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Preparation and functional properties of trypsin modified by carboxymethylcellulose

Reynaldo Villalonga*, Maria L. Villalonga, Leissy Gómez

Center of Biotechnological Studies, Faculty of Agronomy, University of Matanzas, Autopista a Varadero Km 3 1 / 2, Matanzas, C.P. 44740, Cuba

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This paper is dedicated to the memory of Sr. Reynaldo Villalonga Báez, father of R.V. and M.L.V.

Abstract

Trypsin from bovine pancreas was modified by the polyaldehyde derivative of carboxymethylcellulose (CMC) via reductive alkylation with $NaBH_4$. The modified enzyme contained 57% carbohydrate by weight, resulting from the modification of 52% of the amino groups of the protein. In comparison with the native protease, the modified trypsin retained 62% and 42% of the esterolytic and proteolytic activity, respectively. The value of K_m for CMC–trypsin complex was 2.2 times lower than for the native enzyme. The thermostability and pH stability was improved for trypsin by this modification. The conjugate was also more resistant to the action of the anionic surfactant sodium dodecylsulphate and denaturing agents such as 8 M urea and 6 M guanidinium chloride. This modification also protected the enzyme against autolysis at alkaline pH and improved the stability of the enzyme in the presence of methanol.

Keywords: Trypsin; Modified enzyme; Carboxymethylcellulose; Enzyme stability

1. Introduction

Functional stability often limits the practical application of enzymes in medicine and biotechnological processes. Surface protein modification of enzymes by macromolecular substances constitutes a useful tool for improving the stability of these biomolecules [1–4]. In general, the effectiveness of this method depends on choosing the appropriate conditions based on: (i) the type, size and structure of the enzyme; (ii) the structure and size of the modifying polymer; and (iii) the type and conditions for the chemical reaction involved in the modification procedure [5].

Water solubility, biocompatibility and non-toxicity of many polysaccharides, as well as the fact that in naturally occurring glycoenzymes, the carbohydrate residues function as stabilisers of the three-dimensional structure of the protein moiety [6], have favored the use of carbohydrate polymers for modifying enzymes. For example, the covalent attachment of dextran [7–10], polymerised sucrose [5,11] and carboxymethylcellulose (CMC) [12] to enzymes has been successfully reported.

* Corresponding author. Tel.: +53-52-6-1251; fax: +53-52-5-3101.

E-mail address: villa@cdict.umtz.edu.cu (R. Villalonga).

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Proteases are widely used in industrial and biomedical applications. Since thermal, operational and storage inactivation of these enzymes occurs mainly owing to intermolecular self-digestion [13], the development of methods for stabilising their structures and improving their catalytic properties receives increasing attention in enzyme engineering. Among these enzymes, trypsin (EC 3.4.21.4) has been the most extensively studied for this purpose. In this sense, the conjugation of trypsin with several water-soluble polymers such as poly(ethylene glycol) [14,15], dextran [7], polymerised sucrose [5], poly(*N*-vinylpyrrolidone) [16] and poly(ethylene glycol-co-maleic anhydride) [17] has been described.

The present paper reports the effects of chemical modification of trypsin by the anionic polysaccharide CMC on the functional properties of this protease.

2. Experimental

2.1. Materials

Trypsin (bovine pancreas), CMC sodium salt ($M_v = 2.61 \times 10^5$, substitution degree = 0.7, degree of polymerisation = 196) [18] and milk casein, were obtained from BDH (Poole, UK). *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE), sodium borohydride and sodium *m*-periodate, were purchased from Sigma (St. Louis, MO, USA). DEAE Sepharose CL-6B was purchased from Pharmacia Biotech (Uppsala, Sweden). BioGel P30 was obtained from BioRad (Richmond, CA, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and BDH (Poole).

