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Cosolvent-induced inhibition of thermal aggregation of α-amylase

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ABSTRACT

Aggregation of proteins and enzymes plays a crucial role in the formulation of protein based products. Sugar based cosolvents were implicated to reduce the thermal aggregation of α-amylase enzyme. A linear correlation was observed between concentration of cosolvents and inhibition of thermal aggregation. Based on this current study and previously published data it appears that addition of cosolvents enhance preferential hydration of enzyme which resist thermal unfolding and therefore reduce the intermolecular interaction that form visible aggregates.

KEYWORDS

Protein aggregation;
α-amylse;
Hydrophobic interactions;
Cosolvents.

INTRODUCTION

Under certain conditions proteins undergo non specific interactions that results in loss of function or gain of toxic function. The uncontrolled aggregation of protein is known to be associated with various diseases, such as, Alzheimer's disease, Parkinson's disease, and Huntington's disease[1, 2]. Protein aggregation is considered as most problematic in protein formulation as it can occur at almost any stage of production, processing, storage and shipping of protein-based drugs. It can severely influence the pharmacokinetics as well as the safety of the such molecules, as the aggregation of protein is also related with unwanted immunogenicity[3]. Under *in vitro* condition protein aggregation can be induced by several factors, such as, change in temperature, pH and ionic strength of the protein solution. An important factor that compromises conformational stability of proteins and therefore accelerates aggregation in bulk solution is temperature. High temperature unfolds the native protein conformation to a degree that accelerates aggregation[4].

Sugars and polyols form a group of additives that is often referred to as "cosolvents" or "cosolutes", have been used to stabilize proteins under various conditions[5]. In aqueous solution preferential exclusion of cosolvents from the protein surface and preferential hydration of protein is considered to be the major driving force responsible for structure stabilizing effect[6, 7]. It is anticipated that in the presence of such cosolvents the population of partially unfolded aggregation-prone molecules is decreased and aggregation becomes less likely. The present study is an attempt to explore the mechanism of temperature induced aggregation of a carbohydrate cleaving enzyme (i.e., α-amylase) with intention to reduce aggregation of the enzyme by using suitable cosolvents. In this study α-amylse enzyme was used as a model protein.

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MATERIALS

α-Amylase (from *Bacillus amylolequifaciens*), sucrose, sorbitol, trehalose, glycerol, 3,5- dinitrosalicylic acid and starch were commercially procured from Sigma Chemical company St. Louis, MO, USA. Quartz triple distilled water was used for the preparation of all the solutions and 2 mM of $CaCl_2$ was used throughout the experiment.

METHODS

Determination of protein concentration

The absorbance of the enzyme solution was recorded at 280 nm in a Shimadzu UV-1601 UVVisible spectrophotometer (Japan) and the concentration of α-amylase was calculated using the equation;

$$A_{280} = \varepsilon c l$$

Where ε is the extinction coefficient (for α-amylse 14.46)[8], *c* is the concentration of protein in mg/ml and *l* is the path length in cm. Alternatively, the concentration of α-amylase solution was also estimated by the Lowry method[9] using bovine serum albumin (BSA) as standard.

α-Amylse activity assay

The enzyme solution was prepared in 0.02 M citrate buffer, pH 5.9, containing 2 mM of $CaCl_2$. The enzyme and starch solutions were preincubated for 5 min at 37°C. The reaction mixture (2 ml), containing 1 ml of 1% starch solution and 1 ml of enzyme solution, was incubated for 5 min in a temperature-controlled shaking water bath at 37°C. The enzymatic reaction was terminated by the addition of 2 ml of 1% (w/v) alkaline dinitrosalicylic acid solution. The reaction mixture was subjected for 10 min to a boiling water bath for colour development. After cooling, it was diluted five times using triple distilled water, mixed thoroughly, and the absorbance was recorded at 540 nm in a Shimadzu UV-1601 UV-Visible spectrophotometer (Japan). The activity of α-amylase was determined by using the standard plot of maltose. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1.0 μmol equivalence of maltose at pH 5.9 at 37°C in 5 min from 1 ml of 1% (w/v) corn starch solution[10].

