Carbohydrate Polymers 90 (2012) 1811–1817

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Conjugation of α-amylase with dextran for enhanced stability: Process details, kinetics and structural analysis

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ARTICLE INFO

Article history:
Received 7 June 2012
Received in revised form 12 July 2012
Accepted 28 July 2012
Available online 4 August 2012

Keywords:
Stability
Covalent binding
Conjugate
Inactivation rate constant
Activation energy
Secondary structure

ABSTRACT

The influence of enzyme polysaccharide interaction on enzyme stability and activity was elucidated by covalently binding dextran to a model enzyme, α-amylase. The conjugation process was optimized with respect to concentration of oxidizing agent, pH of enzyme solution, ratio of dextran to enzyme concentration, temperature and time of conjugate formation, and was found to affect the stability of α-amylase. α-Amylase conjugated under optimized conditions showed 5% loss of activity but with enhanced thermal and pH stability. Lower inactivation rate constant of conjugated α-amylase within the temperature range of 60–80 °C implied its better stability. Activation energy for denaturation of α-amylase increased by 8.81 kJ/mol on conjugation with dextran. Analysis of secondary structure of α-amylase after covalent binding with dextran showed helix to turn conversion without loss of functional properties of α-amylase. Covalent bonding was found to be mandatory for the formation of conjugate.

1. Introduction

Enzymes are important for biomedical, chemical and industrial applications that are eco-friendly. However, their limited stability under process conditions often becomes a constraint in using them commercially (Iyer & Ananthanarayan, 2008). Techniques such as protein engineering, chemical modification, use of stabilizing additives, and immobilization have been used to overcome these constraints (Fágáin, 2003). Protein engineering can be done by site directed mutagenesis or random mutagenesis for many enzymes to obtain desired advantages (Nielsen & Borchert, 2000). One of the simple ways to stabilize enzymes is by covalently binding it to polysaccharides. This technique encompasses the basic principles of chemical modification, stabilizing additive as well as immobilization. Polysaccharides provide rigidity (Klibanov, 1983) and hydration (Srivastava, 1991) to enzymes on conjugation and enhance its stability.

Under extreme conditions of temperature and pH, irreversible denaturation occurs and inactivates the enzyme. Kinetic stability is influenced both by the state and rate of folding and unfolding of an enzyme. From a thermodynamic perspective also, a decrease in the entropy of unfolded state may stabilize the enzyme (Shaw & Bott, 1996). 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. Binding of penicillin G acylase to various polysaccharides (Misloviacova, Masarova, Bucko, & Gemciner, 2006), invertase and cellulase to chitosan (Darias & Villalonga, 2001; Gomez, Ramirez, & Villalonga, 2000) and horseradish peroxidase and glucose oxidase to dextran by non-covalent linkages (Altikatoglu & Kuzu, 2010) have shown improved thermal and pH stability. Covalent binding of amylase to carboxymethyl cellulose (Villalonga, Gomez, Ramirez, & Villalonga, 1999) and dextran (Lenders & Crichton, 1984) has been reported to be successful, although the conjugate showed lower activity but better stability as compared to free enzyme (Villalonga et al., 1999). A thorough understanding of factors influencing the conjugate formation between enzyme and polysaccharide could enable development of enzymes with retention of activity as well as stability.

Polysaccharides can be activated by an oxidizing agent such as sodium metaperiodate or cyanogen bromide to confer a negative charge on the polysaccharide which could then bind with the enzyme covalently under appropriate conditions of pH. Sodium periodate is preferred over cyanogen bromide as an oxidizing agent. Cyanogen bromide may precipitate polysaccharides during activation and need high pH for coupling which might affect on enzyme structure (Lenders & Crichton, 1984). Dextran is a polysaccharide of choice because of easy commercial availability, water solubility and absence of ionic functionality.

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.07.078

Amylase is an industrially important enzyme for food, paper, textile, distillery and brewing industries all of which can benefit from improved stability of amylases. Although techniques for immobilization of amylase have been reviewed by several authors (Cowan & Fernandez-Lafuente, 2011; Pandey, Soccol, & Soccol, 2000; Sheldon, 2007), we did not find any report on the factors influencing the formation of conjugate of amylase with dextran and the effect on activity and/or stability. Factors such as concentration of oxidizing agent, time of oxidation, pH of enzyme, ratio of enzyme to polysaccharide, time of conjugate formation, and temperature at which the conjugate is formed would be expected to influence the activity as well as stability of the enzyme. This work was undertaken to look in to these factors. We have also evaluated the kinetic and thermal stability of the conjugate, and characterized the changes of the enzyme at the level of secondary structure after conjugation under the conditions that gave maximum retention of activity as well as stability.

2. Experimental

2.1. Materials

Crude α-amylase from *Bacillus licheniformis* (1470 U/mg) was obtained as gift sample from Sigma Chemical industries, Mumbai, India. It was dialyzed against 20 mM of sodium citrate buffer of pH 5.4 at 4 °C overnight and then used for study. Dextran (molecular weight, 40,000) was purchased from HiMedia Laboratory Pvt. Ltd. Mumbai. Sodium metaperiodate were obtained from S.D. Fine Chemicals, Mumbai. All other chemicals were of AR grade and procured from reliable sources.

