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# Modification of α-amylase from *Bacillus licheniformis* by the polyaldehyde derived from β-cyclodextrine and α-amylase thermostability

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The cleavage of β-cyclodextrine by sodium periodate at the seven 2–3 diols of the glucose unit gives rise to the polyaldehyde 1, used to modify α-amylase. The reductive modification of α-amylase from *Bacillus licheniformis* reduced the number of reactive lysine groups from 8 to 3.5 per mol of enzyme with an activity loss of 25% and increased the half-life at 80°C from 4.7 to 7.0 minutes.

α-Amylase; β-Cyclodextrine; Thermostability

## 1. INTRODUCTION

Modification of the amino groups located at the surface by alkylation increased the thermostability of glycogene phosphorylase [1] and of horse liver alcohol dehydrogenase [2].

The need for increased thermostability of α-amylase, an enzyme of great importance in starch industry, arose our interest [3]. The modification of reactive amino groups by a reaction with aldehydes and reduction is an easy reaction to perform. Primary amino groups do not seem to be involved in a number of enzymatic reactions, so modifications of the amino groups may be a way to monitor the properties of the enzymes like thermostability or solubility in organic solvents [4]. Even more polyaldehydes may give rise to intramolecular crosslinking through reactions with several groups and thus increase the thermostability. Glutaraldehyde and its aldol condensation products present in its solution [5] is a reagent commonly used. The polyaldehyde 1 [6] resulting from the selective cleavage of 1.2 diol of β-cyclodextrine [7] is an alternative which does not seem to have been used for protein crosslinking.

## 2. MATERIALS AND METHODS

β-Cyclodextrine was a gift from the firm Roquette Frères (Lestrem). Bidistilled water was used in all experiments and no calcium salt was added. α-Amylase was purified from the commercial preparation Termamyl (*Bacillus licheniformis*) from Novo. The protein concentration was calculated from the absorption at 280 nm with $\varepsilon^{0.1\%}_{1\,cm}=2.45$ [8]. The Termamyl as provided by Novo (50 ml) was dialysed against 20 mM Tris-HCl buffer pH 7.5 (3×2 liters). The solution (170 ml) was concentrated by ultrafiltration (final volume 50 ml, 220 mg/ml). This solution was chromatographed on DEAE-Sepharose (5.0×55 cm) prepared in 0.1 M Tris-HCl buffer pH 7.5. The α-amylase was eluted as a first peak from the column with the same buffer and concentrated by ultrafiltration. The buffer was changed to 22.5 mM Tris-acetate buffer pH 8.2 in three ultrafiltration and dilution steps. The final protein concentration was 50 mg/ml (12 ml).

Half the volume (6 ml) was submitted to chromatofocusing on PBE 94 resine column (0.9×25 cm). The α-amylase was eluted with polybuffer PB 96 (diluted 10 times)/AcOH pH 6.0. The fractions containing the α-amylase were diluted several times with 100 mM acetate buffer pH 5.7 and concentrated by ultrafiltration in order to remove the elution buffer. The final enzyme concentration was 0.1 mM.

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### 2.1. *Preparation of the polyaldehyde 1*

The cleavage of β-cyclodextrine (0.75 mmol) in solution in distilled water (40 ml) by sodium metaperiodate (5.75 mmol; 7.7 equivalents) was followed at room temperature in the dark by the iodometric determination of periodate. After 24 h, the stoichiometric amount of periodate was consumed. Excess reagent and iodate as reaction product were precipitated as barium salt by adding a calculated amount of barium chloride. After filtration of the precipitate, the solution was used as such. This solution was stable for two days at 4°C and with time a precipitate appeared and the solution darkened so that only the freshly prepared 10 mM polyaldehyde 1 solution was used.

### 2.2 *Modification of α-amylase*

To a 24 μM solution (0.2 ml) of α-amylase in 0.25 M phosphate buffer pH 7.7 was added a 10 mM polyaldehyde 1 solution (see Table I) and a 160 mM solution of sodium cyanoborohydride (see Table I). The molar ratio of sodium cyanoborohydride to polyaldehyde was 15. After 24 h at 20°C, the solution was dialysed against water (2×1 liters).

### 2.3 *Activity of α-amylase*

The specific activity of α-amylase was determined using the test of Smith and Roe after chromatofocusing 370 U/mg protein [9].