Determination of thermal aggregation of α-amylse

Light scattering method was used to determine the protein aggregation. The stock solution of the enzyme was prepared in 0.02 M citrate buffer, pH 5.9, containing 2 mM of $CaCl_2$. The absorbance of the protein samples was monitored at 330 nm and 360 nm which arise mainly due to scattering of light by protein aggregates and the soluble proteins usually do not have any absorption at these wavelengths[11, 12]. The protein samples were prepared in the presence of different concentrations of cosolvents, incubated at 20°C for 12 hrs before absorption was recorded at 330 nm and 360 nm after the baseline correction. Alternatively the degree of aggregation was determined by separating the insoluble aggregate by centrifuging at 10000 rpm and measuring the protein concentration in the supernatant.

RESULTS AND DISCUSSION

As shown in Figure 1, α-amylase display optimum activity over the range of temperature from 45-50°C. Increasing the temperature above 50°C leads to decrease in enzyme activity. It was shown previously that addition of cosolvents, specifically sucrose, sorbitol, glycerol and trehalose, prevents heat induced inactivation and helps in retaining the enzyme activity at higher temperatures[13]. The thermal inactivation of the enzyme accompanied with non specific aggregation of unfolded enzyme. The unspecific protein aggregation makes the heat denaturation process thermodynamically irreversible. As shown in Figure 2, heating the enzyme at 60°C for 30 min results in precipitation of approximately 80% of the enzyme from bulk solution. After heat treatment, neither the aggregate nor the supernatant could show any enzyme activity. Thermal aggregation of the enzyme was observed as a major event that concurrently occurred with enzyme inactivation. Considering the fact that in aqueous solution sugar based cosolvents behave as potential protein stabilisers, investigation was made to assess the effect of cosolvents, i.e. sucrose, sorbitol, glycerol and trehalose, on thermal aggregation of the enzyme. The enzyme was dissolved in buffer (0.5 mg/ml), containing different concentrations of each cosolvent, and incubated for 1 hr at 10°C for equilibration. Latter on the enzyme samples containing different concentrations of each cosolvent were incubated simultaneously at 60°C for 30 min. After cooling and centrifugation the

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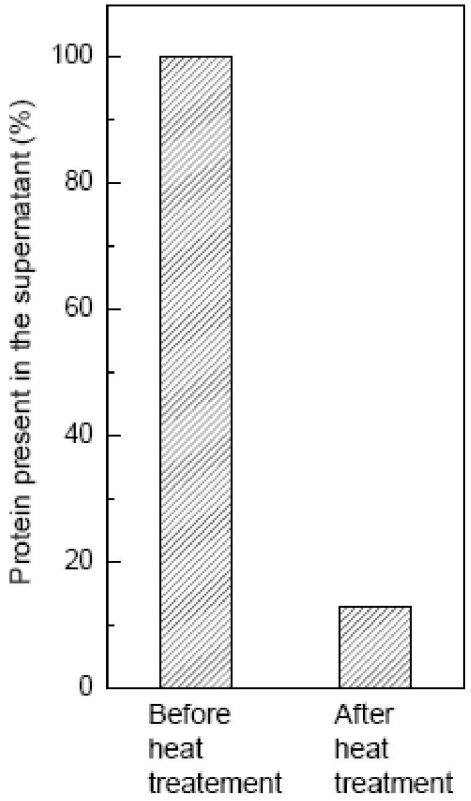

Figure 1 : Thermal aggregation of α-amylase in buffer. The aggregation of the enzyme was quantified by centrifugation and light scattering method.

