Citraconylation and maleylation on the catalytic and thermodynamic properties of raw starch saccharifying amylase from *Aspergillus carbonarius*

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**ABSTRACT**

Amylase capable of raw starch digestion presents a cheap and easier means of reducing sugar generation from various starch sources. Unfortunately, its potential for use in numerous industrial processes is hindered by poor stability. In this work, chemical modification by acylation using citraconic anhydride (CA) and maleic anhydride (MA) was used to stabilize the raw starch saccharifying amylase from *A. carbonarius*. The effect of the anhydrides on the pH and thermal stability of the free amylase was investigated. Enzyme kinetics and thermodynamic studies of the free and modified amylase were also carried out. Blue shifts in fluorescent spectra were observed after modification with both anhydrides. Citraconylation led to increased affinity of the enzyme for raw potato starch, unlike maleylation. The activation energy (kJ mol⁻¹) for enzyme inactivation was increased by 94.8% after modification with CA while only 17.9% increase was noted after modification with MA. Acylation led to an increase in Gibb's free energy and enthalpy while a reduction in entropy was observed. At 80 °C the half-life (h) was 5.92, 11.18 and 14.74 for free, MA and CA enzyme samples, respectively. These findings have potential value in all industries interested in starch conversion to sugars.

1. Introduction

Amylases are used in many bio-industrial processes and account for a sizeable fraction of the world's industrial enzyme market [1]. Amylases (alpha, beta and gluco-) catalyze the degradation of starch to simple sugars used as raw materials for a variety of products making them invaluable in the food and energy sector [2]. The heterogeneity of the starch molecule due to variations in granule morphology and organization results in its resistance to enzymatic attack and incomplete hydrolysis. For efficient hydrolysis of the starch molecule, gelatinization and liquefaction often precede saccharification, incurring a high cost of production and other difficulties [3]. Utilizing native/raw starch hydrolyzing amylases for starch liquefaction and saccharification saves time and cost of heating while increasing process efficiency [4, 5].

Enzyme stability makes it possible to optimally conduct biological processes under a wide range of operating conditions such as high temperatures, extremes of pH and a wide range of solvents [6, 7]. Chemical modification is a powerful and effective means to increase biocatalyst functionality [8, 9]. To overcome the problems which impede the widespread use of raw starch digesting amylases, more research to ascertain their mode of function and stabilization is required [10, 11].

Acylation by the aid of dicarboxylic anhydrides has been reported to improve enzyme activity and stability [9, 12]. The lysine molecules on the amylase surface are very reactive and do not play any role in its activity. It is on the ε-amino groups of these lysine molecules that the α-carboxylic groups from the anhydrides are attached [13]. Since more than half of the residues adjacent to the lysine molecules are hydrophobic amino acids [14], attachment of the bulky carboxylic groups masks the non-polar hydrophobic residues, thereby protecting them from unfavorable interactions with the aqueous environment and further stabilizing the enzyme structure. Acylation is cost-effective, simple, does not require complex equipment, can be carried out singly or in conjunction with other enzyme engineering protocols, does not require in-depth knowledge of the enzyme protein, and can be applied to native, engineered or mutant enzyme [15, 16]. Maleic anhydride (C₄H₄O₃) and citraconic anhydride also called 2-methyl maleic anhydride (C₅H₄O₃) owing to its chemical structure are both cyclic anhydrides of aliphatic carboxylic acids which can be used for the modification of enzymes to achieve stabilization.

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achieve enzyme stabilization [17]. Both anhydrides modify the protein molecule by donating a carboxylic group to replace the positively charged amino group of a lysine molecule resulting to a change in the nature of charge on the enzyme surface but no alteration in the number of charged groups occurs [18]. The reaction with the amino group of the lysine residue leads to the formation of amide linkages which are labile and can be rapidly hydrolyzed at low pH of 3–4 to release the amine [19].

Chemical modification is a proven method for the modification of enzymes; however, reports indicate varying effects on enzyme pH and temperature activity profile, substrate specificity, and even thermo-stabilization depending on the type, chemical groups and concentration of modifier, nature of enzyme, environmental condition for enzyme modification amongst other factors [20, 21, 22, 23]. Also it is very important that enzyme is stabilized using a modifier, there is no huge loss to enzyme activity. In this study, we compared the effect of acylation using either maleic anhydride or citraconic anhydride (2-methylmaleic anhydride) on the properties of raw starch saccharifying amylase from Aspergillus carbonarius including the number of molecules of lysine residues modified, and the effects on the activity and stability of the saccharifying amylase.

