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Preparation of thermostable trypsin–polysaccharide neoglycoenzymes through Ugi multicomponent reaction

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ABSTRACT

A novel synthetic method for preparing enzyme-polysaccharide derivatives is described, based on the use of the Ugi multicomponent reaction. Bovine pancreatic trypsin, the target enzyme, was cross-linked with the anionic polysaccharides *O*-carboxymethylcellulose (CMC) and sodium alginate in the presence of formaldehyde and *t*-butyl isocyanide. The protease retained 69–61% and 43–37% of its initial esterolytic and proteolytic activity after cross-linking. The thermostability of the enzyme was enhanced from 49 °C to 57 °C after modification. The resistance to inactivation at 50 °C was 14- and 6-fold increased, and the activation free energy of thermal inactivation at this temperature was 7.2 kJ/mol and 4.9 kJ/mol higher after modification with *O*-carboxymethylcellulose and sodium alginate, respectively. The enzyme was 15- and 46-fold more resistant to autolytic degradation at pH 9.0 after cross-linking with these polysaccharides.

1. Introduction

In our days, highly stable enzyme forms able to work in homogeneous systems are required for several industrial, biomedical and analytical applications. Covalent cross-linking with polyfunctional compounds, such as polysaccharides, is a well-established method for improving structural rigidity to these biocatalysts [1]. In general, this multipunctual glycosidation also confers increased functional stability to enzymes under extreme denaturing/inactivating conditions for proteins [2].

Several chemical and enzymatic methods have been described for preparing this kind of neoglycoconjugates. Among these, it should be mentioned: (a) reductive alkylation of the enzyme with the peryodate-oxidized polymer derivatives [3,4]; (b) multipoint attachment of CNBr-activated polysaccharides to the protein surface [5]; (c) cross-linking with ionic polymers through a carbodiimide-catalyzed reaction [6,7]; and (d) transglutaminase-catalyzed cross-linking with aminated polysaccharides [8].

In the present work, a novel synthetic procedure was employed for preparing polysaccharide-based neoglycoenzymes, by using an Ugi-four component reaction (U-4CR) as coupling approach. U-4CR is a one-pot multicomponent reaction in which a carboxylic acid, a primary (or secondary) amine, a ketone or aldehyde, and a C-isocyanide are rapidly condensed, yielding an isopeptide derivative in a quasi-quantitative proportion [9]. Although this versatile reaction has been widely employed in organic synthesis since its discovery in 1959 [9], this method has not been previously employed for preparing neoglycoenzymes.

As target enzyme we selected bovine pancreatic trypsin (EC 3.4.21.4), a serine protease widely employed in biomedicine, food and biotechnology industry, as well as in protein analysis [10]. This enzyme has been also extensively employed as a model for the development of new methods for stabilizing enzymes [11–13]. This manuscript describes the covalent modification of the free amino groups located at the protein surface of trypsin by *O*-carboxymethylcellulose (CMC) and sodium alginate (ALG) through a U-4CR, and the effects of these transformations on the catalytic and stability properties of the protease.

2. Experimental procedures

2.1. Materials

Bovine pancreatic trypsin, Fractogel EMD BioSEC (S) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) were obtained from Merck (Darmstadt, Germany). Low viscosity carboxymethylcellulose sodium salt (CMC, $M_v = 3.0 \times 10^4$, substitution degree = 0.7) and sodium alginate from *Laminaria hyperborea*

