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Thermal Stabilization of Immobilized α-Amylase by Coupling with Proline

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α-Amylase was immobilized to Sepharose-4B activated by electrophilic method using cyanogen bromide. The α-amylase coupled was 77% of the total protein added. Further L-proline was covalently linked to the immobilized α-amylase by carbodiimide. Optimum carbodiimide concentration for the coupling of proline to the immobilized α-amylase and the suitable proline concentration for the coupling were determined. Activity of immobilized α-amylase was not altered after coupling to proline. The thermal stability of soluble α-amylase, immobilized α-amylase and immobilized α-amylase–proline conjugates (samples coupled to two different proline concentrations) were studied at 45 and 60°C. Soluble α-amylase lost its total activity in 4 days and 1 day at 45 and 60°C, respectively. Immobilized α-amylase lost its total activity on the 30th and 16th days at 45 and 60°C, respectively. Immobilized α-amylase–proline conjugate (85·35 μg proline/g gel) lost only 78% activity at 45°C on the 30th day while the same preparation took 20 days at 60°C to lose the total activity. On the other hand the immobilized α-amylase–proline conjugate (785·32 μg proline/g gel) lost only 30% of its original activity at 45°C on the 30th day and took 30 days at 60°C to lose its total activity. These results show that the coupling of proline to immobilized enzymes increases their thermal stability.

INTRODUCTION

The use of enzymes, based on their specificity and ability to operate efficiently under mild conditions is continuously expanding. Repeated use of the enzymes has been achieved by immobilization to water insoluble supports.[1,2] Immobilized enzymes show enhanced stability over their soluble counterparts.[1–3] Attempts to increase the stability of soluble[4–6] and immobilized enzymes[7,8] include the addition of polyols such as glycerol and sugars to the medium containing soluble enzyme[4,6] or the coupling of carbohydrates to soluble[9,10] or immobilized enzyme.[8] An increase in the thermal stability of enzymes can be achieved by increasing the viscosity of solutions by adding polymers such as polyethylene glycol.[11] This is due to the formation of multiple hydrogen bonds between the solute and water molecules which reduce water activity and limits considerably the free movement of the mobile parts of the enzyme molecule.[12] Upon immobilization, the chemical binding of an enzyme molecule to the support significantly reduces its freedom to undergo conformational changes. The arrest of the free movement minimizes the number of drastic

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conformational changes which may lead to denaturation.

The major osmolytes in water — stressed eukaryotes are low molecular weight metabolic products such as polyols, free amino acids and amino acid derivatives.[13] Such molecules accumulate in microbial and plant cells during osmotic stress and prevent damage from cellular dehydration by balancing the osmotic strength of the cytoplasm.[14] Proline accumulates and promotes growth under strongly inhibitory levels of osmotic strength.[15] The osmoprotectants influence protein structure and their stability.[14] This had led to the selection of proline to determine its stabilizing effect.

None of the previously reported studies has focused on the immobilization or addition of amino acids to stabilize an enzyme. In this paper thermal stabilization of immobilized α-amylase achieved by coupling to L-proline is described.

MATERIALS AND METHODS

Materials

α-Amylase (Hog Pancreas), 1-ethyl-3(3-dimethyl amino propyl)-carbodiimide hydrochloride and L-proline were from the Sigma Chemical Company (USA) and Sepharose-4B was from Pharmacia Fine Chemicals (Sweden). Cyanogen bromide was prepared in the authors' laboratory.[16]

Analytical methods

α-Amylase activity was determined[17] by measuring the reducing sugars produced by a DNS method.[18] In comparative studies equal amounts of soluble and immobilized enzyme protein (0·96 mg protein, determined by Kjeldhal method[19]) was used. Proline was estimated by the method of Troll and Cannon.[20]

Unit activity of α-amylase

One unit of α-amylase is the amount of enzyme that will liberate 1·0 mg of maltose from starch in 3 min at pH 6·9 and at 45°C.

Immobilization of α-amylase to Sepharose-4B

α-Amylase was immobilized to Sepharose-4B activated by cyanogen bromide.[16]

Coupling of proline to immobilized α-amylase at varying concentrations of carbodiimide

L-Proline (115·1 μg/g wet gel) was mixed with Sepharose-4B immobilized α-amylase at varying concentrations of carbodiimide[21] (0–100 mg/g wet gel) for 18 h at pH 6·0 and 30°C. Proline coupled was estimated[20] indirectly by measuring the unbound proline concentration in the washings. The activity of immobilized α-amylase–proline conjugate was determined as described previously.

Effect of proline concentration

L-Proline, at concentrations from 1·151 μg to $1{\cdot}151 \times 10^4$ μg/g wet gel, was coupled to Sepharose-4B immobilized α-amylase using the optimum concentration of carbodiimide.[21] The amount of proline coupled and the activity of immobilized α-amylase–proline conjugate were determined. In this experiment 1 M ethanolamine (1 ml/g gel) was used as the blocking agent and excess carbodiimide was removed by washing first with distilled water (pH adjusted to 6·0), then with 0·1 M bicarbonate–0·5 M sodium chloride (pH 8·5) and 0·1 M acetate–0·5 M sodium chloride (pH 4·0) buffers alternatively three times and finally with 0·02 M phosphate buffer (6·9).