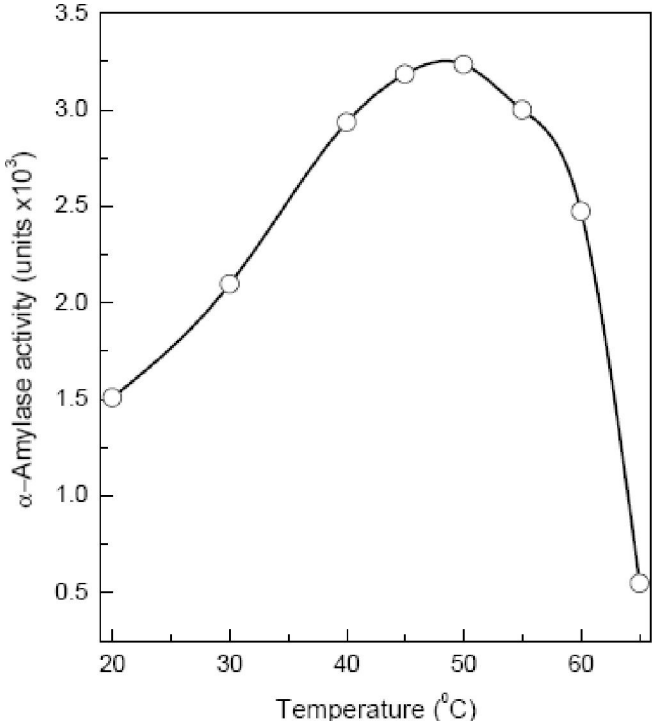

Figure 2 : Activity profile of α-amylase enzyme as a function of temperature. Enzyme solutions were incubated at different temperatures for 5 min and after cooling the enzyme activity were measured at 37ºC using 1% starch solution as substrate.

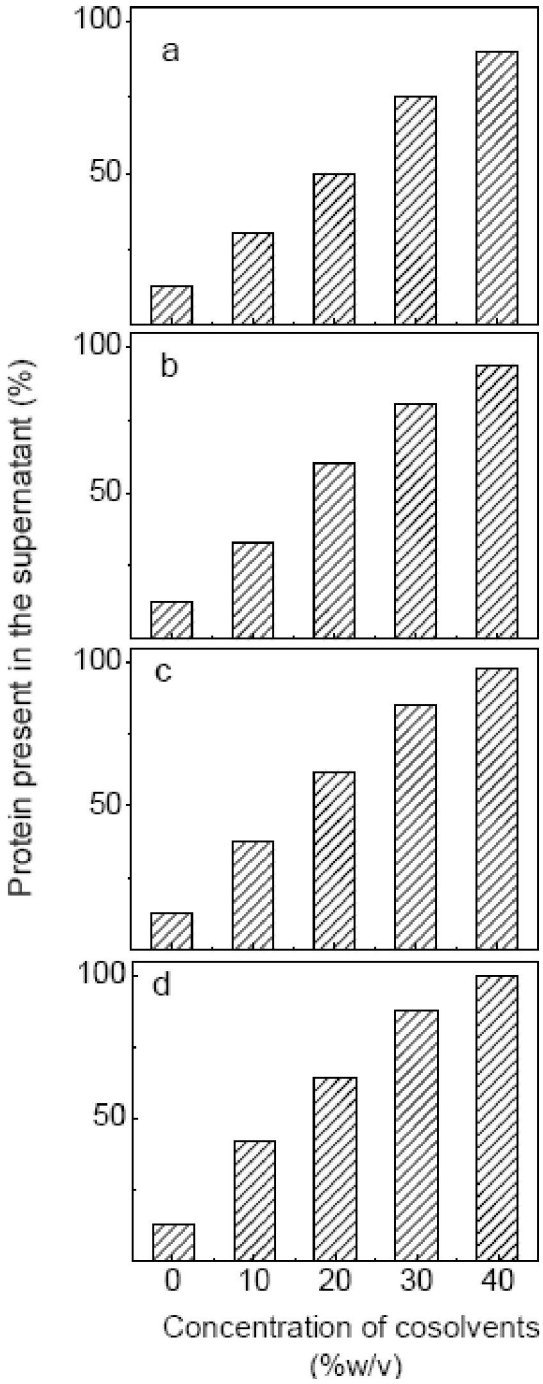

Figure 3 : Thermal aggregation of α-amylase (from *Bacillus amyloliquefaciens)* in the presence of different concentrations of cosolvent. The enzyme solutions were prepared in the presence different concentrations of each cosolvent and subjected for heat treatment (30 min at 60ºC). The aggregation of the enzyme was quantified by centrifugation and light scattering method. The enzyme solution in buffer was considered as control. The amount of protein present as soluble fraction in untreated enzyme sample was considered as 100% and the relative inhibition of aggregation was calculated based on it. The graphs are represented; (a) trehalose (b) glycerol (c) sorbitol and (d) sucrose.

