Protection Afforded by Maltosyl-β-cyclodextrin Against α-Chymotrypsin-Catalyzed Hydrolysis of a Luteinizing Hormone-Releasing Hormone Agonist, Buserelin Acetate

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Purpose. The present study addresses how maltosyl-β-cyclodextrin (G_2-β-CyD) impacts upon the α-chymotrypsin-catalyzed hydrolysis of buserelin acetate, an agonist of luteinizing hormone-releasing hormone with emphasis upon the direct effect of G_2-β-CyD on the activity of the protease.

Methods. Kinetic and solubility studies were performed in isotonic phosphate buffer (pH 7.4) at 25°C and 37°C. The interaction of α-chymotrypsin with G_2-β-CyD in the buffer solution was examined by differential scanning calorimetry.

Results. G_2-β-CyD decelerated the α-chymotrypsin-catalyzed hydrolysis of buserelin acetate to give the 1–3 tripeptide and the 4–9 hexapeptide fragments. This deceleration can be explained solely by a non-productive encounter between a complex of the substrate with G_2-β-CyD and the protease at relatively low CyD concentrations, while the direct inhibitory effect of G_2-β-CyD on the proteolytic activity made a considerable contribution to the overall deceleration of the hydrolysis at higher CyD concentrations. Calorimetric studies indicate the presence of intermediate states in the thermal unfolding of α-chymotrypsin, simultaneously accompanied by the autolysis. By contrast, a two-state thermal unfolding of α-chymotrypsin was observed in the presence of G_2-β-CyD, suggesting reduced proteolytic activity upon binding to G_2-β-CyD.

Conclusions. These results suggest that G_2-β-CyD at higher concentrations inhibits the proteolytic action of α-chymotrypsin through direct interaction with the protease, as well as through the formation of a non-productive complex with the substrate.

KEY WORDS: buserelin acetate; maltosyl-β-cyclodextrin; complexation; α-chymotrypsin-catalyzed hydrolysis; thermal unfolding.

INTRODUCTION

The paradoxical effect of buserelin acetate, a superagonist of luteinizing hormone-releasing hormone (LHRH), has been utilized clinically for the treatment of endocrine-dependent disorders such as endometriosis, precocious puberty, and leiomyoma (1). In the chemical structure of buserelin acetate, a D-amino acid and proline-ethylamide substitute for the glycine residue in the 6th position and for the C-terminal sequence proline-glycinamide, respectively, of the native LHRH sequence. Although these modifications conspicuously decrease the susceptibility of the LHRH agonist to proteolytic enzymes, it is still deactivated at various mucosal administration sites (2).

Our previous studies have demonstrated that chemically-modified cyclodextrins are effective in improving the rate and extent of nasal bioavailability of buserelin acetate in rats (3). The absorption enhancement afforded by the cyclodextrins can be attributed primarily to their ability to reduce the permeation barrier of the nasal epithelium to buserelin acetate. In addition, the cyclodextrins, especially 6-*O*-maltosyl-β-cyclodextrin (G_2-β-CyD), significantly inhibited the enzymatic degradation of buserelin acetate in rat nasal mucosa. On the basis of the inclusion mode of buserelin acetate with the cyclodextrins (4), they may protect buserelin acetate sterically from proteolytic enzymes, by including hydrophobic side chains of the peptide within the cavity, because these binding sites are located near the enzymatic cleavage sites of the peptide. Although some report the inhibitory effects of cyclodextrins on the enzymatic degradation of peptides (5), little is known about the direct interaction of cyclodextrins with proteolytic enzymes and its contribution to the overall deceleration of the proteolysis. Our previous studies indicate that the possibility for the cyclodextrins to inactivate directly the proteolytic enzymes should not be totally dismissed (3,6).

Following up these studies, this paper deals with the effect of G_2-β-CyD on the hydrolysis of buserelin acetate catalyzed by α-chymotrypsin, a typical serine protease, and discusses the mechanisms by which G_2-β-CyD inhibits the hydrolysis with emphasis upon its direct effect on the activity of the protease.

MATERIALS AND METHODS

Materials

Buserelin acetate, its 1–3 tripeptide and 4–9 hexapeptide fragments (Hoechst Japan Ltd., Saitama, Japan) were used without further purification. G_2-β-CyD was a generous gift from Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan). Bovine pancreatic α-chymotrypsin (EC 3.4.21.1, 25 kDa, 50000 U/g) and its substrate, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methyl-coumaryl-7-amide (MCA) (7) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Peptide Institute, Inc. (Osaka, Japan), respectively. Other materials were of reagent grade and deionized double-distilled water was used.

