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Improved functional properties of trypsin modified by monosubstituted amino-β-cyclodextrins

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Abstract

Bovine pancreatic trypsin was chemically modified by several β-cyclodextrin (β-CD) derivatives using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as coupling agent. The modifying agents used were mono-6-amino-6-deoxy-β-cyclodextrin ($CDNH_2$), mono-6-ethylenediamino-6-deoxy-β-cyclodextrin (CDEN), mono-6-propylenediamino-6-deoxy-β-cyclodextrin (CDPN) and mono-6-butylenediamino-6-deoxy-β-cyclodextrin (CDBN). The enzyme–cyclodextrin conjugates contained about 2 mol of oligosaccharide per mol of trypsin. The catalytic and thermal stability properties of trypsin were improved by the attachment of cyclodextrin residues, and these effects were markedly noticeable for cyclodextrin (CD) derivatives having an even number of carbon atoms in the spacer arms. The thermostability of the enzyme was increased by about 2.4–14.5 °C after modification. The conjugates prepared were also more stable against thermal incubation at different temperatures ranging from 45 to 60 °C. In comparison with native trypsin, the enzyme–cyclodextrin complexes were markedly more resistant to autolytic degradation at pH 9.0. Attending to the results here reported, we suggest that conjugation of enzymes with β-CD derivatives might be an useful method for improving the stability and the catalytic properties of these biocatalysts.

Keywords: Trypsin; β-Cyclodextrin; Modified enzyme; Enzyme stability

1. Introduction

It is well known the stabilizing effect conferred by the sugar chains to glycoproteins [1]. Attending to this fact, carbohydrates have historically been used as versatile modifier agents for enzymes in order to increase the functional stability of these biocatalysts [2]. In this regard, a great number of non-ionic and ionic polysaccharides such as dextran [2,3], polymerized sucrose [4], pectin [5], chitosan [6,7], sodium alginate [8] and carboxymethylcellulose [9] have been successfully employed as modifying enzymes. In addition, relevant studies have been devoted to stabilize enzymes by the chemical attachment of mono- and oligosaccharide residues [2,10].

Cyclodextrins (CDs) are a family of cyclic non-reducting oligomers composed of 6, 7 or 8 α-(1 → 4)-linked D-glucopyranose units in the $^4C_1$ chair conformation, which are named α-, β- and γ-CDs, respectively [11]. The structure of these remarkable molecular receptors resembles a truncated annular cone with a central cavity, which is hydrophobic

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in nature and has the appropriate size to include a wide variety of hydrophobic guest compounds [12]. The formation of such adducts has been extensively studied in the latest years due to their potential applications in pharmaceutics, catalysis, chromatography, enzyme mimicking and design of supramolecular architectures [13]. CDs can be chemically modified to generate a variety of derivatives, including amines, thiols, aldehydes, etc. [14]. These modified oligosaccharides can be bond to polymers, peptides and surfaces of different nature or nanoparticles. Several elegant examples of drug-delivery systems, artificial enzymes and sensing devices based in CD conjugates can be found in the literature [11–13].

The possible effects of the covalent modification by chemically activated CDs on the conformational stability of the enzyme have not been previously studied.

As a part of our interest in improving the stability of enzymes [5–9], we here propose the use of several amino derivatives of β-CD, named mono-6-amino-6-deoxy-β-cyclodextrin ($CDNH_2$), mono-6-ethylenediamino-6-deoxy-β-cyclodextrin (CDEN), mono-6-propylenediamino-6-deoxy-β-cyclodextrin (CDPN) and mono-6-butylenediamino-6-deoxy-β-cyclodextrin (CDBN) as modifying agents for enzymes as a novel strategy. These β-CD derivatives can be easily prepared by well-established procedures [15,16], and coupled to aspartic and glutamic acid residues from proteins under very mild conditions. As target enzyme we selected bovine pancreatic trypsin (EC 3.4.21.4), a serine protease that has important industrial and biomedical applications [17].