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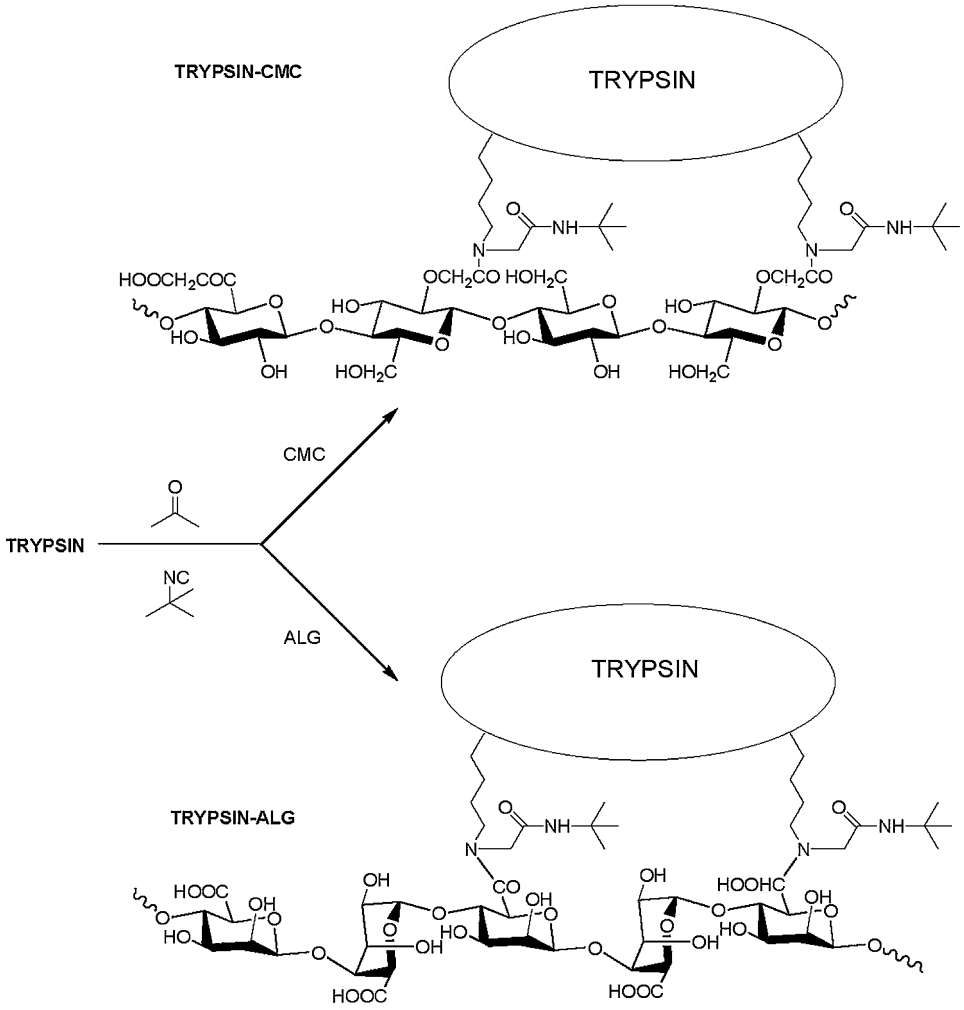

Scheme 1. Preparation of trypsin–CMC conjugate via Ugi-4CR.

(ALG, $M_v = 2.0 \times 10^5$, uronate composition = 37.5% mannuronate and 62.5% guluronate) were obtained from BDH (Poole, UK). All other chemicals were analytical grade.

2.2. Preparation of trypsin–polysaccharide conjugates

Ten milligrams of polymers were dissolved in 5 ml of 50 mM sodium phosphate buffer, pH 5.0, and then 10 mg of trypsin were added. The mixture was stirred at 4 °C for 2 h, and then 23 mmol formaldehyde and 23 mmol *t*-butyl isocyanide were added. The reaction was stirred for 12 h at 4 °C, then exhaustively dialyzed with several changes against 50 mM sodium phosphate buffer, pH 5.0 using Spectra/Por 2 dialysis membranes (cut-off 12–14 kDa), and further applied to a gel filtration column Fractogel EMD BioSEC (S) (2.6 × 60 cm) equilibrated in the same buffer made 100 mM NaCl. The fractions containing the polymer–enzyme complex were pooled and kept at 4 °C.

2.3. Enzymatic assays

Esterase activity of native and modified trypsin preparations was determined at 25 °C in 67 mM TrisHCl buffer, pH 8.0 using BAEE as substrate [14]. One unit of esterolytic activity is defined as the amount of enzyme that hydrolyses 1.0 μmol BAEE per min at 25 °C. Proteolytic activity was determined as described by Laskowski [15] using milk casein as substrate. One unit of proteolytic activity, katal, is defined as the amount of enzyme that releases 1 mol of tyrosine/second at 25 °C.

2.4. Characterization of the conjugates

Total carbohydrates in the conjugates were determined by the phenol–sulfuric acid method [16] using glucose as standard. Protein concentration was estimated as described by Lowry et al. [17] using bovine serum albumin as standard. The degree of modification of amino groups was determined by measuring the amount of free amino groups, in native and modified protein samples, with *o*-phthalaldehyde using glycine as standard [18].

