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## Research Article

Ruth Ololade Amiola, Adedeji Nelson Ademakinwa, Zainab Adenike Ayinla, Esther Nkechi Ezima and Femi Kayode Agboola\*



## Purification and biochemical characterization of a $\beta$ -cyanoalanine synthase expressed in germinating seeds of *Sorghum bicolor* (L.) moench

### *Sorghum bicolor* (L.) Moench'in Çimlenen Tohumlarında İfade Edilen Bir $\beta$ -Siyanoalanin Sentazın Saflaştırılması ve Biyokimyasal Karakterizasyonu

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#### Abstract

**Background:**  $\beta$ -Cyanoalanine synthase plays essential roles in germinating seeds, such as in cyanide homeostasis. **Methods:**  $\beta$ -Cyanoalanine synthase was isolated from sorghum seeds, purified using chromatographic techniques and its biochemical and catalytic properties were determined.

**Results:** The purified enzyme had a yield of 61.74% and specific activity of 577.50 nmol  $H_2S$ /min/mg of protein. The apparent and subunit molecular weight for purified  $\beta$ -cyanoalanine synthase were  $58.26 \pm 2.41$  kDa and 63.4 kDa, respectively. The kinetic parameters with sodium cyanide as substrate were  $0.67 \pm 0.08$  mM,  $17.60 \pm 0.50$  nmol  $H_2S$ /mL/min,  $2.97 \times 10^{-1} s^{-1}$  and  $4.43 \times 10^2 M^{-1} s^{-1}$  for  $K_M$ ,  $V_{max}$ ,  $k_{cat}$  and  $k_{cat}/K_M$ , respectively. With L-cysteine as substrate, the kinetic parameters were  $2.64 \pm 0.37$  mM,  $63.41 \pm 4.04$  nmol  $H_2S$ /mL/min,  $10.71 \times 10^{-1} s^{-1}$  and  $4.06 \times 10^2 M^{-1} s^{-1}$  for  $K_M$ ,  $V_{max}$ ,  $k_{cat}$  and  $k_{cat}/K_M$ , respectively. The optimum temperature and pH for activity were 35°C and 8.5, respectively. The enzyme retained more than

half of its activity at 40°C. Inhibitors such as  $HgCl_2$ , EDTA, glycine and iodoacetamide reduced enzyme activity.

**Conclusion:** The biochemical properties of  $\beta$ -cyanoalanine synthase in germinating sorghum seeds highlights its roles in maintaining cyanide homeostasis.

**Keywords:**  $\beta$ -Cyanoalanine synthase; Cyanide; Sorghum; Detoxification; Characterization; Purification.

#### Özet

**Giriş:**  $\beta$ -siyanoalanin sentaz, çimlenen tohumlarda, siyanür homeostazı gibi önemli roller oynamaktadır.

**Yöntemler:**  $\beta$ -siyanoalanin sentaz, sorgum tohumlarından izole edildi, kromatografik teknikler kullanılarak saflaştırıldı ve biyokimyasal ve katalitik özellikleri belirlendi.

**Bulgular:** Saflaştırılmış enzim, % 61.74'lük bir verim ve 577.50 nmol  $H_2S$ /dak/mg protein spesifik aktivitesine sahipti. Saflaştırılmış  $\beta$ -siyanoalanin sentaz için görünür ve alt birim molekül ağırlığı sırasıyla  $58.26 \pm 2.41$  kDa ve 63.4 kDa idi. Substrat olarak sodyum siyanür ile kinetik parametreler,  $K_M$ ,  $V_{max}$ ,  $k_{cat}$  ve  $k_{cat}/K_M$  için sırasıyla  $0.67 \pm 0.08$  mM,  $17.60 \pm 0.50$  nmol  $H_2S$ /mL/dak,  $2.97 \times 10^{-1} s^{-1}$  ve  $4.43 \times 10^2 M^{-1} s^{-1}$  olarak belirlendi. Substrat olarak L-sistein ile kinetik parametreler  $K_M$ ,  $V_{max}$ ,  $k_{cat}$  ve  $k_{cat}/K_M$  için sırasıyla  $2.64 \pm 0.37$  mM,  $63.41 \pm 4.04$  nmol  $H_2S$ /mL/dak,  $10.71 \times 10^{-1} s^{-1}$  ve  $4.06 \times 10^2 M^{-1} s^{-1}$  idi. Aktivite için optimum sıcaklık 35°C ve optimum pH 8.5 idi. Enzim, aktivitesinin yarısından fazlasını 40°C'de korudu.  $HgCl_2$ , EDTA, glisin ve iyodoasetamid gibi inhibitörler enzim aktivitesini düşürdü.

**Sonuç:** Çimlenmekte olan sorgum tohumlarındaki  $\beta$ -siyanoalanin sentazın biyokimyasal özellikleri, siyanür homeostazının korunmasındaki rolünü vurgulamaktadır.

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**Anahtar Kelimeler:**  $\beta$ -Siyanoalanin sentaz; Siyanür; Sorgum; Detoksifikasyon; Karakterizasyon; Saflaştırma.

## Introduction

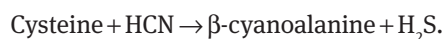
Cyanogenesis, defined as the biological process in which living organisms (plants) release hydrogen cyanide, is a well-defined concept known for several centuries. The idea behind cyanogenesis had been established in most higher plants, ferns, etc. [1–3]. Cyanogenesis is not exclusive to cyanogenic plants, plant species that produce cyanogenic glycosides in storage forms of nitrogen and defensive compounds [4, 5] or plant species accumulating cyanolipids but all higher plants probably form low levels of hydrogen cyanide (HCN) as a co-product of ethylene biosynthesis [6]. Plants use the toxicity of cyanide for protection. Hydrolysis of cyanogenic glycosides in response to attack by herbivores or other tissue damage [7, 8] or during decomposition of plant material in soil [9, 10] leads to the release of cyanide. This makes the degradation of cyanogenic compounds a major source by which cyanide is produced in higher plants.

Cyanide in plants is mostly from biosynthesis of ethylene. The mechanism behind this process occurs via the conversion of 1-amino-cyclopropane-1-carboxylic acid (ACC) to ethylene and the concomitant release of cyanofornic acid. The rapid decarboxylation of cyanofornic acid results ultimately in the release of cyanide [10, 11]. The process leading to the biosynthesis of ethylene occurs throughout the development and growth of the plant. Another contributing factor for the increased cyanide accumulation through ethylene synthesis occurs via stress (biotic and abiotic) in which the plants are exposed to [12, 13], which then leads to increased cyanide production [14, 15]. This theory provides possible explanation as to why there is the presence (albeit in relatively small amount) of this cyanide detoxifying enzymes in hitherto non-cyanogenic plants.

The toxicity of cyanide is well known and the mechanism of action involves the formation of iron/magnesium complex in enzymes hence disrupting vital biologic processes such as respiration, CO<sub>2</sub> fixation and nitrate reduction [16, 17]. It must therefore be rapidly metabolized in biological systems to reduce or prevent its adverse effects. Two fundamental strategies exist for removing cyanide from biological systems: degradation (breakdown to simpler inorganic molecules) or assimilation (incorporation of cyanide into primary metabolites). Degradation is the primary strategy for cyanide detoxification in eubacteria and this includes: hydrolytic, reductive, or oxidative

pathways forming simple nitrogenous compounds such as formamide and ammonium [17, 18].

