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Poly-ε-lysine amylase conjugates to increase the stability of enzyme

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ABSTRACT

Poly-ε-lysine (ε-PL), an unusual naturally occurring homopolyamide of L-lysine having linkage between ε-amino and α-carboxyl groups, is biodegradable, edible and non-toxic towards human and environment. The conjugate of ε-PL with amylase was successfully prepared by ionic interaction. The ε-PL-amylase conjugate showed better temperature and pH stability than native enzyme. The degradation of enzyme by temperature and pH followed first order degradation kinetics. The *k* value (reaction constant), *D* value (decimal reduction time) and activation energy confirmed the better temperature stability of conjugated enzymes than native enzyme. The k_m and V_{max} values were found to be similar for both conjugated and native enzyme suggesting, no change in enzyme configuration which alters the substrate binding.

1. Introduction

Poly-ε-lysine (ε-PL), is a kind of peptide with a linkage between α-carboxyl group and ε-amino group, and obtained from microbial metabolism. It is a cationic polymer and typically produced as a homo-polypeptide of approximately 25–30 L-lysine residues (Nishikawa & Ogawa, 2002). Because of its excellent antimicrobial activity, heat stability and lack of toxicity, ε-PL has attracted a great deal of attention as a natural food preservative. ε-PL when combined with other food additives, its preservative activity against microorganisms is extremely enhanced (Hiraki, 2000). Amino groups of ε-PL are efficiently used for the synthesis of macromolecular gels with high water-binding capacity. The hydrogels are synthesized by chemical cross-linking of polysaccharides using low molecular weight materials and macromolecular cross-linking reagents.

The conjugation of peptides/proteins with polymers is interesting from a structural point of view. It also offers improved properties to polymeric materials, in particular for biomedical applications. Conjugation of suitable biocompatible polymers to bioactive peptides or proteins can reduce the toxicity, prevent immunogenic or antigenic side reactions, enhance blood circulation times and improve the solubility. Conversely, modification of polymers or polymer therapeutics with suitable oligopeptide sequences can prevent random distribution of the drugs in the body which allows active targeting (Guido, Vandermeulen & Klok, 2004).

Polymer conjugation has been reported to reduce the autolysis of proteolytic enzymes and to allow enzymes to dissolve in non-aqueous solvents. There are several examples wherein enzymes were bound to sugars, or sugar-based polymers are stabilized (Novick & Dordick, 1998; Vazquez-Duhalt, Tinoco, D'Antonio, Topoleski & Payne, 2001; Wang et al., 1992). Binding of an enzyme to a polysaccharide provides rigidity (Klibanov, 1983) which is expected to decrease the entropy and consequently improve the stability of enzymes. Conjugation of bioactive peptides to proteins with retention of biological activity requires a method of chemoselective ligation that does not interfere with or

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obscure the essential topochemical features that impart biological activity to the peptide (Phillips et al., 2009).

The main hindrance in using ε-PL in processed foods is its tendency to interact with proteins and acidic polysaccharides, leading to the possible loss of emulsifying properties (Hiraki, 1995; Otsuka, Kuwahara & Manabe, 1992). Hence, significant endeavors have been directed to improve the emulsifying properties of food proteins by conjugating it with polysaccharides. Generally, this is achieved by modifying the protein with a functional group that possesses reactive specificity for a complementary functional group introduced on a specific site on the peptide.

The unique polycationic nature of ε-PL prompted us to employ it for enhancing the stability of an enzyme. Amylase was used as a model enzyme which was expected to form conjugate with ε-PL. α-Amylase plays a central role in the complete degradation of starch to fermentable sugars during the germination or malting of cereal grains. It also finds considerable application, together with starch debranching enzymes, in the production of high maltose syrups. α-Amylase is usually assayed using non-specific reducing sugar assays with starch as a substrate. The present work details the use of ε-PL as a polymer for stabilization of enzyme (α-Amylase) through conjugation and its degradation kinetics study.

