in the presence of CD. In that case, the true $K_m$may be several-fold lower than the observed apparent K_m. Then the actual k_{cal}/K_m will be significantly higher than that shown in Table 1.

While the *Km* and V_{max} values were substantially altered in subtilisin on using CD-anchored substrates, these parameters were found to be unaffected in α-chymotrypsin *(7)*, suggesting that in these two members of the family of serine proteases, the active site topology is quite distinct.

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