### 2.4 *Thermostability of enzymic activity*

Series of samples (10 for instance) of native and modified enzyme solution diluted in 0.2 M acetate buffer pH 5.7 to a final enzyme concentration of 7 μM was heated in a thermostated oil bath at 80°C. At determined times, a sample was removed and cooled to 0°C. The


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activity test was performed using a modified version of the Bernfeld test [10]. A volume (0.1 ml) of the sample solution with the enzyme (about 7 $\mu$M) was added to a 0.5% starch solution. After an incubation at 50°C for 10 min, an alkaline 40 mM solution of sodium dinitrosalicylate (1 ml) was added. A blank was prepared by adding the reagent solution to the starch solution before the addition of the enzyme. The two samples were heated at 100°C for 10 min. After cooling, water (5 ml) was added. The absorption was measured at 540 nm and compared to the result obtained with the non-heated enzyme sample.

2.5 *Determination of the lysine groups*

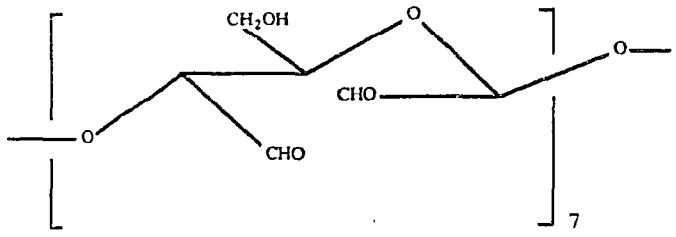
The number of reactive lysine groups was determined by a reaction with 2,4,6-trinitrobenzene sulfonic acid. A solution of the enzyme or modified enzyme was diluted to a final concentration of around 5 $\mu$M in 0.05 M carbonate buffer pH 9.9. To this enzyme solution (800 $\mu$l), a 0.1 M solution of 2,4,6-trinitrobenzene sulfonic acid (20 $\mu$l) was added. The absorption increase at 367 nm was measured as a function of time and the absorption of a sample without enzyme was substracted. A molar absorption coefficient of 11 000 $M^{-1} \cdot cm^{-1}$ was used for calculations.

## 3. RESULTS AND DISCUSSION

Chemical modifications of the α-amylase: reductive methylation (formaldehyde sodium cyanoborohydride) and crosslinking with glutaraldehyde showed that 5 to 6 amino groups could be modified without loss of activity. The thermostability of the modified enzymes was close to that of the native enzyme. Other cross linking reagents: dimethyl adipimidate-, dimethyl suberimidate dihydrochloride or 1,5-difluoro-2,4-dinitrobenzene had no noticeable effect on the thermostability.

The cleavage of β-cyclodextrine by the periodate reagent consumes 7 mol of reagent per mol of β-cyclodextrine [7]. The periodic cleavage of C-2, C-3 diol gives a cyclic polyether with 14 aldehyde groups [6]. A solution of this polyaldehyde 1 was used for the enzyme modification as obtained from periodic cleavage of the β-cyclodextrine in water and after precipitation of periodate anions present in slight excess and of iodate anions as barium salts.

On reductive modification of α-amylase with this polyaldehyde 1 (to the concentration of 0.5 mM), the enzyme activity was sligthly modified. At a higher concentration of polyaldehyde 1, the activity tended to decrease, so that the effect of the modification on the thermostability was studied in the polyaldehyde 1 concentration below 0.5 mM. The number of reactive lysines was determined with 2,4,6-trinitrobenzene sulfonic acid and was found to be close to 8 residues per mol of enzyme [11]. The sequence of the α-amylase from this source: *Bacillus licheniformis* includes 30 lysines [12]. On modification, the number of reactive lysines was reduced (Table I) and the enzymic activity was 75% with 4.5 inactivated lysines per mol of enzyme. The decrease in activity upon incubation at 80°C was a first-order reaction and the thermostability as determined by half-life at 80°C was increased by 45%.




1

Fig. 1.

In contrast, glutaraldehyde and its aldol condensation products had no effect on the thermostability. The easy access to β-cyclodextrine and the periodate cleavage to the polyaldehyde 1 makes this polyaldehyde a useful crosslinking reagent.

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Table I

Experimental conditions and results of the polyaldehyde 1 modification of α-amylase

| Reagent final concentration | Native enzyme | Experiments | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Enzyme concentration ($\mu$M) | | 22.5 | 21 | 18 |
| Polyaldehyde concentration ($\mu$M) | | 350 | 680 | 1250 |
| Cyanoborohydride concentration (mM) | | 5.3 | 10 | 20 |
| Number of modified lysine groups (total 8)/mol enzyme | | 1 | 2 | 4.5 |
| Enzymic activity (% to the activity of the native enzyme) | 100 | 95 | 85 | 75 |
| Half-life (min) at 80°C | 4.7 | 5.6 | 6.2 | 7.0 |

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