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Possible Functional Roles of Exposed Sulfhydryl Groups in Malted and Unmalted African Finger Millet Seed (*Eluesine coracana)* β-amylase

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Abstract: The behavior of exposed sulfhydryl groups of β-amylase from malted and unmalted African finger millet seed was investigated for their possible functional roles by chemical modification studies using 5,5'-dithiobis-(2-nitro-benzoic acid) (DTNB). The malted and unmalted enzyme purified by α-cyclodextrin Sepharose 6B affinity column chromatography was inhibited by the sulfhydryl reagent. The chemical modification study revealed that there were three sulfhydryl and four sulfhydryl groups in β-amylase from both unmalted and malted seed, respectively each having one exposed sulfhydryl group; performing different roles and were essential for catalytic activity. The result shows a distinction between the *in situ* β-amylase from unmalted seed and *de novo* β-amylase in malted seed synthesized during malting. The malted seed β-amylase was oxidatively dimerized by subjecting it to 0.3 M potassium ferricyanide in 3.0 M urea. The dimerized enzyme was inactive. The result from the DTNB chemical modification study of the exposed sulfhydryl group in the β-amylase purified from malted African finger millet seed before oxidation of the group shows that the sulfhydryl group is involved in the formation of dimers.

Key words: β-amylase, *Eluesine coracana*, African finger millet, sulfhydryl groups, malting

INTRODUCTION

β-amylase (EC 3.2.1.2 α-1,4,-D-glucan maltohydrolase), anomer-inverting starch hydrolyzing enzyme which, catalyses the liberation of β-maltose from the non-reducing end of α-1,4-glucans, leaving a β-limit dextrin, when degrading starch and amylopectin (Baba and Kainuma, 1987). Substrates for the enzyme include α- (1,4)-glucans e.g., amylose, amylopectin and limit dextrins with a minimal chain length of four glucose units (Lizotte *et al*., 1990).

In plants, β-amylase occurs in free and combined forms (Evans *et al*., 1997). It has been isolated and studied extensively in different plant species. β-amylase has previously been purified and characterized from sweet potatoes, soybean, barley, rye and pea (Brena *et al*., 1996). This enzyme together with other hydrolytic enzymes are said to be involved in the breakdown of starch during germination of seeds (Guglieminetti *et al*., 1995) and in the development of its embryo.

Five genes encoding β-amylase have been cloned and sequenced from both prokaryotes and eukaryotes (Ziegler, 1999). Three highly conserved sequences are recognized among them and are suggested to comprise the active site (Ziegler, 1999). β-amylases characterized to date are sensitive to various sulfhydryl modifying reagents and are considered to contain sulfhydryl group essential for the activity (Hyun and Zeikus, 1985). The reactivity of free thiol groups (sulfhydryl groups) makes them useful in stability, redox behaviour, metal binding, acidity, nucleophilicity and catalytic activity (Bardwell, 2005; Giles *et al*., 2003). Two cysteine residues form disulphide bond. The disulphide bond

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is useful in protein folding, protein stabilization and regulation of the activity. Reactive oxygen produced during normal metabolism can act to either destroy or regulate the function of cysteine-containing proteins (Bardwell, 2005).

African finger millet (*Eluesine coracana* L. Gaertener) seed displays good malting qualities and considerable industrial potentials (Nzelibe and Agbojo, 2005). The β-amylase activity of the seed is generally high and can serve as alternative for malting processes (Nzelibe and Agbojo, 2005). Determination of the thiol-disulphide status of the malted and unmalted African finger millet seed β-amylase would allow one to predict the number of thiol residues, the possible involvement of the thiol groups in the catalytic activity or a regulatory role and the possible susceptibility of the enzyme to oxidation.

MATERIALS AND METHODS

Materials

3,5 -dinitrosalicylic acid, sodium potassium tartarate, potassium ferricyanide and potato soluble starch were products of BDH Chemical Limited, Poole, England. Epoxyl activated Sepharose 6B was from Pharmacia Fine Chemicals, Uppsala, Sweden. α-Cyclodextrin was from Sigma Chemical Company Limited. St. Loius, Mo., USA. All other chemicals and reagents used were of analytical grade. African finger millet seed (*Eluesine coracana*) was obtained from a local market in Jos, Nigeria. The seed was botanically identified in Crop, Soil and Pest Department, Federal University of Technology, Akure. Nigeria.

