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Characterisation of alpha-amylase inhibitor from *Streptomyces xinghaiensis* AAI2 in solid substrate

Oladotun A. Fatoki[a,b,*], Anthony A. Onilude[b]

[a] *Department of Biology, The Polytechnic, Ibadan, Oyo State, Nigeria*
[b] *Department of Microbiology, University of Ibadan, Nigeria*

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ABSTRACT

Alpha-amylase inhibitors, which play metabolic roles in the organisms that produce them, by impeding the activity of alpha-amylase enzyme, have been isolated from many sources including plants and microorganisms. They vary in characteristics and applications based on their sources though there are characters that appear similar despite the source. Alpha-amylase inhibitor was produced by *Streptomyces xinghaiensis* AAI2 cultured on wheat bran substrate supplemented with a nutrient basal medium which was also used to adjust both the moisture content (73%) and initial pH (6.48) of the medium. The extract obtained was purified using standard purification techniques (ammonium sulphate precipitation, dialysis and column chromatography). Characteristics such as effects of pre-incubation and pH, total protein, specific activity, total activity, percentage yield, molecular weight and kinetics were determined. The alpha-amylase inhibitor produced was found to occur in isoforms having molecular weights of 14.2 and 56 kDa. Total protein, specific activity and percent yield obtained were 0.19 mg/mL, 745.11 AIU and 2.27% respectively. The optimum activity was obtained at pH 8.0 and in the presence of Cobalt and Calcium ions while pre-incubation for 10 min was best with no further improvement in the activity beyond this time. The inhibitor exerted a non-competitive type of inhibition.

Introduction

Enzyme inhibition occurs when there is competition with the enzyme substrate for the enzyme's active site and this leads to reduction in or total inhibition of the activity of the enzyme on the specific substrate. The binding of an inhibitor can stop a substrate from entering the enzyme's active site thereby hindering the enzyme from catalysing its reaction. Substances that inhibit enzymes, especially hydrolases are ubiquitous in nature and carry out their activity by forming complexes that block the active site or alter enzyme conformation, ultimately reducing the catalytic function. Aside their presence in many plant species, they are found in microorganisms and more abundantly in actinomycetes and other bacteria. These inhibitors as a group have been postulated to be glycoproteins [5]. Homology has been observed amongst alpha – amylase inhibitors from closely related organisms including *Streptomyces.* This was observed in the amino acid sequence

* Corresponding author at: Department of Biology, The Polytechnic, Ibadan, Oyo, Nigeria.
E-mail address: fatoki.oladotun@polyibadan.edu.ng (O.A. Fatoki).

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of alpha-amylase inhibitors from *Streptomyces aureofaciens* (AI-3688), *S. tendae* (HOE-467A) and *S. corchorushii* (Paim 1) [3]. Enzyme inhibitors play a metabolic function in the microorganisms where they are synthesised. In addition to this, they have found applications as therapeutic drugs, antibiotics, pesticides and more importantly as anti-diabetic substances. Specific enzyme inhibitors have been found to be biochemical tools useful in the analysis of biological functions and diseases. The preference of solid state fermentation in biosynthesis of important metabolites is as a result of obtaining them using minimum energy requirement, at optimal cost and with almost zero environmental pollution. Hence, there is a need to evaluate the characteristics of alpha-amylase inhibitor produced by solid state fermentation of bacteria, report for which is scarce.

Materials and methods

Fermentation and extraction

Soil-isolated *Streptomyces xinghaiensis* AAI-2 (Genbank accession number KY858944) was cultured in an inoculum medium. Wheat bran was air dried to constant weight while the initial pH and moisture content were adjusted to 6.48 and 73% using a basal medium: cornsteep liquor (0.4% v/v), glucose (1% w/v), $(NH_4)_2HPO_4$ (0.8% w/v), soy flour (0.4% w/v), peptone (1% w/v) at pH 8.3 [8]. The medium was inoculated with the isolate and incubated for 10 days. Ten parts of sodium phosphate buffer (20 mM, pH 6.9) was added to one part of the fermented substrate and extracted at refrigerated temperature as described by Panwar et al. [9].