2. Experimental procedures

2.1. Materials

Bovine pancreatic trypsin, Fractogel EMD BioSEC (S), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) were obtained from Merck (Darmstadt, Germany). β-CD was purchased from Amaizo (Indiana, USA) and used as received. CM-Sephadex C-25 was purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Synthesis of CD derivatives

The β-CD derivatives ($CDNH_2$, CDEN, CDPN and CDBN) were obtained by treating the corresponding mono-6-*O*-tosyl derivative, prepared according to Zhong et al. [18], with 35% aqueous ammonia [15] or the appropriate freshly distilled diamine [16]. The products were purified by ion exchange chromatography on CM-Sephadex C-25 (NH_4^+ form). The purity and identity of these products was checked by TLC, ^1H and ^{13}C NMR and positive-ion FABMS.

$CDNH_2$: ^1H NMR (D_2O) δ 2.75 (dd, 1H, H-6′), 2.98 (dd, 1H, H-6′), 3.40 (t, 1H, H-4′), 3.47–4.05 (m, 39H, H-2, H-3, H-4, H-5, H-5′, H-6), 5.05 (m, 7H, H-1). ^{13}C NMR (D_2O) δ 36.2 (CH_3), 52.8 (C-6′), 61.5 (C-6), 71.5 (C-5′), 72.9, 73.21 (C-3, C-5), 74.2 (C-2), 82.4 (C-4), 85.2 (C-4′), 103.0 (C-1). FABMS *m/z* 1169.3 $(M + H_2O + H)^+$.

CDEN: ^1H NMR (D_2O) δ 2.7–2.9 (m, 3H, H-6′, NCH_2), 3.10 (dd, 1H, H-6′), 3.21 (t, 2H, NCH_2), 3.45 (t, 1H, H-4′), 3.5–4.1 (m, 39H, H-2, H-3, H-4, H-5, H-5′, H-6), 5.1 (m, 7H, H-1). ^{13}C NMR (D_2O) δ 27.6 (CH_2NH_2), 41.1 (CH_2NH), 52.6 (C-6′), 61.2 (C-6), 71.4 (C-5′), 72.9, 73.0 (C-3, C-5), 74.3 (C-2), 82.2 (C-4), 85.5 (C-4′), 103.0 (C-1). FABMS *m/z* 1177.2 $(M + H)^+$.

CDPN: ^1H NMR (D_2O) δ 2.5 (m, 2H, CH_2) 2.7–2.9 (m, 3H, H-6′, NCH_2), 3.11 (dd, 1H, H-6′), 3.23 (t, 2H, CH_2), 3.47 (t, 1H, H-4′), 3.5–4.1 (m, 39H, H-2, H-3, H-4, H-5, H-5′, H-6), 5.1 (m, 7H, H-1). ^{13}C NMR (D_2O) δ 21.5 (CH_2), 26.1 (CH_2NH_2), 40.1 (CH_2NH), 52.9 (C-6′), 60.8 (C-6), 71.5 (C-5′), 72.5–73.0 (C-3, C-5), 74.3 (C-2), 82.9 (C-4), 86.5 (C-4′), 101.9 (C-1). FABMS *m/z* 1191.1 $(M + H)^+$, 1214.2 $(M + Na)^+$.

CDBN: ^1H NMR (D_2O) δ 2.3–2.5 (m, 4H, CH_2) 2.7–2.9 (m, 3H, H-6′, NCH_2), 3.11 (dd, 1H, H-6′), 3.23 (t, 2H, CH_2), 3.47 (t, 1H, H-4′), 3.5–4.1 (m, 39H, H-2, H-3, H-4, H-5, H-5′, H-6), 5.1 (m, 7H, H-1). ^{13}C NMR (D_2O) δ 21.5, 23.4 (CH_2), 26.1 (CH_2NH_2), 40.0 (CH_2NH), 52.7 (C-6′), 61.0 (C-6), 71.8 (C-5′), 72.5–74.0 (C-2, C-3, C-5), 82.8 (C-4), 86.7 (C-4′), 103.1 (C-1). FABMS *m/z* 1205.2 $(M + H)^+$.

2.3. Preparation of trypsin–CD conjugates

Thirty milligrams of EDAC were added to the reaction mixtures containing 20 mg of trypsin dissolved in 10 ml of 50 mM potassium phosphate buffer, pH

6.0, and 100 mg of each CD derivative. The solutions were stirred for 1 h at room temperature and further on for 16 h at 4 °C, and applied to a gel filtration column Fractogel EMD BioSEC (S) (2.6 cm × 60 cm), equilibrated with 20 mM sodium acetate buffer, pH 5.0 made 100 mM of NaCl. The active fractions containing carbohydrates were cooled and kept at 4 °C.

