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Co-conjugation *vis-à-vis* individual conjugation of α-amylase and glucoamylase for hydrolysis of starch

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ABSTRACT

Two enzymes, α-amylase and glucoamylase have been individually and co-conjugated to pectin by covalent binding. Both the enzyme systems showed better thermal and pH stability over the free enzyme system with the complete retention of original activities. Mixture of individually conjugated enzymes showed lower inactivation rate constant with longer half life than the co-conjugated enzyme system. Individually conjugated enzymes showed an increase of 56.48 kJ/mole and 38.22 kJ/mole in activation energy for denaturation than the free enzymes and co-conjugated enzymes, respectively. K_m as well as V_{max} of individually and co-conjugated enzymes was found to be higher than the free enzymes. SDS-polyacrylamide gel electrophoresis confirmed the formation of conjugate and co-conjugate as evident by increased molecular weight. Both the enzyme systems were used for starch hydrolysis where individually conjugated enzymes showed highest release of glucose at 60 °C and pH 5.0 as compared to free and co-conjugated enzyme.

1. Introduction

Enzymatic reactions have many advantages over chemical reactions in terms of low process cost, specificity and release of fewer pollutants. Many industrial applications such as conversion of starch to ethanol (Rattanachomsri, Tanapongpipat, Eurwilaichitr, & Champreda, 2009), methanol oxidation in biofuel cells (Kar, Wen, Li, Minteer, & Barton, 2011) and enzymatic deracemisation of 2-naphthyl alanine (Caligiuri et al., 2006) need multi enzymatic and multistep reactions which involves lots of energy, cost and time (Findrik & Vasic-Racki, 2009). Multistep enzymatic processes can be carried out simultaneously to make them more economical. Simultaneous enzymatic actions can be carried out just by simply using mixture of two enzymes as well as by co-immobilization of two enzymes on one support (Deshpande, D'Souza, & Nadkarni, 1987; Minakshi & Pundir, 2008). Co-immobilization provides both the enzymes at the same time in the reaction and also stabilizes them with respect to temperature and pH. In most of the serial enzymatic processes, product of first reaction is the substrate of the next reaction. Immobilization of two enzymes on one support brings them in close proximity which enables better chances for a product of the first reaction to become a substrate for second before diffusing into the reaction environment. This also efficiently utilizes the initial substrate (Shimizu & Lenhoff, 1979).

In recent times, significant attention is being paid to utilize agricultural waste efficiently for the production of biofuels, biochemicals and biomaterials. Bio-ethanol production is gaining momentum over the past few years because of increasing energetic requirements. Bio-ethanol production needs starch hydrolysis to release glucose which involves a two-step process. The process starts with liquefaction of starch with amylase to produce soluble oligosaccharides which in turn becomes the substrate for saccharification by glucoamylase to release glucose (Park, Haam, Jang, Ahn, & Kim, 2005). Both the enzyme can be easily mixed together with the optimization of operating temperature, pH and time for application. These enzymes have been used simultaneously and separately for the saccharification of cassava starch, although simultaneous saccharification showed better results (Ruiz, Sanchez, Torres, & Molina, 2011). Simultaneous action of enzymes has been compared with step by step action for degrading starch granules, where simultaneous enzymatic action proved to be better (Slominska, Klisowska, & Grzeskowiak, 2003). High temperature is normally preferable for industrial processes due to higher rates of reaction and better process economics. Hence simultaneous use of enzymes having high thermal and pH stability would be expected to have an edge over conventional processes. Co-immobilization of α-amylase and glucoamylase had been done on hydrophilic silica gel and DEAE-cellulose for starch hydrolysis (Park et al., 2005).

Amongst many techniques for stabilization of enzymes, simple process is covalent binding of enzymes to polysaccharides.

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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. Enzyme–polysaccharide conjugate have been successfully formed using optimized process and shown to have better thermal and pH stability (Jadhav & Singhal, 2012). These results propelled us to investigate the possibility of conjugating a polysaccharide with two different enzymes so as to use them in simultaneous enzymatic processes. Two different approaches can be used to achieve such a conjugation. Both the enzymes can be covalently bound to the polysaccharides to prepare a co-conjugate where enzymes and polysaccharide will covalently entangle with each other. This co-conjugation may provide stability to both the enzymes by providing enough rigidity while maintaining their respective activities. Another approach could be conjugation of the two enzymes separately on the polysaccharides and then mixing two conjugates together to use them simultaneously.