2. Materials and methods

2.1. Materials

Raw potato starch was prepared in our laboratory according to a method earlier outlined [24]. All chemicals were purchased from Wako pure chemicals, Japan. Raw starch digesting amylase (RSDA, 43 U/mg) was obtained from Aspergillus carbonarius (Bainier) Thom IMI 366159 [25].

2.2. Modification of RSDA with acid anhydrides

The method described by Nwagu et al. [23] was used. The protein was used at 6 mg/ml concentration in 5 ml of 100 mM borate buffer (pH 8.0) and the process was followed at room temperature by step-wise addition of different concentrations of the acid anhydrides (citraconic anhydride [μg] and maleic anhydride [mg]) while maintaining the pH of the stirred solution at 8.0 by the addition of 2 M NaOH throughout the reaction. Aliquots of the suspension were collected at intervals. The reaction mixture was allowed to stand for 1 h at room temperature after which the sample was dialyzed extensively with 0.2 M phosphate buffer pH 6.0.

2.3. Enzyme assay

RSDA activity was assayed using a reaction mixture containing 0.2 ml of 1% raw potato starch in 0.2 M citrate-phosphate buffer, pH 5.0. The kinetic determination of enzyme activity was assayed using a reaction mixture containing 0.2 ml of 1% raw potato starch solution for 20 min at 40 °C, incubated with 1% raw potato starch solution for 20 min at 40 °C. The kinetic parameters, enzyme activity was assayed in reaction mixtures containing different concentrations (0.2–1 mg ml⁻¹) of raw potato starch in 0.2 M citrate-phosphate buffer, pH 5.0. The kinetic constants (Km/Vmax) were estimated using Lineweaver-Burk plot.

2.4. Amino acid analysis

The enzyme preparation was hydrolyzed with 6 M HCl at 110 °C under nitrogen. Triplicate samples were collected after 24 h, 48 h or 72 h. Re-drying agent (methanol:triethylamine:water = 2:1:1) was used and neutralized samples were dried under vacuum. Analysis of the samples was done using an AminoTac JLC-500/V automated amino acid analyzer according to the manufacturer’s instructions.

Determination of modified lysine groups was carried out with trinitrobenzene sulphuric acid (TNBS) according to the method reported by Fields [27]. A blank which did not contain the enzyme solution was used as control.

2.5. Properties of the modified and non-modified RSDA

2.5.1. Fluorescence spectra

Fluorescence measurements were carried out using a LS 55 spectrofluorimeter. The intrinsic tryptophan fluorescence on excitation at 295 nm was recorded for emission from 320-550 nm. The slit widths for both the excitation and the emission monochromators were set at 10 nm, the scan speed at 540 nm/min and the resolution at 10 nm. RSDA concentration was approximately 15 μg/ml in 0.2 M phosphate buffer pH 5.

2.5.2. Effect of pH on RSDA stability

The pH stability of the soluble and modified RSDA was studied by storing the enzyme in buffers of pH ranging from 3.5-7.0 using 0.2 M citrate-phosphate buffer and at pH 8.0–9.0 using 0.1 M Tris/HCl buffer 2 h at 10 °C. RSDA assay was carried out at 40 °C using the DNS method as earlier stated.

2.5.3. Effect of temperature on RSDA activity

The effect of temperature on the activity of the modified enzymes was determined by incubation of the enzyme/starch preparations in 0.2 M citrate-phosphate buffer of optimum pH at temperatures ranging from 30-80 °C for 20 min, followed by the determination of raw starch digesting amylase activity using the DNS method.

2.5.4. Kinetics of the RSDA

Thermoinactivation kinetics was determined by heating of native and modified RSDA samples in 0.2 M citrate-phosphate buffer of optimum pH at various temperatures (60, 70 and 80 °C) in the absence of substrate. Samples were withdrawn at different time intervals, promptly cooled on ice, incubated with 1% raw potato starch solution for 20 min at 40 °C.