Two pathways possibly exist for which assimilation of cyanide can occur. Sulfur transferases mediate the first pathway where these enzymes (3-mercaptopyruvate transferase and rhodanese) are involved in the transfer to a recipient a thiol (-SH) group. The recipient in terms of cyanide detoxification is cyanide. The product of this process is a thiocyanate [19–24]. Cyanide assimilation also occurs via a second pathway where there is the incorporation of cyanide into nitrogen metabolism through synthesis of aspartate and asparagine.  $\beta$ -Cyanoalanine pathway is the most prevalent example of this type of cyanide assimilation [25, 26] in which the first step is catalyzed by  $\beta$ -cyanoalanine synthase ( $\beta$ -CAS), an enzyme that mediates a reaction which substitutes the sulfhydryl moiety of cysteine (or another alanyl donor like serine) with cyanide, forming the non-protein amino acid,  $\beta$ -cyanoalanine (or the nitrile cyanoalanine) with the concomitant release of hydrogen sulfide [27–29].



The second step is catalyzed by a bi-functional enzyme, nitrilase 4 (NIT4) [E. C. 3.5.5.1], which is capable of carrying out both nitrilase and nitrile hydratase activities simultaneously, using  $\beta$ -cyanoalanine as the substrate. The nitrile hydratase activity leads to the conversion of cyanoalanine to asparagine while the nitrilase activity forms aspartate and ammonium [30, 31].

Cyanogenesis has been studied extensively in higher plants. As a matter of fact, it was in sorghum that the pathway necessary for cyanogenic glycoside production was initially identified [32, 33]. Sorghum [*Sorghum bicolor* (L.) Moench] is an upright, quick growing grass which is a member of the Poaceae family. The grass blades are flat, stems are rigid, and there are no creeping rhizomes. The grain is predominately red or reddish brown. Sorghum is valued for its grain, stalks and leaves, which makes it one of the world's major cereal crops and an important fodder crop [34]. Sorghum is a well-known cyanogenic plant which releases cyanide upon hydrolysis of its stored cyanogenic glycosides [35]. Another instance where cyanide production increases is during germination, where there is increased production of ethylene. The increased production of ethylene further increases cyanide production [15]. The presence and need for the cyanide detoxifying enzyme,  $\beta$ -cyanoalanine synthase can therefore not be overemphasized.

Hence this study investigated the biochemical and catalytic properties of a purified cyanide detoxifying enzyme,  $\beta$ -cyanoalanine synthase, in germinating sorghum seeds. This will provide a broader perspective

in the use of  $\beta$ -cyanoalanine synthase in possible cyanide bioremediation.

## Materials and methods

### Materials

Reagents used were of analytical grade. L-cysteine, sodium cyanide, Tris-base, hydrochloric acid, ferric chloride, N,N-dimethyl-*p*-phenylenediamine sulfate was obtained from Fisher Scientific, Loughborough, UK. Glycerol, Borate, boric acid, citric acid, trisodium citrate, were obtained from BDH Chemicals Limited, Poole, England. Sodium hydroxide was obtained from Merck Millipore International, Darmstadt, Germany. Glycine, ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Company Limited, St. Louis, MO, USA. DEAE-cellulose and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sorghum grains were obtained from “Oja tutun” market in Ile-Ife and identified as *S. bicolor* (L.) Moench at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife.

### Steeping and germination

About 50 g of carefully sorted *S. bicolor* seeds (red cultivar grains) was thoroughly washed with distilled water and then soaked in the different growth media: distilled water, 10 mM Tris-HCl buffer (pH 7.0, pH 7.5, pH 8.0, pH 8.5 and pH 9.0) for approximately 24 h at room temperature (25°C) to break seed dormancy and speed up the germination process [25, 36] with the soaking water/buffer being changed at 12-h interval so as to prevent fermentation. After 24 h of steeping, the seeds were evenly spread on damp papers and covered to ensure darkness. The germination process was carried out in the dark at room temperature and sprinkled with the respective growth media: distilled water, 10 mM Tris-HCl buffer (pH 7.0, pH 7.5, pH 8.0, pH 8.5 and pH 9.0) twice per day. The seeds were allowed to germinate for 3, 4 and 5 days (1 day of steeping inclusive).

### Enzyme extraction

After the third day, a portion of the sprouted seeds, germinated with different media was collected.  $\beta$ -Cyanoalanine

synthase was extracted by homogenizing the germinating seeds in 2.5 volumes of 50 mM Tris-HCl buffer, pH 8.5. The homogenate was stirred and passed through a sieve cloth to remove the chaff. The resulting solution was centrifuged at  $10,000 \times g$  for 20 min at 4°C in a cold centrifuge. The supernatant recovered was used as the crude extract. The same procedure was repeated for the sprouted seeds collected on the fourth and fifth days for seeds germinated with the different growth media which include distilled water, 10 mM Tris-HCl buffer (pH 7.0, pH 7.5, pH 8.0, pH 8.5 and pH 9.0).  $\beta$ -Cyanoalanine synthase activity was carried out on each crude extract obtained from different growth media and each day of germination; the crude extract with the highest activity was used for purification and characterization.

### Enzyme and protein assay

$\beta$ -Cyanoalanine synthase assay was according to the method of Ogunlabi and Agboola [37] which is a modification of the methods of both Hendrickson and Conn [28] and Yip and Yang [38]. This is based on the rate of formation of hydrogen sulfide ( $H_2S$ ), one of the end-products; sulfide liberated from cysteine was assayed by spectrophotometry after conversion to methylene blue.

Assay was performed in a 10-mL serum bottle with the rubber cork. The assay mixture contained 1 mL of the substrate solution (25 mM L-cysteine and 25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5) and 1 mL of appropriately diluted enzyme solution. The mixture was incubated at 30°C for 10 min and the reaction terminated by the addition of 0.5 mL of 20 mM N,N-dimethyl-*p*-phenylenediamine (in 7.2 N HCl) and 0.5 mL of 30 mM  $FeCl_3$  in (1.2 N HCl) through the rubber cork using a calibrated syringe and needle. The mixture was shaken vigorously and placed in the dark for 20 min. The solution was clarified by centrifugation and the absorbance was read at 650 nm. The amount of sulfide produced was calculated using the correlation by Hendrickson and Conn [28] in which  $A_{650}$  of 1.0 is equivalent to 0.5  $\mu$ mol of sulfide produced under the assay condition. One unit of enzyme activity was defined as the amount of enzyme yielding 1.0 nmol of  $H_2S$ /min under the assay condition.

The protein concentration was determined by the method described by Bradford using BSA as standard [39].

### $\beta$ -Cyanoalanine synthase purification

The crude  $\beta$ -cyanoalanine synthase was concentrated by dialysis against 50% glycerol in 50 mM Tris-HCl buffer,

pH 8.5 and was left in the refrigerator overnight. The concentrated enzyme was used for ion-exchange chromatography on DEAE-cellulose.