Many reports have been published for immobilization of glucoamylase and amylase using different support materials to enhance thermal, pH and storage stability (Jadhav & Singhal, 2012; Milosavic, Prodanovic, Jovanovic, & Vujcic, 2004; Reshmi, Sanjay, & Sugunan, 2006; Storey, Duncan, & Chakrabarti, 1990). Here, we have attempted co-conjugation of α-amylase and glucoamylase on pectin to stabilize them for using in simultaneous reactions. We have selected pectin as polysaccharide for this study based on the previously published results (Jadhav & Singhal, 2013). Pectin showed more oxidized sites and enhanced thermal and pH stability of α-amylase on conjugation. We also conjugated both the enzymes individually on pectin and mixed them together. Both enzyme systems were used for starch hydrolysis to find the best possible enzyme system for such an application.

2. Experimental

2.1. Materials

Crude α-amylase from *Bacillus licheniformis* 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. Glucoamylase from *Aspergillus niger* was obtained from Sigma Aldrich, Mumbai, India. Pectin was purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Sodium metaperiodate was obtained from S. D. Fine Chemicals, Mumbai. Gluocose oxidase-peroxidase (GOD-POD) kit was purchased from Accurex Biomedical Pvt. Ltd. Mumbai, India. All other chemicals were of AR grade and procured from reliable sources.

2.2. Preparation of α-amylase–glucoamylase–pectin co-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. Pectin (25 mg/ml) 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 pectin solution was dialyzed against 0.1 M sodium acetate buffer of pH 5.0 at 4 °C overnight. α-Amylase solution (2.5 mg/ml protein) was prepared in buffer of pH 5.0 (5 ml) and mixed with oxidized pectin solution (10 ml) and kept for conjugate formation for 20 h at room temperature (∼28 ± 2 °C). Glucoamylase solution (2.5 mg/ml protein) was prepared in buffer of pH 5.0 (5 ml), added to the above solution and kept for 20 h at room temperature. Sodium borohydrate (40 mg) was then added to 20 ml of conjugate mixture to reduce remaining oxidized sites of pectin and kept for 4 h. Finally, the prepared conjugate solution was dialyzed against 20 mM of sodium citrate buffer of pH 5.0 at 4 °C overnight (Ahmed, Saleh, & Abdel-Fattah, 2007; Jadhav & Singhal, 2012; Srivastava, 1991; Villalonga, Gomez, Ramirez, & Villalonga, 1999). This conjugate was considered as co-conjugate of α-amylase and glucoamylase (CAG) and used for further studies. Control enzyme solution of free amylase (FA) and free glucosidase (FG) in a mixture (FA + FG) was prepared by mixing α-amylase (5 ml) and glucoamylase (5 ml) with sodium citrate buffer of pH 5.0 (10 ml) and used for study.

2.3. Preparation of individual conjugates of α-amylase–pectin and glucoamylase–pectin

Oxidized pectin solution (25 mg/ml) was prepared as described in Section 2.2. For preparation of α-amylase–pectin conjugate (CA), oxidized pectin (5 ml) was mixed with α-amylase (2.5 mg/ml protein, 5 ml) and kept at room temperature (∼28 ± 2 °C) for 20 h. Sodium borohydrate (20 mg) was then added to 10 ml of conjugate mixture to reduce remaining oxidized sites of pectin and kept for 4 h. Finally, the prepared conjugate solution was dialyzed against 20 mM of sodium citrate buffer of pH 5.0 at 4 °C overnight. Similarly glucoamylase–pectin conjugate (CG) was prepared and both the conjugates were mixed together in equal amount for further study. This was considered as mixture of conjugated α-amylase and conjugated glucoamylase (CA + CG).

2.4. α-Amylase and glucoamylase combined activity assay

Prepared mixture of α-amylase and glucoamylase enzyme sample (100 μl, 125 μg/ml of total protein concentration where each enzyme at concentration of 62.5 μg/ml) was added to 1% starch (100 μl) solution prepared in 0.02 M sodium-citrate buffer of pH 5.0. Mixture was kept at 50 °C for incubation; after 15 min it was kept on ice and 5% tricarboxylic acid (300 μl) was added to stop the reaction. The released glucose was detected by a glucose oxidase-peroxidase (GOD-POD) kit (Sathish, Madhavan, Vasanthi, & Amuthan, 2012). Above sample (10 μl) was added to GOD-POD working enzyme solution (1 ml) and kept for 30 min at room temperature (∼28 ± 2 °C). The absorbance was measured at 505 nm using UV-Vis spectrophotometer (Hitachi U2001, Japan). The combined enzyme activity of α-amylase and glucoamylase was calculated using a standard curve plotted using glucose in the range of 0.0–1.0 mg/ml. One unit of combined enzyme activity was defined as the micromole of glucose released per minute per ml per mg of mixed enzyme solution at 50 °C. Protein in the sample was detected using Folin-Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and it was calculated using bovine serum albumin as a standard in the range of 40–200 μg/ml.