2.2. Preparation of α-amylase–dextran conjugate

Sodium metaperiodate (0.1 M) solution was prepared in oxidation buffer (0.1 M sodium acetate buffer of pH 5.0) and used as the oxidizing solution. Dextran (250 mg) was oxidized in 10 ml of oxidizing solution in dark for 90 min, after which the oxidation was stopped by adding 0.3 ml of ethylene glycol, and kept for 1 h in dark. Oxidized dextran solution was dialyzed against 0.1 M Sodium acetate buffer of pH 5.0 at 4 °C overnight. Amylase solution was prepared in buffer of pH 5.0 and mixed with equal volume of oxidized dextran solution and kept for conjugate formation for 20 h at room temperature (~28 ± 2 °C). Sodium borohydrate (20 mg) was then added to 10 ml of conjugate mixture to reduce remaining oxidized sites of dextran and kept for 4 h. Finally, the prepared conjugate solution was dialyzed against 20 mM of sodium citrate buffer of pH 5.4 at 4 °C overnight (Ahmed, Saleh, & Abdel-Fattah, 2007; Srivastava, 1991; Villalonga et al., 1999). This conjugate was used for further studies.

2.3. Effect of concentration of sodium metaperiodate on oxidation of dextran

Sodium metaperiodate (3.2 g) was dissolved in 3 ml of sulphuric acid and volume made to 15 ml with oxidation buffer to prepare 1 M stock of sodium metaperiodate. Different concentrations of sodium metaperiodate (0.05, 0.1, 0.2, 0.4, and 0.6 M) were prepared using stock solution and oxidation buffer as a diluents. Dextran (250 mg) was oxidized using 10 ml of each concentration of metaperiodate under dark. Samples were withdrawn after every 30 min for 120 min and immediately analyzed for presence of aldehyde group after oxidation of dextran using 3,5-dinitrosalicylic acid (DNSA) test (Lenders & Crichton, 1984).

2.4. Effect of conjugation process parameters on stability of α-amylase

2.4.1. α-Amylase activity assay

Enzyme sample (0.5 ml, appropriately diluted) was added in 0.5 ml of 1% starch solution prepared in 0.02 M sodium citrate buffer of pH 5.4. Mixture was kept at 50 °C for 10 min, DNSA reagent (0.5 ml) added and kept in a boiling water bath for 15 min. Samples were chilled quickly followed by addition of 4.5 ml of distilled water. The absorbance was measured at 540 nm. Enzyme activity was calculated using a standard curve plotted using maltose in the range of 0.0–1.0 mg/ml. One unit of enzyme activity was defined as the micromole of maltose released per minute per ml of enzyme solution at 50 °C. The protein content of the enzyme solutions was assayed by Folin–Lowry method using bovine serum albumin as a standard in the range of 200–1000 μg/ml (Lowry, Rosebrough, Farr, & Randall, 1951).

2.4.2. Effect of pH of enzyme solution

Oxidized dextran solution (25 mg/ml) was prepared using 0.1 M sodium metaperiodate prepared in oxidation buffer. Oxidation was completed in 60 min and stopped by adding 0.3 ml of ethylene glycol as quenching agent. Solution was dialyzed overnight at 4 °C. Appropriate concentration of α-amylase solution was prepared using 0.1 M sodium acetate buffer of different pH (4.6, 5.0, 5.4, and 5.8) which was lower than p*I* of 5.8 of α-amylase. The α-amylase solutions (5 ml) were mixed with 5 ml of oxidized dextran and incubated for 20 h for conjugate preparation at room temperature. After incubation, 20 mg of sodium borohydrate was added in 10 ml of conjugate mixture to reduce remaining oxidized sites of dextran and it was kept for 4 h. Finally, prepared conjugate solutions were dialyzed against 20 mM sodium citrate buffer of pH 5.4 at 4 °C overnight. Free α-amylase and conjugated α-amylase solutions (5 μg/ml of protein) were prepared and incubated at 80 °C for 30 min. The samples were then immediately chilled by placing the test tubes in an ice bath, and assayed for α-amylase activity. The percentage residual activity was calculated with respect to their individual original activities.

2.4.3. Effect of ratio of dextran to enzyme concentration

To oxidized dextran solutions (2.5–50 mg/ml), 10 ml of enzyme solution (2.5 mg/ml protein) was added so that the final ratio of dextran to enzyme concentration was maintained at 1:1–20:1. The formation of the conjugate and stability assay was carried out as described in Section 2.4.2.

2.4.4. Effect of temperature

Conjugates were prepared by keeping 10 ml mixture of α-amylase and oxidized dextran at different temperatures (4, 20, 30, 40, and 50 °C) for 20 h. The conjugates so prepared were incubated at 80 °C for 30 min and evaluated for activity and stability as described in Section 2.4.2.

