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Functional properties and application in peptide synthesis of trypsin modified with cyclodextrin-containing dicarboxylic acids

Michael Fernández[a], Alex Fragoso[b], Roberto Cao[b], Maysa Baños[b], Marion Ansorge-Schumacher[c], Winfried Hartmeier[c], Reynaldo Villalonga[a,*]

[a] *Enzyme Technology Group, Center for Biotechnological Studies, University of Matanzas, Matanzas, C.P. 44740, Cuba*
[b] *Laboratory of Bioinorganic Chemistry, Faculty of Chemistry, University of Havana, Havana 10400, Cuba*
[c] *Department of Biotechnology, Faculty of Biology, Aachen University (RWTH-Aachen) Worringerweg 1, D-52056 Aachen, Germany*

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Abstract

Bovine pancreatic trypsin was chemically modified with several β-cyclodextrin (CD) derivatives containing adipic, pimelic and dodecanodioic acids using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) as coupling agent. The modifying agents used were mono-6-(4-carboxybutane-1-carboxamidoyl)-6-deoxy-β-cyclodextrin, mono-6-(5-carboxypentane-1-carboxamidoyl)-6-deoxy-β-cyclodextrin and mono-6-(10-carboxydecane-1-carboxamidoyl)-β-cyclodextrin. The modified enzymes contained about 6 mol of oligosaccharide per mol of trypsin. The catalytic and stability properties of trypsin were improved after the chemical modification. The thermostability profile of CD-modified trypsins was increased by about 10–14 °C. The conjugates were also more stable against thermal incubation at different temperatures ranging from 45 to 60 °C. In comparison with native trypsin, the cyclodextrin–enzyme conjugates were markedly more resistant to autolytic degradation at pH 9.0. Furthermore, the CD–trypsin conjugates gave higher aminolysis rates in kinetically controlled peptide synthesis reactions. The results reported here suggest that conjugation of enzymes with β-cyclodextrin derivatives is a useful method for improving the stability and the catalytic properties of these biocatalysts.

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

1. Introduction

Proteases are hydrolytic enzymes able of cleaving peptide bonds between the amino acid residues of peptides and proteins [1]. There are well-characterized proteases with high cleavage specificities that have a great importance in life science and biotechnology [2]. In the last decades, such enzymes have been widely used as biocatalysts for peptide synthesis because they are able to catalyze the reverse reaction of peptide bond formation in high ionic strength media and low water-containing systems [2]. In comparison with chemical methods, biocatalytic approaches are more suitable because the production of racemic mixtures is reduced giving higher yields of enantio-pure products [3]. However, the use of proteases as catalysts in condensation reactions shows several drawbacks such as partial product hydrolysis and low enzymatic activities at high temperatures. Moreover, organic solvents are often required as reaction media for hydrophobic reactants [3]. To overcome these operational limitations, different strategies for improving the catalytic and stability behaviors of native enzymes have been developed. The most common methods for protease stabilization are site-directed mutagenesis [4], chemical modification with low molecular weight compounds [5] and polymeric molecules [6,7], immobilization in solid supports [8] and the use of polysaccharide additives as stabilizing agents [9,10].

Trypsin is a well-studied serine-protease involved in the digestion processes of mammals that cleaves specific peptide bonds having a lysine or arginine residue [1]. This pro-

* Corresponding author. Tel.: +53 45 26 1251; fax: +53 45 25 3101.
E-mail address: reynaldo.villalonga@umcc.cu (R. Villalonga).

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tein has been used as biocatalyst mainly in dipeptide synthesis using arginine or lysine esters as acyl donors and different amino acids as nucleophilic agents [2]. In order to improve trypsin functional properties in different media and conditions it has been previously modified with low molecular weight compounds such as ethyleneglycol bis(succinic acid *N*-hydroxysuccinimide)-ester [5], polymers such as polyethylenglycol [11], poly(*N*-vinylpyrrolidone) [12], carboxymethylcellulose [13] and oligosaccharides such as amine and aldehyde derivatives of cyclodextrins (CDs) [14-17].