2.5. Effect of conjugation and co-conjugation of α-amylase and glucoamylase with pectin on thermal and pH stability

The mixture of free enzymes (FA + FG), co-conjugate (CAG) and mixture of conjugated α-amylase and conjugated glucoamylase (CA + CG) were evaluated for thermal stability by incubating (125 μg of total protein where each enzyme was 62.5 μg) at 40 °C, 45 °C, 50 °C, 55 °C and 60 °C in 20 mM sodium citrate buffer of pH 5.0 for 15 min followed by quick chilling on ice. The enzyme activity was then assayed and percent residual activity was calculated with respect to their individual original activities. Individual original activities were referred to as activities of FA + FG, CAG and CA + CG under standard assay conditions without incubation at high temperatures and pHs.

The mixture of free enzymes (FA + FG), co-conjugate (CAG) and mixture of conjugated α-amylase and conjugated glucoamylase (CA + CG) (25 μg of total protein where each enzyme was 12.5 μg)

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 residual activity.

2.6. Effect of conjugation and co-conjugation on combined kinetics of thermal inactivation of α-amylase and glucoamylase

The mixture of free enzymes (FA + FG), co-conjugate (CAG) and mixture of conjugated α-amylase and conjugated glucoamylase (CA + CG) were incubated at 40 °C, 45 °C, 50 °C in 20 mM sodium citrate buffer of pH 5.0. Samples were withdrawn after every 30 min for 180 min, chilled quickly, and then assayed for combined 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 $0.693/k$.

2.7. SDS-polyacrylamide gel electrophoresis

Free α-amylase (FA), free glucoamylase (FG), co-conjugates (CAG) and mixture of conjugated α-amylase and conjugated glucoamylase (CA + CG) were run on 10% SDS-polyacrylamide gel and stained with silver staining method to estimate the molecular weight using suitable markers to confirm the presence of conjugate and co-conjugate.

2.8. Kinetics of mixture of free enzymes, co-conjugates and mixture of individually conjugated enzymes

Three enzyme systems including mixture of free enzymes (FA + FG), co-conjugate (CAG) and mixture of individually conjugated enzymes (CA + CG) were evaluated for determination of kinetic constants K_m and V_{max} using substrate concentration from 1 to 14 mg/ml. Lineweaver–Burk plot was used to calculate kinetic constants.

2.9. Application of conjugates and co-conjugates for glucose release by starch hydrolysis

Starch solution (5%) was prepared in 0.02 M sodium citrate buffer of pH 5.0. Starch solution (950 μl) was mixed with prepared enzyme systems (50 μl) and kept at 40 °C, 50 °C, 60 °C and 70 °C to determine the efficiency of free, conjugated and co-conjugated enzymes to release glucose. Samples were removed after every 30 min for 180 min to monitor release of glucose. Released glucose was detected using glucose oxidase-peroxidase (GOD-POD) kit. Appropriately diluted sample (10 μl) was mixed with GOD-POD working enzyme solution, kept at room temperature for 30 min and absorbance was taken at 505 nm using UV-Vis spectrophotometer (Hitachi U2001, Japan). The effect of change in pH of the reaction on the efficiency of enzyme systems to release glucose was also evaluated. 0.02 M citric acid/sodium citrate buffer of pH 3.0–6.0 was used to monitor glucose release at 60 °C.

3. Results and discussion

3.1. Combined enzyme activity of α-amylase and glucoamylase after conjugation and co-conjugation

α-Amylase and glucoamylase were present in a mixture of enzymes in free, conjugated and co-conjugated form, making it difficult to determine their individual activities. Hence starch was used as substrate to detect combined activity of both these enzymes. α-Amylase release oligosaccharides and glucose, whereas glucoamylase release glucose using oligosaccharides as well as starch as a substrate. Finally, combined enzyme activity was calculated from total glucose released in the reaction mixture detected by glucose oxidase-peroxidase (GOD-POD) kit. Combined activity of free enzymes (FA + FG), co-conjugate (CAG) and individually conjugated enzymes (CA + CG) were 217 U/mg ± 3.620, 201 U/mg ± 2.974 and 207 U/mg ± 2.782, respectively. Combined activity of enzyme system was found to be unaltered after co-conjugation and individual conjugation of enzymes with pectin. Retention of original activity is a major advantage of conjugation over immobilization.