### **Ion-exchange chromatography on DEAE-cellulose**

DEAE-cellulose was prepared according to the Whatman instruction manual and packed into a 1.5×20 cm column. A sample of the concentrated enzyme was layered on the column. The column was then washed with the buffer to remove the unbound protein, followed by a stepwise elution with 0.5–1.0 M NaCl in 50 mM Tris-HCl buffer, pH 8.5. Fractions of 5 mL were collected at a flow rate of 40 mL/h. Protein was monitored spectrophotometrically at 280 nm. The fractions were also assayed for  $\beta$ -cyanoalanine synthase activity. The active fractions were pooled and dialysed against 50% glycerol in 50 mM Tris-HCl buffer, pH 8.5.

### **Gel filtration chromatography on Sephacryl S-200**

Sephacryl S-200 was packed into a 1.5×40 cm column. The post DEAE-cellulose sample dialysed against 50% glycerol in 50 mM Tris-HCl buffer, pH 8.5 was layered on the column. The column was eluted with 200 mL of 50 mM Tris-HCl buffer, pH 8.5. Fractions of 5 mL were collected from the column at a flow rate of 20 mL/h. Protein was monitored spectrophotometrically at 280 nm. The fractions were also assayed for  $\beta$ -cyanoalanine synthase activity. The active fractions were pooled and stored by dialysis against 50% glycerol in 50 mM Tris-HCl buffer, pH 8.5.

### **Characterisation of the purified $\beta$ -cyanoalanine synthase**

#### **Determination of kinetic parameters**

The kinetic parameters ( $K_M$  and  $V_{max}$ ) of  $\beta$ -cyanoalanine synthase were determined by varying the concentrations of L-cysteine between 0.5 mM and 5 mM at fixed concentration of 10 mM sodium cyanide (NaCN). Also, the concentration of NaCN was varied between 0.5 mM and 5 mM at fixed concentration of 10 mM L-cysteine. Plots of the reciprocal of initial reaction rate ( $1/V$ ) versus reciprocal of the varied substrates  $1/[S]$  at each fixed concentrations of the other substrate were made

according to Lineweaver and Burk [40] using Graph Pad Prism 5.

### **Determination of molecular weight of *S. bicolor* $\beta$ -CAS**

#### **Determination of native molecular weight of *S. bicolor* $\beta$ -CAS on Sephacryl S-200**

The native molecular weight of the enzyme was estimated by gel filtration on a Sephacryl S-200 column (1.5×40 cm) using the following protein markers: lysozyme (14,000 Da)  $\alpha$ -chymotrypsinogen (25,000 Da), peroxidase (44,000 Da) and bovine serum albumin (66,000). The marker proteins at 2 mg/mL were run separately and the column was eluted with 100 mM Tris-HCl buffer, pH 8.5 at a flow rate of 20 mL/h. Fractions of 5 mL were collected and monitored spectrophotometrically by taking absorbance at 280 nm and the elution volume of each protein was estimated. The pure enzyme (5 mL) was then passed through the same column. A plot of logarithm of the molecular weight of the standard proteins against  $K_{av}$  (partition coefficient) was made. The molecular weight of *S. bicolor*  $\beta$ -CAS was interpolated from the curve.

### **Determination of molecular weight of *S. bicolor* $\beta$ -cyanoalanine synthase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was carried out according to the method of Weber and Osborn [41] using Tris-glycine buffer system to determine purity as well as subunit molecular weight. The enzyme preparation was denatured by boiling for 5 min in boiling water and was loaded on different wells of the gel (4% stacking, 12% resolving) slab. The standard protein was also applied to a different well on the same gel along with the sample.

### **Effect of temperature on enzyme activity**

The enzyme was assayed at temperatures between 10°C and 60°C to investigate the effect of temperature on the activity of the purified enzyme and also to determine the optimum temperature. The reaction mixture containing 1 mL of the substrate solution (25 mM L-cysteine and

25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5) was incubated at the indicated temperature and initiated by the addition of an aliquot of the enzyme. The residual enzyme activity was plotted against the different temperatures.

### Thermal stability

The thermal stability of the enzyme was studied by incubating 1 mL of the enzyme at temperatures around the optimum temperature (35°C, 40°C, 45°C and 50°C) for 1 h. 0.1 mL was withdrawn at 15 min interval and assayed for residual activity. The residual activity at each temperature was expressed as a percentage of the activity at zero time which was taken to be 100%. The percentage residual activity was plotted against incubation time.

### Effect of pH on enzyme activity

The activity of purified  $\beta$ -cyanoalanine synthase in different buffers at different pH values ranging between pH 5.0 and pH 11.0 was investigated. All buffers were 100 mM in concentration. The buffers used were sodium citrate buffer (pH 5.0–7.5), Tris-HCl (pH 7.5–9.0), Borate buffer (pH 8.5–10.0) and Glycine-NaOH buffer (pH range 10.0–11.0). The reaction mixture contained the substrate (25 mM L-cysteine and 25 mM NaCN) in respective buffers and 1 mL of appropriately diluted enzyme.

### Effect of salts on enzyme activity

Effect of salts on  $\beta$ -cyanoalanine synthase activity was studied using the following salts in final concentrations of 1 mM, 5 mM and 10 mM: NaCl, KCl,  $\text{NH}_4\text{Cl}$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$  and  $\text{CaCl}_2$ . The salt was incorporated into the substrate solution, containing 25 mM L-cysteine and 25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5. The reaction was initiated by the addition of 1 mL appropriately diluted enzyme.

Reaction mixtures without salts were taken as control with 100% activity.

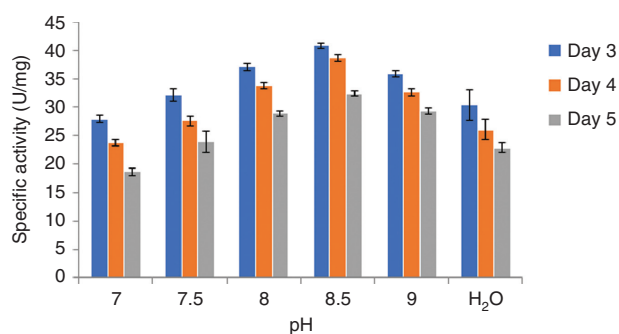
### Effect of inhibitor on enzyme activity

The effect of some representative inhibitory compounds was examined. These include glycine, EDTA, iodoacetamide, HgCl and  $\beta$ -mercaptoethanol in final concentrations of 1.0 mM, 5 mM and 10 mM, incorporated into the substrate solution of 25 mM L-cysteine and 25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5. One milliliter of appropriately diluted enzyme was added to initiate the reaction. Reaction mixture without inhibitor was taken as control with 100% activity.

## Results

### Germination optimization for $\beta$ -cyanoalanine synthase activity

Sorghum grains grown with 10 mM Tris-HCl buffer, pH 8.5 for 3 days has the highest  $\beta$ -cyanoalanine synthase activity (Figure 1). The crude extract obtained from this was used for purification and characterization of the enzyme.