The inactivation rate constants (k), half-life (t1/2) and decimal inactivation rate constants (Kd) were determined by the expression:

\[ t_{1/2} = \frac{(\ln 2)_d}{k} \]  \hspace{1cm} (2)

\[ \text{Eq. (3), of Espachs-Barroso and co-workers [28] was used to determine} \]

D-values at all temperatures for the native and modified raw starch saccharifying amylase.

\[ D = \frac{(\ln 10)_d}{K_d} \]  \hspace{1cm} (3)

The activation energy for the irreversible activation Ea(d) was determined through the algebraic method using the rate constant from two different temperatures

\[ \ln K_d = \frac{E_a(d)}{RT_1} + \ln A \]  \hspace{1cm} (4)

\[ \ln K_d = \frac{E_a(d)}{RT_2} + \ln A \]  \hspace{1cm} (5)

Taking the difference of the two equations

\[ \ln K_d = \frac{E_a(d)}{RT_2} - \frac{E_a(d)}{RT_1} = \frac{E_a(d)}{R} \ln \left( \frac{T_2}{T_1} \right) \]

\[ E_a(d) = \frac{R \ln \left( \frac{T_2}{T_1} \right)}{\left( \frac{1}{T_1} - \frac{1}{T_2} \right)} \]  \hspace{1cm} (6)

where T is the absolute temperature, \( R \) is the gas constant, A is the frequency factor and Kd is the decimal inactivation rate constant.
The degree of modification was observed to be inversely proportional to the activity retention of the raw starch saccharifying enzyme. While some authors have noted the loss of enzyme activity following chemical modification with anhydrides [14, 32], others reported increased activity [17, 33, 34]. A decrease in the amylase activity following modification is attributed to a few factors including the molecular size of the modifier or the reduced accessibility of substrate to the active site due to conformational changes after modification [34]. In the current case, another factor is the possible distortion of any of the two binding sites in the starch binding domain (SBD) of the raw starch saccharifying amylases. SBD is required for the interaction between the raw starch molecules and the saccharifying amylase, and also the onward movement of the substrate to the catalytic domain for product formation [35, 36, 37]. Enhanced substrate binding improves starch absorptivity of raw starch digesting enzymes and facilitates increased hydrolytic activity [11]. Moreover, variation in the location of the lysine residues attacked by the carboxylic group of the anhydride could bring about differences in the enzyme activity.

It is expected that the lysine groups which are located on the enzyme surface and therefore are more easily accessible will be the first targets for acylation. It is worthy to note that a higher quantity of MA was required to achieve the same percentage of modification compared to CA. However, increasing the concentration of MA to achieve such a level of modification as seen in the CA modified derivative led to a higher loss in the enzyme activity. Isumaya et al. [38] also observed a correlation between the degree of modification and enzyme activity. Chemical modification of raw starch digesting amylase from *Saccharomycopsis fibuligera* using acid anhydride led to 90–95% modification, however, the specific activity of the modified enzyme was reduced by 60%.

According to Xue [32], lower residual activity was observed for the derivative obtained using the higher molecular weight (MW) modifier. In the present study, CA with a molecular weight of 112.08 g/ml has a higher MW than MA with a molecular weight of 98.06 g/ml. Though the CA was measured in μl and MA in mg, in terms of activity loss and modification degree, these units had a similar impact on the enzyme in question. It was observed that the activity retention was 75% when 15 µg was used and 72% when 15 mg MA was used; with a further increase of modifier concentration to 20 µg or 20 mg as the case may be, activity retention dropped to 75% for CA modified (CAM) and 68% for MA modified (MAM). In this case, the CAM had higher enzyme activity retention, even with a higher molecular weight. The amylase activity loss was, therefore, observed to be more a factor of the concentration of the modifier used, than the molecular weight of the modifier.

After the modification, preliminary studies were carried out to determine the best enzyme derivative in each category (CAM & MAM), in terms of enzyme thermostabilization (results not shown). The enzyme samples modified with 10 µl citraconic anhydride and 20 mg maleic anhydride exhibited the best potential. These were applied for further studies.

From the above results, it can be deduced that the modification of approximately 10 lysine residues with citraconic and maleic anhydrides (an equivalence of 52–54% of the total lysine residues of the saccharifying amylase), was required for the stability of the enzyme. The similar

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### Table 1. Modification of the raw starch saccharifying amylase using acid anhydrides.

| Acid Anhydride | Citraconic (µl) | Maleic (mg) |
|---------------|----------------|-------------|
|               | 10            | 15          | 20          | 10          | 15          | 20          |
| Modification (%) | 54.1 ± 0.3    | 64.6 ± 0.7  | 82.4 ± 1.0  | 27.5 ± 0.2  | 47.9 ± 1.1  | 52.2 ± 0.6  |
| Lysine residues modified | 10          | 12          | 16          | 5           | 9           | 10          |
| Activity Retention (%) | 81.9 ± 1.9    | 76.3 ± 0.9  | 75.2 ± 1.2  | 94.0 ± 3.7  | 72.1 ± 2.8  | 67.9 ± 1.5  |