Purification of alpha-amylase inhibitor

Crude extract of alpha-amylase inhibitor was purified by heating in a water bath at 70 °C for 2 min, ammonium sulphate precipitation to 30%, overnight dialysis against the extraction buffer at 4 °C and gel chromatography using Sephadex G-100. One millilitre (1 mL) fractions were taken by continuous elution with the equilibrating buffer and inhibitory activity was assayed for in the entire fraction collected as described by Bernfeld [2]. The fractions with inhibitory activity were pooled together.

Characterisation

Purified alpha-amylase inhibitor was added to commercial alpha-amylase, pre-incubated at 28±2 °C for varying durations (5, 10, 20 and 30 min). Assay for residual amylase activity was determined using the DNSA assay method [2]. The quantity of protein in the extract was determined with the aid of a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Wilmington, USA). Total protein (mg), total activity (Units), specific activity (Unit/mg), purification fold and percentage yield were determined.

Molecular weight determination

Molecular weight determination was carried out using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The marker was run along with the samples. The gel was thereafter stained in coomasie blue dye overnight. The gel was destained with distilled water until the protein bands are visualised. The protein bands obtained were compared with the markers [4].

Effect of metal ions

Solutions of metal ions (Ca^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Na^{2+}, Mg^{2+}, K^{+}, Zn^{2+} and Hg^{2+}) were prepared by dissolving weighted amounts of the requisite salts in distilled water to obtain the desired concentration (5 mM, 10 mM and 20 mM). The metal ion solutions were incubated at room temperature with an equal volume of the alpha-amylase inhibitor extract for 20 min [12]. Alpha-amylase was added to the reaction mixture and incubated for a further ten minutes. Soluble starch solution was added to this mixture and incubated for five minutes. The assay for residual alpha-amylase activity was carried out by DNSA assay described by Bernfeld [2].

Inhibition kinetic studies

The kinetic study was done by carrying out an assay as described earlier using varying starch concentration and varying inhibitor concentration. Three concentrations (0.048 mg/mL, 0.095 mg/mL and 0.190 mg/mL) of the purified inhibitor was prepared by diluting the purified alpha-amylase inhibitor in sodium phosphate buffer while five starch concentrations (mg/ml) were prepared (0.01, 0.02, 0.04, 0.08 and 0.10). The Michaelis-Menten curve and Lineweaver-Burke plot were constructed.

Results and discussion

In the different stages of purification of the extract shown in Table 1, a progressive decrease in the protein concentration was observed. The extract (lysate) obtained by centrifugation and application of heat (75 °C) had the highest protein concentration (13.033 mg/ml) while the least was obtained after the same extract had been purified to column chromatography

Table 1
Purification table of alpha amylase inhibitor.

Fraction	ID	Vol (mL)	Total Protein (mg)	Activity (AIU*)	Total activity	Specific activity (AIU/mg)	Purification fold	% Yield
Lysate	A	100	13.03	186.93	18,693	14.35	1.0	100
Protein Precipitate	B	32	2.35	56.99	1823.68	24.25	1.69	9.8
Dialysis	C	16	1.51	51.11	817.76	33.85	2.36	4.5
Sephadex G-100	D	3	0.19	141.57	424.71	744.58	51.89	2.3

* AIU – Amylase Inhibitor Units.

Table 2
Effect of pre-incubation time on activity of alpha amylase inhibitor from *S. xinghaiensis*.

Time (mins)	Activity (Units/min/ml)	Percentage Inhibition (%)
5	0.0384	73.52
10	0.0192	86.76
20	0.016	88.97
30	0.016	88.97

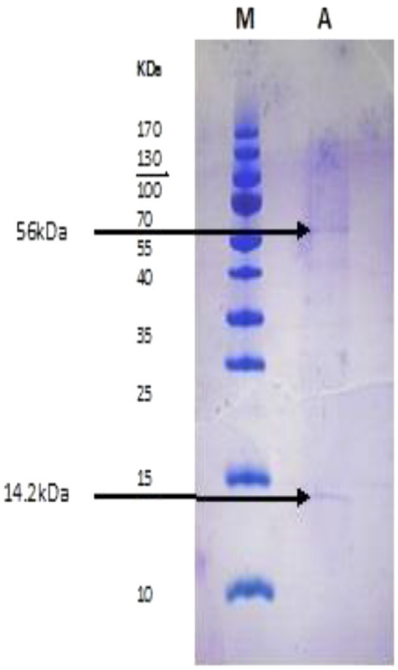

Fig. 1. Electrophoretogram of α-amylase inhibitor from *S. xinghaiensis* AAI2 **Key:**M – Markers; A – Alpha-amylase inhibitor.