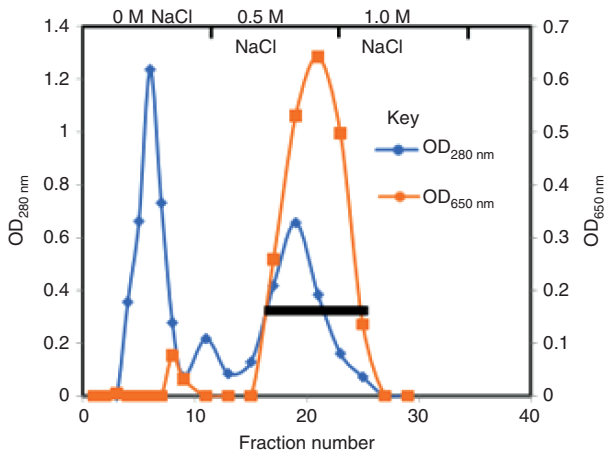
**Figure 1:** Germination optimization for  $\beta$ -cyanoalanine synthase activity.

**Table 1:** Summary of purification procedure for *S. bicolor*  $\beta$ -cyanoalanine synthase.

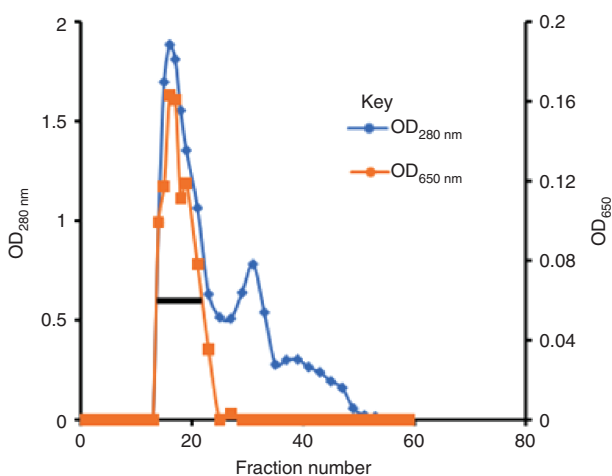
	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	41	3423.50	39.36	86.98	100.00	1.00
Ion-exchange on DEAE-cellulose	40	2560.00	24.00	106.67	74.80	1.23
Dialysis against 50% glycerol	18	2151.00	19.80	108.64	62.83	1.25
Gel filtration on Sephacryl S-200	61	2113.65	3.66	577.50	61.74	6.64

## Purification of *S. bicolor* $\beta$ -cyanoalanine synthase by ion-exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sephacryl S-200

A summary of a typical purification procedure of  $\beta$ -cyanoalanine synthase from *S. bicolor* is presented in Table 1. The elution profile of the enzyme on ion exchange chromatography on DEAE-cellulose is shown in Figure 2. A single peak of activity was obtained which was pooled and dialysed against 50% glycerol and then layered on gel filtration chromatography on Sephacryl S-200. The elution profile on Sephacryl S-200 is shown in Figure 3. A single peak of activity was obtained with a yield of 61.74% and a purification fold of 6.64.



**Figure 2:** Ion-exchange chromatography of *S. bicolor*  $\beta$ -cyanoalanine synthase on DEAE-cellulose.



**Figure 3:** Gel filtration chromatography of *S. bicolor*  $\beta$ -cyanoalanine synthase on Sephacryl S-200.

## Molecular weight determination of *S. bicolor* $\beta$ -cyanoalanine synthase

### Native molecular weight of *S. bicolor* $\beta$ -cyanoalanine synthase by gel filtration chromatography on Sephacryl S-200

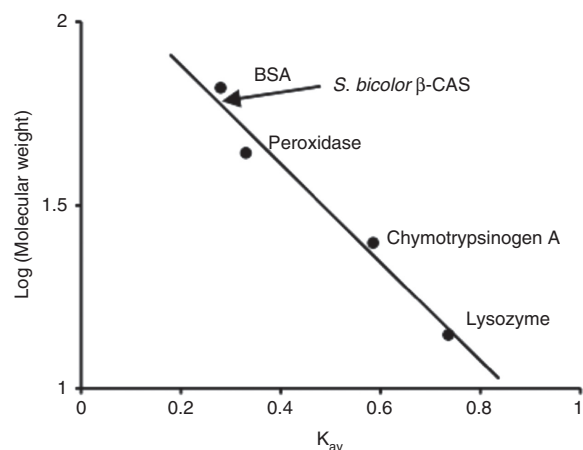
The native molecular weight obtained from the plot of the logarithms of molecular weight of standard proteins against the partition coefficient was 58.3 kDa. The plot of the  $K_{av}$  values against the logarithm of the molecular weight is shown in Figure 4.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *S. bicolor* $\beta$ -cyanoalanine synthase

A distinct band was obtained whose molecular weight is estimated to be 63.4 kDa (Figure 5A). The plot of the logarithms of molecular weight of standard proteins against the relative mobility  $R_f$  is shown in Figure 5B.

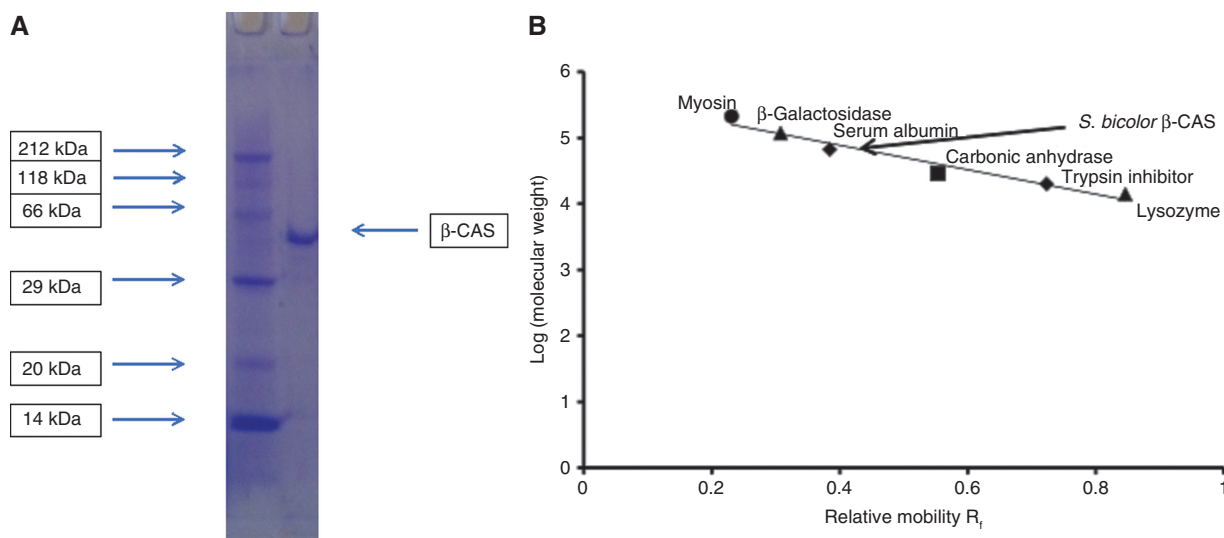
### Kinetic parameters for *S. bicolor* $\beta$ -cyanoalanine synthase