Cyclodextrins are a familly of cyclic non-reducing oligomers composed of 6, 7 or 8 -(1→4)-linked D-glucopyranose units in the $^4C_1$ chair conformation, which are named α-, β- and γ-cyclodextrin, respectively [18]. The structure of these remarkable molecular receptors resembles a truncated annular cone with a central cavity. The cavity is hydrophobic in nature and has the appropriate size to include a wide variety of lipophilic guests [19]. The formation of such adducts has been extensively studied in recent years due to their potential applications in pharmaceuticals, catalysis, chromatography, enzyme mimicking, design of supramolecular architectures, etc. [20]. Cyclodextrins can be chemically modified to generate a variety of more reactive derivatives, including amines, thiols, aldehydes, carboxylates, etc. [21].

In this work we chemically modified the lysine residues of bovine pancreatic trypsin with three CD derivatives bearing a carboxylic group connected through spacers of different length. We report the influence of the spacer length on the improved kinetic and stability properties of the trypsin–CD conjugates as well as in their higher efficiencies for dipeptide synthesis in comparison with the native enzyme.

2. Material and methods

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). CDs were purchased from Amaizo (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 carboxylic CD derivatives

Succinyl (CD1), heptanodioyl (CD2) and dodecanodioyl (CD3) CD derivatives were synthesized as follows: dicyclohexylcarbodiimide (6.2 g, 30 mmol) was added to a cold solution of the appropriated dicarboxylic acid (20 mmol) in dry N,N′-dimethylformamide (25 mL), and the solution was stirred at 5 °C for 30 min. To this solution, mono-6-amino-6-deoxy-β-CD (2.3 g, 2 mmol) [22] was added in one portion and the solution was stirred at room temperature. After 24 h, the insoluble precipitate of dicyclohexylurea was filtered off and the remaining solution was precipitated by addition over acetone (300 mL), washed several times with acetone and dried under high vacuum to give the target products in 60–70% yield. The products were fully characterized by conventional NMR techniques and MALDI-MS.

2.3. Preparation of trypsin–CD conjugates

Thirty milligrams of EDAC were added to 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 derivatives. The solutions were stirred for 1 h at room temperature and for 16 h at 4 °C, and further applied to a gel filtration column Fractogel EMD BioSEC (S) (2.6 × 60 cm) (104– 4 × 106, fractionation range), equilibrated in the same buffer containing 100 mM of NaCl. The active fractions containing carbohydrates were pooled and kept at 4 °C.

2.4. Assays

Esterolytic activity of native and modified trypsins was determined at 25 °C in 67 mM Tris–HCl buffer, pH 8.0 using BAEE as substrate [23]. One unit of esterolytic activity is defined as the amount of enzyme that hydrolyses 1.0 μmol of BAEE per min at 25 °C. Total carbohydrates were determined by the phenol-sulfuric acid method [24] using glucose as standard. Protein concentration was estimated as described by Lowry et al. [25] using bovine serum albumin as standard.

2.5. Temperature optimum

The enzyme activities of native and CD-modified trypsin preparations were measured at different temperatures ranging from 30 to 80 °C. The corresponding values of optimum temperature were calculated from Arrhenius plots.

2.6. Thermostability

Native and modified trypsin preparations were incubated at different temperatures in 20 mM sodium acetate buffer, pH 5.0, in order to minimize autolysis. Aliquots were removed after 10 min incubation, diluted in cold 0.1 M Tris–HCl buffer, pH 8.0, and assayed for esterolytic activity.

2.7. Thermal inactivation

Native and modified trypsin preparations were incubated at different temperatures ranging from 45 to 70 °C in 50 mM sodium acetate buffer, pH 5.0, in order to minimize autolysis. Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity. The first-order rate constants of inactivation, ki, were obtained from linear regression in logarithmic coordinates.