2.4. Enzymatic assay

The esterolytic activity of the native and modified trypsins was determined at 25 °C in 67 mM Tris–HCl buffer, pH 8.0 using BAEE as substrate [19]. One unit of esterolytic activity is defined as the amount of enzyme that hydrolyses 1.0 μmol of BAEE per minute at 25 °C. Proteolytic activity was determined as described by Laskowski [20] using milk casein as a substrate. One unit of proteolytic activity, katal, is defined as the amount of enzyme that releases 1 mol of tyrosine per second at 25 °C. Protein concentration was estimated as described by Lowry et al. [21] using bovine serum albumin as standard.

2.5. Analyses

Total carbohydrates were determined by the phenol–sulphuric acid method [22] using glucose as standard. For FABMS peptide mapping, native and CD-modified trypsins were reduced and (*S*)-carboxymethylated [23], and further digested for 6 h at 37 °C with sequential grade trypsin (1:100 (w/w)) in 20 mM NH_4HCO_3 buffer, pH 8.5. The peptide mixtures were purified by reverse-phase chromatography on a Vydac C18 column (5 μm, 4.6 mm × 250 mm) using a linear CH_3CN gradient (0–80%) in 0.1% CF_3COOH. FAB mass spectra were recorded on a Jeol HX-110 spectrometer using glycerol–thioglycerol as matrix. The association constant between BAEE and the native β-CD was determined by ^1H NMR spectrometry [24] using a Bruker AC 250 spectrometer at 250.13 MHz. A 0.01 M solution of β-CD in D_2O at pD 8.0 and 25 °C was titrated with BAEE at different BAEE:CD molar ratios (usually from 0 to 2) and the induced chemical shift differences of the inner H-3 protons of CD were determined. The value of the association constant was determined by least-squared fitting of the experimental values to the theoretical equation obtained considering 1:1 complexation stoichiometry [24].

2.6. Autolysis

Native and modified enzyme preparations were incubated at 30 °C in 50 mM Tris–HCl buffer, pH 9.0. Aliquots were removed at different times, diluted in cold 0.1 M Tris–HCl buffer, pH 8.0, and assayed for esterolytic activity.

2.7. Thermostability profile

Native and modified enzyme forms were incubated at different temperatures in 20 mM sodium acetate buffer, pH 5.0. Aliquots were removed after 10 min of incubation, diluted in cold 0.1 M Tris–HCl buffer, pH 8.0, and assayed for esterolytic activity. The values of T_{50}, defined as the temperature at which 50% of the initial activity was retained, were determined from the graphics.

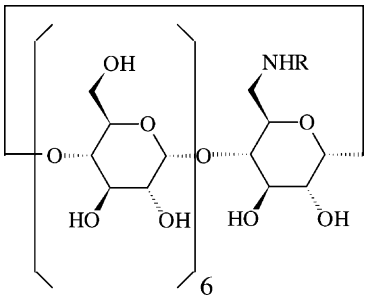
2.8. Kinetics of thermal inactivation

Native and modified enzyme preparations were incubated at different temperatures ranging from 45 to 60 °C in 50 mM sodium acetate buffer, pH 5.0. Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity. The first-order rate constants of inactivation, k_i, were obtained from linear regression in logarithmic coordinates.

3. Results and discussion

3.1. Preparation and structural properties of trypsin–CD conjugates

The strategy employed in this work for the functionalization of the β-CD core involves the synthesis of the well known mono-6-*O*-tosyl derivative, followed by a reaction with excess of ammonia or the appropriate diamine. The monotosylation reaction is one of the most effective methods to activate one hydroxyl group of CD, since the *p*-toluenesulphonate groups can be readily substituted by many nucleophiles [18]. In our case, the reaction of mono-6-*O*-tosyl-β-CD with excess of ammonia at room temperature overnight afforded the mono-6-amino-derivatives [15], while the reaction with each diamine at room temperature under argon gave the corresponding mono-6-alkyldiamino



$CDNH_2$, R = H

CDEN, R = -$(CH_2)_2NH_2$

CDPN, R = -$(CH_2)_3NH_2$

CDBN, R = -$(CH_2)_4NH_2$

Scheme 1. Structure of the β-CD derivatives.

derivatives in good yield [16]. The CD amines were purified by cation exchange chromatography over CM-Sephadex C-25 and all the products gave satisfactory NMR and mass spectra. Scheme 1 shows the structure of the CD derivatives synthesized by the procedure cited above.