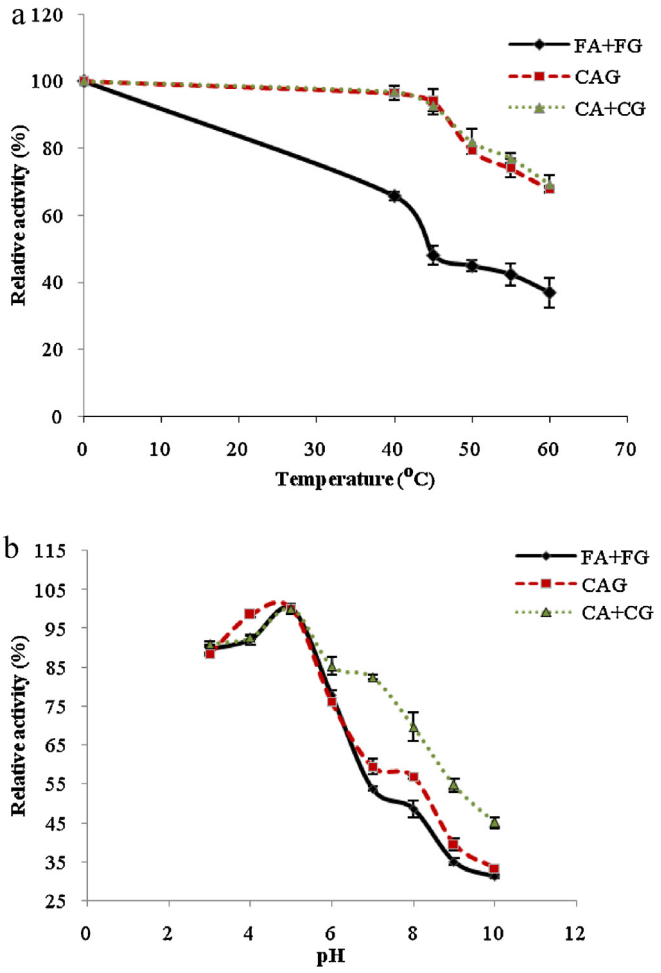

Fig. 1. Profile of mixture of free enzymes (FA + FG), co-conjugated enzymes (CAG) and mixture of individually conjugated enzymes (CA + CG) with respect to (a) thermal stability and (b) pH stability. A: α-amylase and G: glucoamylase.

3.2. Effect of conjugation and co-conjugation of α-amylase and glucoamylase with pectin on thermal and pH stability

The thermal stability of mixture of free enzymes (FA + FG), co-conjugate (CAG) and mixture of individually conjugated α-amylase and glucoamylase (CA + CG) was studied by incubating enzymes at 40–60 °C for 15 min in the absence of substrate. The activity was found to decrease with an increase in temperature for all the three enzyme systems (Fig. 1a). The mixture of individually conjugated enzymes (CA + CG) and co-conjugated enzyme complex (CAG) was about 30% more stable than free enzyme mixture at each temperature. Similarly, pH stability study showed CA + CG and CAG enzyme systems are more stable as compared to free

enzyme mixture (FA + FG) (Fig. 1b). CAG enzyme system showed little enhancement of stability in acidic as well as in alkaline pH as compared to free enzyme system. However, CA + CG enzyme system has more enhancement of stability in alkaline but not in acidic conditions. A comparison of stability between CA + CG and CAG showed CA + CG to clearly have at least 10–15% higher stability at pH 8, 9 and 10 however, CAG showed 6% more stability in acidic condition of pH 4. If the enzyme has bound to polyanionic carrier, pH optimum may shift to alkaline directions (Costa et al., 2001). Pectin is polyanionic in nature so it might be one of the reasons for getting higher stability in alkaline but not in acidic condition.