2.2. Preparation of trypsin–CMC conjugate

One gram of CMC was dissolved in 100 ml of distilled H_2O, and 1.07 g of $NaIO_4$ was added. The reaction mixture was stirred in the dark for 48 h. One millilitre of ethylene glycol was added and the reaction mixture was left for 1 h. The solution was further exhaustively dialysed against distilled H_2O and kept at −20°C. For coupling activated CMC to trypsin, 120 mg in 15 ml of polymer solution was mixed with 3 ml of 1.0 M sodium phosphate buffer, pH 7.9, and then 30 mg of trypsin dissolved in 5 ml of 1 mM HCl was added. Distilled water was added to the reaction mixture up to 30 ml of final volume and stirred in the dark for 48 h. $NaBH_4$ was added up to 200 mM of final concentration, and the reaction mixture was left overnight under continuous stirring. The solution was further dialysed against 20 mM Tris–HCl buffer, pH 8.0, centrifugated at $14{,}000 \times g$, and applied to a DEAE Sepharose CL-6B column (1.5×5.0 cm), equilibrated with 20 mM Tris–HCl buffer, pH 8.0. The modified enzyme was eluted with a NaCl gradient (0–0.5 M) in the same buffer. The active fraction with carbohydrates was pooled and applied to a gel filtration column BioGel P30 (1.6×90 cm), equilibrated with 0.15 M NaCl, 50 mM sodium phosphate buffer, pH 7.9. The active fraction was again pooled and kept at −20°C.

All reactions and enzyme purification were carried out at 4°C.

2.3. Assays

Esterolytic activity of native and modified trypsin 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 substrate. Total carbohydrates were determined by the phenol-sulphuric acid method [21], using glucose as standard. The degree of modification of amino groups was determined by measuring the amount of free amino groups with *o*-phtalaldehyde using glycine as standard [22]. Protein concentration was estimated as described by

Lowry et al. [23] using bovine serum albumin as standard.

2.4. Thermostability

Native and modified enzyme preparations were incubated at different temperatures in 0.1 M Tris–HCl buffer, pH 8.0. 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.

2.5. pH stability

Native and modified enzyme preparations were incubated at 30°C in the following buffers: 50 mM sodium acetate, pH 2.0–6.0, 50 mM sodium phosphate, pH 7.0 and 50 mM sodium borate, pH 8.0–12.0. Aliquots were removed after 30 min of incubation, diluted in 0.1 M Tris–HCl buffer, pH 8.0 and assayed for esterolytic activity.

2.6. Autolysis

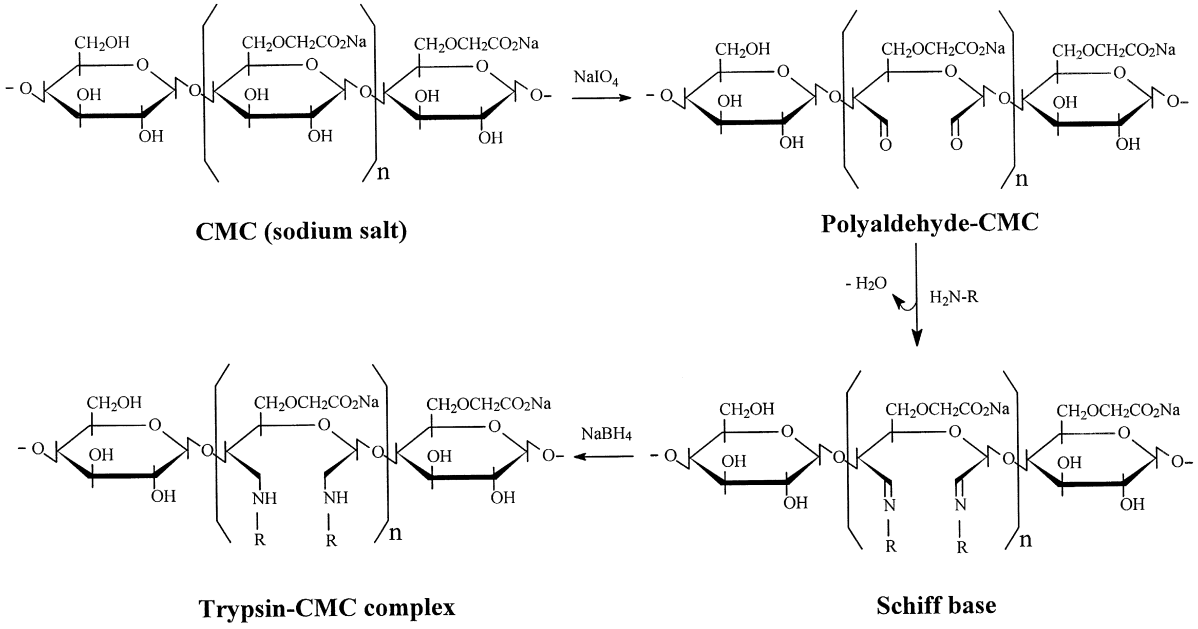
Native and modified enzyme preparations were incubated at 30°C in 50 mM sodium borate buffer, pH 12.0. Aliquots were removed at different times, diluted in cold 0.1 M Tris–HCl buffer, pH 8.0 and assayed for esterolytic activity.