Kinetic Studies

Alpha-chymotrypsin (2 mU) was dissolved in 0.2 ml of 40 mM phosphate buffer (pH 7.4) containing 116 mM NaCl. The protease solution was added to the buffer solution (0.8 ml, pH 7.4) containing buserelin acetate and G_2-β-CyD at various concentrations and incubated at 37°C. At an appropriate interval, a 0.1 ml aliquot of the mixture was withdrawn and added to 0.1 N hydrochloric acid solution (1 ml) to terminate the enzymatic reaction. The residual buserelin in the reaction mixture was determined by high performance liquid chromatography (HPLC) as previously described (3). The reaction products were identified by the retention times with authentic samples.

The proteolytic activity of α-chymotrypsin (10 U/l, 8 mM) in the absence and presence of G_2-β-CyD (10–80 mM) was

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determined by monitoring the hydrolysis of the MCA substrate (20–200 μM) by the protease in the buffer solution (pH 7.4) at 25°C. The concentration of the MCA fluorophore released was determined fluorometrically, using a Hitachi F-4010 spectrofluorometer (Tokyo, Japan). The excitation and emission wavelengths for the MCA fluorophore were 380 and 440 nm, respectively. The apparent 1:1 stability constant for the complex of the MCA substrate with G_2-β-CyD was determined by the fluorescence method and use of the Scott's equation (8).

Solubility Studies

A constant and excess amount (10 mg) of buserelin acetate was added to the buffer solution (1 ml, pH 7.4) containing a given concentration of G_2-β-CyD. After being shaken vigorously for 6 h, an aliquot was filtered through a cellulose acetate membrane, Advantec Dismic 13CP045AN (Toyo-Roshi Co., Tokyo, Japan). The concentration of buserelin acetate in the filtrate was determined spectrophotometrically at 280 nm. The apparent 1:1 stability constant for the complex of buserelin acetate with G_2-β-CyD was calculated from the linear portion of the solubility diagram according to the method of Higuchi and Connors (9).

Calorimetric Studies

Scanning calorimetric measurements were made using an MC-2 adiabatic differential microcalorimeter (MicroCal, Inc., Northampton, MA, U.S.A.) using the MicroCal ORIGIN software package for data acquisition and analysis. All solutions were degassed under a vacuum before being loaded into the calorimeter cells. The calorimetric scans were performed at a rate of 1°C/min in the temperature range from 20 to 70°C under an excessive N_2 pressure at about 2.2 kg/cm^2. After subtracting the reference buffer data, the raw data in the form of heat capacity as a function of measuring temperature were converted to excess molar heat capacity using the scan rate and the protease concentration.

For a strictly two-state reversible process, the van't Hoff enthalpy (ΔH_v) can be estimated from the differential scanning calorimetric (DSC) curve according to the following equation:

$$\Delta H_v = 4RT^2_{max}(c_{max}/\Delta h_d) \quad (1)$$

where R is the gas constant, T_{max} is the absolute temperature at which the excess specific heat reaches the maximal value, c_{max} is the maximal value of the excess specific heat, and Δh_d is the specific enthalpy change (10).

RESULTS AND DISCUSSION

Figure 1 shows typical HPLC chromatograms of reaction mixtures of buserelin acetate after a 4 h incubation with α-chymotrypsin (2 U/l) in the absence and presence of G_2-β-CyD (32 mM) in isotonic phosphate buffer (pH 7.4) at 37°C, where the initial concentration of buserelin acetate was 160 μM. Alpha-chymotrypsin is known to catalyze the hydrolysis of peptide bonds in which the reactive carbonyl group belongs to the L-amino acids such as tryptophan, tyrosine, phenylalanine and, to a lesser extent, leucine and methionine (11). Under the present condition, irrespective of the presence of G_2-β-CyD, the major metabolites were the 1–3 tripeptide and the 4–9 hexapeptide fragments of buserelin acetate, i.e., the predominant cleavage of the L-tryptophan-L-serine bond in the nonapeptide as shown in Fig. 1.