(0.187 mg/ml). After protein precipitation, a decrease in the residual amylase activity which corresponds to an increase in the activity of the alpha-amylase inhibitor was observed. These parameters had an effect on the specific activity which was highest in the sample obtained after column chromatography was done. This same sample recorded the highest purification fold though with a percentage yield of only 2.27%.

Heat treatment at 75 °C for a microbial metabolite which has been reported to be peptide in nature did not lead to a complete loss of activity, rather there was improved inhibitory activity of the metabolite up to 56.08% though there was a decline in activity with progression of this treatment with time. This stability is usually a characteristic of metabolites produced by microorganisms through solid state fermentation [13]. Previous early researches on alpha amylase inhibitor have obtained similar results [6,8].

The effect of pre-incubation of alpha-amylase inhibitor with commercial alpha-amylase enzyme at 30 °C temperature is shown in Table 2. While the least inhibition (73.52%) was obtained at 5 min of pre-incubation, the inhibitory activity remained steady until 30 min.

The determination of molecular weight of the metabolite by SDS-PAGE yielded more than one protein band on the gel corresponding to 14.2 kDa and 56 kDa respectively when compared to markers (Fig. 1). This confirmed the existence of alpha amylase inhibitors in isoforms. The occurrence of alpha amylase inhibitor from culture filtrate of *Streptomyces tendae*

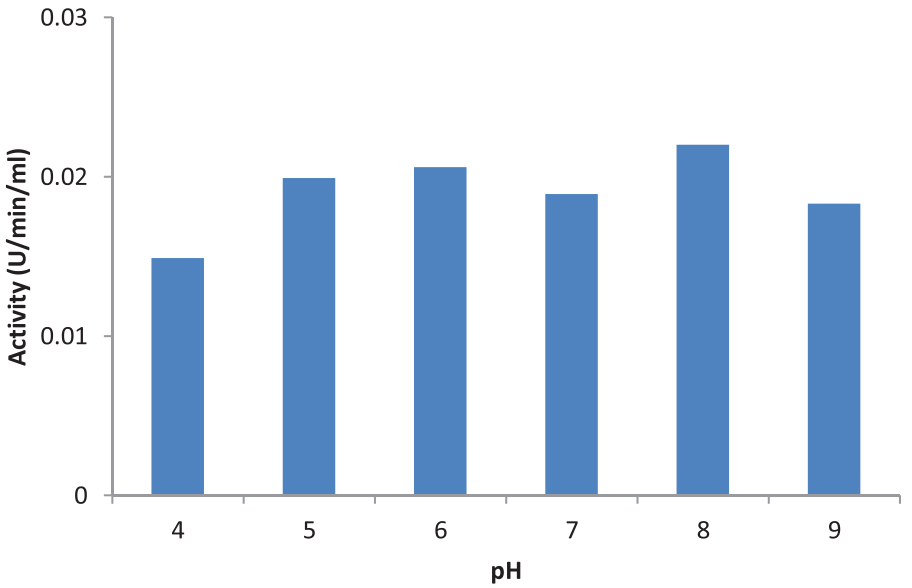

Fig. 2. Effect of pH on activity of alpha amylase inhibitor from *S. xinghaiensis*.

