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Thermal stabilization of trypsin with glycol chitosan

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Abstract

Glycol chitosan was evaluated as thermoprotectant additive for trypsin in aqueous solutions. Maximal stabilization was achieved by using a polymer/protein ratio of 2 (w/w). The catalytic properties of trypsin were not affected by the presence of the polysaccharide. The enzyme thermostability was increased from 49 °C to 93 °C in the presence of the additive. Trypsin was also 37-fold more stable against incubation at 55 °C and its activation free energy of thermal inactivation was increased by 9.9 kJ/mol when adding glycol chitosan.

Keywords: Trypsin; Glycol chitosan; Enzyme stability; Additive

1. Introduction

During last decades, increasing attention has been devoted to prepare stabilized enzyme forms. In special, the improvement of thermorresistance to industrial enzymes has received considerable attention, in order to design more economic and efficient production processes catalyzed for these biomolecules. Strategies for thermostabilizing enzymes in aqueous media have been mainly addressed to produce genetically [1,2], chemically [3–6] or enzymatically [7,8] modified enzyme variants, as well as to use water-soluble compounds as thermoprotectant additives [9–11]. Among these, the later appears as the most economic approach, taking into account the simplicity and low cost of this method.

Addition of substances such as polyols can increase stability of enzymes through different mechanisms. This protective effect can be mediated by the formation of new hydrogen bonds and polar interactions at the surface of the protein, the increase in the free energy of protein denaturation and changes in the structure of water [10,12,13]. In recent reports, we described the use of β-cyclodextrin branched polysaccharides as thermostabilizing agents for enzymes through supramolecular-mediated interactions [14,15]. On the other hand, enzymes can be stabilized by electrostatic interactions between the additive substance and the tertiary protein structure when charged macromolecules are used as protecting agents [16].

Chitosan, a copolymer of β(1-4)-D-glucosamine and β(1-4)-*N*-acetyl-D-glucosamine prepared by alkali deacetylation of chitin [17], is a non-toxic, biodegradable and positive charged polysaccharide widely used as support for enzyme immobilization [18,19]. Chitosan has been also employed as modifying agent for preparing thermostable neoglycoenzymes [4,20]. However, low solubility at neutral and basic pH [21] has limited the use of this polysaccharide as thermoprotectant additive for enzymes. In spite of this, water-soluble derivatives prepared by chemical transformation of chitosan can be evaluated for these purposes. This work is focusing on the application of glycol chitosan as additive for the stabilization of bovine pancreatic trypsin in aqueous media.

2. Experimental procedures

2.1. Materials

Glycol chitosan was purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and bovine pancreatic trypsin

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(30 U/mg versus BAEE [22], 3.0×10^{-2} katal/kg versus casein [23]) and were obtained from Merck. All other chemicals were of analytical grade.

2.2. 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 [22]. One unit of esterolytic activity was 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 [23] using milk casein as substrate. One unit of proteolytic activity, katal, is defined as the amount of enzyme that releases one mole of tyrosine per second at 25 °C. Protein concentration was estimated from $\varepsilon_{280\,nm} = 3.7 \times 10^4\ M^{-1}$ [24].

2.3. Thermal stability profile

Trypsin preparations were incubated at scheduled temperatures in 50 mM sodium acetate buffer, pH 5.0 (0.02 mg protein/ml), in the absence and presence of the additive solutions (0.04 mg polymer/ml). Aliquots were removed after 10 min of incubation, chilled quickly and assayed for esterolytic activity.

2.4. Kinetics of thermal inactivation

Trypsin preparations were incubated at different temperatures ranging from 45 °C to 70 °C in 50 mM sodium acetate buffer, pH 5.0 (0.02 mg protein/ml), in the presence and absence of the polysaccharide solutions (0.04 mg polymer/ml). Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity. The first-order rate constants, k_i, of inactivation 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:

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

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

3. Results and discussion

In order to determine the optimum concentration of glycol chitosan on the thermal stability properties of trypsin, the half-life times ($t_{1/2}$) of the enzyme at 50 °C was determined in the presence of different concentrations of the additive. We defined the thermoprotective effect of the polysaccharides as the ratio between $t_{1/2}$ of trypsin in the presence and in the absence of this additive. As is illustrated in Fig. 1, $t_{1/2}$ values increase progressively when the concentration of the polymer increases, reaching maximal stabilization at ratio higher than 2.0 mg polymer/mg protein, corresponding to 0.04 mg/ml polymer concentration. Consequently, this value of optimum polymer concentration was selected for further experiments.