2.7. Stability in presence of sodium dodecylsulphate (SDS)

Native and modified enzyme preparations were incubated at 30°C in 0.3% SDS in 0.1 M Tris–HCl buffer, pH 9.0. Aliquots were removed at scheduled times, diluted in 0.1 M Tris–HCl buffer, pH 8.0 and assayed for esterolytic activity.

2.8. Stability in concentrated urea and guanidinium chloride (GdmCl) solutions

Native and modified enzyme preparations were incubated at 30°C in 0.1 M Tris–HCl



Scheme 1. Synthesis of trypsin–CMC conjugate. H_2N-R indicates protein.

buffer, pH 8.0, containing 8 M urea or 6 M GdmCl. Aliquots were removed at different times, diluted in 0.1 M Tris–HCl buffer, pH 8.0 and assayed for esterolytic activity.

2.9. Activity in methanol / water system

Three millilitres of a mixture, composed of 0.14 units of native or modified enzyme preparations, water (final concentration 0.93–8.33 M) and 0.167% BAEE solution in methanol (w/v), were incubated at 25°C. Esterolytic activity was determined by the increase of absorbance at 253 nm.

3. Results

The anionic polysaccharide CMC, previously activated by periodate treatment, was attached to trypsin as shown in Scheme 1. CMC is a linear and partially *O*-carboxymethylated β(1-4) glucan. For this reason, only the glucosidic units in which both hydroxyl groups at C-2 and C-3 positions are not substituted, can be activated by periodate oxidation.

The extent of modification of amino groups for the trypsin–CMC complex was determined as 52% of the native enzyme. This result showed that eight out of 15 amino groups from trypsin were modified with polyaldehyde–CMC. The carbohydrate content of CMC-modified trypsin was calculated as 57% by weight of modified enzyme. This value represented 237 mol of carbohydrates (as glucose) per mole of trypsin in the CMC–enzyme complex.

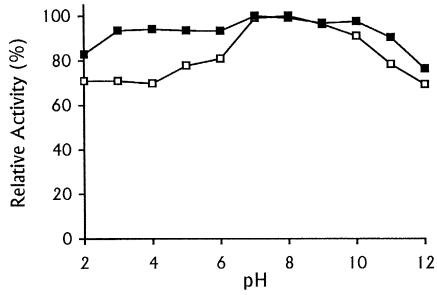


Fig. 2. pH stability of native (□) and CMC-modified trypsin (■).

According to the degree of polymerisation of CMC, an average of 1.21 mol of polymer was attached to each mole of enzyme. This result, compared with the modification rate of amino groups, shows a high degree of intramolecular cross-linking for trypsin–CMC complex.

The specific activity of the modified enzyme toward casein was 42% of that of the native enzyme. Toward a small substrate, BAEE, the modified trypsin retained 62% of the original esterolytic activity.

The modification enhanced the affinity of the enzyme for the substrate BAEE: native and modified forms gave K_m values of 39.4×10^{-6} and 17.8×10^{-6} M. The optimum range of pH for esterolytic activity was increased for trypsin from 7.5–9.5 to 7.5–12.0 by conjugation with CMC.

Fig. 1 shows that the thermostability was improved for trypsin by the conjugation with CMC: the value of T_{50} for trypsin–CMC complex, defined as the temperature at which 50%

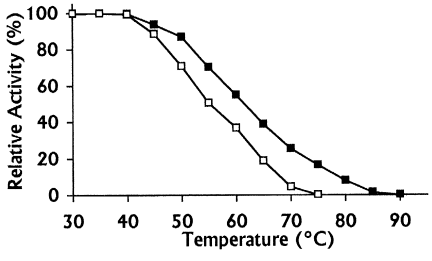


Fig. 1. Thermal stability of native (□) and CMC-modified trypsin (■).

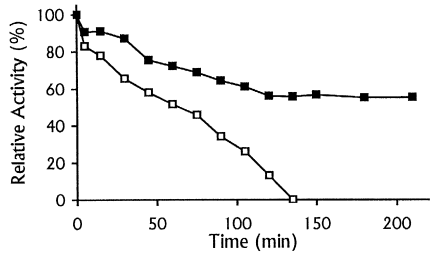


Fig. 3. Stability profile of native (□) and CMC-modified trypsin (■) against autolysis at alkaline pH.