2.4.5. Effect of incubation time

The conjugates were prepared and evaluated for activity and stability as detailed in Section 2.4.2 but with the time of conjugation varying from 4 to 24 h.

2.5. Characterization of free and conjugated α-amylase

2.5.1. Optimum temperature and optimum pH of free and conjugated α-amylase

The enzyme activities of free and conjugated α-amylase were analyzed at different temperatures ranging from 30 °C to 90 °C to find its optimum temperature. The effect of pH on free and conjugated enzyme activity was determined using

20 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 20 mM NaH_2PO_4/Na_2HPO_4 buffer of pH 7.0–8.0, 20 mM glycine/NaOH buffer of pH 9.0–10.0. α-Amylase activity was assayed as explained in Section 2.4.1.

2.5.2. Effect of conjugation under optimized conditions on thermal and pH stability

Based on the experiments reported in Sections 2.3 and 2.4, the conjugate was prepared under optimized conditions. The free and the conjugated α-amylase so prepared (0.5 μg/ml of protein) were evaluated for thermal stability by incubating at 50, 60, 70, 80, 90 °C in 20 mM sodium citrate buffer of pH 5.4 for 15 min followed by quick chilling on ice. The enzyme activity was then assayed from which percentage residual activity was calculated with respect to their individual original activities.

Free and conjugated α-amylase (1 μg/ml of protein) were incubated at room temperature in 50 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 50 mM NaH_2PO_4/Na_2HPO_4 buffer of pH 7.0–8.0, 50 mM glycine/NaOH buffer of pH 9.0–10.0. The samples were incubated for 1 h, diluted appropriately in assay buffer and tested for α-amylase activity.

2.5.3. Effect of conjugation on kinetics of thermal inactivation of α-amylase

Free and conjugated α-amylase preparation (0.5 μg/ml of protein) were incubated at 60, 70, and 80 °C in 20 mM sodium citrate buffer of pH 5.4. The samples were withdrawn every 10 min for 60 min, chilled quickly, and then assayed for residual enzymatic activity. A semi-log plot of percent residual activity vs. time was plotted from which the inactivation rate constant, k, was calculated as the slope, and $t_{1/2}$, the time required for the activity to decrease to half its original activity was calculated as $0.693/k$.

2.5.4. Size exclusion chromatography and SDS-polyacrylamide gel electrophoresis of free and conjugated α-amylase

Conjugated α-amylase was purified using Sephadex g 200 column of bead height 30 cm. The fractions were eluted at flow rate 0.5 ml/min with 20 mM sodium citrate buffer of pH 5.4 containing 0.15 M NaCl. All the fractions were analyzed for presence of protein at 280 nm. Enzyme activity and dextran content of all fractions were determined. These fractions were then run on 10% SDS-polyacrylamide gel to estimate the molecular weight of free and conjugated α-amylase using suitable markers.

2.5.5. Effect of conjugation on secondary structure of α-amylase as evaluated by circular dichroism spectroscopy

Changes in secondary structure of α-amylase due to conjugation with dextran was analyzed by circular dichroism spectroscopy (CD) on Jasco J 810 spectrophotometer at Tata Institute of Fundamental Research, Mumbai. Fractions collected from size exclusion chromatography were used as samples. Three scans were conducted and average of each sample from 200 nm to 300 nm at interval of 0.1 nm at a rate of 50 nm/min and a response time of 1 s. The optical path was 0.1 cm.

2.6. Evaluation of nature of bonding on conjugate formation and enzyme stability

Conjugate A was prepared as per above described protocol which involved covalent bonding between α-amylase and dextran. Conjugate B was prepared similarly as Conjugate A by using dextran without any oxidation to exclude only covalent bonding but including all possible non-covalent bonding. Conjugates A and B as well as free α-amylase (0.5 μg/ml of protein) were evaluated for thermal stability by incubating at 40–80 °C for 15 min and measuring the residual enzyme activity.

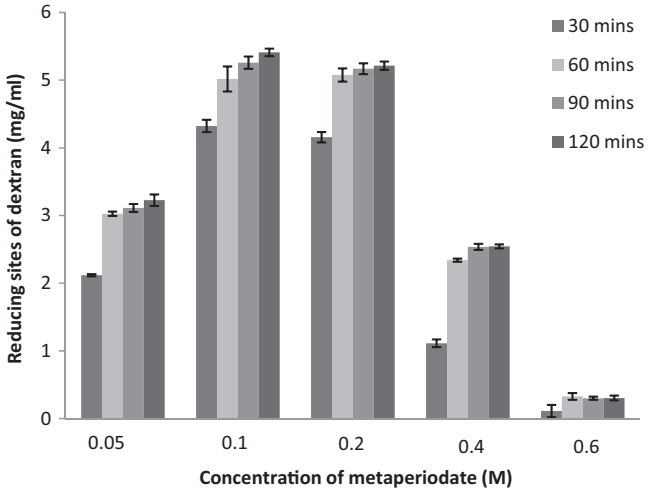

Fig. 1. Reducing sites of dextran formed at different concentration of sodium metaperiodate.