2.8. Autolysis

Native and modified trypsin forms were incubated at 35 °C in 50 mM Tris–HCl buffer, pH 9.0. Aliquots were removed at different times, diluted in 0.1 M sodium acetate buffer, pH 5.0, and further assayed for esterolytic activity.

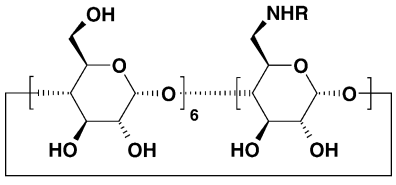
2.9. Dipeptide synthesis

Native and modified trypsins were evaluated as biocatalysts in dipeptide synthesis by adding 0.05 mg of each enzyme adduct to different reaction mixtures containing 100 mM BAEE and 200 mM phenylalaninamide in 1 mL 250 mM borate buffer pH 9.0. The reactions were continuously stirred at 10 °C and the dipeptide formation was followed by scheduled sampling each 30 min during 5 h. Reaction samples were diluted in methanol and 10 μL were taken and analyzed by HPLC using a Beckmann Coulter System Gold apparatus and Nucleosil 105 C18 column with 0.4 mL/min flow rate, detection at 225 nm and mobile phase 50:50 (v/v) aqueous methanol with 0.05% aqueous trifluoroacetic acid (50/50 by volume). Both substrates and products were identified and quantified by comparison with standards of each.

3. Results

3.1. Structural and catalytic properties of native and modified trypsins

The reactions of mono-6-amino-6-deoxy-β-CD with an excess of succinic, heptanodioic and dodecanodioic acids yielded three carboxylic monofunctionalizated CD derivatives in good yields (Scheme 1). The obtained derivatives mono-6-(4-carboxybutane-1-carboxamidoyl)-6-deoxy-β-cyclodextrin (CD1), mono-6-(5-carboxypentane-1-carboxamidoyl)-6-deoxy-β-cyclodextrin (CD2) and mono-6-(10-carboxydecane-1-carboxamidoyl)-β-cyclodextrin (CD3) were covalently attached to bovine pancreatic trypsin using EDAC as coupling agent. The trypsin–CD conjugates contained 6 mol of oligosaccharide per mol of protein (Table 1). As a result of the modification, the kinetic parameters for BAEE esterolysis at pH 8.0 and 30 °C were improved. In comparison with native enzyme, the specific



CD1: R = –CO–$(CH_2)_4$–COOH
CD2: R = –CO–$(CH_2)_5$–COOH
CD3: R = –CO–$(CH_2)_{10}$–COOH

Scheme 1. Structure of the β-CD derivatives.

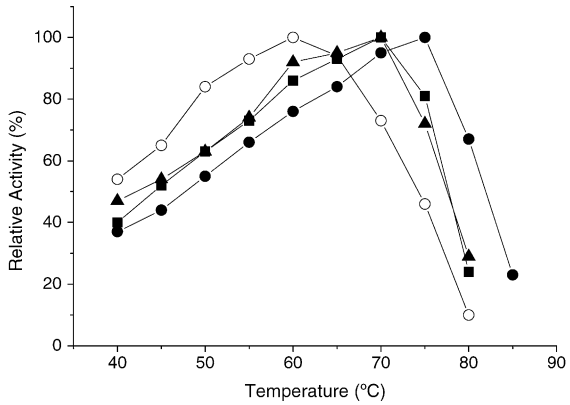


Fig. 1. Optimum temperature profile for BAEE esterolysis of native (◯) and modified trypsin with CD1 (●), CD2 (■) and CD3 (▲) derivatives.

activities of the CD-modified trypsins were 2-, 1.3- and 1.4-fold higher and the catalytic efficiencies were 245, 185 and 185% higher for the proteins modified with CD1, CD2 and CD3, respectively (Table 1).

Fig. 1 depicts the optimum temperature for BAEE esterolysis of native trypsin and their conjugates. As can be seen, the trypsin–CD1 conjugate having the shortest spacer showed the highest optimum temperature increment of about 15 °C, while those conjugates having CD2 and CD3 gave 10 °C increments in comparison with native trypsin.