Methods

Preparation of α-Cyclodextrin Sepharose 6B (Affinity Gel)

α-Cyclodextrin was immobilized on epoxyl activated Sepharose 6B according to the method of Vretbald (1974). Epoxyl activated Sepharose 6B (8.0 g) was swollen in distilled water and then transferred to 0.1 M NaOH. After removal of excess liquid by suction, the gel was reacted with 600 mg of α-cyclodextrin in 24 mL of 0.1 M NaOH with shaking at 45±3°C for 24 h. The immobilized α-cyclodextrin-was washed and then used.

Enzyme Extraction

Ground seed (100 g) was mixed and stirred in cold 300 mL 50 mM sodium acetate buffer pH 4.5 for 24 h. The homogenate was filtered using cheesecloth. The filtrate was centrifuged at 14000 g for 10 min with Automatic Superspeed centrifuge at 4°C. The supernatant was filtered through a loose plug of glass wool to remove the floating lipids. The filtrate served as the crude extract. Another 100 g of the seed was soaked in distilled water for 24 h. Excess water was drained and the seeds were allowed to malt for 4 days on moist paper and crude extract was got through the same process as described earlier.

Purification

The method of purification of Totsuka and Fukazawa (1993) was adopted. Crude extract (5.0 mL) was applied to a column (1.0×10 cm) packed with the affinity gel that had previously been equilibrated with 50 mM acetate buffer pH 4.5 containing 1.0 M ammonium sulphate. The concentrated extracts were earlier mixed with 1.0 M ammonium sulphate. The unadsorbed proteins were eluted. Fractions of 5.0 mL were collected at a flow rate of 10 mL h^{-1}. The bound proteins were eluted with the same eluting buffer containing no ammonium sulphate. The fractions containing β-amylase activity were pooled and stored at -5°C. Unless otherwise stated all purifications were carried out at 4°C in the cold Laboratory.

Enzyme Assay

β-amylase activity was measured as described by Oboh and Ajele (1997) using 3,5-dinitrosalicylic acid (DNSA) colour reagent. The appropriately diluted enzyme solution (0.5 mL) of protein concentration of 0.07 mg mL^{-1}) was added to 0.5 mL of 1 % (w/v) soluble starch dissolved in the appropriate buffer. Control consisted of 0.5 mL buffer plus 0.5 mL soluble starch solution. Reaction tubes were incubated at the appropriate temperature for 3 min. DNSA reagent (1.0 mL) was added to each tube and the tube was placed in a boiling water bath for 5 min. The tubes were cooled and 10 mL of distilled water was added and mixed and the absorbance was taken at 540 nm. The unit of β-amylase activity was defined as that amount of enzyme, which released one micromole of maltose from starch per minute at 25°C and pH 6.0. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of Freely Reactive and Buried Sulfhydryl Groups

Freely reactive and buried sulfhydryl groups was determined with Ellman's reagent, 5,5-dithiobis-(2-nitrobenzoate) (DTNB) as described by Creighton (1990) and Afolayan (1989). The buried sulfhydryl groups were unmasked with 6.0 M guanidine HCl. The number of reactive sulfhydryl groups was calculated using 13880 M^{-1} cm^{-1} as extinction coefficient of thionitrobenzoate in 6.0 M guanidine HCl and 13600 M^{-1} cm^{-1} as the extinction coefficient in aqueous solution at 412 nm. These spectrophotometric measurements were made using Cecil CE 395 double beam digital U.V. spectrophotometer.

The Oxidation and Reduction of the Active β-amylase Thiol Group

The purified and active β-amylase (protein concentration of 0.92 mg mL^{-1}) from malted African finger millet was oxidized with 0.3 M potassium ferricyanide in the presence of 3.0 M urea at pH 7.0 and 4°C for 90 h as described by Matsui *et al.* (1977) with slight modification. After dialysis of the reaction mixture, 100 µL was subjected to 10% Polyacrylamide gel electrophoresis (PAGE) as described by Weber and Osborn (1975). The electrophoresis was carried out in Tris-HCl buffer (pH 8.0) at 8 mA per tube for 7 h. R_m, which expresses the ratio of mobility of protein to bromophenol blue as marker was determined.