Thermal stability of different α-amylase preparations

Soluble α-amylase, immobilized α-amylase and immobilized α-amylase–proline conjugates A and B in 0·02% (w/v) sodium azide–0·02 M phosphate buffer (pH 6·9) were stored at 45°C and 60°C. Activities of the enzyme preparations were monitored for 30 days at 45°C.

RESULTS AND DISCUSSION

Coupling of proline at varying concentrations of carbodiimide

The α-amylase coupled to Sepharose-4B was 3·85 mg/g wet gel (Fig. 1). As the carbodiimide concentration recommended for coupling was 10–100 times greater than the concentration of the reactants,[21] the concentration of carbodiimide required for coupling the carboxyl group of proline to the amino group in the amino acid residues of α-amylase immobilized to Sepharose-4B was studied. When 115·1 μg of proline was used per 3·85 mg α-amylase immobilized on 1 g Sepharose-4B, the optimum carbodiimide concentration required for coupling was 60 mg (Fig. 1). In all the cases when different concentrations of proline were coupled while using different con-

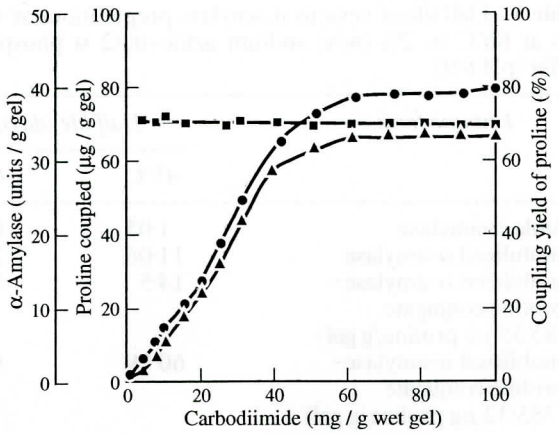

Fig. 1. Effect of carbodiimide concentration on the coupling of proline to α-amylase immobilized on Sepharose-4B. ●, Proline coupled; ▲, coupling yield of proline; and ■, α-amylase activity of the conjugates. Coupling yield of proline = [Proline coupled/g gel]/[Proline added/g gel] × 100.

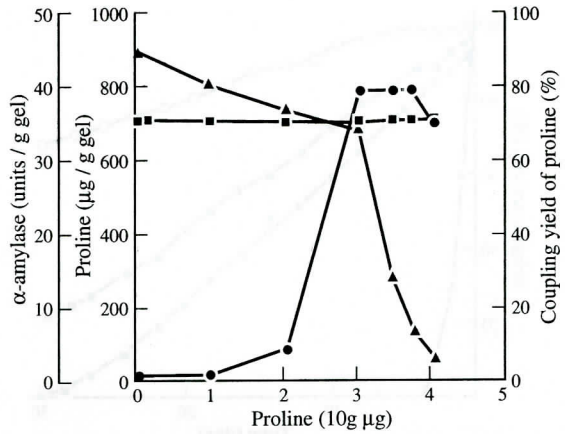

Fig. 2. Effect of proline concentration on the coupling of proline to α-amylase immobilized on Sepharose-4B. ●, Proline coupled; ▲, coupling yield of proline; and ■, α-amylase activity of the conjugates. Coupling yield of proline was calculated as described in Fig. 1.

centrations of carbodiimide, the activity of immobilized α-amylase did not alter. Thus 60 mg/g wet gel was selected in experiments to find the maximum proline that could be coupled with the immobilized α-amylase. No buffers were used in this experiment since buffers having amino, phosphate or carboxyl groups need to be avoided while using carbodiimide as a coupling agent.[21] The pH was maintained at 6·0 (by the addition of 1 N NaOH) and not at pH 4·5 when incubated for a longer period (18 h).[22]

Effect of proline concentration

Proline concentrations in the range $1{\cdot}151\text{–}1{\cdot}151 \times 10^4$ μg (0·01–100 μmole)/g wet gel were used for coupling. The amount of proline coupled increased with proline concentration and when the proline concentration was $1{\cdot}15 \times 10^4$ μg/g wet gel the maximum amount of proline coupled was 785 μg/g wet gel (Fig. 2). When the proline concentration was increased further the amount of proline coupled remained almost constant (785·0–790·2 μg/g wet gel) while the coupling yield decreased. This decrease probably indicates that the available amino groups in the amino acid residues of α-amylase immobilized on Sepharose-4B had all been utilized by the 790 μg of proline. In these experiments the proline coupling did not alter the activity of α-amylase conjugates. These results also probably indicate that amino groups of α-amylase that are not involved in catalytic activity or the amino groups in or around the catalytic site of the enzyme were not involved in proline coupling.