2. Materials and methods

2.1. Materials

ε-PL used in present study was generously provided by Handary Bio-Engineering B.V. Netherlands. Deionized water was used for experimental protocol and during analysis. All the chemicals used in present study were of the AR grade and were purchased from S.D. Fine Chemicals, Mumbai, India. α-Amylase (1200 U/mg) was a gift sample from Sigma Industries, Mumbai, India. One unit of amylase liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 and 20 °C. All the chromatographic separations were performed using Biologic Duo Flow System from Bio-Rad, California, USA.

3. Methods

3.1. Preparation of enzyme – ε-PL conjugate

Standard ε-PL sample was dissolved in deionized water to prepare 5 mg/ml solution. Amylase (5 mg/ml) was suitably diluted to produce 1 mg/ml protein concentration. The pH of amylase was increased gradually above its pI (5.5) to achieve negative charge on it. ε-PL and amylase (pH 7.5) were mixed in 100 ml flask with 5:1 w/w ratio with continuous stirring. This mixture was allowed to incubate overnight at 25±2 °C. The conjugate thus formed was subjected to various extreme temperatures (50 °C–80 °C) and pH's (7.0–13) for different time intervals (5, 15, 30, 45 and 60 min). Both conjugated and native enzymes were analyzed for their temperature and pH stability. Enzyme kinetics was performed with time dependent enzyme degradation. Arrhenius equation and Lineweaver–Burk plot were used to calculate the temperature degradation kinetic parameters.

3.2. Kinetic data analysis

Enzyme activity was described using the reaction rate and the dependence of reaction rate on temperature. Parameters used in the study were reaction rate constant (k) and the Arrhenius activation energy (E_a). The kinetic data was analyzed as described by Van Boekel (1996). Enzyme degradation is generally considered to follow first order reaction kinetics.

$$-\frac{dc}{dt} = kC \tag{1}$$

where C is the enzyme activity, t is the time and k is the reaction constant (1/min).

Integrating above equation with C_0 (activity of enzyme C at time t)

$$C = C_0.e^{-kt}$$

Taking natural logarithm,

$$\ln \frac{C}{C_0} = -kt \tag{2}$$

For the first order reaction, a plot of $\ln C/C_0$ against time t gives a straight line, and the rate constant is represented by the slope. Temperature dependence of a reaction is described by the Arrhenius equation:

$$k = k_0 e^{(-E_a/RT)} \tag{3}$$

where k_0=frequency factor or the Arrhenius constant (1/min), R is the universal gas constant (8.3145 J/mol K), and T is the absolute temperature (K). A slope of graph $\ln k$ and $1/T$ gives the value for activation energy (E_a).

The decimal reduction time (D value) is the time required to reduce the enzyme activity by 90% was related to reaction rate constants as D=2.303/k

3.3. Michaelis–Menten kinetic parameter (K_m and V_{max}) calculation

Measurements of enzymatic reactions were used to characterize enzymes with regard to their substrate affinities and maximal reactions rates. By measuring the rate of substrate utilization (v) at different substrate concentrations (s), K_m and V_{max} can be calculated by the methods of Lineweaver–Burk, Eadie/Hofstee, or Wilkinson methods (Counotte & Prins, 1979). Lineweaver–Burk plot was used in the present study to calculate K_m and V_{max} for conjugated and native enzyme.

3.4. Gel filtration chromatography

The ε-PL-amylase conjugate was separated from the unreacted ε-PL by gel filtration chromatography with Sephadex G-150 column (2.0 cm × 20 cm). Elution was carried out with phosphate buffer (pH 7.0; 0.01 M; flow rate of 0.4 ml/min) and fractions (3 ml) were collected automatically with Biologic Duo Flow System. ε-PL was detected at 215 nM while amylase was detected at 280 nM followed by specific assay explained in the analytical methods section.