3. Results and discussion

3.1. Effect of concentration of sodium metaperiodate on oxidation of dextran

Effect of sodium metaperiodate concentration and time of reaction on the oxidation of dextran was studied by using sodium metaperiodate of 0.05–0.6 M (Fig. 1). Oxidation of dextran generates aldehydes which are reactive functional groups (Sanderson & Wilson, 1971) and can be analyzed using DNSA. The oxidized form of dextran can be calculated in terms of mg/ml of reducing aldehyde groups present on dextran. Maximum oxidation was observed at 0.1 M sodium metaperiodate after 60 min of reaction. At higher concentrations of 0.4 M and 0.6 M sodium metaperiodate, oxidation was found to be reduced. This may be due to higher content of acid that was added during the preparation of sodium metaperiodate stock. Acids are known to inhibit oxidation. Longer reaction times did not give a significant increase in oxidation. Hence, further work was carried out using dextran that was oxidized for 60 min using 0.1 M sodium metaperiodate. Many investigators have used sodium metaperiodate for oxidation of dextran and other polysaccharides such as carboxymethyl cellulose under random conditions, but without any measurement of the extent of oxidation. In some cases, the time of reaction has been reported to be as long as 24 h (Altikatoglu, Arioz, Basaran, & Kuzu, 2009) to 48 h (Villalonga et al., 1999). The extent of oxidation is important since it increases the possibilities of binding of enzymes at the sites of oxidation.

3.2. Effect of conjugation process parameters on stability of α-amylase

3.2.1. Effect of pH of enzyme solution

The enzyme has a positive charge at pH below its p*I*. A positive charge on the enzyme and negative charge on the oxidized dextran would favour electrostatic attraction along with covalent bonding (carbinolamines formation) between α-amylase and dextran (Sanderson & Wilson, 1971). Hence, we evaluated the effect of pH of α-amylase solution during conjugate formation on the stability of the resultant conjugate (Fig. 2a). It was observed that the conjugate prepared at pH 4.6 and 5.0 showed better stability than