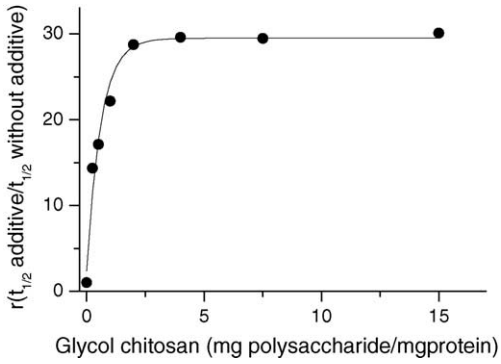

Fig. 1. Effect of glycol chitosan concentration on the thermal stabilization of trypsin at 50 °C.

In general, the structure and activity of water is changed after addition of polyols such as polysaccharides [25]. This effect leads to increase thermal stabilization to enzyme protein structure in the presence of such kind of additive. In addition, it is expected that the formation of new hydrogen bonds between glycol chitosan chains and hydrophilic residues at the surface of trypsin molecules could increase conformational rigidity to this enzyme, and consequently improve its resistance to elevated temperatures. Both of these effects depend of the amount of additive in the enzyme protein solution, and could justify the thermal stability behaviour of trypsin in the presence of different concentration of glycol chitosan (Fig. 1).

However, in the present study we determined that the improved thermostabilization showed by trypsin after adding glycol chitosan is mainly mediated by electrostatic forces. In this sense, both the occurrence of direct electrostatic interactions between the positive charged polysaccharide and the protein structure as well as changes in the electrostatic potential at the microenvironment of the enzyme after adding the cationic polymer could be involved in this stabilizing mechanism. Experimental evidences about this thermostabilizing "electrostatic effect" of glycol chitosan on trypsin molecules are described below.

Catalytic properties of trypsin were not affected by adding the positive charged polymer at the above-cited concentration. The specific proteolytic and esterolytic activities, as well as the catalytic constants, remained the same in the presence and absence of glycol chitosan. This result suggests that both the tertiary structure of the enzyme as well as the ionization of the amino acid residues at the active site were not substantially affected by the introduction of the polymeric molecules of glycol chitosan in the catalytic reaction media.

On the other hand, the addition of glycol chitosan to trypsin solutions not protected the enzyme against autolytic inactivation processes at pH 9.0 (data not shown).

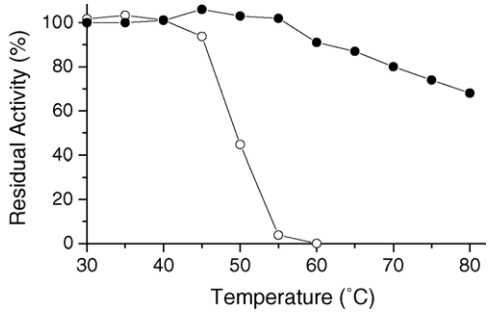

Fig. 2. Thermal stability profile of trypsin in the absence (◯) and presence (●) of glycol chitosan (0.04 mg polymer/ml).

Fig. 2 shows the thermal stability profile of trypsin after 10 min of incubation at different temperatures in the absence and presence of the additive. The enzyme preparations containing glycol chitosan were remarkably more stable at temperatures higher than 45 °C, in comparison with the free enzyme. In this sense, the value of T_{50}, defined as the temperature at which 50% of the initial activity was retained, was increased for trypsin from 49 °C to about 93 °C after addition of this modified polymer. It should be notify that the later value was calculated from the slope of the decay temperature–activity curve for trypsin between 45 °C and 80 °C showed in Fig. 2, and assuming that the shape of the curve is not changing at temperatures higher than 80 °C. However, this calculated value helps us to predict higher catalytic stabilization for the protease–glycol chitosan system at elevated temperatures.

It should be noted that this thermostabilizing effect conferred to trypsin by adding glycol chitosan was noticeably higher than those previously achieved by using β-cyclodextrin branched polysaccharides [14,15].

The time course of thermal inactivation of trypsin preparations was studied in the range of temperatures between 45 °C and 70 °C. The results of these experiments are reported in Table 1. Half-life time values at each temperature evaluated were significantly higher for the enzyme in the presence of the polymer, in comparison with the corresponding to native protease. This result demonstrates that glycol chitosan molecules play a stabilizing effect over the active structure of trypsin at elevated temperatures. This effect was remarkably higher at 55 °C, temperature at which $t_{1/2}$ of trypsin was 37-fold higher after addition of the polymer.