3.3. Effect of conjugation and co-conjugation on combined kinetics of thermal inactivation of α-amylase and glucoamylase

Thermal inactivation of FA + FG, CAG and CA + CG showed the activity of all the samples to decrease with time at each temperature. Kinetics of enzyme inactivation was evaluated by a semi-log plot of percent residual activity vs. time (Fig. 2) in terms of inactivation rate constant, k and half life $t_{1/2}$. The k values of the mixture of free enzymes (FA + FG) at 40 °C, 45 °C and 50 °C were 0.00139 min^{-1}, 0.00429 min^{-1} and 0.0137 min^{-1}, respectively, corresponding to $t_{1/2}$ of 498.56, 161.53 and 50.58 min at their respective temperatures. The co-conjugated enzyme (CAG) had k values of 0.00099 min^{-1}, 0.00328 min^{-1} and 0.0121 min^{-1} at 40 °C, 45 °C and 50 °C corresponding to their $t_{1/2}$ of 700 min, 211.28 min and 57.13 min, respectively. The CA + CG enzyme system showed k values of 0.00058 min^{-1}, 0.00238 min^{-1} and 0.0112 min^{-1} at 40 °C, 45 °C and 50 °C corresponding to their $t_{1/2}$ of 1194.82 min, 291.17 min and 61.87 min, respectively. It can be seen that the inactivation rate constant of both CAG and CA + CG enzyme systems were lower than the FA + FG at each temperature indicating conjugated enzyme systems to be more stable against denaturation as compared to free enzyme mixture (Amini, Sorouraddin, & Rashidi, 2011). CA + CG showed lower inactivation rate constant with high half life than CAG implying CA + CG to be more stable than CAG at each temperature. When two enzymes bound to pectin for co-conjugation, they may have competitive binding for the pectin and form covalent cross linking structure. As two enzymes compete for binding and they might interfere with each other while interaction with pectin, there may not be perfect covalent interactions of both the enzymes with pectin. This cross linking may provide enough binding force and rigidity to both the enzymes to be stable over free enzyme system but comparably lower than individually conjugated enzyme system. While in individual conjugation, there might be a perfect covalent binding of each of the enzyme with pectin and then mixing of two conjugate for further use. This may be attributed to lesser rigidity provided by pectin to α-amylase and glucoamylase on co-conjugation but significantly high in individual conjugation.

Further, activation energy of all enzyme systems was calculated from Arrhenius plot (Fig. 3) as product of slope and R, where R is the constant (8.314 J mol^{-1} K^{-1}.). The activation energy of FA + FG, CAG and CA + CG was found to be 192.29 kJ/mole, 210.55 kJ/mole and 248.77 kJ/mole, respectively. The activation energy is an index of the thermal stability of protein with higher activation energy for denaturation implying a more stable enzyme (Sundaram & Venkatesh, 1998). Higher activation energy of CAG and CA + CG over FA + FG suggested their better thermal stability than that of FA + FG. However, CA + CG needed 38.22 kJ/mole more energy to denature as compared to CAG. Hence, it can be concluded that CA + CG may be a better enzyme system to explore both enzymes simultaneously for industrial applications.