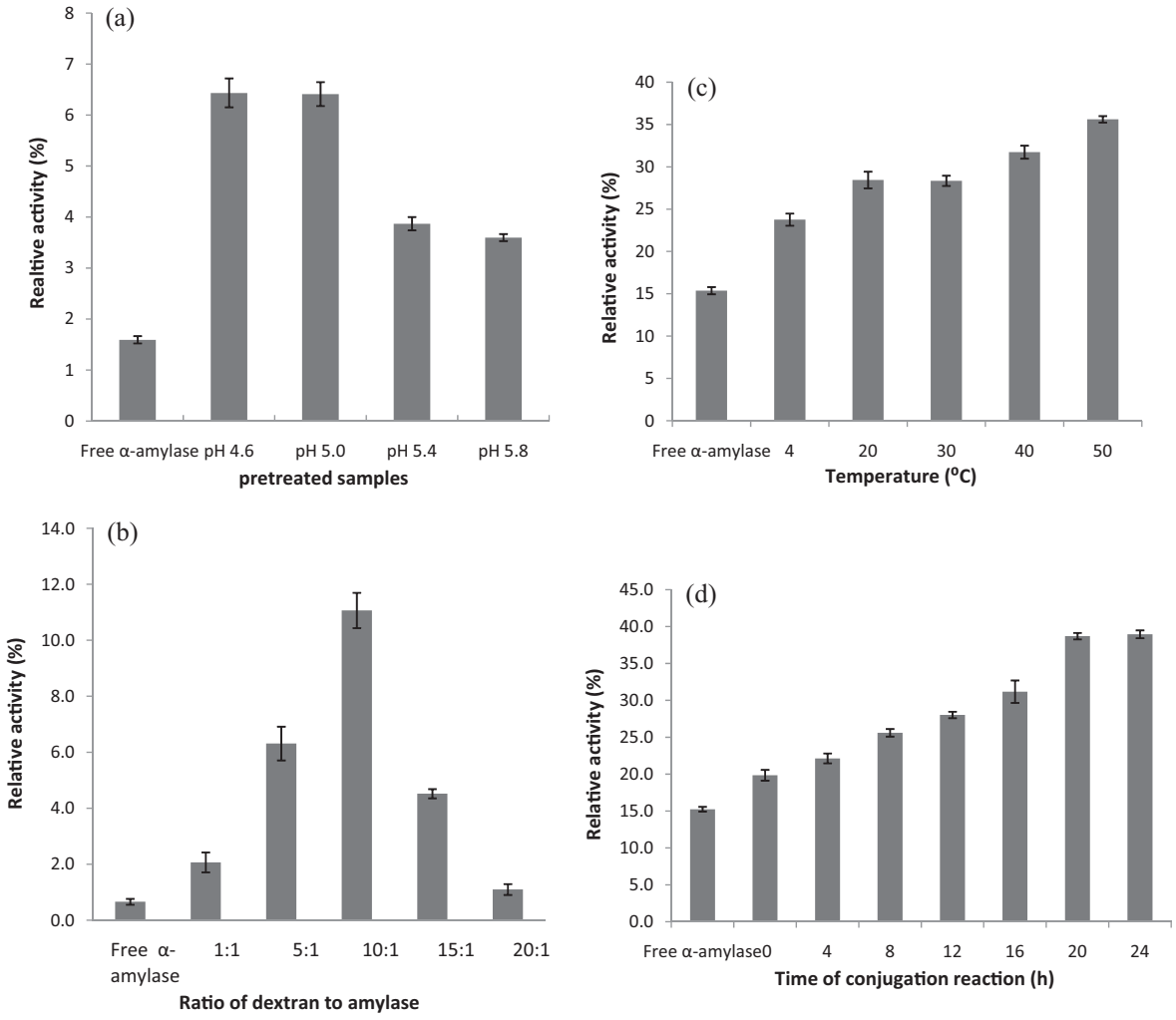

Fig. 2. Effect of conditions of conjugation on the relative activity of free and conjugated enzyme (a) pH of the enzyme solution, (b) ratio of dextran to α-amylase concentration, (c) temperature of conjugation, and (d) time of conjugation.

other pH's used in the study. We selected a pH of 5.0, since a pH of 4.6 was very close to pH 4.0 at which enzyme gets denatured with loss of activity.

3.2.2. Effect of ratio of dextran to enzyme concentration

The effect of ratio of dextran to α-amylase (protein concentration) on the stability of the conjugate was studied (Fig. 2b). A lower ratio of 1:1 and 5:1 showed lower stability, and maximum stability was seen at a ratio of 10:1 beyond which there was decrease in its stability. Lower concentration of dextran may be insufficient to give adequate rigidity and molecular crowding to enhance stability of conjugate, whereas higher concentration may give more rigidity and also block the active sites of enzyme causing decreased activity and stability. Hence, a critical ratio of dextran to α-amylase (10:1) was required to be maintained during conjugate preparation.

3.2.3. Effect of temperature

Amylase was conjugated at different temperatures at pH 5.0 and using a 10:1 ratio of oxidized dextran to α-amylase. The conjugated enzymes so prepared were evaluated for their stability by incubating at 80 °C for 30 min. We found the stability of the conjugate to be better when the reaction was carried out at the optimum temperature of 50 °C of α-amylase (Fig. 2c). Here, we hypothesis that the enzyme is in its catalytically active form at its optimum temperature; if dextran covalently binds to α-amylase at this temperature, it may provide maximum stability by maintaining favourable form of enzyme with enough flexibility. Although the conjugate formed at 50 °C showed maximums stability, its actual activity was found to decrease by 27%. α-Amylase used in the study was completely denatured above 50 °C as confirmed by the precipitation occurring during the reaction. Hence, to achieve an optimum activity and stability of the conjugate, it is recommended that the reaction be carried out between 20 and 40 °C. Hence all further reactions were carried out at room temperature (28 ± 2 °C).

3.2.4. Effect of incubation time

Conjugated α-amylases were prepared by reaction of the α-amylase and oxidized dextran for varying time period of 4–24 h, followed by evaluation of the stability of conjugate at 80 °C for 30 min (Fig. 2d). The stability of the conjugate was found to increase with an increase in the reaction time with maximum stability being observed after 20 h and remaining unchanged thereafter. It is postulated that 20 h is sufficient for proper covalent bonding between oxidized dextran and α-amylase which led to maximum stability of the conjugated α-amylase.

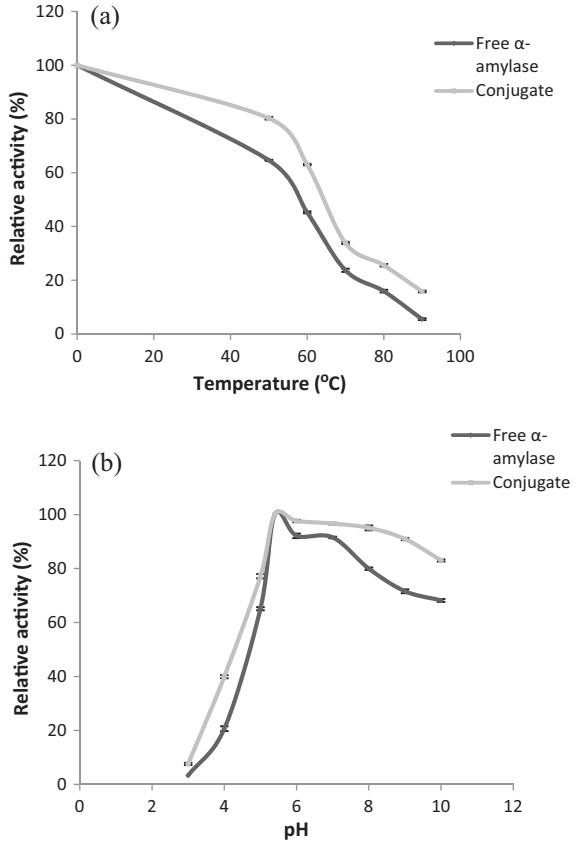

Fig. 3. Profile of free and conjugated α-amylase with respect to (a) thermal stability and (b) pH stability.