Significant difference between based on the concentration of anhydride (P < 0.05).

$$E_{a(d)} = RT_sT_2/(T_2-T_1) \ln K_d/{K_d}$$ (6)

Thermodynamics of irreversible inactivation of the native and modified RSDA was determined using the Eyring's absolute rate equation [29] as reported by Riaz et al. [30].

$$K_d = (k_bT / h)e^{-(\Delta H^*/R)}e^{(\Delta S^*/R)}$$

To calculate the enthalpy ($\Delta H^*$), Gibbs free energy ($\Delta G^*$), and entropy of enzyme inactivation ($\Delta S^*$) of irreversible inactivation were calculated by applying Eqs. (7), (8), and (9) below

$$\Delta H^* = E_{a(d)} - RT$$ (7)

$$\Delta G^* = -RT \ln ((k_bh)/(kbT))$$ (8)

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T$$ (9)

Where $k_b = (6.6262 \times 10^{-34} \text{J.s}^{-1})$ is the Planck's constant, $k_b = (1.3806 \times 10^{-23} \text{J/K})$ is the Boltzmann's constant.

### 2.5.6. Effect of ethylenediamine tetracetic acid (EDTA) on enzyme activity

To determine the effect of EDTA on the amylase activity, 0.2 M RSDA was stored for 2 h at 10 °C in 0.1 ml of various concentrations of EDTA. After storage, 0.2 ml 1% starch solution prepared in 0.2 M citrate phosphate buffer pH 5 was added to the mixture and incubated for 20 min. Subsequently, the amylase activity was determined as earlier described.

All experiments were done in triplicates and results represent mean values with less than 5.0 % error.

### 3. Results and discussion

#### 3.1. Modification and catalytic activity of the native and modified amyloses

Research has shown that acid anhydrides are protein modifiers and the rate or effectiveness of modification depends on the nature of the enzyme and the concentration of the modifier [31]. To determine the right concentration of citraconic anhydride (CA) and maleic anhydride (MA) required for the chemical modification of the raw starch saccharifying amylase, different concentrations of the acid anhydrides were applied to equal amounts of the enzyme. Table 1 shows that the rate of modification was influenced by the type of modifier used and its concentration.

Using 10, 15, and 20 µl CA led to 54%, 65%, and 82% modification degree while 10, 15, 20 mg of MA led to 28%, 48%, and 52% modification degree, respectively. We observed that CA led to the modification of more lysine residues compared to MA. The lowest concentration of CA used led to the modification of approximately 10 lysine residues, while that of MA only modified 5 lysine residues. The highest number of lysine residues were modified using 20 µl CA (16 residues) and 20 mg MA (10 residues). This is an indication that the higher the concentration of the modifier, the higher the modification degree and therefore the greater the number of lysine residues modified. A similar trend was earlier reported by Xue et al. [32] while chemically modifying bromelain with poly(maleic) anhydride.
number of residues required by both modifiers may be due to the similarity in the chemical structure of the modifiers used (see Scheme 1).

3.2. Intrinsic fluorescence spectra

A fluorescence emission spectrum is a dependable tool for the evaluation of conformational changes in protein. It is based on the fluorescence energy transfer between the tryptophan molecule present in the enzyme-polymer to the heme present in the enzyme-polymer. In the current experiment, a blue shift in the fluorescence spectrum was observed (Figure 1). From the figure, it was obvious that the modification using both citraconic and maleic anhydrides led to a slight reduction in the intensity of the emission from the tryptophan spectra (λmax). Similarly, a reduction in λmax was observed after the chemical modification of papain with dicarboxylic anhydrides maleic and citraconic anhydrides [17]. Modifying the enzyme using acid anhydrides such as citraconic and maleic anhydrides leads to an alteration of the positive charges on lysine residues with negatively charged carboxyl groups [39]. According to Eaton et al. [40], anhydrides have non-polar groups which help to strengthen the hydrophobic interaction amongst protein residues, therefore improving the packing of the protein hydrophobic core, leading to further burial of the tryptophan residue. Therefore, the addition of the bulky groups to the lysine surface may have enhanced the compactness of the enzyme, while further masking the tryptophan, thereby preventing its exposure to the solvent [22]. This will explain the tryptophan emission at a shorter wavelength. However, λmax was observed to be lower for MA compared to CA. It is not clear why this is so.