Lineweaver-Burk plots for varying concentration of sodium cyanide at fixed concentration of L-cysteine gave  $K_M$  and  $V_{max}$  of  $0.67 \pm 0.08$  mM and  $17.60 \pm 0.50$  nmol  $H_2S$ /mL/min, respectively (Figure 6) while that of



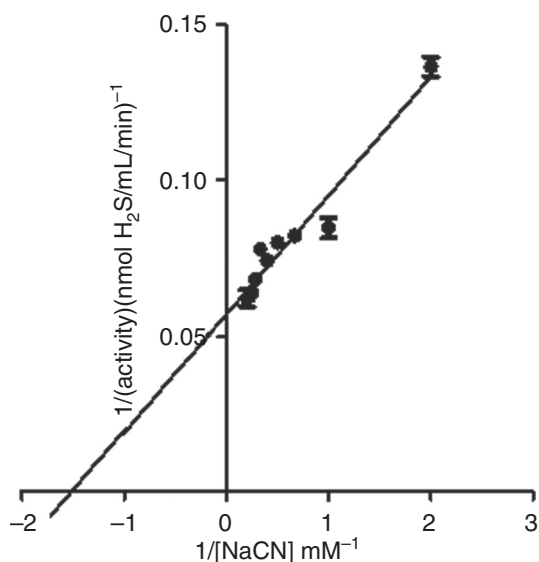
**Figure 4:** Plot of logarithm of molecular weight against partition coefficient.

Marker proteins used include lysozyme (14 kDa), chymotrypsinogen A (25 kDa), peroxidase (44 kDa) and BSA (66 kDa). The position of *S. bicolor*  $\beta$ -cyanoalanine synthase is indicated by the arrow.



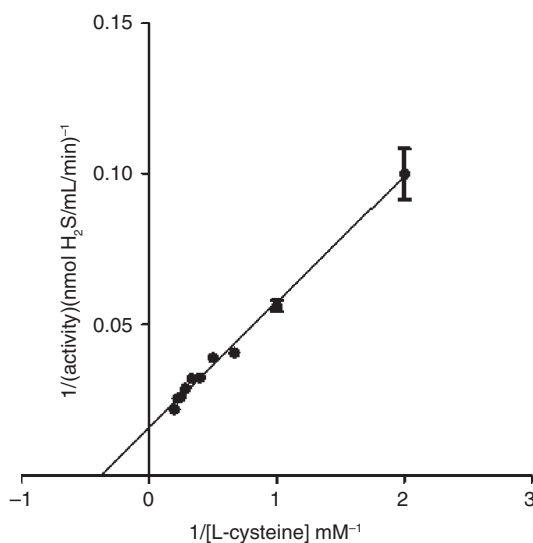
**Figure 5:** A distinct band whose molecular weight is estimated to be 63.4 kDa and the plot of the logarithms of molecular weight of standard proteins against the relative mobility.

(A) Electrophoregram of SDS-polyacrylamide gel electrophoresis of *S. bicolor*  $\beta$ -cyanoalanine synthase. Proteins were stained with Coomassie brilliant Blue R-250. Lane 1 is the molecular weight ladder while lane 2 is *S. bicolor*  $\beta$ -cyanoalanine synthase. (B) Plot of logarithm of molecular weight of protein standards against the relative mobility ( $R_f$ ). The relative mobility of protein standards and *S. bicolor*  $\beta$ -CAS on the SDS-PAGE gel were determined. The molecular weight of *S. bicolor*  $\beta$ -CAS was interpolated from the plot of the logarithm of molecular weight of protein standards against the relative mobility. The protein mixture for the protein standard include myosin (212 kDa),  $\beta$ -galactosidase (118 kDa), serum albumin (66 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa).



**Figure 6:** Lineweaver-burk plot for *S. bicolor*  $\beta$ -cyanoalanine synthase by varying concentration of NaCN between 0.5 mM and 5 mM at fixed concentration of 10 mM L-cysteine. The value shown represents the average from triplicate experiment. Error bar represent the standard deviation.

varying concentration of L-cysteine at fixed concentration of sodium cyanide are respectively  $2.64 \pm 0.37$  mM and  $63.41 \pm 4.07$  nmol  $H_2S/mL/min$  (Figure 7). The values of the kinetic parameters of the Lineweaver-Burk plots are presented in Table 2.



**Figure 7:** Lineweaver-burk plot for *S. bicolor*  $\beta$ -cyanoalanine synthase by varying concentration of L-cysteine between 0.5 mM and 5 mM at fixed concentration of 10 mM NaCN. The value shown represents the average from triplicate experiment. Error bar represent the standard deviation.

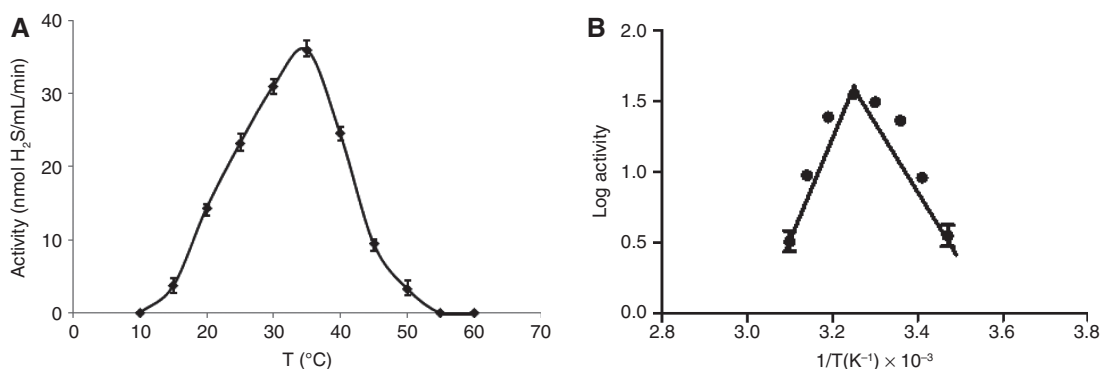
### Effect of temperature on the activity of *S. bicolor* $\beta$ -cyanoalanine synthase

The effect of temperature on the activity of *S. bicolor*  $\beta$ -CAS is shown in Figure 8A. The enzyme showed optimum



**Table 2:** Summary for the kinetic parameters for *S. bicolor*  $\beta$ -cyanoalanine synthase.

Substrate	$K_M$ (mM)	$V_{max}$ (nmol $H_2S$ /mL/min)	$k_{cat}$ ( $s^{-1}$ ) $\times 10^{-1}$	$k_{cat}/K_M$ ( $M^{-1} s^{-1}$ ) $\times 10^2$
NaCN	$0.67 \pm 0.08$	$17.60 \pm 0.50$	2.97	4.43
L-cysteine	$2.64 \pm 0.37$	$63.41 \pm 4.04$	10.71	4.06



**Figure 8:** The effect of temperature on the activity of *S. bicolor*  $\beta$ -cyanoalanine synthase and the Arrhenius plot for the effect. (A) The activity-temperature profile of  $\beta$ -CAS from *S. bicolor* was obtained by varying the temperature between 10°C and 60°C. The values shown represent the average from triplicate experiments. Error bars represent the standard deviation. (B) From the Arrhenius equation, the logarithm of activity was plotted against the inverse of temperature in Kelvins. The activation energy was obtained from the slope of the graph which is  $-E_a/(2.303R)$ , where  $R=8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ . The activation energy of the reaction was then calculated from the slope of the linear portion with negative slope. The positive slope indicates the onset of protein denaturation.