The amino CD derivatives were further attached to the carboxylate groups located at the protein surface of bovine pancreatic trypsin via amide bonds, using EDAC as coupling agent. Two mole of oligosaccharides were found to be attached to each mole of trypsin molecule in the synthesized complexes (Table 1). The amino acid sequence of bovine pancreatic trypsin reveals that the enzyme contains five glutamic and four aspartic acid residues, and its C-terminal residue is asparagine [25]. According to this information, the degree of modification of the enzyme was calculated as 20% in the prepared conjugates. This result was not surprising, taking into account that two aspartic acid residues are less exposed and involved in the catalytic site of trypsin (Asp102 and Asp189) [26], and two glutamic acid residues (Glu70 and Glu80) are bond to a calcium ion [27]. On the other hand, the tree-dimensional structure of trypsin reveals that Asp194 is buried into the protein structure and the accessibility of Asp71 and Glu77 to modification with CDs could be affected by their vicinity to the modified Asp153 residue, as reported later.

In order to determine the amino acid residues transformed by the CD derivatives, the conjugated trypsins were proteolytically digested and further analysed by reverse-phase chromatography-FABMS. These analyses revealed that, in all the cases, the modifying agents were located at Asp153 and Glu186 residues (Fig. 1). None of these amino acid residues participates in the active site of the enzyme [26].

3.2. Catalytic properties

Reported in Table 1 are the catalytic characteristics of the CD–trypsin adducts. Trypsin retained high proteolytic activity after modification with the CD moieties. On the other hand, the esterolytic activities of the $CDNH_2$ and CDEN conjugates are similar to that of the native form. On the contrary, the trypsin modified with the CDPN derivative retained about 86% of the initial esterolytic activity. In this case, it is clear that the affinity of trypsin by BAEE was affected by the attachment of the CD residues: K_m value was increased from 38.7 μM for trypsin to 44.8 μM for CDPN-modified enzyme.

The esterolytic activiy of trypsin was increased to about 20% after covalent modification with CDBN. This behaviour can be explained by the increased affinity of the modified enzyme for the substrate,

Table 1
Structural and catalytic properties of trypsins modified by CD derivatives[a]

Enzyme	CD content (mol/mol of protein)	Esterolytic activity (U/mg)	Proteolytic activity (katal/kg)	K_m (μM)	$k_{catalyst}$ (s^{-1})	$k_{catalyst}/K_m$ ($M^{-1}\,s^{-1}$)
Native	–	36	3.0×10^{-2}	38.7	12.4	3.3×10^5
Trypsin–$CDNH_2$	2	34	2.8×10^{-2}	26.6	12.7	4.8×10^5
Trypsin–CDEN	2	35	2.9×10^{-2}	24.6	13.0	5.3×10^5
Trypsin–CDPN	2	31	2.8×10^{-2}	44.8	12.2	2.7×10^5
Trypsin–CDBN	2	44	3.0×10^{-2}	22.6	13.9	6.2×10^5

[a] The data represented are the means from at least triplicate measurements with standard error <5%.

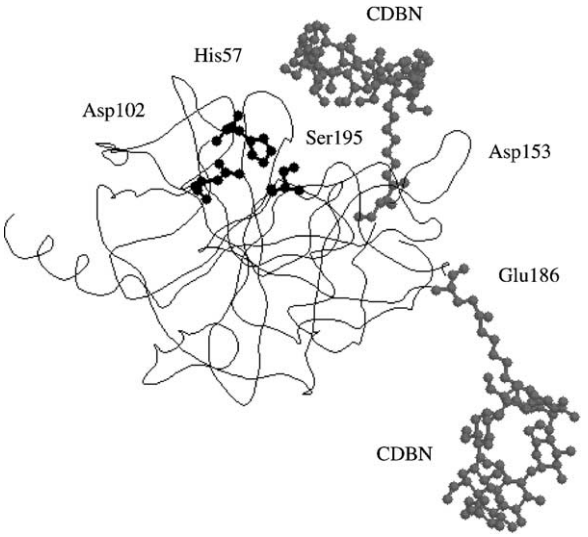


Fig. 1. Structure of bovine pancreatic trypsin chemically modified with CDBN derivative.