3.3. pH and temperature profile of the modified and non-modified amylase

Acylation is a simple inexpensive but practical method that can be employed to enhance the resistance of industrial enzymes especially hydrolases against inactivating agents including very extreme pH and temperatures, enzyme inhibitors and metal chelators [33]. The modification of amylase with citraconic anhydride and maleic anhydride had varying effects on the optimum pH for the activity of the saccharifying amylase. The optimum pH of the amylase changed from pH 5 to the alkaline pH 8 when citraconic anhydride was used but decreased to pH 4 with the use of maleic anhydride. Recall that during acylation, the negatively charged carboxyl groups replace the positive charges on the lysine groups of the enzyme protein [33]; therefore, the shift of the optimum pH to the alkaline side after citraconylation may be an indication of the negatively charged carboxyl groups introduced. The positive shift in pH is expected if these groups create a net overall anionic charge in the enzyme protein. Earlier research showed that acylation of the raw starch saccharifying amylase using phthalic anhydride altered the pH from 5 to 7 [23]. The drop in optimum pH for the MA was unexpected, considering that it functions in a manner similar to CA even though it has a lower molecular weight. However, Ismaya et al. [38] reported that 90–95% modification of amino acid residues on the amylase of *Saccharomyces fibuligera* did not lead to a change in optimum pH. The optimum pH for the activity of mesophilic amylase from *Bacillus amyloliquefaciens* and thermophilic amylase from *B. licheniformis* was unaltered following chemical modification with citraconic anhydride [41]. Other factors in the enzyme environment may also influence its optimum pH after modification and these include the surface charges on the modifying agent as well as the condition of the immediate environment of the enzyme. It is possible that the slight difference in the structure of the anhydrides and the variation in the exact lysine molecules which they modified, especially as regards their locations on the enzyme structure may have partly attributed to it. Yandri et al. [42] reported that the modification of α-amylase using varying concentrations of citraconic anhydride led to a decrease of 0.5 units in the optimum pH.

Modification with citraconic and maleic anhydrides did not improve the acid stability of the amylase. However, increased stability of the enzyme in alkaline pH range was observed (Figure 2). When stored at pH 10, the native enzyme retained about 56% residual activity while CAM had above 93% of its residual activity. According to available literature [33], linkages established by the carboxylic anhydrides are stable at neutral to alkaline pH and are hydrolyzed at a faster rate under acidic condition of pH of pH 3–4. Therefore, the instability at acidic pH of 3 may be due to deacylation or reduction in hydrophilicity of the carboxyl group as a result of its protonation. A reduction in hydrophilicity will restore the initial character of the enzyme protein surface even though deacylation may not occur [14].

There was no change in the optimum temperature (30 °C) of the amylase following immobilization (Figure 3). Similarly, acylation did not lead to a change in the optimum temperature for stem bromelain activity but increased the activity at higher temperatures [32]. The slight change in the optimum temperature for enzyme activity and the increased
resistance to higher temperatures, especially as regards the CAM may be due to enzyme rigidi-

fication. Enzyme rigidi-

fication could be due to physical, hydrophobic or covalent interactions between the enzyme active groups and the carboxylic anhydrides. Modification with dicar-

boxylic anhydrides leads to hydrophilization by the introduction of one negatively charged carboxylic group to each lysine residue modified. This replaces one positively charged amino group leading to a decrease in hydrophobicity, corresponding increase in hydrophilicity and electro-

static repulsion. The current work suggests that slight changes in enzyme hydrophobicity can lead to improved stability of the enzyme protein.

3.4. Kinetics of the modified and non-modified enzyme

Figure 4 shows the Lineweaver Burk plot of the native and modified enzymes. The modification of the amylase with citraconic anhydride led to a reduction in the Km while modification with maleic anhydride led to an increase in Km. Reduction in Km of the amylase indicates positive conformational changes leading to increased affinity of the enzyme for its substrate which in this case is raw potato starch. There was no corre-

sponding increase in Vmax even though the Km decreased after modification with CA, meaning that there was a favorable change in the substrate binding site of the CA modified enzyme where the enzyme substrate (E-S) complex is formed. However, a slight increase in Vmax was recorded for MAM compared to the native enzyme (Table 2). This probably implies that the catalytic site of the enzyme was affected by modification of the lysine groups/amino groups present or by the con-

figurational changes in the enzyme structure owing to the modifica-

tion, in such a manner to increase the rate of conversion of the substrate to product.