3.2. Stability properties of native and modified trypsins

CD-modified trypsins were more resistant toward thermal inactivation at different temperatures, ranging from 30 to 70 °C, as shown in Fig. 2. The T_{50} (temperature at which the trypsin preparations retain 50% of their initial activity after 10 min incubation) was increased after the chemical modification from 50 to 61–63 °C, approximately. Additionally, thermal inactivation kinetics of native and modified enzymes were followed at 45, 50, 55 and 60 °C and $t_{1/2}$ of inactivation (time at which the enzymatic preparations retain 50% of their initial activity) are reported in Table 2. All enzymatic

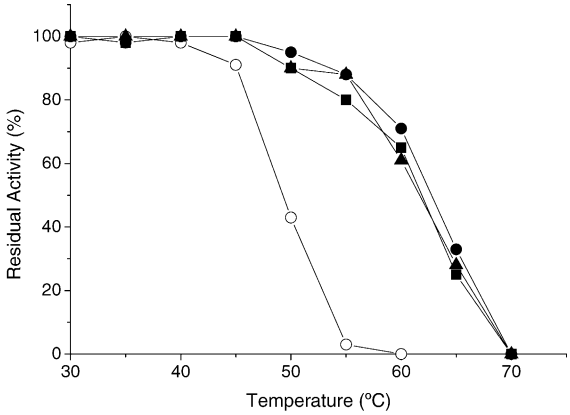


Fig. 2. Thermal stability profile of native (◯) and modified trypsin with CD1 (●), CD2 (■) and CD3 (▲) derivatives.

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

Enzyme	CD content (mol/mol protein)	Specific activity (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Native	–	36	38.7	12.4	3.3×10^{-5}
Trypsin–CD1	6	70.0	29.6	24.1	8.1×10^{-5}
Trypsin–CD2	6	51.3	28.6	17.7	6.1×10^{-5}
Trypsin–CD3	6	57.8	31.8	19.9	6.2×10^{-5}

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	2264 ± 40	187 ± 2	125 ± 3	59.3 ± 0.9
Trypsin	890 ± 30	76 ± 1	42.2 ± 0.6	37.6 ± 0.8
Trypsin	417 ± 10	104 ± 6	53.4 ± 0.8	40.8 ± 0.7

preparations had first-order kinetic behaviors, but the inactivation rates for the conjugates were lower in comparison with the native trypsin. Remarkably, the $t_{1/2}$ of inactivation at 45 and 60 °C for trypsin–CD1 were about 19- and 25-fold longer than those of native trypsin. At the same time, CD2- and CD3-modified enzymes showed values about 8- and 15-fold longer at 45 °C and 16- and 18-fold longer at 60 °C than the native trypsin, respectively.

Furthermore, autolytic inactivation kinetics of the native and modified trypsins were followed during 3 h (Fig. 3). The values of $t_{1/2}$ of the modified proteases were markedly higher in comparison with the native enzyme. The highest increment, 80-fold higher in comparison with native trypsin, was achieved for the trypsin–CD3 conjugate, while the enzymatic conjugates having CD2 and CD3 showed values about 10- and 18-fold higher, respectively.

3.3. Dipeptide synthesis

Comparative dipeptide synthesis experiments were developed in kinetic-controlled systems using native and modified trypsin as biocatalysts. Benzoyl-arginine-ethyl-ester and phenylalaninamide were used as acyl donor and nucleophylic agent, respectively, for the synthesis of benzoyl-arginine-phenylalaninamide (BAPheNH) dipeptide in borate buffer pH 9.0 at 10 °C (Fig. 4). The conjugates were more efficient biocatalysts as evidenced by the fact that the reactions catalyzed by 0.05 mg of conjugate reached the maximum yields at lower reaction times than the reaction with 0.05 mg of native trypsin. Consequently, higher dipeptide concentrations in the reactions catalyzed by the modified enzymes were achieved faster in comparison to the reaction with the native trypsin. After 2 h, the conjugate-catalyzed reactions showed maximum conversions of about 70%. In contrast, the native trypsin-catalyzed reaction reached only 50% conversion after 2 h and a maximum conversion ratio of about 72% was achieved only after 3 h of reaction time.