At an initial concentration of 40 μM buserelin acetate in isotonic phosphate buffer (pH 7.4) at 37°C, the α-chymotrypsin (2 U/l)-catalyzed hydrolysis of the peptide followed pseudo-first order kinetics. As shown in Fig. 2-A, the apparent rate constant (k_{obs}) for the hydrolysis of buserelin acetate decreased with an increase in the concentration of G_2-β-CyD added. The dependence of the k_{obs} value on the G_2-β-CyD concentration was quantitatively analyzed according to the following equation (12), to obtain the stability constant (K_C) and rate constant (k_C) of a 1:1 complex of buserelin acetate with G_2-β-CyD:

$$\frac{[G_2\text{-}\beta\text{-}CyD]_t}{k_0 - k_{obs}} = \frac{1}{k_0 - k_C} \cdot [G_2\text{-}\beta\text{-}CyD]_t + \frac{1}{K_C \cdot (k_0 - k_C)} \quad (2)$$

where k_0 and $[G_2\text{-}\beta\text{-}CyD]_t$ are the rate constant in the absence of G_2-β-CyD and the total concentration of G_2-β-CyD, respectively. The plots according to equation (2) gave a good straight line with a correlation coefficient of 0.999 (Fig. 2-B) and the K_C and k_C values were determined to be 510 M^{-1} and 1.2 × 10^{-3} h^{-1}, respectively. The catalytic cycle of α-chymotrypsin is known to be initiated by the binding of a substrate molecule to the active site in such a way that its hydrophobic positioning group fits into a hydrophobic pocket at the active site of the protease (13). Our previous studies indicate that the aromatic side chains of buserelin acetate such as L-tryptophan and L-tyrosine residues are incorporated into the cavity of G_2-β-CyD (3,4), a situation which may prevent the access of these positioning groups to the hydrophobic pocket of α-chymotrypsin, eventually leading to the deceleration of the hydrolysis. Furthermore, conformational changes in buserelin acetate, upon binding to G_2-β-CyD, may alter the susceptibility of the peptide to α-chymotrypsin (4).

Figure 3 shows the phase solubility diagram obtained for buserelin acetate with G_2-β-CyD in isotonic phosphate buffer (pH 7.4) at 25°C. The solubility of buserelin acetate increased linearly with a rise in the G_2-β-CyD concentration. From the linear portion of the phase solubility diagram, the apparent 1:1 stability constant for the complex of buserelin acetate with G_2-β-CyD was determined to be 38 M^{-1}. In addition, our previous studies (3) have provided an estimate of the K_c value of 59 ± 4 M^{-1} for the buserelin acetate:G_2-β-CyD complex, which was determined by the fluorescence method under the conditions similar to those in Figs. 2 and 3. Nevertheless, the apparent K_c value (510 M^{-1}) estimated from the kinetic data is much greater than that obtained by the solubility and fluorescence methods. This discrepancy may be due in part to the the direct effect of G_2-β-CyD on the proteolytic activity of α-chymotrypsin.

To evaluate the contribution of the interaction between G_2-β-CyD and α-chymotrypsin to the overall deceleration of the hydrolysis of buserelin acetate catalyzed by the protease, the following kinetic studies were carried out. Instead of buserelin acetate, the MCA substrate was chosen here for the convenience of kinetic measurement and for avoiding two complications; namely the fact that buserelin acetate showed substrate inhibi-