Kcat regarded as the turnover time stands for the number of times a molecule of the substrate is converted to product by an individual enzyme site per second. The highest Kcat was observed for the native enzyme, followed by MAM. About 22.1% reduction in Kcat (compared to the native enzyme) was observed for the CAM. The CAM and MAM were observed to have similar speci-

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city constant (Kcat/km), approximately 3.0 units higher than the non-modified amylase. From Table 2, it appears that the native amylase has better catalytic ef-

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ciency (Kcat/Km) compared to the CAM and MAM. However, reports have shown that the use of Kcat/Km, to evaluate catalytic efficiency is often misleading, and does not hold when the enzyme operates under high substrate condition which is often obtainable under industrial conditions where the enzymes are applied for biotechnological purposes [43]. Liu and co-workers [20] while evaluating the kinetic constants of horseradish peroxidase treatment on the dyes, bromophenol blue and methyl orange reported that citraconic anhydride-modified horseradish peroxidase had a greater af-

finity and catalytic efficiency compared to the native enzyme.

3.5. Stability and thermo-inactivation kinetics of the modified and non-

modified enzyme

To evaluate the kinetics of the native and modified amylase, thermoinactivation studies were conducted at varying temperatures as shown in Figure 5(a-c). Inactivation of the enzyme followed the first-order
kinetics as can be seen in the figures. The inactivation rate constant, the enzyme half-life and D-value of the enzyme when incubated at 60, 70 and 80 °C were calculated as shown in Table 3. The half-lives (h) of the native enzyme at 60, 70 and 80 °C were 8.77, 8.35 and 5.92 respectively; for the CA modified they were 69.3, 30.1 and 14.74 while for MAM the half-lives were 33.0, 16.9, 11.8, respectively. The above results show that the half-life of the enzyme which is a factor of the enzyme stability, in this case under high temperatures, was remarkably increased following the modification using CA and MA. Modification of lipase with ficollic, benzoic anhydride and polyethylene glycol improved the half-lives of thermal inactivation for all modified variants from 40 to 166% at 50, 60 and 70 °C relative to unmodified lipases [13]. More dramatic increase in half-life was observed in the current experiment for both CA and MA modified amylase enzymes. Using citraconic anhydride and trimellitic anhydride for the modification of horseradish peroxidase (HRP), Hassani [22] observed that at a lower temperature (50–60 °C), the enzyme derivative of the higher molecular weight compound trimellitic anhydride was more stable than at higher temperatures (70–80 °C). At higher temperatures, the citraconic anhydride HRP derivative was more stable. The authors suggested that the effect of hydrophilization on HRP stability was a factor of the temperature. In the current study, CAM which has the higher MW had a more stabilizing effect on the raw starch saccharifying amylase investigated compared to the MAM at all the temperatures investigated. The D-value here connotes the time required to lose 90% of the enzyme activity at the investigated temperatures of 60, 70 and 80 °C. Modification with CA led to an approximately 800% increase in the D-value of the native enzyme at 60 °C. However, the D-value for MA modified was only 109.65, an approximately 400% increase in the initial D-value of the enzyme (native enzyme). At 80 °C, there was a reduction in the D-value of all enzyme samples; D-value for the CAM and MAM were 248.9% and 188.7% higher than the native enzyme, respectively. From the above results, it is evident that positive conformational changes have occurred which resulted in a more rigid and stable enzyme structure. Enzyme thermostability of the modified amylase can be attributed to attractive electrostatic interactions between the surface charges on the enzyme molecule leading to a compactness of the enzyme structure [22].

### Table 2. Kinetic constants of native and modified amylase.

| Enzyme | Vmax (mol/min/mL) | Kcat (1000/sec) | Km (mg/ml) | Kcat/Km (1000 mM/s) |
|--------|-------------------|-----------------|-----------|---------------------|
| Native | 33.75             | 8.27            | 0.34      | 24.32               |
| CAA    | 26.32             | 6.45            | 0.29      | 21.94               |
| MAA    | 34.48             | 8.45            | 0.39      | 21.95               |

Significant difference between values of Vmax, Kcat and Km (P < 0.05).