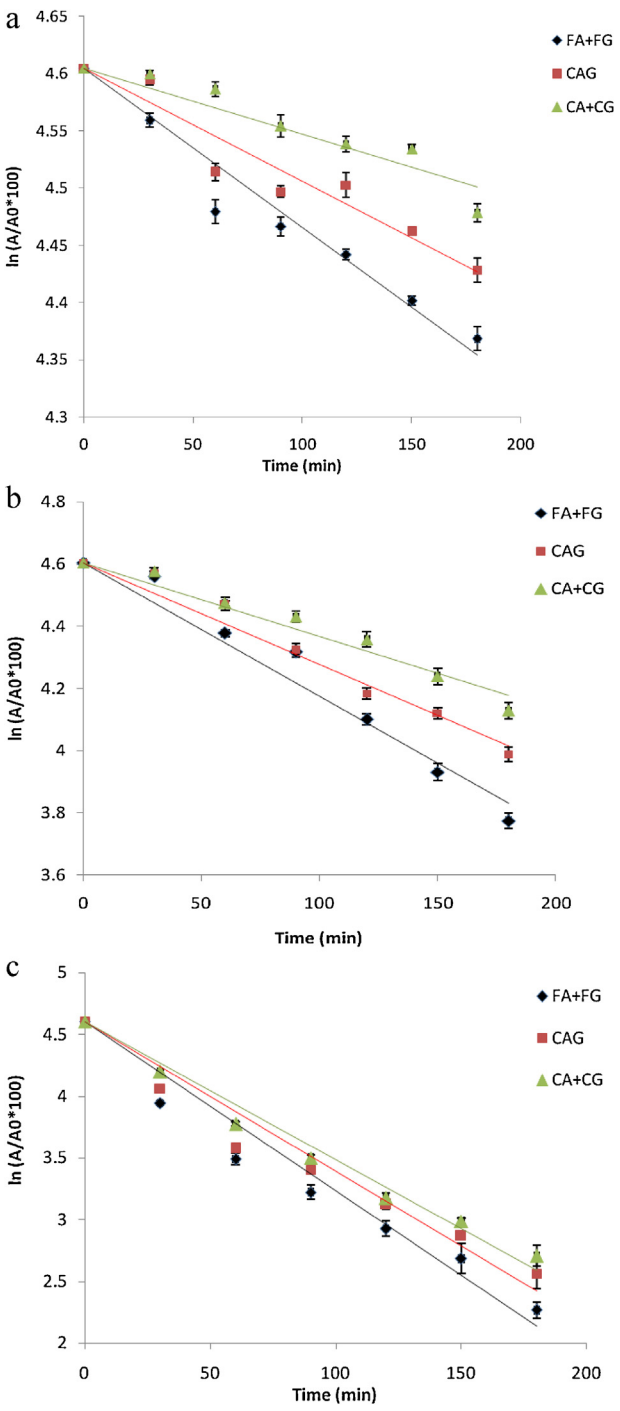

Fig. 2. Kinetics of thermal inactivation of mixture of free α-amylase and glucoamylase, co-conjugated α-amylase and glucoamylase and individually conjugated α-amylase and glucoamylase at (a) 40 °C, (b) 45 °C and (c) 50 °C.

3.4. SDS-polyacrylamide gel electrophoresis

The formation of individual and co-conjugates was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Silver staining of free α-amylase run on polyacrylamide gel showed the presence two isozymes having molecular weights of 130 and 170 kDa in lane 1. Similar staining of free glucoamylase showed a band of molecular weight of 26 kDa in lane 2. CAG and CA + CG showed band of an increased molecular weight in lanes 3 and 4, respectively, which stack on the resolving gel indicating anomalous behaviour of enzyme after binding with polysaccharides and hence confirmed

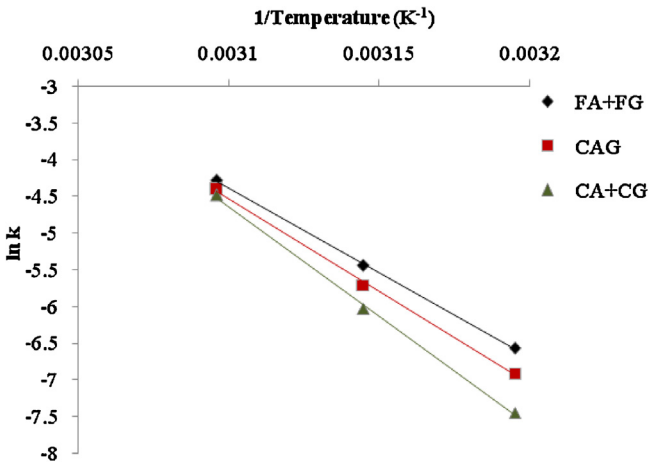

Fig. 3. Arrhenius plot for inactivation of free, co-conjugated and individually conjugated α-amylase and glucoamylase.

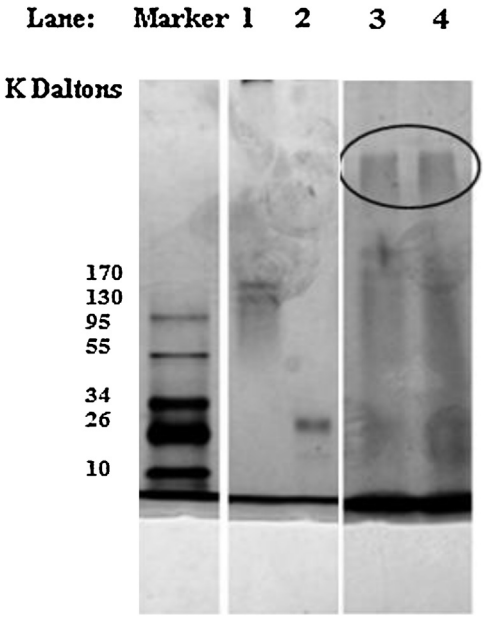

Fig. 4. Polyacrylamide gel (10%) stained with silver staining. Lane 1: Free α-amylase, lane 2: Free glucoamylase, lane 3: Co-conjugated enzymes and lane 4: Mixture of individually conjugated enzymes.

the conjugate formation. Glycoproteins are reported to show relatively low free electrophoretic mobility and peculiar conformation in SDS (Marciani & Papamatheakis, 1978).

