Thermostabilization of *Bacillus amyloliquefaciens* α-amylase by chemical cross-linking

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Abstract

Chemical cross-linking of a mesophilic α-amylase from *Bacillus amyloliquefaciens* (BAA) was carried out. Intra-molecular cross-links between lysine residues upon treatment of the enzyme with ethylene glycol bis(succinic acid *N*-hydroxy succinimide ester) resulted in enhancement of thermostability as indicated by irreversible thermoinactivation experiments. Enhancement of thermostability coincided with a dramatic protection against aggregation, combined with a decrease in surface hydrophobicity. Deamidation, another important mechanism of irreversible thermoinactivation, was also diminished upon modification. While no significant changes in the kinetic parameters are evident, rigidification of the protein structure is suggested by circular dichroism (CD) and fluorescence studies.
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Keywords: α-Amylase; Chemical cross-linking; Thermostability; Aggregation; Hydrophobicity; Deamidation

1. Introduction

α-Amylases are endo-acting enzymes which belong to the glycoside hydrolase family 13 (GH13) (Henrissat, 1991) and hydrolyse cleavage of α-1,4-glycosidic bonds in starch and related oligosaccharides and polysaccharides at random. They have been characterized from a wide variety of organisms and almost all of them have similar structures and catalytic mechanisms (MacGregor et al., 2001). They are also among the most important commercial enzymes with a number of applications, including their use in starch processing, baking, paper and textile industries. In some of these processes, α-amylases must resist extreme conditions, such as elevated temperatures and alkaline/oxidizing environments containing surfactants, chelating agents and proteases (Nielsen and Borchert, 2000). Two principal methods have

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been used for stabilizing α-amylases: isolation of thermophilic forms and modification of existing structures, either chemically, or by employing protein engineering methods. Our own recent studies involving chemical modification of lysine residues in BAA indicated improved stability of the enzyme (Khajeh et al., 2001a). In the present study, we have investigated the effect of introducing intra-molecular cross-links between lysine residues in the same enzyme. Four homobifunctional cross-linkers have been used, the product of one of which showed greater stabilization and was further characterized.

2. Materials and methods

Mesophilic α-amylase from *Bacillus amyloliquefaciens* (BAA), dimethyl suberimidate, dimethyl pimelimidate, suberic acid bis(*N*-hydroxy succinimide ester), ethylene glycol bis(succinic acid *N*-hydroxy succinimide ester) were purchased from Sigma (St. Louis, MO, USA). Ethylidene ρ-nitrophenyl-α-D-maltoheptaoside (EPS) was provided by Roche (Mannheim, Germany) and all other chemicals were obtained from Merck (Darmstadt, Germany). Reproducibility of the data presented in this manuscript was confirmed by repeating the experiments at least twice.

2.1. Methods

2.1.1. Modification with dimethyl pimelimidate and dimethyl suberimidate

Two milligrams per milliliter of each cross-linker was separately added to 10 mg/ml of the enzyme in 0.1 M phosphate buffer pH 8 (Ryan et al., 1994; De Renobales and Welch, 1980). After completion of the reaction at room temperature, sodium borohydride was added in order to reduce the resulted Schiff's base (Blanco and Guisán, 1989). Samples thus obtained were then extensively dialyzed at 4 °C against 20 mM Tris buffer pH 7.5.

2.1.2. Modification with suberic acid bis(N-hydroxy succinimide ester) and ethylene glycol bis(succinic acid N-hydroxy succinimide ester)

2.5 mg/ml suberic acid bis(*N*-hydroxy succinimide ester) or 3 mg/ml ethylene glycol bis(succinic acid

N-hydroxy succinimide ester) previously dissolved in dimethyl sulfoxide, was added to a mixture of 10 mg/ml of enzyme in 0.1 M phosphate buffer pH 8 (Ryan et al., 1994; Massague et al., 1981). Upon completion of the reaction at room temperature, the products were extensively dialyzed against 20 mM Tris buffer pH 7.5 at 4 °C. Control samples were prepared following the same procedure in the absence of the modifier.

To determine the number of modified lysine residues, the number of free amino groups was measured following the TNBS method (Fields, 1971; Cayot and Tainturier, 1997). Samples were first dialyzed in 0.1 M borate buffer, pH 9.5 and the final protein concentration was adjusted to 0.5 mg/ml.

Protein concentration was determined by the use of pyrogallol red (Watanabe et al., 1986) and Bradford (Bradford, 1976) methods.

2.1.3. Determination of enzymatic activity

The amylase activity (3 μg/ml of the enzyme) was determined at 37 °C, using 1% soluble starch as substrate at pH 7.5 in 20 mM Tris and the Bernfeld method to determine reducing sugars (Bernfeld, 1955). For maltosidase activity, α-amylase (6 μg/ml) was assayed at 37 °C, using EPS (2 mM) as substrate in 20 mM Tris buffer pH 7.5. This procedure is based on the progressive hydrolysis of the substrate, gradually releasing ρ-nitrophenol with an absorption maximum at 405 nm (Hägele et al., 1982).

2.1.4. CD measurements

CD spectra were obtained using a Jasco-J-715 spectropolarimeter (Japan). Protein concentrations were 0.2 mg/ml (far-UV spectra) and 1 mg/ml (near-UV spectra). Results are expressed as molar ellipticity, $[\Theta]$ ($^{\circ}$ cm^2 dmol^{-1}), based on a mean amino acid residue weight (MRW) assuming average weight of 113.4 for BAA. The molar ellipticity was determined as $[\Theta]_{\lambda} = (\theta \times 100 \ \text{MRW})/(cl)$, where c is the protein concentration in mg/ml, l the light path length in cm, and θ is the measured ellipticity in degrees at a wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\Theta]_{291} = 7820^{\circ}$ cm^2 dmol^{-1}, and with JASCO standard non-hydroscopic ammonium (+)-10-camphorsulfonate, assuming $[\Theta]_{290.5} = 7910^{\circ}$ cm^2 dmol^{-1} (Protasevich et al., 1997). The data were smoothed using the JASCO-J-715 software, including