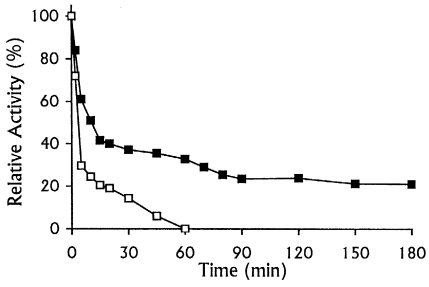


Fig. 4. Effect of 0.3% SDS on the stability of native (□) and CMC-modified trypsin (■).

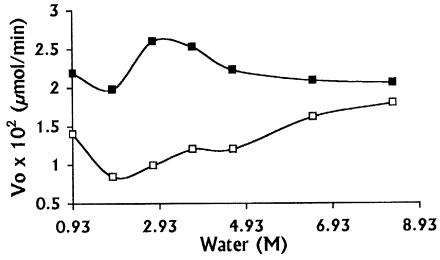


Fig. 6. Stability of native (□) and CMC-modified trypsin (■) in methanol/water system.

of the initial activity was retained under the conditions described above, was increased from 55°C to 62°C, in comparison to the native protease. It was further demonstrated that the pH stability was increased for the modified trypsin in the range of pH between 2.0 and 12.0 (Fig. 2). At lower values of pH, the improved stability was larger than at alkaline pH.

The time-course of autolysis for native and modified enzyme at pH 12.0 is shown in Fig. 3. Under these conditions, the native enzyme showed a first-order-like degradation with a kinetic constant $k = 0.64\ \text{min}^{-1}$. Similar behaviour was obtained for CMC–trypsin complex until 120 min of incubation, but the lower value of the kinetic constant $k = 0.34\ \text{min}^{-1}$ for this process indicates the improved stability of the conjugated enzyme towards autolysis. At longer periods of incubation, the modified enzyme retained over 55% of its initial activity.

Fig. 4 shows that the stability in the presence of 0.3% SDS was improved for the CMC–trypsin complex. The CMC-modified trypsin was also more resistant to the action of denaturant agents such as 8 M urea and 6 M GdmCl as shown Fig. 5.

The activity of modified trypsin in monophasic system methanol/water is shown in Fig. 6. Under these conditions, the enzyme stability was improved for CMC–trypsin complex with a maximum activity obtained at 3.05 M water.

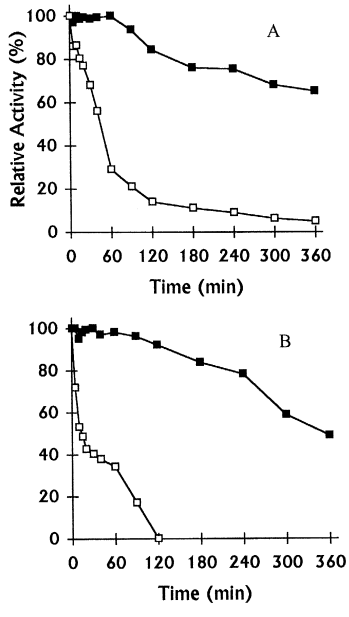


Fig. 5. Effect of 8 M urea (A) and 6 M GdmCl (B) on the stability of native (□) and CMC-modified trypsin (■).

4. Discussion

Trypsin from bovine pancreas was covalently linked to the polyaldehyde derivative of CMC via reductive alkylation with $NaBH_4$ (Scheme 1). In this process, the N-terminal α-amino group, as well as the ε-amino groups from lysines, could be transformed into acid unstable Schiff bases, which yield secondary alkyl amines by $NaBH_4$ treatment [24]. Trypsin contains 14 lysine residues and its N-terminal residue is an isoleucine [25]. None of these amino acid residues participates in the active site of the enzyme, a classic Asp102–His57–Ser195 triad from mammalian serine proteases [26].

The covalent attachment of CMC to the surface of trypsin results in several changes in the structural and functional properties of the enzyme. The value for optimum pH and K_m for esterolytic activity changed after this modification. These results suggest that the attachment of the anionic polymer affects the ionisation of the amino acid residues at the active site of trypsin.