The Ead (activation energy for thermal inactivation) of the CAM was 63.9 kJ mol⁻¹, approximately 2 fold that of the native enzyme (32.8 kJ mol⁻¹); however, the Ead for MA modified derivative was 37.0 kJ mol⁻¹, showing only 4.2 kJ mol⁻¹ increase in Ead (Table 4). A slight increase in Gibb’s free energy was observed after the modification of the amylase, but a higher Gibb’s free energy was noted for CAM (95.375 kJ mol⁻¹) compared to MAM (93.64 kJ mol⁻¹). A higher enthalpy change was observed for CAM (61.01 kJ mol⁻¹) compared to 34.09 kJ mol⁻¹ noted for MAM and 29.9 kJ mol⁻¹ obtained for the native amylase. The increase in enthalpy and Gibb’s free energies is an indication that higher free energies will be required before MAM and CAM can be spontaneously inactivated [48]. In the current study, the stabilization could be attributed to a slight decrease in hydrophobicity and an increase in hydrophilicity, electrostatic repulsion, and also hydrogen bond formation leading to better enzyme conformation and rigidification of the enzyme structure. The rigidification of the enzyme protein often leads to enzyme stabilization by reducing the rate constant at which the saccharifying amylase is inactivated.

Negative entropy values were obtained for all samples including the native and modified amylase, but only a slight entropy change occurred (from an initial value of −179.7 J mol⁻¹ K⁻¹ to 176. J mol⁻¹ K⁻¹) following modification with citraconic anhydride. A rapid drop in entropy (−94.68 J mol⁻¹ K⁻¹) was observed following maleylation of the saccharifying amylase. The entropy value provides information on the compactness of an enzyme protein; reduction in entropy observed during the current study shows that the enzyme is less disordered and better packed. This is expected due to improved opportunities for hydrogen bond formation which would have occurred based on the groups introduced by the modifer [41, 45] (see Table 4).

#### 3.7. Effect of EDTA on modified and non-modified amylase

Figure 6 shows the effect of increasing concentration of the inhibitor, ethylene diamine tetraacetic acid (EDTA) on the activity of the modified and non-modified raw starch saccharifying amylase. The native and modified enzymes lost their saccharifying activities with increasing concentrations of the inhibiting agent. CAM was most stable and retained about 58% of its saccharifying activity after incubation in 1% EDTA. MAM, on the other hand, retained less than 20% of its activity while the native non-modified amylase lost its saccharifying activity following exposure to 1% EDTA for 2h.

The results obtained from the effect of EDTA on the saccharifying amylase further shows that CAM was the most stable of the two derivatives. Both of the anhydrides are dicarboxylic with the methyl group on citraconic anhydride being the only difference between the two. It may be possible that the methyl group introduced during citraconylation provides additional chances for hydrogen bond formation. Though methyl groups are weakly acidic and are therefore considered weak proton donors for hydrogen bonds to occur, they are capable of doing so under the right conditions [46]. Hydrogen bonds have immense stabilizing effects on the enzyme molecule; according to available literature, each hydrogen bond donates about 0.5–1.8 kcal mol⁻1 energy to the binding energy of proteins with the exact energy gain depending on its distance and geometry [47, 48].

From this result, it is obvious that these anhydrides especially citraconic anhydride can be used for the stabilization of this enzyme. Irrespective of the similarities in the structures, both anhydrides produced enzymes of similar but distinct properties. The reason for this is not very clear, however contrary to earlier notions, the hydrogen in methyl group when under the right orientation can serve as a proton donor, facilitating the formation of an additional hydrogen bond. The hydrogen bond is proven to contribute remarkably to the stability of the enzyme structure.

The increased stability and improved half-life of the modified raw starch saccharifying amylase of A. carbonarius increase its potential for use in starch saccharifying industries as well as other processes where amylases are required. Increased stability of the amylase affords greater flexibility during processing. One of the downsides of utilizing raw starch digesting amylases is that the operations are carried out at a relatively low temperature which makes contamination of the process easier. The modified enzyme can be used in a continuous process of starch saccharification at varying temperatures depending on the gelatinization temperature of the grain/tuber starch used in a given process (operating...
温度应该低于凝胶化温度。凝胶化和糖化阶段淀粉的运动速度的巨大差异使得连续淀粉糖化过程的设计非常困难 [49]。在这种情况下，应用稳定生淀粉消化酶有望节能并简化加工过程，尤其是因为α-淀粉酶在广泛范围的谷物和块茎淀粉中有效地水解之 [50]。在碱性pH和对螯合剂EDTA的稳定性表明，CAM修饰的α-淀粉酶在工业上具有重要意义。

图5. 温度对原位、草酰乙酸修饰和maleic酸修饰生淀粉糖化酶的影响。第一阶反应，(A) 60 °C，(B) 70 °C 和 (C) 80 °C。

在这一温度下，应用α-淀粉酶有望节能并简化加工过程。因为它在多种基质中有效，特别是在谷物和块茎淀粉中。特别是在碱性pH和对螯合剂EDTA的稳定性表明，CAM修饰的α-淀粉酶在工业上具有重要意义。
also has potential for use in detergent, pulp and paper industries as well as textile industries.