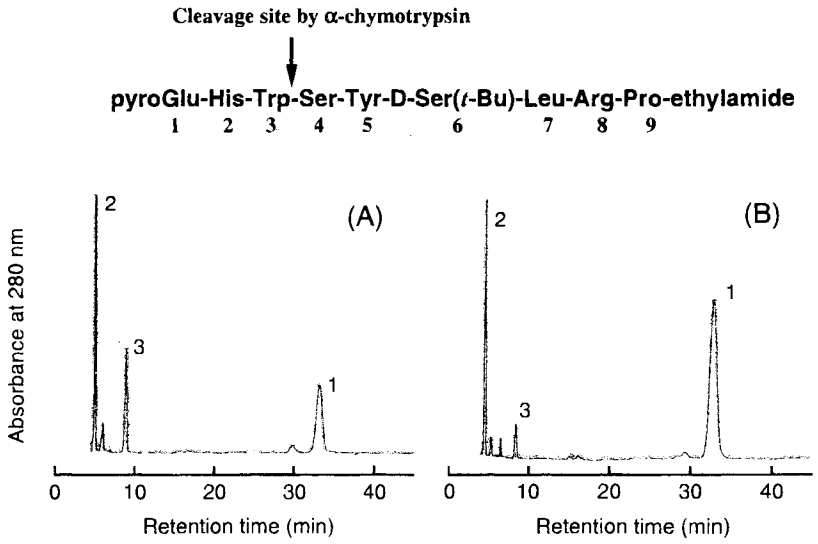

Fig. 1. Typical HPLC chromatograms of reaction mixtures of buserelin acetate after 4 h incubation with α-chymotrypsin (2 U/l) in the absence and presence of G_2-β-CyD (32 mM) in isotonic phosphate buffer (pH 7.4) at 37°C. The initial concentration of buserelin acetate was 160 μM. (A):buserelin acetate alone, (B): with G_2-β-CyD. (1):buserelin, (2): the 1–3 tripeptide fragment, (3): the 4–9 hexapeptide fragment.

tion under the present conditions and also, its CyD complex had the structural heterogeneity (4). Figure 4 shows Lineweaver-Burk plots for the inhibitory effect of G_2-β-CyD on the hydrolysis of the MCA substrate catalyzed by α-chymotrypsin. In Fig. 4-A, the reciprocal of the total concentration of the substrate, $[S]_t^{-1}$, was used in an abscissa. A family of straight lines converged to a common intercept on a vertical axis, showing competitive inhibition.

On the other hand, in Fig. 4-B, the reciprocal of the free concentration of the MCA substrate, $[S]_f^{-1}$, was used in an abscissa. Assuming a non-productive encounter between the complex of the MCA substrate with G_2-β-CyD and α-chymotrypsin, the kinetic data can be analyzed according to the following equation:

$$\frac{1}{v} = \left(1 + \frac{[G_2\text{-}\beta\text{-}CyD]_t}{K_i}\right) \cdot \frac{K_m}{V_{max}} \cdot \frac{1}{[S]_f} + \left(1 + \frac{[G_2\text{-}\beta\text{-}CyD]_t}{K_i'}\right) \cdot \frac{1}{V_{max}} \quad (3)$$

$$[S]_f = [S]_t \cdot \left(1 - \frac{K_c \cdot [G_2\text{-}\beta\text{-}CyD]_t}{1 + K_c \cdot [G_2\text{-}\beta\text{-}CyD]_t}\right) \quad (4)$$

where v is the initial hydrolysis rate, V_{max} is the maximum hydrolysis rate, K_m is the Michaelis constant, K_i is the dissociation constant of the α-chymotrypsin:G_2-β-CyD complex, and K_i' is the dissociation constant of a ternary complex of α-chymotrypsin, the MCA substrate, and G_2-β-CyD, respectively.

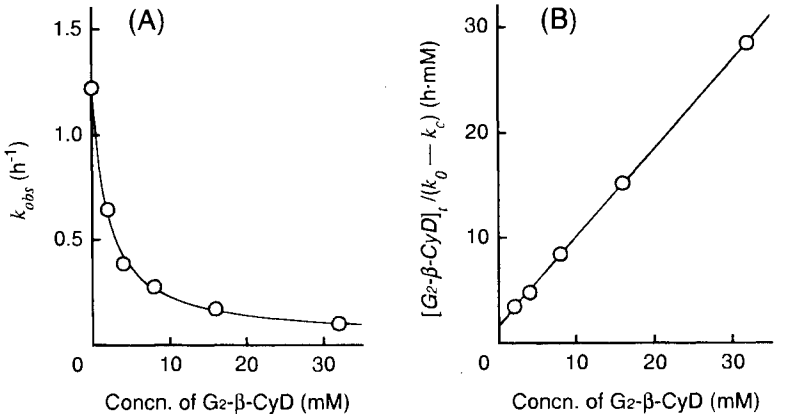

Fig. 2. Effect of G_2-β-CyD on the hydrolysis of buserelin acetate by α-chymotrypsin (2 U/l) in isotonic phosphate buffer (pH 7.4) at 37°C. The initial concentration of buserelin acetate was 40 μM. Each value represents the mean of duplicate experiments, which coincide with each other within ±5%.