as is reflected by the minor value of K_m. Similarly, the affinity for BAEE of the $CDNH_2$- and CDEN-modified trypsins was also higher than the corresponding to the native counterpart. This result suggests that the attached CD moieties stabilize the complex enzyme–BAEE in the transition state. A plausible explanation for this fact could be the formation of inclusion complexes between the CD residues and the BAEE molecules, but also with the *N*-α-benzoyl-L-arginine formed by the hydrolytic action of trypsin. In fact, we determined by ^{1}H NMR that the association constant for the inclusion complex between β-CD and BAEE at pD 8.0 and 25 °C is $180 \pm 25\,M^{-1}$. On the other hand, analysing the tree-dimensional structure of trypsin, it can be noted that the modified Asp153 residue is located very close to the active site of the enzyme [28], as is illustrated in Fig. 1.

According to this information, it is clear that the location of a CD moiety near to the catalytic residues of trypsin can increase the concentration of BAEE at the microenvironment of this active site, increasing the affinity of the modified enzyme for the substrate. This effect is markedly noticeable in the trypsin–CDBN complex, in which the affinity of the enzyme for BAEE was increased to 170% relative to the native counterpart.

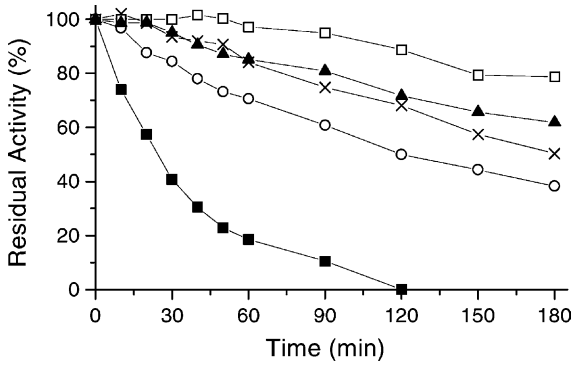


Fig. 2. Kinetic of autolytic degradation for (■) native and modified trypsin with (○) $CDNH_2$, (×) CDEN, (□) CDPN and (▲) CDBN derivatives.

In addition, it should be noted the influence of the spacer arms on the catalytic efficiency of the conjugates prepared, as is reported in Table 1 for the corresponding values of $k_{catalyst}/K_m$. Modification of trypsin with $CDNH_2$, CDEN and CDBN derivatives increased this catalytic parameter, and this effect increases when the length of the spacer arms increases. On the contrary, the catalytic efficiency of trypsin was reduced after conjugation with CDPN derivative.

3.3. Stability properties

The autolysis behaviour of the CD–trypsin complexes at pH 9.0 and 30 °C is depicted as shown in Fig. 2. This result indicates that the covalent attachment of the oligosaccharide moieties prevents the autolytic degradation in the modified trypsins. It is also clear that the structure of the spacer arms of the modifying agents is able to affect this stabilizing effect. In this regard, a higher length of the spacer arms on CD derivatives having an even number of carbon atoms corresponds to a higher resistance to autolytic degradation, as shown in Fig. 2. On the other hand, the resistance to autolysis was markedly more pronounced for the trypsin–CDPN conjugate, probably associated to the lower enzymatic activity showed by this complex, in comparison with the other trypsin adducts prepared.

A plausible explanation for the lower autolysis patterns showed by the transformed enzyme could be the steric hindrance caused by the oligosaccharide moieties to the cleavage sites in the protein. In fact, the

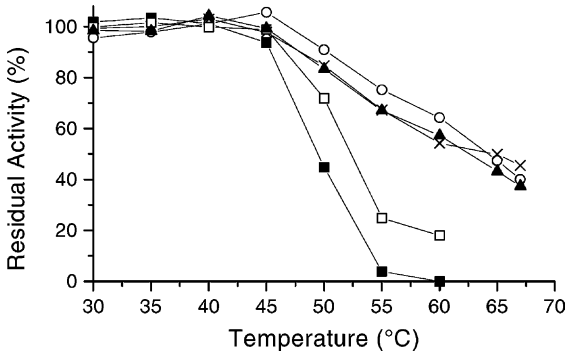


Fig. 3. Thermal stability profile of (■) native and modified trypsin with (○) $CDNH_2$, (×) CDEN, (□) CDPN and (▲) CDBN derivatives.

structure of bovine pancreatic trypsin [25] reveals that the CD-modified amino acid residues are closed to several potential cleavage sites in the enzyme. In that case, the residues Glu186–Lys188 and Lys156–Asp153 are very near in the amino acid sequence of trypsin. In addition, the pair Glu186–Lys222 is spatially closed in the tree-dimensional structure of the enzyme [28].