Table 1
Half-life times of trypsin in the absence and presence of glycol chitosan

Temperature (°C)	Half-life time (min)	
	Trypsin	Trypsin + glycol chitosan
45	61 ± 3	825 ± 24
50	15.8 ± 0.4	455 ± 18
55	8.2 ± 0.6	305 ± 9
60	5.2 ± 0.2	117 ± 11
70	–	42 ± 3

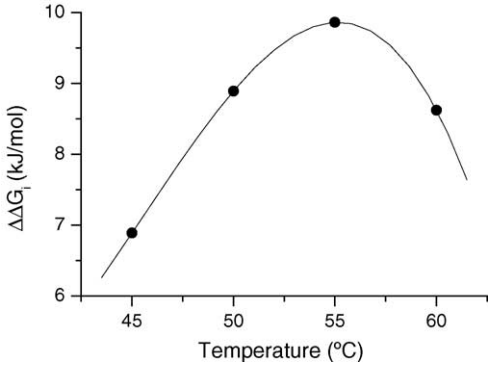

Fig. 3. $\Delta\Delta G_i$ vs. temperature profile between the free and the glycol chitosan containing trypsin preparations (0.04 mg polymer/ml).

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. As is illustrated in Fig. 3, ΔG_i was significantly increased for the protease after addition of glycol chitosan in all range of temperatures studies. A maximum increase of 9.9 kJ/mol was observed for ΔG_i at 55 °C. This increment in ΔG_i represents good stabilization for trypsin in the presence of the additive, considering that the net free energy for stabilization of globular proteins is, in general, small [25].

Preservation of trypsin activity at high temperatures in the presence of glycol chitosan could be justified by the contribution of the different thermostabilizing mechanisms cited above as classics for polyols [10,12,13]. In addition, a possible factor contributing to the thermostabilization of trypsin in the presence of glycol chitosan could be the formation of electrostatic interactions at the surface of the enzyme between the negative charged groups of the protein and the amino groups from the polysaccharide. By this mechanism, the polysaccharide chains could increase the rigidity of the enzyme structure through multipoint electrostatic cross-links, reducing the protein chain mobility of trypsin and preserving then its active conformation at high temperatures.

In order to confirm this hypothesis, the inactivation pattern of trypsin preparations at 55 °C was determined by incubating

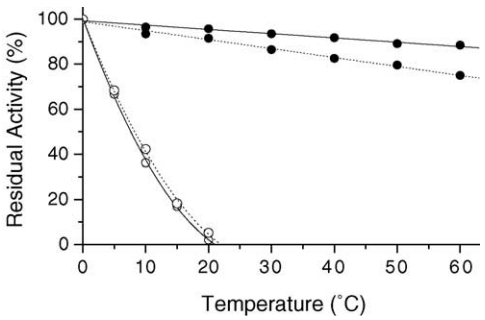

Fig. 4. Kinetics of thermal inactivation of trypsin (◯) and trypsin + glycol chitosan (●) preparations (0.7 mg polymer/ml) at 50 °C in the presence (····) and the absence (——) of 1.0 M CH_3CO_2Na.

the enzymes during 1 h at pH 5.0 in the presence and in the absence of 1.0 M CH_3CO_2Na. It is well known that polyelectrolyte interactions are energetically affected in high ionic strength media [26]. For this reason, it is expected that the presence of this salt in the incubating solutions could disrupt any stabilizing electrostatic interaction occurring between the protease and the polymer.

As is illustrated in Fig. 4, the stability of trypsin was not significantly affected by the addition of CH_3CO_2Na. On the contrary, the $t_{1/2}$ of the enzyme in the presence of glycol chitosan was 2.4-fold reduced after addition of the salt. This result demonstrated that the stabilization showed by the enzymes in the presence of the chitosan derivative is partially mediated by an electrostatic mechanism.

At this point, it should be notify that not difference in the kinetics of thermal inactivation of trypsin preparations was found when similar experiment was performed by using 1.0 M NaCl as dissociating salt. This phenomenon could be explained by the lower affinity that chitosan shows for Cl^- ions, in comparison with $CH_3CO_2{}^-$ [17].

4. Conclusions

In this work, we propose the use of a water-soluble derivative of chitosan, glycol chitosan, as additive for increasing the stability properties of bovine pancreatic trypsin in aqueous solutions. Independent of any mechanistic conclusions, the remarkable stabilization conferred to this protease in the presence of the cationic polyelectrolyte at high temperatures is relevant from a practical point of view.

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