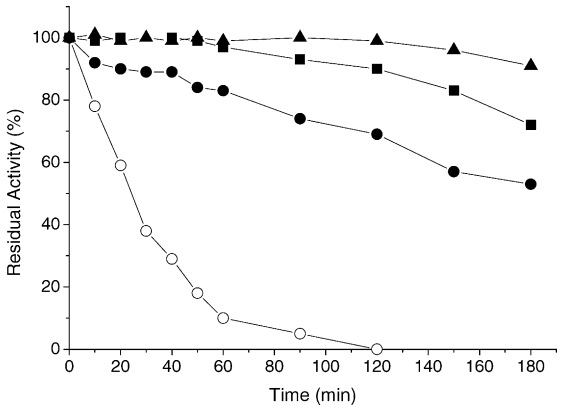


Fig. 3. Kinetics of autolytic degradation for native (◯) and modified trypsin with CD1 (●), CD2 (■) and CD3 (▲) derivatives.

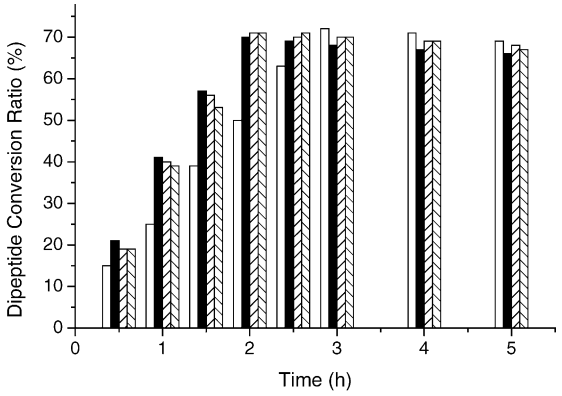


Fig. 4. Dipeptide conversion ratios for native (□) and modified trypsin with CD1 (■), CD2 (▨) and CD3 (▧) derivatives.

4. Discussion

The reactions of trypsin with carboxylic CD derivatives modified 6 out of the 15 lysine residues present in the trypsin primary structure, including the *N*-terminal residue. This corresponds to a 30% of carbohydrate per weight of modified protein. The mass ratio is higher in comparison with previous results of about 15 and 20% per weight of modified trypsin achieved for the modifications of trypsin carboxylic and amino residues with amino- and aldehyde–CD derivatives, respectively [14–17]. This result is clearly understandable taking into account that the amount of reactive carboxylic groups in aspartic and glutamic residues of trypsin is lower

than the amount of reactive lysins. On the other hand, the spacer arm lengths of the CD derivatives used in this work are much larger (n = 6–12) than that of the aldehyde–CD (n = 1) previously used for modification of lysine residues [17]. No difference in reactivity with trypsin, using EDAC as coupling agent, was observed for the three carboxylic–CD derivatives used here, probably because some lysine residues are not exposed enough on the trypsin surface for the chemical reaction with the bulky oligosaccharides even having long spacer arms. Therefore, the 40% of modified lysines obtained here is 2-fold lower in comparison with the 85% reported by Murphy [3] for the modification of trypsin with bis(succinic acid *N*-hydroxysuccinimide)-ester, as expected considering the higher bulkiness of the CD moieties.