With the aim of evaluating whether the covalent attachment of the cyclodextrin derivatives at the protein surface of trypsin can induce thermostabilization, and also to determine whether there is a correlation between the length of the spacer arms of these modifying agents and the thermostability of the conjugated enzyme preparations, two different types of experiments were performed. In both the cases, the stability experiments were carried out at acid values of pH in order to avoid any interference caused by the autolytic degradation of the proteases.

Fig. 3 shows the thermal stability profile of the native and the modified trypsins after 10 min of incubation at different temperatures. A significant increase in the thermal resistance was observed for trypsin after modification with the CD derivatives, expressed in the higher values of T_{50} of these adducts. According to the results reported in Fig. 3, a remarkable thermostabilization was obtained for trypsin modified with the CD derivatives having an even number of carbon atoms in the spacer arms. On the contrary, the T_{50} values of the conjugate of trypsin with CDPN was only 2.4 °C higher than that of the corresponding the native enzyme.

In previous reports, Murphy and Fágáin [29] described the covalent modification of trypsin with acetic acid *N*-hydroxy-succinimide ethyl ester, and only an increase of 5 °C was obtained for T_{50}. Similar stabilization was also reported for trypsin modified with carboxymethylcellulose, obtaining an increase of 7 °C for T_{50} under similar experimental conditions [30]. In comparison with these results, the conjugates with $CDNH_2$, CDEN and CDBN were 12.5–14.5 °C more thermostable than the native trypsin.

In other sets of experiments, the residual activities of the native and modified trypsin preparations were periodically evaluated after incubation at different temperatures from 45 to 60 °C, as shown in Fig. 4. It should be noted that all the enzyme forms lost activity with time of incubation at each temperature according to the same pattern, corresponding to first-order inactivation kinetics. However, the half-life times of the enzyme modified with $CDNH_2$, CDEN and CDBN were higher than the corresponding to the native counterpart, as reported in Table 2, indicating that these transformations increased the resistance of trypsin to heat inactivation processes.

Based on the values of half-life times for the different trypsin forms as shown in Table 2, it should be noted that the increased thermoresistance was markedly higher at 60 °C. In order to evaluate the

Table 2
Half-life times of native and CD-modified trypsins at different temperatures

Enzyme	Half-life (min)			
	45 °C	50 °C	55 °C	60 °C
Native	117 ± 6	17 ± 1	8.3 ± 0.1	2.3 ± 0.2
Trypsin–$CDNH_2$	112 ± 2	57 ± 7	50 ± 4	37 ± 1
Trypsin–CDEN	268 ± 10	42 ± 2	33 ± 3	28 ± 1
Trypsin–CDPN	39 ± 1	19 ± 1	5.3 ± 0.5	5.2 ± 0.3
Trypsin–CDBN	408 ± 4	128 ± 11	47 ± 3	27 ± 1