4. Conclusion

The advent of more modern techniques in genetic and protein engineering notwithstanding, the use of chemical modification to achieve enzyme stability remains very appealing due to its simplicity, reliability, unlimited use, and cost-effectiveness. Starch hydrolysis in industries remains expensive due to time, efforts and energy needed for gelatinization and cooling before saccharification of the starch molecules. Alkaline stable amylases are relevant in the paper, textile and detergent industries.

Acylation using citraconic anhydride led to a more alkaline and thermostable enzyme derivative of the raw starch saccharifying amylase from A. carbonarius. Though both processes (citraconylation and maleylation) improved the stability of the saccharifying amylase, citraconylation had a more pronounced effect evident from thermoinactivation studies at high temperatures. It is evident from the current study that though chemical modification is a welcome step towards the stabilization of raw starch digesting enzymes, there is the need for a prior study of the appropriate modifiers and concentration, necessary to achieve the desired yield. Our studies also indicate that citraconylation can be used to tailor the optimum pH of this enzyme, meaning that digestion of starch at ambient temperature is possible at a wider pH range. The resistance of CAM to EDTA an enzyme inhibitor also underlines its potential in many biindustrial processes which involve starch hydrolysis.

Table 3. Kinetic parameters of thermal inactivation of native and modified RSDA.

| Temp (°C) | Enzyme  | K (h⁻¹) | t ½ (h⁻¹) | D value |
|----------|---------|---------|-----------|---------|
| 60       | NA      | 0.08    | 8.77      | 29.14   |
|          | CAM     | 0.01    | 69.3      | 230.26  |
|          | MAM     | 0.02    | 33        | 109.65  |
| 70       | NA      | 0.08    | 8.35      | 27.74   |
|          | CAM     | 0.02    | 30.13     | 100.11  |
|          | MAM     | 0.04    | 16.90     | 56.16   |
| 80       | NA      | 0.12    | 5.92      | 19.68   |
|          | CAM     | 0.05    | 14.74     | 48.99   |
|          | MAM     | 0.06    | 11.18     | 37.14   |

NA: native amylase; CAM: citraconic anhydride modified; MAM: Maleic anhydride modified. No significant difference between levels (P > 0.05).

Table 4. Thermodynamic properties of thermal inactivation of native and modified RSDA.

| Parameter | NA       | CAM      | MAM      |
|-----------|----------|----------|----------|
| Eₜₐₜ (kJ mol⁻¹) | 32.8    | 63.91    | 36.98    |
| G (kJ mol⁻¹)       | 92.0    | 95.38    | 93.96    |
| H (kJ mol⁻¹)       | 29.9    | 34.09    | 61.01    |
| S (J mol⁻¹ K⁻¹)    | -179.7  | -176.11  | -94.68   |

NA: native amylase; CAM: citraconic anhydride modified; MAM: Maleic anhydride modified. No significant difference between levels (P > 0.05).

Figure 6. Effect of varying concentration of EDTA on native (NE), citraconic anhydride modified (CA) and maleic anhydride modified (MA) raw starch saccharifying amylase.

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| Temp (°C) | Enzyme  | K (h⁻¹) | t ½ (h⁻¹) | D value |
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Acylation using citraconic anhydride led to a more alkaline and thermostable enzyme derivative of the raw starch saccharifying amylase from A. carbonarius. Though both processes (citraconylation and maleylation) improved the stability of the saccharifying amylase, citraconylation had a more pronounced effect evident from thermoinactivation studies at high temperatures. It is evident from the current study that though chemical modification is a welcome step towards the stabilization of raw starch digesting enzymes, there is the need for a prior study of the appropriate modifiers and concentration, necessary to achieve the desired yield. Our studies also indicate that citraconylation can be used to tailor the optimum pH of this enzyme, meaning that digestion of starch at ambient temperature is possible at a wider pH range. The resistance of CAM to EDTA an enzyme inhibitor also underlines its potential in many biindustrial processes which involve starch hydrolysis.

Declarations

Author contribution statement

Tochukwu N. Nwagu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hideki Aoyagi, Shigeki Yoshida: Contributed reagents, materials, analysis tools or data.

Bartholomew Okolo: Conceived and designed the experiments; Wrote the paper.

Anene Moneke: Analyzed and interpreted the data.

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Additional information

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