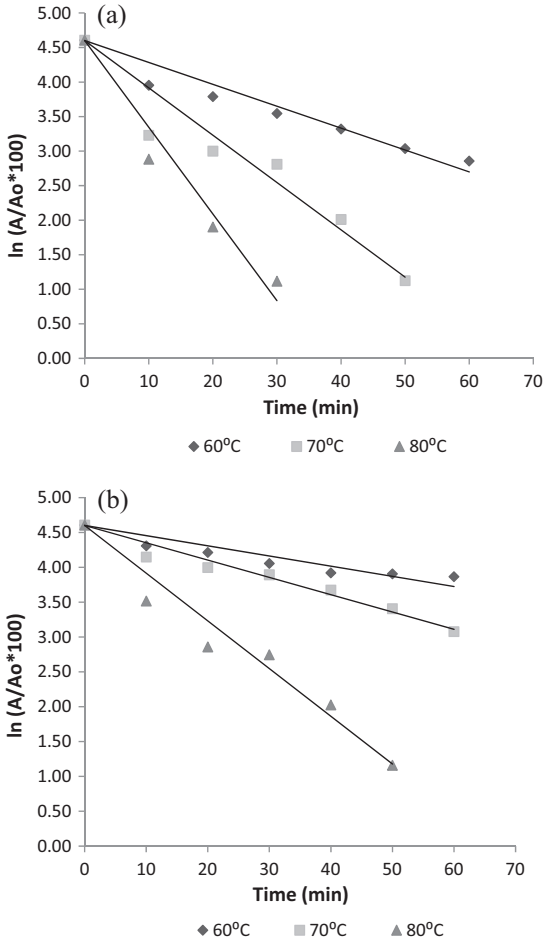

Fig. 4. Kinetics of thermal inactivation of (a) free α-amylase and (b) conjugated α-amylase.

3.3. Characterization of free and conjugated α-amylase

3.3.1. Optimum temperature and optimum pH of free and conjugated α-amylase

Conjugated α-amylase was prepared successfully under the optimized conditions as indicated above in Sections 3.1 and 3.2. The pH and temperature optima of α-amylase remained unaltered on conjugation at 5.4 and 50 °C, respectively (data not shown), although the conjugated α-amylase showed 5% loss of activity as compared to free α-amylase under these conditions. A retained activity to the extent of 39.8% has been reported for chitosan–cellulase system (Darias & Villalonga, 2001) and 54% for α-amylase–carboxymethylcellulose (Villalonga et al., 1999). Marshall (1976) evaluated the effect of conjugation of α-amylase with dextran using cyanogen bromide as a coupling agent and found 70% loss of activity with no change in pH optimum.

3.3.2. Effect of conjugation under optimized conditions on thermal and pH stability

The thermal stability of free and conjugated α-amylase was carried out by incubating the enzymes at 40–90 °C for 15 min. The activity was found to decrease with an increase in temperature for both the free and conjugated α-amylase (Fig. 3a). Conjugated α-amylase was almost 10% more stable than free α-amylase at each temperature. Similarly, pH stability study showed the conjugated α-amylase to be more stable toward extreme acidic and alkaline conditions of pH 4 and 10, respectively (Fig. 3b). The difference of stability between free and conjugated α-amylase was about 20% at pH 4, while it was 15% at pH 10. Hence dextran amylase conjugate may show promising industrial applications. Cross-linking of α-amylase to dextran using cyanogens bromide as coupling agent has been reported to show 7% and 20% improvement in stability at pH 4.4 and 3.5, respectively, and no effect on stability in the alkaline pH range (Marshall, 1976).