3.5. Kinetics of mixture of free enzyme, co-conjugates and mixture of individually conjugated enzymes

Kinetic constants of FA + FG, CAG and CA + CG were calculated using Lineweaver–Burk plot (Table 1). K_m of individually conjugated and co-conjugated enzyme system was slightly higher than the free enzyme mixture, showing lower affinity of conjugated and co-conjugated enzymes to the substrate. This low affinity may be due to increased rigidity and interference by the covalently attached polysaccharide. Moreover, CAG and CA + CG also

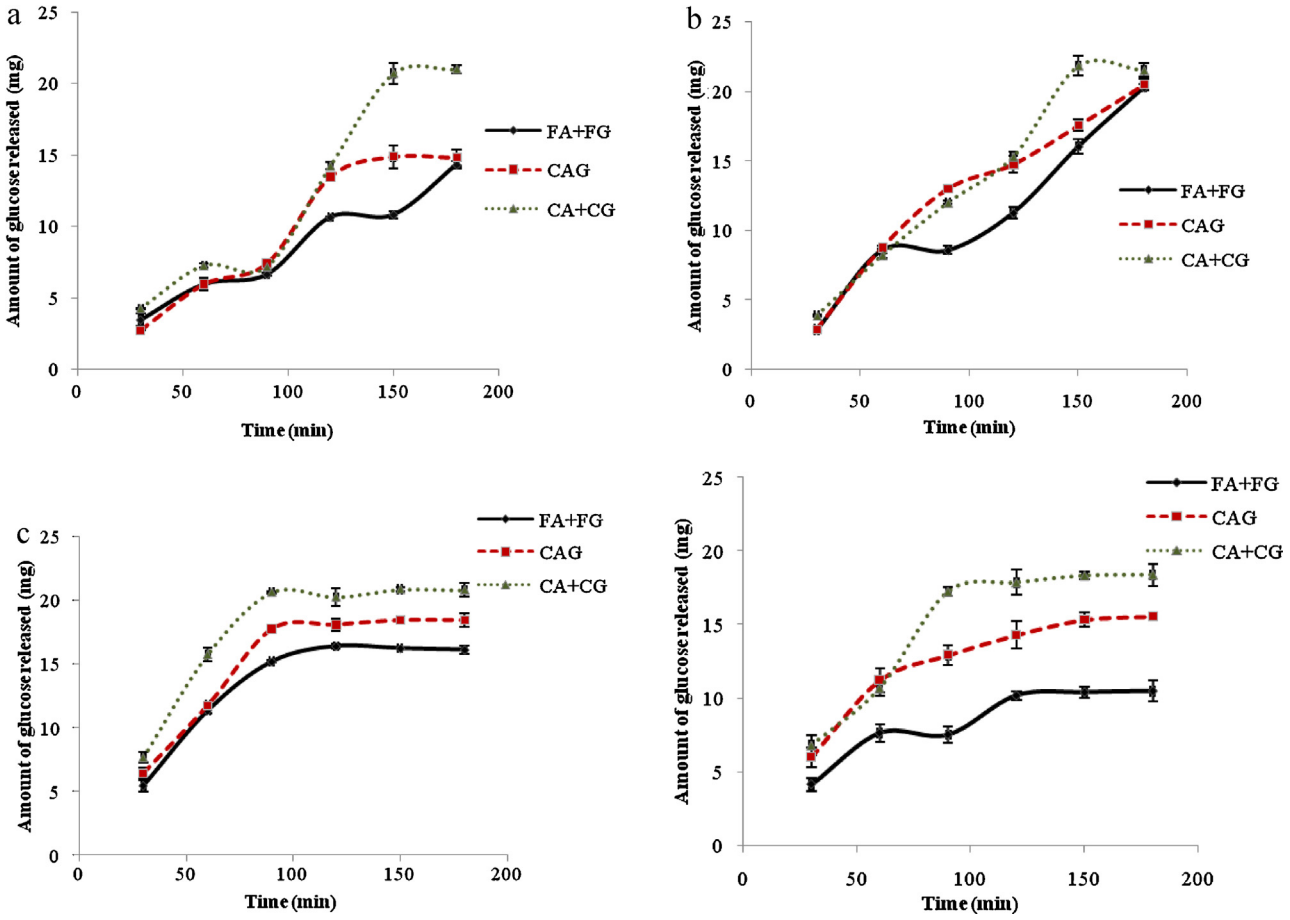

Fig. 5. Hydrolysis of starch to glucose using free, co-conjugated and individually conjugated enzyme systems at (a) 40 °C, (b) 50 °C, (c) 60 °C and (d) 70 °C.

