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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.

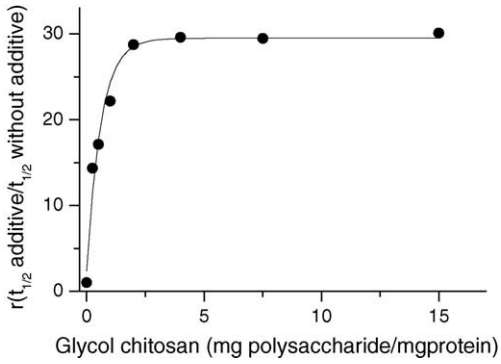

Fig. 1. Effect of glycol chitosan concentration on the thermal stabilization of trypsin at 50 °C.

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).