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protein concentration in the supernatants was determined according to the method described. As shown in Figure 3 addition of cosolvents, significantly reduced the degree of enzyme aggregation compared to in the absence of cosolvents. A complete inhibition of enzyme aggregation was observed in the presence of 40% (w/v) sucrose. The ability of cosolvents to inhibit the aggregation was found be concentration dependent. Although all the cosolvents displayed inhibitory effect on thermal aggregation of the enzyme, the degree of inhibition varied between cosolvents. Such differences could arise due to the variation in the preferential interaction of cosolvents with protein and water. It is stated in various findings that cosolvents usually stabilize the enzyme again denaturation by a phenomena known as preferential interaction[14, 15]. Presence of cosolvents results in preferential hydration of enzyme[14-16]. In other words, the protein surface will have more water concentration compared to the bulk solution. In a globular, soluble protein the amino acid residues of different hydrophobic index are organized in such a fashion that the hydrophobic residues are sequestered inside and constitute the core, to avoid thermodynamically unfavourable interaction with water, where as the hydrophilic residues are mostly distributed on protein surface. Such rational arrangement is necessary to provide stability and functional characteristics of a protein. Unfolding of the proteins usually accompanies with exposure of core hydrophobic residues to aqueous environments. Since, such interactions are thermodynamically unfavourable, the exposed hydrophobic clusters tend to minimize their contact with water. In aqueous solution, when such hydrophobic clusters approach closer they interact with each other. Such interactions perpetuate over time and results in the formation of large size visible aggregates. It was found that these aggregates were completely solution in low concentration of sodium dodecyl sulphate solution, which establishes that the aggregates formed from thermal denaturation of α-amylase are hydrophobic in nature. As, it was mentioned previously that the presence of cosolvents helps in preferential hydration of proteins and the denatured proteins are relatively more hydrated compared to the native one[17], it could be possible that additional layers of water on protein surface shield the hydrophobic face of the unfolded proteins and inhibit the hydrophobic intermolecular interactions. At this juncture there could be two possibilities which might be responsible for cosolvent induced inhibition of aggregation of α-amylase. Either, the added cosolvents may resist the protein unfolding due to preferential hydration of enzyme or these cosolvents may shift the folding-unfolding equilibrium towards to the native state of the enzyme. It was noticed that the addition of cosolvents had no influence once the aggregation was initiated. This shows that cosolvent can not reverse the preformed aggregates into native conformation and close to native state. Therefore it is clear from above observation that enzyme is thermodynamically more stable in the presence of cosolvents. Nevertheless the role of cosolvents becomes limited once the enzyme is denatured or aggregated.

REFERENCES

[1] R.W.Carrell, B.Gooptut; Curr.Opin.Struct.Biol., **8**, 799 **(1998)**.
[2] G.Hoffner, P.Djian; Biochimie, **84**, 273 **(2002)**.
[3] A.S.Rosenberg; Effects of Protein Aggregates: An Immunologic Perspective, AAPS Journal, **8**, **(2006)**.
[4] J.F.Carpenter, B.S.Kendrick, B.S.Chang, M.C.Manning, T.W.Randolph; Methods Enzymol., **309**, 236 **(1999)**.
[5] S.N.Timasheff; Adv.Prot.Chem., **51**, 355 **(1998)**.
[6] J.F.Carpenter, M.C.Manning; Rational Design of Stable Protein Formulations - Theory and Practice.Ed., **(2002)**.
[7] E.Y.Chi, S.Krishnan, T.W.Randolph, J.F.Carpenter; Pharm.Res., **20**, 1325 **(2003)**.
[8] S.Rajendran, C.Radha, V.Prakash; Int.J.Pept.Protein Res., **45**, 122 **(1995)**.
[9] O.H.Lowry, N.J.Rosenborough, A.L.Farr, R.J.Randall; J.Biol.Chem., **193**, 265 **(1951)**.
[10] P.Bernfeld; Methods Enzymol., **1**, 149 **(1955)**.
[11] M.Vrkljan, T.M.Foster, M.E.Powers; Pharm.Res., **11**, 1004 **(1994)**.
[12] C.C.Hsu, H.M.Nguyen, D.A.Yeung; Pharm.Res., **12**, 69 **(1995)**.
[13] J.K.Yadav, V.Prakash; J.Biosci., **34**, 377 **(2009)**.
[14] D.W.Bolen, I.V.Baskakov; J.Mol.Biol., **310**, 955 **(2009)**.
[15] S.N.Timasheff; Proc.Nat.Acad.Sci., **99**, 9721 **(2002)**.
[16] S.N.Timasheff; Biochemistry, **41**, 13473 **(2002)**.
[17] S.Baier, J.McClements; J.Agri.Food Chem., **49**, 2600 **(2001)**.