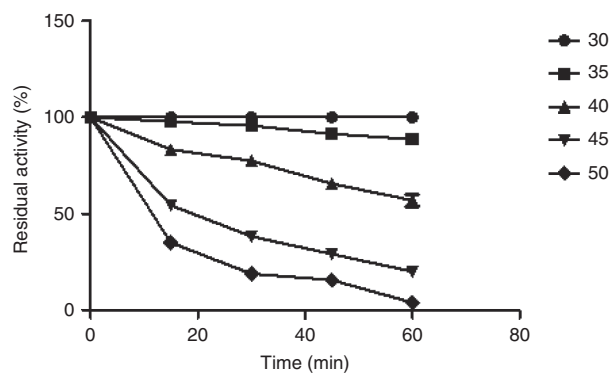
activity at 35°C. The activation energy,  $E_a$  obtained from the Arrhenius plot are 131.75 J/mol/K and  $-103.54 \text{ J/mol/K}$  (Figure 8B).

### Heat stability of *S. bicolor* $\beta$ -cyanoalanine synthase

$\beta$ -Cyanoalanine synthase from *S. bicolor* was stable at 30–40°C as it retained about 50% or more of its activity after incubating for 1 h at this temperature (Figure 9). At increased temperatures ranging from 45 to 50°C, the enzyme lost about 90% of its activity after incubating for 1 h.

### Effect of pH on the activity of *S. bicolor* $\beta$ -cyanoalanine synthase

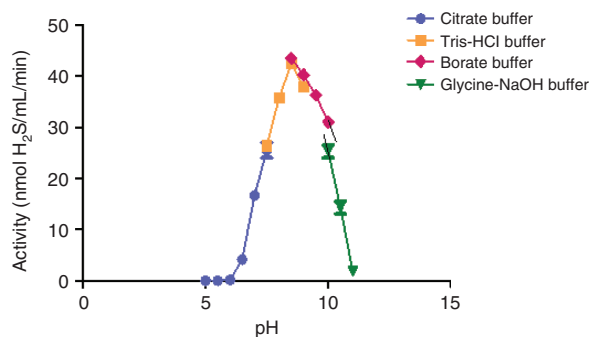
The effect of pH on the activity of *S. bicolor*  $\beta$ -CAS is shown in Figure 10. The enzyme showed no activity at pH 5.0–6.0. There was a stable increase in activity between pH 6.5–8.5 followed by a gradual decrease. The enzyme showed optimum activity at pH 8.5.



**Figure 9:** Heat stability of *S. bicolor*  $\beta$ -cyanoalanine synthase. Aliquots of  $\beta$ -CAS were incubated at different temperatures (35°C–50°C) for 1 h. An aliquot of the enzyme solution was taken at every 15-min interval and assayed for  $\beta$ -CAS activity and the residual activity was determined under the standard reaction conditions. The activity at zero time was taken as 100%. The residual activity was plotted against the time of incubation.

### Effect of salts on the activity of *S. bicolor* $\beta$ -cyanoalanine synthase

Salts of monovalent ions ( $Na^+$ ,  $K^+$  and  $NH_4^+$ ) had little effect, with 1 mM–10 mM concentrations causing about



**Figure 10:** Effect of pH on the activity of *S. bicolor*  $\beta$ -cyanoalanine synthase.

Optimum pH was obtained using 100 mM citrate buffer (pH 5.0–7.5), 100 mM Tris-HCl buffer (pH 7.5–9.0), 100 mM borate buffer (pH 8.5–10.0) and 100 mM glycine-NaOH buffer (pH 10.0–11.0).

15%–20% stimulation. The divalent ions ( $Mg^{2+}$ ,  $Ca^{2+}$  and  $Mn^{2+}$ ) also had little effect, with 10 mM concentration causing about 10% stimulation of activity (Table 3).

**Table 3:** Effect of salts on the activity of *S. bicolor*  $\beta$ -cyanoalanine synthase.

Salts	Relative activity (%)		
	1.0 mM	5.0 mM	10 mM
MnCl <sub>2</sub>	100.57 ± 2.91	102.0 ± 1.2	109.7 ± 2.4
MgCl <sub>2</sub>	102.9 ± 2.4	107.1 ± 3.6	111.9 ± 3.1
CaCl <sub>2</sub>	103.7 ± 1.2	106.3 ± 2.4	110.6 ± 1.2
KCl	115.7 ± 1.2	122.6 ± 3.6	123.4 ± 2.4
NH <sub>4</sub> Cl	117.4 ± 3.6	120.0 ± 2.4	118.3 ± 2.4
NaCl	120.0 ± 2.4	124.3 ± 3.6	117.4 ± 1.2

Assays were carried out in final concentrations of 1.0–10.0 mM of chloride salts of manganese, magnesium, calcium, potassium, ammonium and sodium. The relative activity was determined by measuring  $\beta$ -CAS activity in the control that contained no chloride salt and taken as 100%. The values shown represent the average from triplicate experiments.

**Table 4:** Effect of inhibitors on the activity of *S. bicolor*  $\beta$ -cyanoalanine synthase.

Inhibitor	Relative activity (%)		
	1.0 mM	5.0 mM	10 mM
Mercuric chloride	88.14 ± 3.7	5.65 ± 2.1	0
EDTA	7.34 ± 2.1	0	0
Glycine	96.35 ± 1.7	84.86 ± 0.9	66.43 ± 2.9
Iodoacetamide	62.84 ± 1.5	22.14 ± 0.8	0

Assays were carried out in final concentrations of 1.0–10.0 mM of inhibitors. The relative activity was determined by measuring  $\beta$ -CAS activity in the control that contained no inhibitor and taken as 100%. The values shown represent the average from triplicate experiments.

## Effect of inhibitors on *S. bicolor* $\beta$ -cyanoalanine synthase

Activity of *S. bicolor*  $\beta$ -cyanoalanine synthase was greatly affected by mercuric chloride, EDTA, and Iodoacetamide with about 80% loss of activity even at 1 mM concentration of EDTA (Table 4).