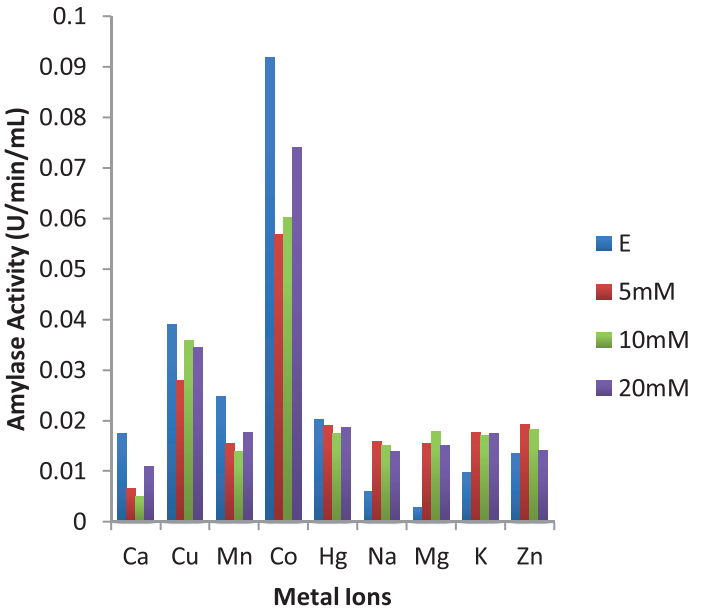

Fig. 3. Effect of metal ions on activity of alpha amylase inhibitor from *S. xinghaiensis* against commercial alpha-amylase.

[15] as well as *Albizia lebeck* [11] amongst others in isoforms, confirms that this phenomenon is a common occurrence in this metabolite.

Fig. 2 shows the effect of pH on the activity of alpha amylase inhibitor. Alpha amylase inhibitory activity was retained at various pH studied. The optimum activity (0.0222 ± 0.0004 U/min/ml) was obtained at pH 8 while the minimum was obtained at pH 4 – an acidic pH. The conformation and activity of enzymes are altered as the pH varies. This change impacts the binding of alpha amylase inhibitor to the enzyme, hence the activity. The highest inhibitory activity was obtained around neutral pH which corresponds to the optimum pH for growth and metabolism of this group of microorganisms [14].

The effect of metal ions is shown in Fig. 3. The residual activity of alpha-amylase enzyme was measured. Since there is an inverse relationship between enzyme activity and inhibitor activity, this served as the measure of inhibitory activity. Enzyme activity was highest in the presence of Cobalt while enzyme activity was also recorded in the presence of Copper, Manganese and Calcium. High inhibitory activities were observed in the presence of 5 mM concentration of Calcium, Cobalt and Copper. In comparison to the control, the inhibitor activity was retarded in the presence of Sodium, Potassium, Magnesium and Zinc

ions. Alpha-amylase being a metalloenzyme has improved activity in the presence of certain metal ions including Calcium and Magnesium ions [10]. Metal ions that activate enzyme activities have been found to do so by assisting in the anchoring of the substrate to the enzyme, in stabilising charges and transition state as well as in the relay of charges [1].

The effect of substrate concentration on enzyme velocity at various concentration of alpha amylase inhibitor was determined. The highest V_{max} and K_m (0.1677±0.05 and 0.025±0.02 respectively) were obtained at inhibitor concentration of 0.048 mg/mL while the least were obtained at 0.095 mg/mL inhibitor concentration (V_{max} 0.0999±0.072 and K_m 0.0043±0.002). The results obtained indicate that the alpha-amylase inhibitor exerts a non-competitive type of inhibition on alpha-amylase enzyme [16]. This further suggests that in the presence of inhibitor, the substrate binds to the active site of the enzyme while the inhibitor can bind to a secondary site other than the active site of the enzyme [7]. Michaelis-Menten constant (K_m) was calculated as 0.027 mM. A low K_m value implies and further confirms that there is a "tight-binding" with the enzyme. This same phenomenon was also observed in tendamistat (an inhibitor produced by *S. tendae*) [15]. *S. tendae* however, is found to be evolutionarily related to the isolate used for the production of this alpha amylase inhibitor.

Conclusion

Alpha-amylase inhibitor protein with high inhibitory activity and stability was recovered in wheat bran culture of *S. xinghaiensis.* The protein occurs in isoforms with a non-stringent requirement for pre-incubation and showing optimal activity at neutral pH and in the presence of metal ions.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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