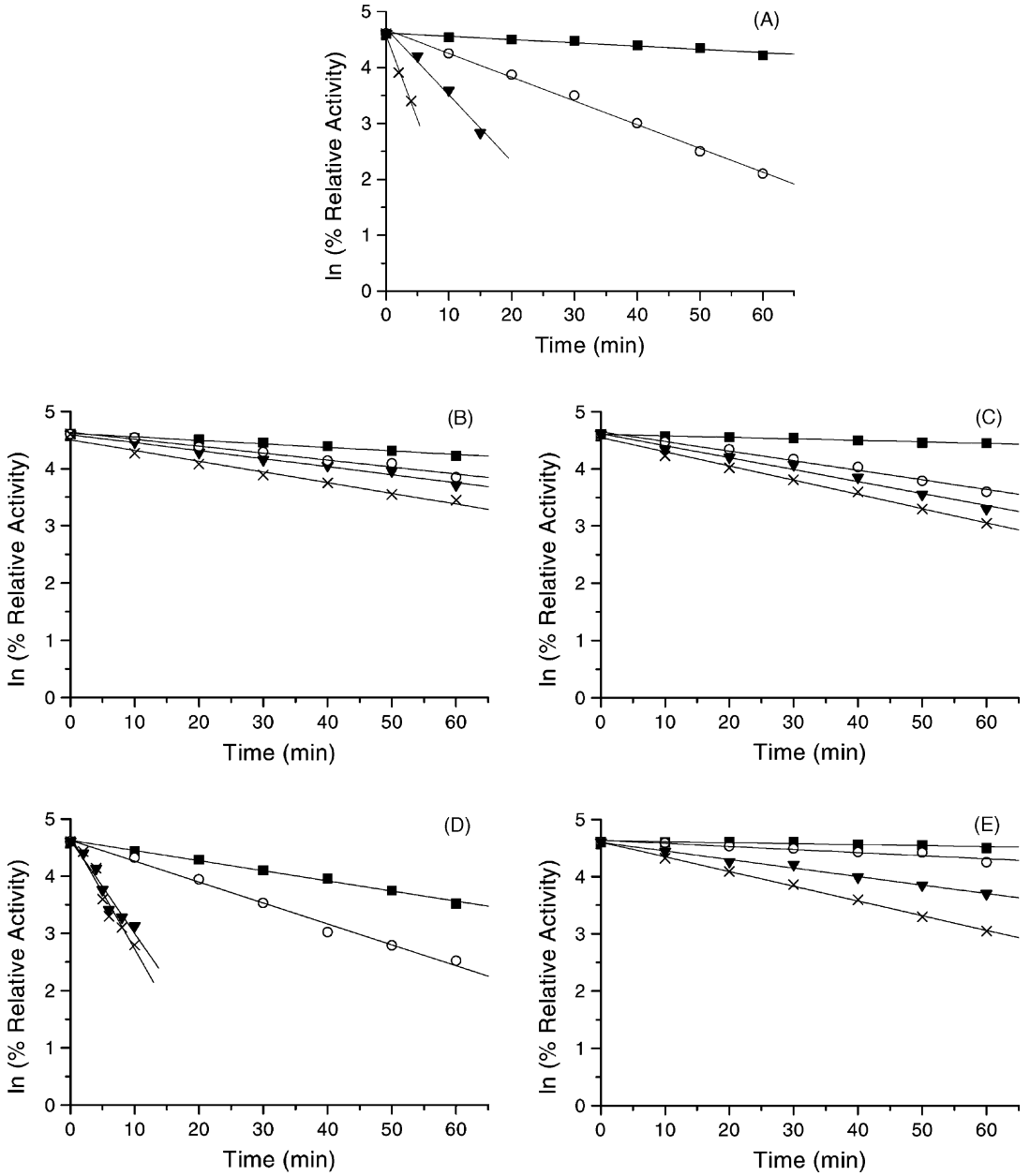


Fig. 4. Kinetic of thermal inactivation of (A) native and modified trypsin with (B) $CDNH_2$, (C) CDEN, (D) CDPN and (E) CDBN derivatives at (■) 45 °C, (○) 50 °C, (▼) 55 °C and (×) 60 °C.

influence of the spacer arms of the CD derivatives used to improve heat resistance showed by the conjugates synthesized, the increase in activation Gibbs energy of inactivation ($\Delta\Delta G_i$) between the modified and non-modified trypsin preparations at 60 °C were determined. These values of $\Delta\Delta G_i$ were calculated according to the following equation:

$$k_i = \left(\frac{k_B T}{h}\right) \exp\left(\frac{-\Delta G_i}{RT}\right)$$

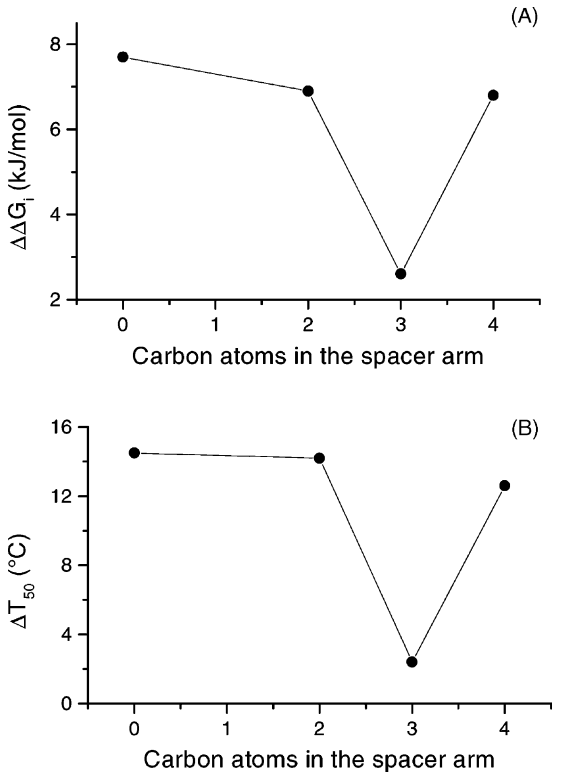


Fig. 5. Influence of the spacer arms on (A) the $\Delta\Delta G_i$ at 60 °C and (B) the ΔT_{50} for trypsin–CD conjugates.

where k_i is the first-order inactivation rate constant (h^{-1}), k_B Boltzmann's constant ($J\,K^{-1}$), h Planck's constant (J h), R the gas constant ($J\,mol^{-1}\,K^{-1}$) and T is the absolute temperature.

In all the cases, the increased thermal stability of the CD–trypsin conjugates were confirmed by the positive values obtained for $\Delta\Delta G_i$, as shown in Fig. 5A. According to the results reported in Fig. 5A, it is clear that the thermal resistance conferred to trypsin at 60 °C was higher after covalent coupling with the CD derivatives having an even number of carbon atoms in the spacer arms. Similar behaviour was found for the values of T_{50} of these conjugates, as shown in Fig. 5B.

Taking into account that the Gibbs energy of stabilization of globular proteins in solution is about 5–7 kcal/mol (21–29.4 kJ/mol) [31], the $\Delta\Delta G_i$ values of 6.8, 7.0 and 7.7 kJ/mol obtained for the enzyme modified with $CDNH_2$, CDEN and CDBN at 60 °C, respectively, represent a good stabilization for these adducts.

Regarding the increased thermal resistance observed in the CD–trypsin complexes, a possible explanation could be the conformational stabilization of trypsin molecules due to intramolecular cross-links caused by the formation of inclusion complexes between the CD moieties and the aromatic amino acid residues located near to the covalent modification points. According to this hypothesis, it is expected that the hydrophobic nature of these cross-links must confer resistance to the enzyme at higher temperatures, because it has been previously demonstrated that in globular proteins the stabilizing effect caused by the hydrophobic interactions increases with the increase of temperature [32].

On the other hand, the results shown in Fig. 5A and B demonstrate the importance of the spacer arm in the thermal stabilization of the synthesized adducts. It is clear that trypsin modified with CDPN derivative showed a minor thermostability compared to that of the corresponding derivatives with an even number of carbon atoms in the spacer arms. This fact suggests that the CD moieties may need an optimal spatial orientation for their interaction with the hydrophobic amino acid residues in the protein backbone.

It is well known that the interaction between hydrophobic clusters at the protein surface of enzymes and the surrounding waters is energetically unfavourable, and contributes significantly to the thermal inactivation of enzymes in aqueous media [33]. In the present case, the attachment of highly hydrophilic CD moieties to the surface of trypsin can significantly contribute to the increased thermoresistance exhibited by the modified forms of the enzyme. Similar effect has been previously reported by Mozhaev et al. [34] for chymotrypsin modified with several hydrophilic compounds.

4. Conclusions

In the present paper, we described the use of several monosubstituted amino derivatives of β-CD as modifying agents for enzymes. The results presented in this work for trypsin demonstrates the efficacy of the transformation strategy used for improving the stability properties of this enzyme. Significantly, this stabilizing effect resulted from a very small chemical change in the protein structure, which was reflected

in the higher catalytic properties of these adducts. In addition, an advantage in the use of this kind of derivatives in protein modification is the possibility to design most efficient enzyme–CD conjugates varying the spacer arms of the modifying agents.

Attending to the results presented in this paper, we suggest that the manipulation of surface protein structures by covalent attachment of CD residues might be a useful method for enhancing the stability and the catalytic properties of enzymes.

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