The mechanism of catalysis for trypsin reveals that the His57 imidazole ring acquires a proton from catalytic Ser195 in the transition state [26]. Consequently, one role for the conserved Asp120 is to provide electrostatic compensation for the developing positive charge in His57. In fact, site-directed mutagenesis experiments have demostrated that a negative charge, adjacent to His57, is essential for efficient catalysis in trypsin [27,28]. According to this mechanism, we can expect that anionic groups from CMC could contribute to the electrostatic stabilisation of His57 in the transition state, then affecting the kinetic parameters as well as the optimal conditions for enzyme catalysis.

On the other hand, the three-dimensional structure of trypsin reveals that Lys188, which is located at the surface loop 1 near the substrate binding pocket [26,29], is one of the most available residues for conjugating to CMC. This modification could contribute to increased optimum pH range showed by the trypsin–CMC complex because the presence of a lysine charge at position 188 plays an important role in regulating the pH range of catalysis for trypsin, as was demostrated by site-directed mutagenesis studies [30,31].

The stability of the enzyme under various denaturing conditions was improved for the trypsin–CMC complex. This stabilisation results from the contribution of several factors in the maintenance of the active conformation of the enzyme. Among these, the most important factor is the conformational stabilisation of trypsin molecules due to intramolecular cross-linking caused by the multipoint attachment of one molecule of polymer to several amino groups from the same polypeptide chain [1,7,32]. In our study, a high degree of intramolecular cross-linking was estimated for trypsin–CMC complex.

Protein aggregation plays an important role in the thermal denaturation mechanism of trypsin [33]. For this reason, other factors contributing to the improved thermostability of the modified enzyme (Fig. 1) could be the electrostatic repulsion between the enzyme molecules attached to the anionic polysaccharide.

Another factor contributing to the stabilisation of modified trypsin is the ionic interaction between positively charged groups at the surface of the protein and carboxylate groups from CMC. Intramolecular salt bridges are, in fact, one of the principal forces contributing to the maintenance of the active conformation of enzymes [34]. In the present work, the hypothesis cited above is supported by the fact that the relative activity for modified trypsin was reduced from 94% to 83%, when all carboxylate groups from CMC were converted into the acidic form by changing the pH from 3.0 to 2.0 [35]. In contrast, no significant variation on the activity was observed for native enzyme at this limb of pH (Fig. 2).

At alkaline values of pH, the improved stability for CMC–trypsin conjugate was lower than at acid pH. This is because at alkaline pH, the ionogenic groups from His, Lys and Arg residues are dissociated to neutral groups, resulting in a disruption of the intramolecular salt bridges with carboxylate groups from CMC. For this reason, the stabilisation of CMC–trypsin complex at alkaline pH is only provided by the reduction of the autolytic degradation rate, as explained below.

In general, autolytic processes are involved in all denaturation mechanisms of proteases [13] because the unfolded protein molecules are more prone to proteolysis than their corresponding folded forms [36,37]. Modification of lysine residues, as well as the steric hydrance caused by the bulky polysaccharide substituents, are indeed, determinant factors in the improved sta-

bility against proteolytic autodegradation shown by the modified trypsin (Fig. 3).

Other factors, such as the influence on protein hydration, the formation of hydrogen bonds between the covalently attached polysaccharide and the hydrophilic amino acid residues at the surface of the enzyme, are also important factors contributing to trypsin stabilisation by covalent modification with carbohydrate polymers [38,39].

Proteolytic enzymes are widely used as active components in industrial and laundry detergents [40]. That is why, the development of new and inexpensive methods for stabilising proteases in the presence of surfactants receives increased attention. Fig. 4 shows that the stability against the anionic surfactant SDS was improved for the CMC–trypsin complex. Two simultaneously occurring processes might be assumed for trypsin deactivation by SDS at alkaline pH: (1) an increased binding of SDS to the protein, increasing the fraction of unfolded enzyme and (2) an increased proteolytic rate as a result of increased susceptibility of the substrate (i.e., unfolded trypsin molecules) [41]. According to this mechanism, the stabilisation of modified trypsin could be caused by the multipoint attachment of CMC, which protects the enzyme against proteolytic attack and prevents the unfolding of trypsin molecules, as was explained above. This stabilisation suggests that CMC-modified proteases could be valuable products for detergent formulations.

In this work, we have reported the use of CMC, a non-toxic and biocompatible polymer, for modifying trypsin. The results suggest that the covalent modification of serine proteases by the anionic polysaccharide CMC might be a useful method for improving the enzyme stability under various denaturing conditions.

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