2.5. 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.

2.6. Kinetics of thermal inactivation

Native and modified enzyme preparations were incubated at different temperatures ranging from 45 °C to 60 °C in 50 mM sodium

Table 1
Structural properties of trypsin–polysaccharide conjugates*.

Enzyme	Polymer content (mol/mol protein)	Free amino groups (mol/mol protein)	Degree of cross-linking (mol/mol protein mole polymer)
Native	–	15	–
Trypsin–CMC	1.6	6	5.6
Trypsin–ALG	1.0	9	6.0

* The data represented are the means from at least triplicate measurements with standard error less than 5%.

Table 2
Catalytic properties of trypsin–polysaccharide conjugates*.

Enzyme	Esterolytic activity (U/mg)	Proteolytic activity (katal/kg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
Native	36	3.0×10^{-2}	38.7	12.4	3.3×10^5
Trypsin–CMC	25	1.3×10^{-2}	49.2	10.6	2.2×10^5
Trypsin–ALG	22	1.1×10^{-2}	52.0	8.2	1.6×10^5

* The data represented are the means from at least triplicate measurements with standard error less than 5%.

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. The activation Gibbs energy of inactivation (ΔG_i) for all enzymes forms was calculated according to the following equation:

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

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

2.7. 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.

3. Results and discussion

The free amino groups of trypsin were covalently linked to the carboxylate groups of CMC and ALG through a U-4CR, forming a stable isopeptide structure (Scheme 1). The modified enzyme samples were further purified by dialysis and gel filtration chromatography on Fractogel EMD BioSEC (S) gels. The structural properties of the enzyme–polymer complexes are summarized in Table 1 . Analytical characterization of the conjugates revealed an average of 1.6 and 1.0 mol of CMC and ALG attached to each mole of modified enzyme, corresponding to modification of 57% and 43% of the free amino groups from trypsin, respectively. According to these values, the degree of intramolecular cross-linking for trypsin–CMC and trypsin–ALG complexes was estimated as 5.6 mol and 6.0 mol of modified amino groups per mol of polymer in each mol of enzyme, respectively. It should be mentioned that the lower polymer content in the trypsin–ALG complex could be caused by the higher molecular weight of this polymer, in comparison with CMC. According with this hypothesis, the high molecular weight ALG chains attached to the protein should avoid the subsequent interactions of other polysaccharide molecules due to steric hindrance, then reducing the occurrence of further modification reactions.

The catalytic properties of the glycosidated enzymes are reported in Table 2. The esterase activity of the modified enzyme represented about 69% and 61% of the corresponding to native trypsin after cross-linking with CMC and ALG, respectively. Lower activity was shown by the conjugates towards a macromolecular substrate, casein. In this regards, the enzyme retained 43% and 37% of the initial proteolytic activity after glycosidation with the polyanions CMC and ALG, respectively. The kinetics constants for the hydrolysis of BAEE with trypsin were also affected by this glycosidation process. The affinity of the enzyme for BAEE, as well as the catalytic constant and the turnover number, was lower for both modified enzymes in comparison with the native counterpart.

This reduction in the catalytic properties of the enzyme could be explained by the high hydrodynamic volume of the anionic polysaccharides attached to the protease surface, reducing the interaction of trypsin which its substrates due to steric hindrance phenomena. Similar reduction in the catalytic behavior of trypsin modified with other polysaccharides has been previously reported [7,8,11]. It should be also noted that the enzyme could be partially inactivated during the coupling process, mainly by the presence of formaldehyde in the reaction medium.

Despite these effects on the catalytic properties of trypsin, glycosidation with the polymers via U-4CR yields enzyme derivatives with noticeable stability against several inactivating conditions for the protease. Fig. 1 shows the thermal stability profiles for native and modified trypsin forms after 10 min of incubation at different temperatures. Native enzyme was significantly inactivated at temperatures higher than 45 °C showing a value of T_{50}, defined as the temperature at which 50% of the initial activity was retained, equal to 49 °C. On the other hand, both modified trypsins showed similar inactivation patterns, with improved resistance to thermal treatment in comparison with the native counterpart. In this regards, trypsin–CMC and trypsin–ALG complexes showed the same value of T_{50} = 57 °C.