3.3.3. Effect of conjugation on kinetics of thermal inactivation of α-amylase

Thermal inactivation of free and conjugated α-amylase showed the activity of both samples to decrease with time. Kinetics of enzyme inactivation was evaluated by a semi-log plot of percent residual activity vs. time (Fig. 4) in terms of inactivation rate constant, k, obtained as the slope of the semi-log plot and $t_{1/2}$, the time required for the enzyme activity to decrease to half of its original activity. The k values of the free α-amylase at 60 °C, 70 °C and 80 °C were 0.031 min^{-1}, 0.068 min^{-1} and 0.125 min^{-1}, respectively, corresponding to $t_{1/2}$ of 22.35, 10.19 ad 5.54 min at their respective temperatures. The conjugated α-amylase had k values of 0.014 min^{-1}, 0.025 min^{-1} and 0.068 min^{-1} at 60 °C, 70 °C and 80 °C corresponding to their $t_{1/2}$ of 49.5 min, 27.72 min and 10.19 min, respectively. It can be seen that the inactivation rate constant of conjugated α-amylase was lower than the free α-amylase at each temperature indicating conjugated α-amylase need more time to denature as compared to free α-amylase and hence implied to be more stable (Amini, Sorouraddin, & Rashidi, 2011). This may be attributed to the rigidity and molecular crowding provided by dextran to α-amylase on conjugation. Further, activation energy of the free and conjugated enzyme was calculated from Arrhenius plot (Fig. 5) as product of slope and R, where R is the constant (8.314 J/mol). The activation energy of free and conjugated α-amylase was found to be 68.19 kJ/mol and 77.00 kJ/mol,

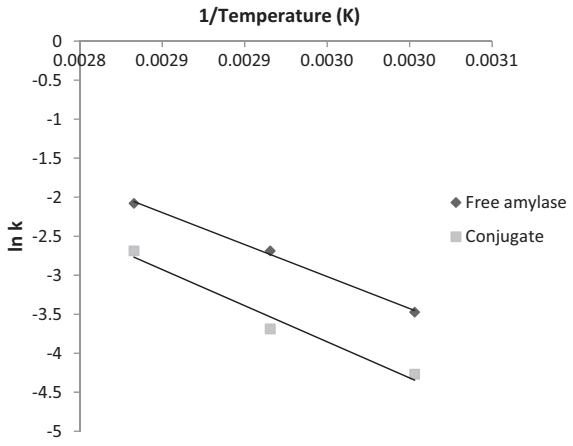

Fig. 5. Arrhenius plot for inactivation of free and conjugated α-amylase.

respectively. The activation energy is an index of the thermal stability of protein with higher activation energy for denaturation implying a more stable enzyme. Higher activation energy of conjugated α-amylase over free α-amylase suggested its better thermal stability than that of free α-amylase. Marshall (1976) showed the α-amylase cross-linked with dextran using cyanogens bromide as a coupling agent to lose 30% activity at 65 °C in 30 min as compared to total loss of activity of the free enzyme under similar conditions. However, the author did not report the effect of conjugation on the kinetics or activation energy of the enzyme.

3.3.4. Size exclusion chromatography and SDS-polyacrylamide gel electrophoresis of free and conjugated α-amylase

Fractions collected from size exclusion chromatography were analyzed for protein, enzyme activity and dextran content. Fraction nos. 15, 16 and 17 showed both enzyme activity and presence of dextran indicating the presence of conjugated α-amylase in those fractions. Silver staining of free α-amylase run on polyacrylamide gel showed the presence two isozymes having molecular weights of 130 and 170 kDa, respectively. Similar staining of conjugated enzymes showed an increased molecular weight (beyond the range of 170 kDa of markers used in the work) (Fig. 6). This also emphatically confirmed the presence of conjugated α-amylase. Marshall (1976) did the gel permeation chromatography of α-amylase cross-linked with dextran using cyanogens bromide as the coupling agent and found a band of the conjugate on top of the gel and inferred the conjugate to be of higher molecular weight.

Table 1
Analysis of the secondary structure of free and conjugated α-amylase by using circular dichroism spectrophotometer.

Sample	Free α-amylase (%)	Conjugated α-amylase (%)
Helix	25.9	22.3
Beta	9.9	9.2
Turn	23.6	28.8
Random	40.5	39.7
Total	100.0	100.0

3.3.5. Effect of conjugation on secondary structure of α-amylase as evaluated by circular dichroism spectroscopy

All the three fractions showing conjugated α-amylase were pooled together and used as the sample for conjugated α-amylase, and the remaining fractions containing free enzyme was used for structural analysis using circular dichroism spectrophotometer. The results were compared with reference of Yang-Us wtdilcor.JWR and used to analyse changes in secondary structure of conjugated α-amylase as compare to free α-amylase. The conjugated α-amylase showed a 3.6% decrease in helix and a 5.2% increase in turn as compare to free α-amylase (Table 1). This helix to turn conversion in conjugated α-amylase may be a result of covalent binding of dextran to α-amylase. When water molecule is inserted into alpha helix, it displays a variety of beta turn conformation. Hence water molecule could mediate conformational interconversion between helices and turns (Perczel, Foxman, & Fasman, 1992). In this case, dextran could have possibly been inserted into helix and caused the conversion of helices to beta turns. Though secondary structure of conjugated α-amylase was found to be altered as compared to free α-amylase, the bound dextran did not affect the enzyme activity. Marshall (1976) showed the conjugation of α-amylase with dextran using cyanogen bromide as a coupling agent but did not comment on the structural changes.