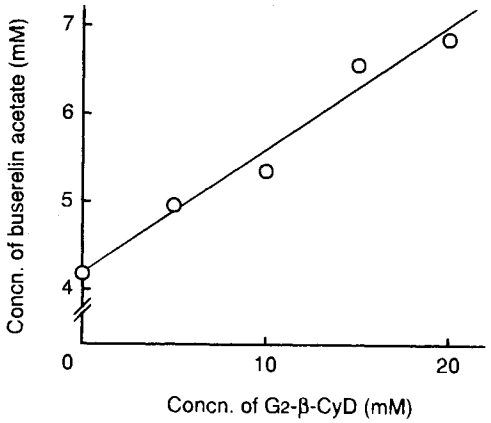

Fig. 3. Phase solubility diagram of buserelin acetate:G_2-β-CyD system in isotonic phosphate buffer (pH 7.4) at 25°C.

Under the present condition, the K_c value for the substrate:G_2-β-CyD complex was determined to be 319 ± 13 M^{-1}, and was used for estimating the concentration of the free substrate, $[S]_f$, according to the equation (4). In Fig. 4-B, when the concentration of G_2-β-CyD was less than 30 mM, the plots for all the systems fell into a common straight line, indicating that the G_2-β-CyD mediated inhibition of the hydrolysis can be solely explained by a non-productive encounter between the complexed substrate and α-chymotrypsin. Upon further addition of G_2-β-CyD, the plots showed a positive deviation, suggesting the contribution of the direct inhibitory effect of G_2-β-CyD on the α-chymotrypsin activity, giving a K_i value of 231 ± 87 mM (Fig. 4-B). Under the present condition, high cncentrations (500 mM) of glucose and maltose, which are the constituent unit and the branched sugar moiety of G_2-β-CyD, do not affect the α-chymotrypsin-catalyzed hydrolysis of the MCA substrate, indicating the crucial role of the macrocyclic cavity of G_2-β-CyD for the deceleration of the hydrolysis. This view is supported by a recent study in which the enthalpy of activation for the α-chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester increased when the substrate was solubilized in β-CyD, although other kinetic parameters were changed (14).

Further insight into the direct interaction between G_2-β-CyD and α-chymotrypsin was gained by differential scanning calorimetry (DSC). Figure 5 shows the effects G_2-β-CyD (0.1 M) and maltose (1 M) have on the excess heat capacity curve for thermal unfolding of α-chymotrypsin (80 μM) in isotonic phosphate buffer (pH 7.4). Rescanning of the heated sample showed the denaturation to be completely irreversible under the present condition. The mean unfolding temperature (T_m) and the calorimetric enthalpy (ΔH_c) were estimated from the DSC curves, with the results given in Table I. On the other hand, the van't Hoff enthalpy (ΔH_v) can be estimated from the DSC curves, assuming a strictly two-state reversible process (Table I). In the case of α-chymotrypsin alone, the ΔH_v value was about half of the ΔH_c value, suggesting the presence of intermediate states in the unfolding process, presumably due to the autolysis (10).

By contrast, these quantities for the unfolding of α-chymotrypsin with G_2-β-CyD were nearly the same, indicating that the denaturation is very close to a two-state process. A similar two-state denaturation of α-chymotrypsin has been observed in the pH range 2–4, in which the protease is no longer reactive (15). It is well known that maltose stabilizes proteins against thermal denaturation through its structure-making effect on the surrounding water molecules (16). In fact, maltose increased the T_m value of α-chymotrypsin by approximately 5°C, but did not affect the $\Delta H_v/\Delta H_c$ ratio. In the case of G_2-β-CyD, the conformational energy of the unfolded α-chymotrypsin is likely to be reduced by incorporating the exposed hydrophobic groups in the unfolding protein into the CyD cavity (17), which may compensate fully for the thermal stabilization arising from the branched sugar moiety. Rather small ΔH_c value of α-chymotrypsin with G_2-β-CyD may be ascribable to the binding of G_2-