The catalytic efficiency of the modified trypsins, although up to 2-fold higher than the native trypsin, confirmed that modification of lysine residues provokes no decrease in trypsin esterase activity, as was reported by Labouesse [26]. The specific activity of 70 U/mg obtained for trypsin–CD1 is the highest achieved by us for trypsin forms modified with CD derivatives [14-17]. On the other hand, the K_m values of CD-modified trypsins increased 1.2-fold. Taking into account the aromatic nature of the benzoyl residue of BAEE, the increment in trypsin–CD kinetic parameters could be caused by the formation of inclusion complexes between the substrate and the CD cavity attached to the protein that could cause an increase of the substrate concentration in the enzyme microenvironment. At the same time, the 10–15 °C increase in optimum temperatures for BAEE esterolysis is also remarkable. We have previously associated this active site stabilization with a reduction of trypsin side chain mobility caused by the attached CDs that may interact with the protein surface via hydrogen bonding or by forming supramolecular inclusion complexes with the hydrophobic side chains of trypsin [14–17]. These intramolecular interactions could produce non-covalent multipoint crosslinks between the carbohydrate moieties and the protein surface that reduces the degrees of freedom of trypsin tertiary structure and keeps the enzyme active at higher temperatures.

The trypsin–CD conjugates retained in a larger degree their initial activity, in comparison with the native enzyme, during thermal inactivation experiments at temperatures ranging from 35 to 70 °C. The T_{50} value of 64 °C obtained for trypsin–CD1 is 5 °C higher than the value reported by Murphy for ethyleneglycol bis(succinic acid *N*-hydroxysuccinimide)-ester modified trypsin [3]. In another set of experiments, the thermal inactivation rates obtained at temperatures ranging form 45 to 60 °C were drastically decreased after chemical modification. Consequently, we suggest that the attached CD moieties participate in supramolecular-mediated interactions that could prevent trypsin aggregation at high temperatures, a mechanism that has been reported to play a main role in trypsin thermal inactivation processes [27].

Since trypsin inactivation at basic pH has been associated with protein self-digestion [28], it has been reported that modifying lysine residues involved in trypsin autolysis reduces the protein hydrolysis rates [3]. Since the CD-modified trypsins have 40% of modified lysine residues, a higher autolysis resistance was expected with respect to the native enzyme. On the other hand, the conjugate having the longest spacer (trypsin–CD3) showed the lower autolysis rate. This could be provoked by the partial burying of the unmodified lysine and arginine residues by the bulky moieties attached to the protein surface. These facts agree well with previous results in which the covalent attachment of carbohydrate moieties to trypsin increased the resistance toward autolytic degradation [13–17].

As pointed out previously, trypsin has been used in the synthesis of arginine and lysine-containing dipeptides. Therefore, it was interesting to test the effectiveness of the prepared conjugates in such reactions, also considering that they show improved functional properties. Since we suppose that non-polar supramolecular interactions could play a main role in the performance of the modified trypsins, we avoided to use low water-containing systems in these assays that may disrupt CD host-guest complexes. Therefore, dipeptide synthesis was carried out in high ionic strength aqueous media, following the protocol reported by Riechmann [29]. The reactions of BAEE with phenylalaninamide catalyzed by modified and native trypsins were followed until the highest dipeptide concentration was reached. The maximum aminolysis/hydrolysis ratios for modified and native trypsins were 2.2 and 2.5, respectively. In spite of the similarity of the aminolysis/hydrolysis ratios for both forms, the aminolysis rates were approximately 1.7-fold higher for the conjugates, as shown in Fig. 4. Therefore, CD-modified trypsins reached approximately 70% of maximum conversion ratio after 2 h, while native trypsin needed 3 h for reaching a maximum of 72% conversion ratio. However, it is important to point out that BAEE hydrolysis rates for the conjugates were approximately 1.5 higher, agreeing well with the higher BAEE esterolysis rates found in spectrophotometric assays. These results also support the hypothesis that supramolecular interactions between BAEE and attached CD moieties increase trypsin catalytic efficiency.

5. Conclusions

In this paper we report the chemical modification of trypsin with three carboxylic CD derivatives via covalent attachment to lysine residues using a water-soluble carbodiimide as coupling agent. The modified enzymes showed enhanced catalytic properties and were more resistance to thermal and autolytic inactivations. At the same time, modified trypsins were more efficient than native trypsin when were assayed as biocatalysts for dipeptide synthesis. These results confirm that the previously reported strategy of chemical modification with cyclodextrin derivatives constitute an effective approach for improving the functional properties of enzymes.

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