## Discussion

$\beta$ -Cyanoalanine synthase ( $\beta$ -CAS) is an enzyme that catalyses the conversion of cyanide and cysteine to  $\beta$ -cyanoalanine [25]. It is the key enzyme for cyanide detoxification in plants [37, 42]. It has been detected in all plants examined though levels of activity vary considerably between species and between different tissues of the same plant. In plant physiology,  $\beta$ -CAS is regarded as the main cyanide detoxifying enzyme necessary for the removal of cyanide that is produced in the life cycle of the plant [28, 42–45]. Ethylene biosynthesis is the ubiquitous source of cyanide in plants. Conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene releases cyanofornic acid, which spontaneously decarboxylates to release  $CN^-$  [10, 11]. Ethylene synthesis occurs throughout plant growth and development, but increases significantly when plants are subjected to either biotic or abiotic stress [12, 13], which then leads to increased cyanide production [14, 15].

This research work reported the existence of  $\beta$ -cyanoalanine synthase from germinating seeds of *S. bicolor* ((L.) Moench). After extensive literature search, there has been no known report exclusively on the purification and characterization of this enzyme in *S. bicolor*. The highest activity for  $\beta$ -cyanoalanine synthase in germinating seeds of sorghum was observed on the third day of germination with Tris-HCl buffer, pH 8.5 (Figure 1). Plant species, such as cocklebur, sorghum, barley and almond, store cyanogenic compounds as a source of nitrogen; there is increased evolution and release of endogenous cyanide immediately prior to and during germination, to help break seed dormancy and promote germination [46–48]. As a result,  $\beta$ -CAS activity increases considerably during imbibition following an ethylene burst [49].

In the purification method adopted in this study,  $\beta$ -cyanoalanine synthase was purified about seven-fold from germinating seeds of *S. bicolor*. The purification fold is lower compared to  $\beta$ -CAS purified from previous studies. For example, Hendrickson and Conn [28] purified  $\beta$ -cyanoalanine synthase 140-fold from mitochondrial acetone powder of blue lupine seedlings using a

combination of ammonium sulfate precipitation, acetone precipitation, fractionation on Sephadex G-100 column chromatography and preparative gel electrophoresis.  $\beta$ -CAS was purified 6200-fold from fresh spinach leaves by a procedure including the preparation of acetonized mitochondria, ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephadex A-50, gel filtration on Sephadex G-100 or Ultrogel AcA 44, hydrophobic chromatography on AH-Sepharose 4B and preparative polyacrylamide gel electrophoresis [28].  $\beta$ -CAS was purified 17-fold from the cytosolic fraction of the gut of grasshopper *Zonocerus variegatus* (L.) by ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-100 columns [37].  $\beta$ -CAS from *S. bicolor* has a specific activity of 577.50 nmol  $\text{H}_2\text{S}/\text{min}/\text{mg}$  and a yield of 61.74%. Ikegami et al. [50] reported a specific activity of 33.8 mmol/mL/mg and a yield of 9.8% from 16.5 g of acetone powder extract of blue lupine shoots while [42] obtained a yield of 24.3% and specific activity of 43.5 mmol/mL/mg from 2.5 g of protein of blue lupine seedlings. Yields of 12%, 15% and 12% and specific activities of 13.8, 6.3 and 16.5 mmol/mL/mg, respectively were obtained by [51] starting with 83.2, 69.8 and 85.3 mg of protein of the leaf, rind and tuber of cassava, respectively. The specific activity of  $\beta$ -CAS purified from the cytosolic fraction of the gut of grasshopper, *Z. variegatus* was 37.5 nmol  $\text{H}_2\text{S}/\text{min}/\text{mg}$  and had a yield of 25.6% [37]. The specific activity obtained for  $\beta$ -CAS from *S. bicolor* may be as a result of difference in the nature of starting material, cytosolic fractions as against the mitochondrial fractions reported from other plant sources.

The native molecular weight obtained for *S. bicolor*  $\beta$ -CAS was 58.3 kDa (Figure 4) and the sub-unit molecular weight was estimated to be 63.4 kDa (Figure 5A) which shows it is a monomeric enzyme. In plants, two classes of  $\beta$ -CAS have been identified, based on differences in amino acid composition and protein structure [50] with molecular weight which varies from 50 kDa to 62 kDa [28, 50, 51]. In blue lupine,  $\beta$ -CAS is a monomeric enzyme, with a molecular weight of about 52 kDa, and contains one mole pyridoxal phosphate per mole of protein, which is essential for the catalytic activity [42]. In spinach (*Spinacia oleracea*) and *Lathyrus latifolius*, the enzyme contains two identical subunits of 28 kDa–30 kDa, each containing one molecule of pyridoxal phosphate, similar to the  $\beta$ -CAS of the cyanide-producing eubacterium *Chromatium violaceum* [52, 53].

The kinetic parameters for *S. bicolor*  $\beta$ -CAS compared very well with reported values of  $\beta$ -CAS from other plant sources, showing that *S. bicolor*  $\beta$ -CAS has a high affinity for cyanide than for L-cysteine and is able to rapidly detoxify cyanide that is produced during germination

[54, 55]. Lineweaver-Burk plots gave apparent  $K_M$  values of 2.64 mM and 0.67 mM for L-cysteine and cyanide, respectively (Figures 6 and 7). The  $K_M$  value for L-cysteine was almost the same value as that determined for blue lupine, 2.5 mM [28], spinach (*S. oleracea*), 2.3 mM [50] and particulate fractions of potato tubers, 2.82 mM. It was however lower than that determined for *Vicia angustifolia*, which has a  $K_M$  value of 3.6 mM for L-cysteine [50]. The  $K_M$  value for sodium cyanide was lower than that determined for (*S. oleracea*) which has a  $K_M$  value of 0.73 mM but higher than that determined for blue lupine, 0.55 mM [28] *V. angustifolia*, 0.5 mM [50] and potato tubers, which has a  $K_M$  value of 0.235 mM for sodium cyanide. The maximum velocity,  $V_{\max}$ , obtained for *S. bicolor*  $\beta$ -CAS was 63.41 nmol  $\text{H}_2\text{S}/\text{mL}/\text{min}$  and 17.60 nmol  $\text{H}_2\text{S}/\text{mL}/\text{min}$  for L-cysteine and sodium cyanide, respectively [37] reported  $V_{\max}$  values of 2.17 nmol  $\text{H}_2\text{S}/\text{mL}/\text{min}$  and 20.0 nmol  $\text{H}_2\text{S}/\text{mL}/\text{min}$  for L-cysteine and NaCN, respectively for  $\beta$ -CAS from *Z. variegatus*.  $K_M$  is equivalent to the substrate concentration at which the reaction rate is half maximal and is often used as an indicator of the affinity of an enzyme for its substrate [57]; a high  $K_M$  indicates weak binding, that is, low affinity of the enzyme for the substrate while a low  $K_M$  indicates strong binding that is high affinity of the enzyme for the substrate [57]. The maximal rate,  $V_{\max}$ , reveals the turnover number of an enzyme, which is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate [57]. The turnover number,  $k_{\text{cat}}$ , for *S. bicolor*  $\beta$ -CAS was  $10.71 \times 10^{-1} \text{ s}^{-1}$  for L-cysteine and  $2.97 \times 10^{-1} \text{ s}^{-1}$  for sodium cyanide. The specificity constant,  $k_{\text{cat}}/K_M$  is the rate constant for the interaction of substrate and enzyme and can be used as a measure of catalytic efficiency, to know how efficient an enzyme converts a substrate into product. The  $k_{\text{cat}}/K_M$  obtained for *S. bicolor*  $\beta$ -CAS was  $4.06 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  and  $4.43 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  for L-cysteine and sodium cyanide, respectively. The  $k_{\text{cat}}/K_M$  ratios of the enzymes superoxide dismutase, acetylcholinesterase, and triose phosphate isomerase are between  $10^8$  and  $10^9 \text{ M}^{-1} \text{ s}^{-1}$ , which represent the upper limit for  $k_{\text{cat}}/K_M$ . Enzymes such as these that have  $k_{\text{cat}}/K_M$  ratios at the upper limits are said to have achieved catalytic perfection [56, 57].