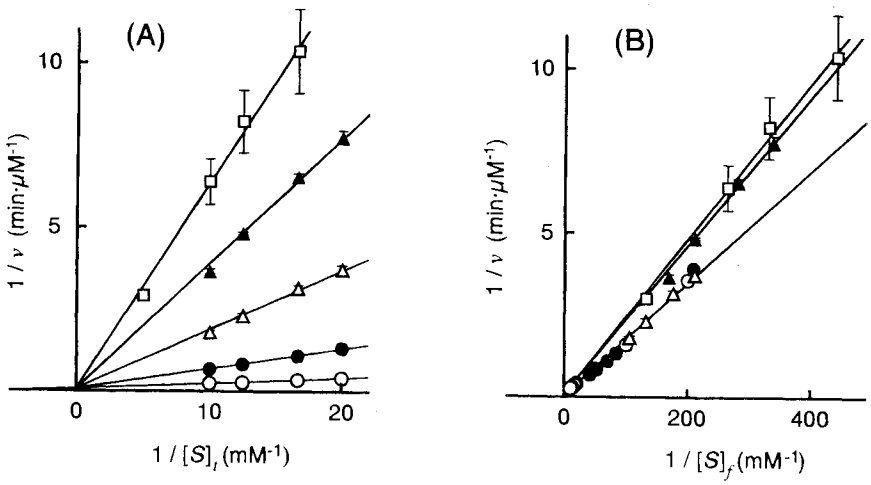

Fig. 4. Lineweaver-Burk plots for the hydrolysis of the MCA substrate by α-chymotrypsin (10 U/l, 8 nM) in the absence and presence of G_2-β-CyD in isotonic phosphate buffer (pH 7.4) at 25°C. (A): the reciprocal of the total concentration of the substrate was used in an abscissa, (B): the reciprocal of the free concentration of the substrate was used in an abscissa. (○): the substrate alone, (●): with 10 mM G_2-β-CyD, (△): with 30 mM G_2-β-CyD, (▲): with 50 mM G_2-β-CyD, (□): with 80 mM G_2-β-CyD. Each value represents the mean ±S.E. of 2–5 experiments.

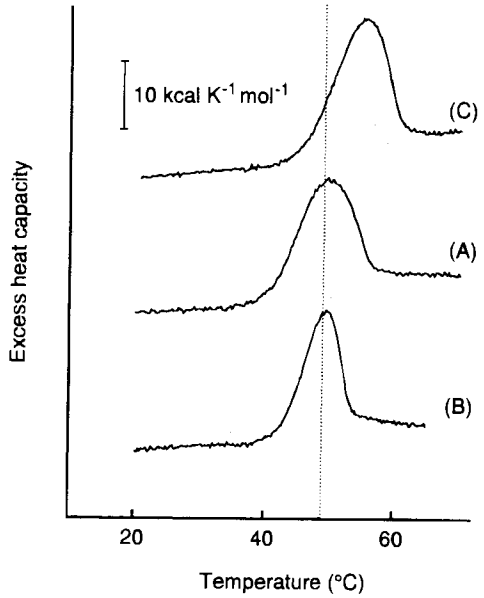

Fig. 5. Effects of G_2-β-CyD (0.1 M) and maltose (1 M) on the excess heat capacity curve for the thermal unfolding of α-chymotrypsin (80 μM) in isotonic phosphate buffer (pH 7.4). The dotted line represents the T_m value for α-chymotrypsin alone. (A):α-chymotrypsin alone, (B): with G_2-β-CyD, (C): with maltose.

β-CyD to aromatic amino acid residues of the protease, a process which is known to be endothermic (18). These results indicate that G_2-β-CyD reduces the catalytic activity of α-chymotrypsin in such a way that the accessible hydrophobic side chains of the protease may be incorporated into the CyD cavity, a situation which should produce some localized distortion and/or steric hindrance near the catalytic site of the protease.

Table I. Thermodynamic Parameters of α-Chymotrypsin (80 μM) in the Absence and Presence of G_2-β-CyD (0.1 M) and Maltose (1 M) in Isotonic Phosphate Buffer (pH 7.4)

System	T_m (°C)	ΔH_c (kcal/mol)	ΔH_v (kcal/mol)	$\frac{\Delta H_v}{\Delta H_c}$
α-chymotrypsin alone	48.9 ± 0.2	167 ± 2	81 ± 1	0.49 ± 0.02
With G_2-β-CyD	48.7 ± 0.1	126 ± 6	116 ± 2	0.93 ± 0.06
With maltose	54.2 ± 0.4	188 ± 14	86 ± 3	0.46 ± 0.05

Note: Each value represents the mean ± S.D. of 3 experiments.

CONCLUSIONS

The results obtained suggest that G_2-β-CyD at higher concentrations inhibits both the proteolytic action of α-chymotrypsin through direct interaction with the protease, as well as the formation of a non-productive complex with the substrate, however further studies are required to gain an insight into the amino acid residues or segments responsible for the observed reduction in the catalytic activity of the protease.

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