The time-course of thermal inactivation of the biocatalysts prepared was studied by incubating all trypsin preparations in 50 mM sodium acetate buffer, pH 5.0 at different temperatures, ranging

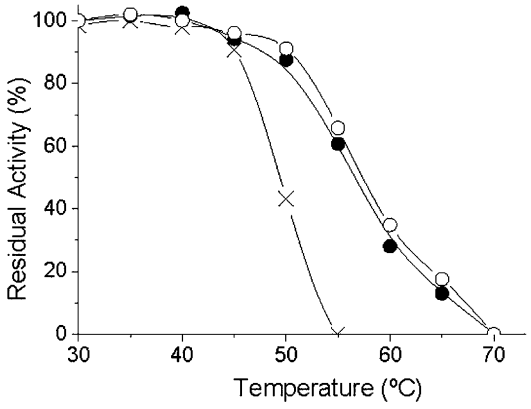

Fig. 1. Thermal stability profile of native (×) and modified trypsin with CMC (○) and ALG (●).

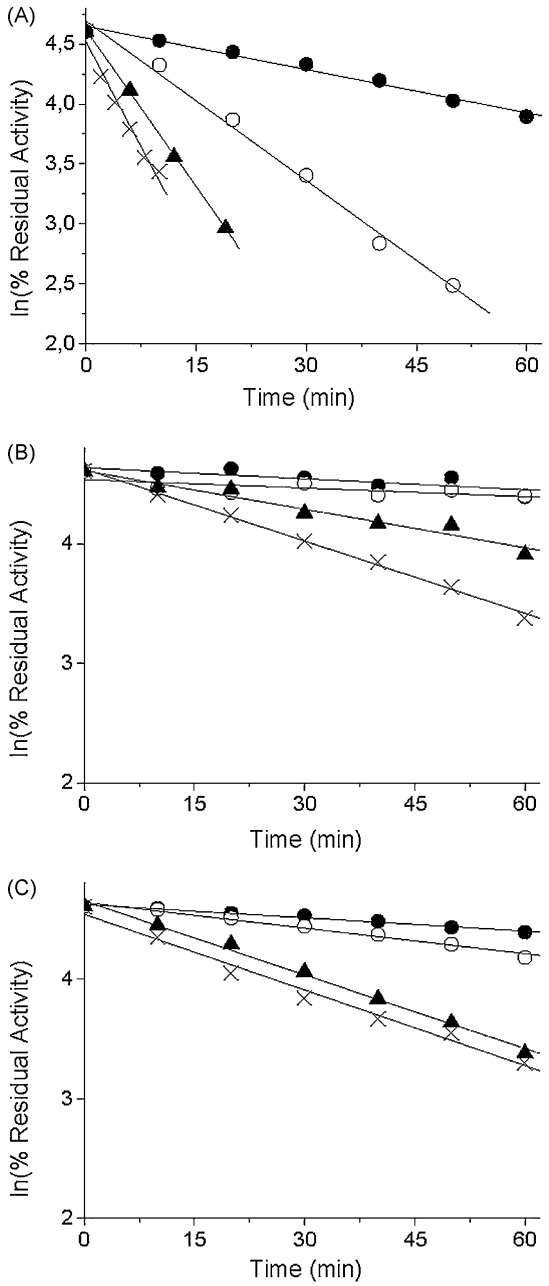

Fig. 2. Kinetics of thermal inactivation of native (A) and modified trypsin with CMC (B) and ALG (C) at 45 °C (×), 50 °C (▲), 55 °C (○) and 60 °C (●).

from 45 °C to 60 °C (Fig. 2). Under these conditions, all enzyme preparations lost catalytic activity progressively with the time of incubation according to a first-order kinetics process. However, the half-life time values at each temperature evaluated were significantly higher for the enzyme–polymer complexes, in comparison with the corresponding to native protease (Table 3). This effect was

Table 3
Half-life times of native and modified trypsins at different temperatures*.

Enzyme	Half-life (min)			
	45 °C	50 °C	55 °C	60 °C
Native	58 ± 6	16 ± 1	9.0 ± 0.3	6.0 ± 0.5
Trypsin–CMC	348 ± 12	231 ± 17	64 ± 9	35 ± 4
Trypsin–ALG	173 ± 8	99 ± 10	35 ± 4	31 ± 5

* The data represented are the means from at least triplicate measurements.