RESULTS

The number of sulfhydryl groups from the unmalted and malted seeds that were freely reactive with DTNB at pH 7.0 was estimated to be one per mole of the enzyme. The number of sulfhydryl groups in the completely unfolded β-amylase from the unmalted and malted seeds were three per molecule (two buried, one exposed) and four per molecule (three buried, one exposed), respectively. Chemical modification of the exposed sulfhydryl groups in β-amylase from unmalted seed with DTNB resulted in the loss of activity. So also, the activity was lost during unmasking of the enzyme buried sulfhydryl groups. In the β-amylase from malted seed the DTNB chemical modification resulted in the increase in the km from 2.81 to 4.14 mg mL^{-1}. It decreases the value of km Kcat^{-1} from 18.67 to 9.45 sec^{-1} mg^{-1} mL^{-1}).

The sulfhydryl group in β-amylase from malted African finger millet was subjected to oxidation using potassium ferricyanide in the presence of 3 M urea. The Rm values were 0.18, 0.08 and 0.19 for rods 1, 2 and 3, respectively. The oxidation allowed the enzymes to form a dimer based on the level of migration in non SDS-PAGE as shown in Fig. 1. This oxidative dimerization leads to inactivation of the enzyme. The exposed sulfhydryl group was subjected to chemical modification using DTNB. Oxidative dimerization was allowed to occur. The migration rate was almost the same with the untreated β-amylase (lanes 1 and 3).

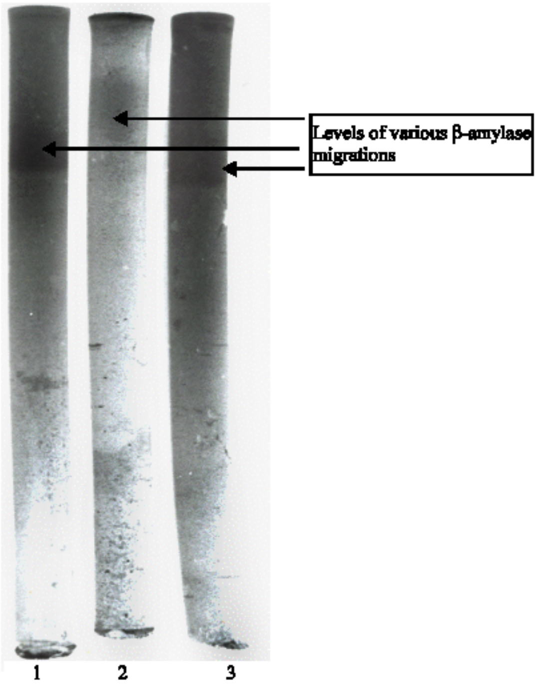

Fig. 1: The PAGE was performed in the absence of SDS on 10% gel using the Sodium phosphate buffer system according to the method Weber and Osborn (1969). The electrophoresis was carried out in Tris-HCl buffer (pH 8.0) ay 8 mA per tube. Coomassie Blue R-250 was used for staining. 1: native enzyme (untreated active β-amylase. 2: active β-amylase incubated with 0.3 M $K_3Fe(CN)_6$ in the presence of 3.0 M urea at pH 7.0 and 4°C for 90 h. 3: The exposed -SH group modified before oxidation

DISCUSSION

It is generally known that β-amylase from various origins are sulfhydryl enzymes (Matsui *et al*., 1977). The result from this study has shown that β-amylase from both malted and unmalted African finger millet seed is a sulfhydryl enzyme. It was inhibited by DTNB, a sulfhydryl reagent. The inactivation of the enzyme by sulfhydryl reagent is an indication that the cysteine residue might be indispensable for the β-amylase enzymatic reaction through redox behaviour or nucleophilic reaction. It has been posited that the essential sulfhydryl group in soybean β-amylase does not participate in the catalysis, but that it is situated near the binding site of maltose (Mikami *et al*., 1980). It might be possible with both malted and unmalted β-amylase from African finger millet seed. Whatever would affect the sulfhydryl residue affects the structural integrity of the β-amylase and ultimately the catalytic efficiency. The modification of sulfhydryl group causes a structural conformational change of β-amylase (Morita *et al*., 1976). The absolute inactivation of the exposed sulfhydryl group in the β-amylase from unmalted seed might indicate that the sulfhydryl group is involved in zymogen formation. The reduction of the β-amylase catalytic efficiency (km and Kcat km^{-1}) in the malted seed upon the modification of the exposed sulfhydryl group suggests its possible role in regulation. This exposed sulfhydryl group is therefore, very susceptible to oxidation.