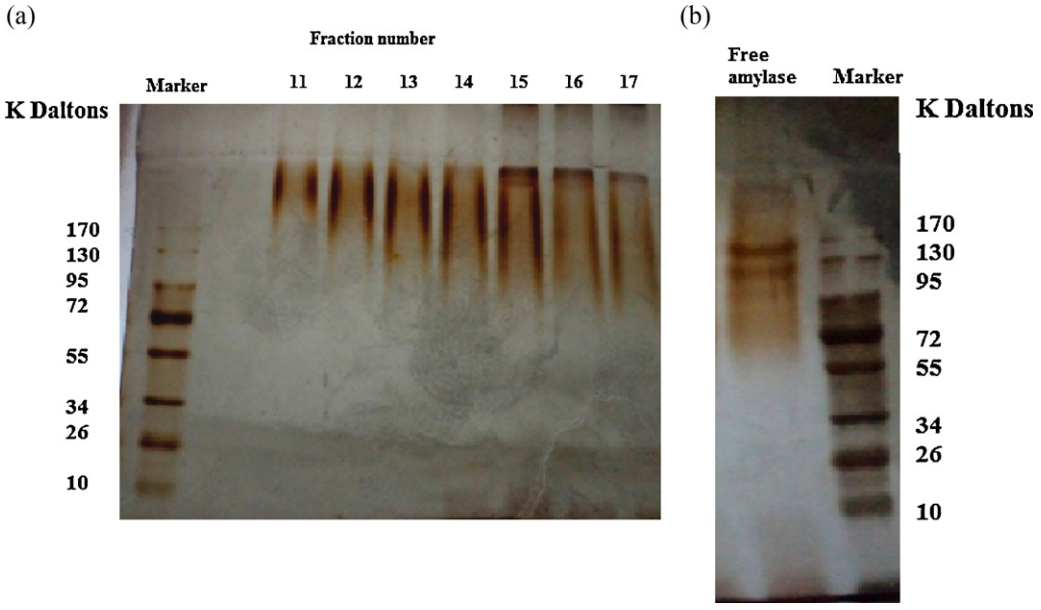

Fig. 6. Polyacrylamide gel (10%) stained with silver staining (a) presence of high molecular weight conjugated α-amylase in fraction nos. 15, 16 and 17. (b) Free α-amylase with two isozymes of molecular weight of 130 and 170 kD.

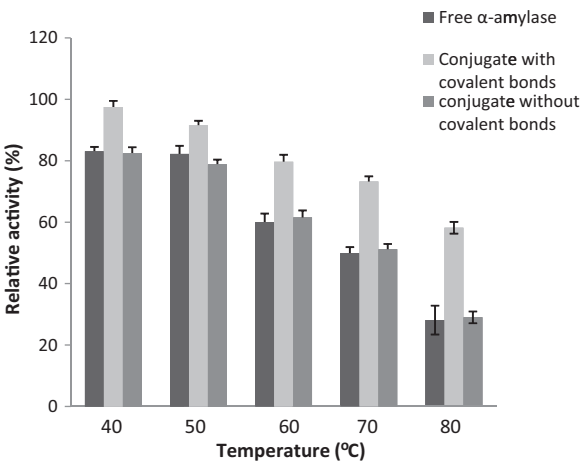

Fig. 7. Effect of covalent bonding between α-amylase and dextran as seen from thermal stability profile of free and conjugated α-amylase with and without covalent bonds.

3.3.6. Evaluation of nature of bonding on conjugate formation and enzyme stability

In order to understand the possibility of bonding other than covalent bonding on the stability of α-amylase, conjugates were prepared using oxidized and native dextran along with α-amylase under identical conditions. Conjugated α-amylase formed with native dextran i.e. without covalent bond formation, showed similar thermal stability as free α-amylase. However, conjugated α-amylase with covalent bonds showed enhanced stability over both free and conjugate formed with native dextran (Fig. 7). This clearly highlights the significance of covalent bonding for giving rigidity and hence the stability to α-amylase. Report was found on the significance of electrostatic interaction on the activity and stability of glucuronidase conjugated with carboxymethyl cellulose (Li et al., 2010). This may be due to the different enzyme and/or polysaccharide used by these researchers. To the best of our knowledge, there is no such report on the conjugation of amylase with any polysaccharide or any enzyme with dextran.

4. Conclusion

Amylase–dextran conjugate was formed using optimized process parameters with 95% retention of activity and enhanced stability against extreme temperature and pH. Conjugated α-amylase showed lower inactivation rate constant, a 50% increase in half life at 60–80 °C, and an increase in activation energy for denaturation by 8.81 kJ/mol than the free α-amylase. Conjugated α-amylase had a higher molecular weight than the free enzyme, confirming the process of conjugation of the two biopolymers. Analysis of the secondary structure showed helix to turn conversion in conjugated α-amylase. The role of covalent bonding between α-amylase and dextran was confirmed experimentally. Such conjugated enzymes can be explored for industrial applications.

Acknowledgements

Authors are grateful to Department of Biotechnology, Government of India, for providing financial assistance during the course of this investigation. The help extended in carrying out the CD analysis of the samples at Tata Institute of Fundamental Research, Mumbai, is also gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.07.078.

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