Thermal stability of α-amylase preparations

The soluble α-amylase, the immobilized α-amylase and the immobilized α-amylase–proline conjugates (a) with 85·35 μg proline/g gel and (b) with 785·32 μg proline/g gel were stored in 0·02% (w/v) sodium azide–0·02 M phosphate buffer (pH 6·9) at 45°C for one month. Sodium azide was added to avoid microbial contamination and to ensure that the activity measured was solely from the enzyme preparations.

The coupling of proline to soluble α-amylase was not carried out due to the problems associated with the separation of soluble α-amylase–proline conjugate from excess uncoupled proline. At 45°C the total activity of the soluble enzyme was lost on the fourth day, while all three immobilized enzyme preparations retained activity for much longer time periods (Fig. 3). The immobilized α-amylase–proline conjugates A and B retained 22 and 70% of the initial activities, respectively, on the 30th day by which time the immobilized α-amylase had lost all activity. When these enzyme preparations were stored at 60°C in 0·02% (w/v) sodium azide–0·02 M phosphate buffer (pH 6·9), the soluble enzyme lost all activity on the first day itself while the immobilized preparations retained activity for more than 10 days (Fig. 4). The immobilized α-amylase had lost total activity by the 16th day while immobilized α-amylase–proline conjugates A and B completely lost their activities only after the 20th and 30th days, respectively (Fig. 4). These results show that the immobilization of α-amylase to Sepharose-4B improves the thermal

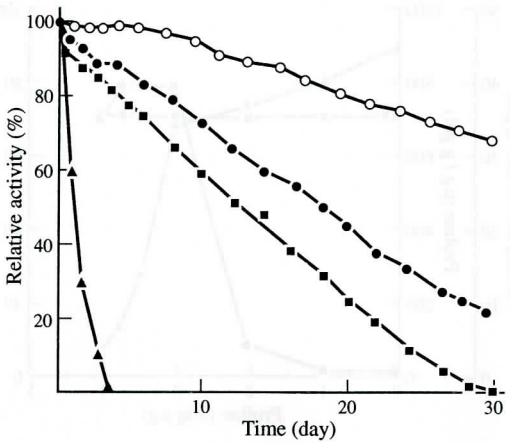

Fig. 3. Thermal stability at 45°C of (▲) soluble α-amylase; (■) immobilized α-amylase and immobilized α-amylase–proline conjugate (●) A, 85·35 μg proline/g gel and (○) B with 785·32 μg/g gel in 0·02 M phosphate buffer (pH 6·9).

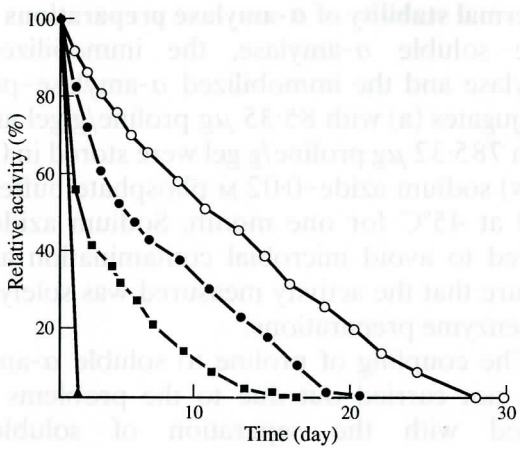

Fig. 4. Thermal stability at 60°C of (▲) soluble α-amylase; (■) immobilized α-amylase and immobilized α-amylase–proline conjugate (●) A, 85·35 μg proline/g gel and (○) B with 785·32 μg/g gel in 0·02 M phosphate buffer (pH 6·9).

stability of α-amylase and that this thermal stability can be further improved by conjugating with proline (Table 1). Among the two immobilized α-amylase–proline conjugates, preparation B showed higher stability than preparation A. This indicates that coupling of proline to immobilized α-amylase can improve its thermal stability (Table 1).

CONCLUSION

The optimum amount of carbodiimide required to couple proline was 60 mg/g gel while the maximum proline that could be coupled to 3·85 mg α-amylase immobilized in 1 g Sepharose-4B was 785·35 μg. The soluble enzyme showed least stability at both temperatures studied. Immobilization improved enzyme stability and further improvement was brought about by coupling proline to the immobilized enzyme. This effect of proline was confirmed by studying the stability of immobilized α-amylase–proline conjugate containing two different concentrations of proline.

Table 1. Half life of various α-amylase preparations at 45°C and at 60°C in 2% (w/v) sodium azide–0·02 M phosphate buffer (pH 6·9)

Enzyme form	*Half life (days)*	
	45°C	*60°C*
Soluble α-amylase	1·05	0·5
Immobilized α-amylase	11·06	2·34
Immobilized α-amylase–proline conjugate (85·35 μg proline/g gel)	14·5	5·74
Immobilized α-amylase–proline conjugate (785·32 μg proline/g gel)	60·79	8·18

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