The discrepancy in the number of the cysteine residues between the β-amylase from malted and unmalted African finger millet seed clearly shows the distinction between *in situ* β-amylase inherent in the unmalted seed and β-amylase from the malted seed. The difference in the number might indicate difference in structural conformation between β-amylase from malted and unmalted seeds. Daussant and Corrazier (1970) reported that the increase in β-amylase activity on germination is due to the activation of a latent form of the enzyme and that *de novo* synthesis of the enzyme does not occur. It has been generally accepted that the inactive latent form of β-amylase associated with protein molecules in ungerminated seeds is activated either enzymically or nonenzymically and free enzyme is subsequently released (Ziegler, 1999). The difference in the number of sulfhydryl residues from the β-amylases from malted (4) and unmalted (3) African finger millet seed is suggestive of the fact that there is *de novo* synthesis of the β-amylase during malting with a distinct change in conformation different from that of the β-amylase from unmalted African finger millet seed to meet their respective physiological role. It is plausible, also, to hypothesize that the bulky β-amylase molecules derived from the *in situ* β-amylase in the unmalted African finger millet becomes obscured with germination and malting that *de novo* synthesis becomes significant which is reflected in the malted African finger millet seed β-amylase.

Three and four sulfhydryl residues derived from β-amylase from unmalted and malted African finger millet seed respectively is in agreement with the three cysteine residues from *Bacillus cereus* β-amylase (Nomura *et al*., 1995), five from soybean β-amylase (Morita *et al*., 1976). The thiol of a cysteine residue occasionally acts as a ligand of metal ions (Tatara *et al*., 2005).The sulfhydryl residue of β-amylase in both malted and unmalted African finger millet seed might serve as a ligand for K^+, Na^+ and Ca^{2+} that generally enhance the activity and stability of the enzyme. Cysteine residues frequently form disulphide bonds. The formation of disulphide bonds plays a crucial role in the folding of proteins (Wedemeyer *et al*., 2000). The buried sulfhydryl residues of β-amylase in both malted and unmalted African millet seed are likely to be involved in formation of buried disulphide bridge and indispensable to the β-amylase catalytic activity-probably to maintain the correct conformation of the folded protein through correct hydrophobic packing of the enzyme. Buried and exposed sulfhydryl residues increase irreversible unfolding to form incorrect disulphide bond (Bonaccorsi *et al*., 2000). If it is applicable to African millet seed beta amylase, this is very likely with three buried sulfhydryl residues in beta amylase from malted seed but less likely with two buried sulfhydryl residues in unmalted seed.

The possible functional role of exposed sulfhydryl residues of β-amylase in malted African finger millet seed was further explored. The β-amylase was oxidatively dimerized by treatment with 0.3 M ferricyanide in 3.0 M urea. The dimer was inactive when checked for β-amylase catalytic activity. The enzyme must have lost its activity as a result of dimerization. It has been reported that the treatment of β-amylase from malt with either potassium ferricyanide or hydrogen peroxide in the presence of urea at pH 7.0 led to inactivation of the β-amylase (Matsui *et al*., 1977). Present result is not an exception. The dimerization appeared to be the result of formation of intermolecular disulphide bond. The question is during unfolding in 3 M urea in the presence of 0.3 M ferricyanide is it possible for the dimer to be formed with either buried or exposed sulfhydryl residues? The result has indicated that the exposed sulfhydryl residue is involved in the formation of dimer. It therefore shows that an exposed sulfhydryl residue is sensitive to oxidation and could account for unstable nature of β-amylase from malted African finger millet seed.

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