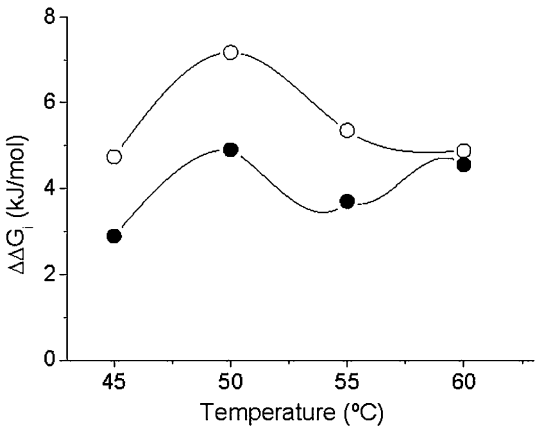

Fig. 3. $\Delta\Delta G^{\ddagger}$ *vs* temperature profiles between the native and modified trypsin CMC (○) and ALG (●).

remarkably higher at 50 °C, temperature at which $t_{1/2}$ of trypsin was 14- and 6-fold higher after modification with CMC and ALG, respectively.

The data obtained from kinetics of thermal inactivation experiments were processed in order to determine the influence of the additive on the activation Gibbs energy of inactivation at each temperature. Fig. 3 shows that ΔG_i was significantly increased for trypsin after modification with the polymers. A maximum increase of 7.2 kJ/mol and 4.9 kJ/mol at 50 °C was observed for ΔG_i of the enzyme modified with CMC and ALG, respectively. This increment in ΔG_i represents good stabilization for the modified trypsin forms, taking into account the net free energy for stabilization of globular proteins [19].

Preservation of the catalytic activity at high temperatures of the trypsin–polymer complexes could be justified by the contribution of the different covalent and non-covalent interactions between the protein structure and the polysaccharide molecules [2–8]. Among these, covalent and ionic cross-linking should be considered the most important contributing forces in the thermostabilization showed by the neoglycoenzyme prepared.

The time-course of autolysis for native and modified enzyme was evaluated in Tris buffer at pH 9.0 and 30 °C. As shown in Fig. 4, the native enzyme showed a first-order-like degradation kinetic pattern with a half-life time of 15 min. On the contrary, modification of trypsin with CMC and ALG yield derivatives that showed noticeable stabilization against autolytic degradation in alkaline conditions, showing half-life time values of 3.9 h and 11.6 h, respectively. The stabilization conferred to this enzyme against autolytic processes should be mainly associated with the

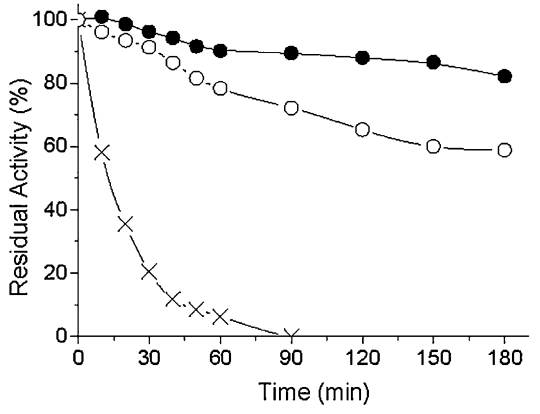

Fig. 4. Kinetics of autolytic degradation for native (×) and modified trypsin with CMC (○) and ALG (●).

formation of isopeptide structures involving the lysine residues, and then avoiding their recognitions by other trypsin molecules. In addition, the structural rigidity provoked by the covalent cross-linking with the polymers as well as the steric hindrance caused by these macromolecules should also significantly contribute to stabilize to enzyme molecule against autolytic degradation.

4. Conclusions

In this work, we propose a novel synthetic route for preparing enzyme-polysaccharide neoglycoderivatives via U-4CR. By employing bovine trypsin as target enzyme, we demonstrated that a remarkable stabilization against thermal treatment and autolytic degradation was conferred to the protease after this glycosidation process. Experiments are now in progress to generalize this conjugation strategy for other enzymes and proteins.

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