Table 1
Kinetic parameters of free, co-conjugated and individually conjugated enzyme systems using Lineweaver–Burk plot.

Enzyme sample	V_{max} (U/ml)	K_m (mg/ml)
FA + FG	1495	0.942
CAG	1840	1.273
CA + CG	1923	1.333

FA + FG: mixture of free α-amylase and glucoamylase. CAG: co-conjugated α-amylase and glucoamylase with pectin. CA + CG: mixture of conjugated α-amylase with pectin and conjugated glucoamylase with pectin.

showed higher V_{max} than FA + FG. K_m of glucoamylase has been reported to increase after its entrapment in alginate beads and fibres (Tanriseven, Uludag, & Dogan, 2002).

3.6. Application of mixture of free enzyme, co-conjugates and mixture of individually conjugated enzymes for glucose release by starch hydrolysis

The results obtained as above confirmed CA + CG and CAG to have better stability at high temperature and pH than FA + FG. Moreover, CA + CG showed comparatively higher stability than CAG. To explore their industrial use, starch hydrolysis was carried out using all the three enzyme systems. It was done at different temperatures to investigate the enzyme system that could work best for starch hydrolysis. The amount of glucose released was chosen as the parameter to decide which of the three enzyme systems was best suited for the intended application. The glucose released increased with progress of time at 40 °C, 50 °C, 60 °C and 70 °C with CAG and CA + CG showing higher release of glucose as compared to FA + FG. CA + CG was better than CAG in terms of release of glucose (Fig. 5). At 60 °C, glucose release was found to be maximum with almost 16–20 mg/ml of glucose obtained by CA + CG enzyme system within 120 min. Rate of glucose release was found to decrease at 70 °C which may be due to initiation of denaturation of enzyme at high temperature. Glucoamylase was thermally unstable as compared to α-amylase and hence denatured earlier. α-Amylase remains comparatively rigid at high temperature but alone could not release enough glucose (data not shown). Hydrolysis of starch using a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads has been reported to be more efficient than sequential use of two enzymes (Roy & Gupta, 2004).

Effect of pH on the enzyme efficiency for glucose release was studied at 60 °C for 90 min. As described in Section 3.2 combined enzyme activity was high in acidic pH compared to alkaline. Hence, only acidic pH range was selected for this study. A pH 5 was better for all three enzyme systems to hydrolyse starch and release glucose with CA + CG showing highest glucose release (Fig. 6). Hence it can be concluded that CA + CG can be explored industrially for glucose release by starch hydrolysis at pH 5 and 60 °C to save time and cost of production.

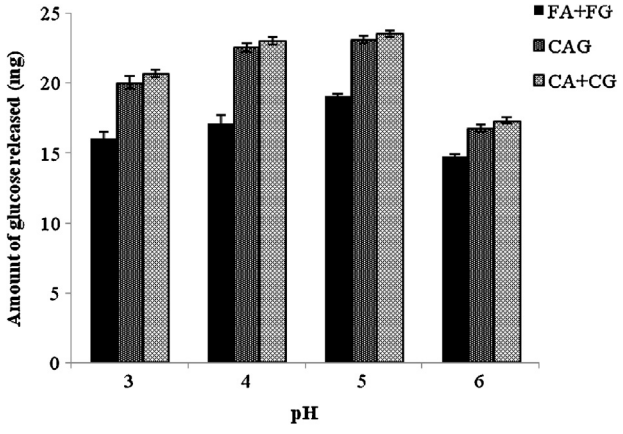

Fig. 6. Hydrolysis of starch to glucose using free, co-conjugated and individually conjugated enzyme systems at 60 °C using buffers of different pHs.

4. Conclusion

α-Amylase and glucoamylase showed better thermal and pH stability on individual and co-conjugation with pectin compared to mixture of free enzyme system. Mixture of individually conjugated enzymes showed lower inactivation rate constant, higher half life and an increase of 38.22 kJ/mole in activation energy for denaturation than the co-conjugated enzymes. Both the co-conjugated and individually conjugated enzyme systems showed better starch hydrolysis efficiency over free enzymes with higher and rapid hydrolysis at 60 °C and pH 5.0. Hence, it can be concluded that a mixture of individually conjugated amylase and glucoamylase can be utilized in rapid hydrolysis of starch to glucose with an added advantage of enhanced thermal and pH stability.

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