The optimum temperature obtained for *S. bicolor*  $\beta$ -CAS was 35°C (Figure 8A). The optimum temperature for  $\beta$ -CAS from the grasshopper *Z. variegatus* was 30°C with activity reducing to near zero at 45°C. An optimum temperature of 30°C has been reported for both immobilized and dissolved  $\beta$ -CAS [57]. Also,  $\beta$ -CAS from cassava had maximum activity at 30°C when studied over a temperature range of 20–45°C [51]. It can be concluded that this

enzyme is most active at around 30–35°C and loses activity as temperature increases above 35°C. The energy required to form the transition state from the substrate, that is, a minimum amount of energy reactants must first acquire to transform into products, is called the activation energy,  $E_a$ . Enzymes therefore function to lower the activation energy; in other words, enzymes facilitate the formation of the transition state. The lower activation energy means that more molecules have the required energy to reach the transition state, and hence, a faster reaction [57, 58]. The Arrhenius plot of the effect of temperature on the reaction rates for *S. bicolor*  $\beta$ -CAS consists of two linear segments with a break occurring at 35°C. The apparent activation energy values obtained from these slopes were 131.75 J/mol/K and -103.54 J/mol/K (Figure 8B).

Thermal stability studies on the purified enzyme showed that the enzyme was relatively stable at 30–40°C (Figure 9) as it retained about 50% or more of its activity after incubating for 1 h at this temperature. At increased temperatures ranging from 45 to 50°C, the enzyme lost nearly 90% of its activity after incubating for 1 h. This is similar to the  $\beta$ -CAS from cassava where studies over a temperature range of 20–45°C showed that the enzyme activity decreased significantly above 40°C [51]. A sharp decline in enzyme activity for *S. bicolor*  $\beta$ -CAS at 35°C could imply that this enzyme is sensitive to thermal inactivation, a process which apparently results from thermally induced transitions of the native structure which leads to the exposure of hydrophobic surfaces and irreversible protein association. Consequently, an enzyme loses its compact three-dimensional structure at extremely high temperature leading to loss of activity.

The optimum pH values reported for  $\beta$ -CAS from different sources fall within the alkaline pH of 8.0–10.0. Hendrickson and Conn [28] reported an optimum pH of about 9.5 for  $\beta$ -CAS from 10-day-old etiolated blue lupine seedlings. The optimum pH obtained for  $\beta$ -CAS from the cyanide-producing bacterium, *Chromobacterium violaceum* was found to be pH 9.15, with diethanolamine-HCl as the preferred buffer [59]. Similarly,  $\beta$ -CAS purified from the leaves of spinach (*S. oleracea*) exhibited a single pH optimum at around pH 9.0–9.5 with Tris-HCl buffer [50] and pH optimum for  $\beta$ -CAS from immature seeds of *V. angustifolia* was found to be pH 9.4–9.5, also with Tris-HCl buffer [50].  $\beta$ -CAS from potato tubers was reported to have an optimum pH of 8.0–9.0 [61]. An alkaline pH of 9.0 for optimum activity was reported for  $\beta$ -CAS from grasshopper (*Z. variegatus*) [37]. The optimum pH obtained for *S. bicolor*  $\beta$ -CAS was 8.5, which also falls within the alkaline pH range.

Hendrickson and Conn [28] investigated the effect of salts on the activity of  $\beta$ -CAS purified from the

mitochondria of 10-day-old etiolated blue lupine seedlings and no effect was observed when  $\text{NH}_4^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , acetate, or chloride ions were incubated with the enzyme. However, salts of  $\text{NH}_4^+$  and metal ions such as  $\text{Na}^+$ ,  $\text{K}^+$  were reported to relatively stimulate the activity of  $\beta$ -CAS purified from the cyanide producing bacteria, *C. violaceum* with 1 mM  $\text{K}^+$  causing 15%–20% stimulation of activity; divalent ions such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  also had little effect, with 10 mM  $\text{Ca}^{2+}$  causing 10% stimulation of activity [59]. Similar observation was made for  $\beta$ -CAS from germinating seeds of *S. bicolor* with salts such as NaCl, KCl, and  $\text{NH}_4\text{Cl}$  causing about 15%–20% stimulation of activity at 1 mM–10 mM concentrations.  $\text{MgCl}_2$ ,  $\text{CaCl}_2$  and  $\text{MnCl}_2$  which are divalent metal salts also stimulated the enzyme activity, with 10 mM concentration causing about 10% stimulation. The influence of these metals on enzyme activity is probably due to the fact that they enhance the protein folding thereby enhancing catalysis. Also, there is also the possibility that  $\beta$ -CAS is a metal activated protein as the inclusion of these metals allowed for enhanced biocatalysis.

Activity of *S. bicolor*  $\beta$ -CAS was greatly affected by iodoacetamide and mercuric chloride which are known site-specific inhibitors (Table 4). Iodoacetamide can inactivate an enzyme by reacting with a critical cysteine residue [57]. The reaction catalyzed by  $\beta$ -CAS begins with binding of cysteine to the active site; pyridoxal-5-phosphate (PLP) co-factor in the  $\beta$ -CAS structure identifies the active site. In the first half reaction, the  $\alpha$ -amine of the cysteine reacts with the Cys-49 of the PLP-Lys-95 Schiff base to release the Lys residue. Formation of PLP-Cys allows Lys-95 to act as a general base in the  $\alpha$ ,  $\beta$ -elimination of sulfide resulting in the formation of the  $\alpha$ -aminoacrylate intermediate [60–62]. The chelator EDTA caused more than 90% of inhibition at 1 mM concentration. Total inhibition of activity was observed for  $\beta$ -CAS from *C. violaceum* at 380  $\mu\text{M}$ . The inhibition was 85% relieved by 2 mM  $\text{Mg}^{2+}$  suggesting that a divalent metal was required for activity [59].

## Conclusion

Pathways for the detoxification of cyanide have been studied in many plants. The results obtained from this study shows that the purified  $\beta$ -cyanoalanine synthase from the germinating sorghum seeds is present to maintain cyanide homeostasis produced during germination. The purified  $\beta$ -cyanoalanine synthase in this study has biochemical and catalytic properties similar to that from other sources and these characteristics makes it unique

in the cyanogenesis during germination of the sorghum. The possible role of  $\beta$ -cyanoalanine synthase in cyanide removal via bioremediation will be further exploited as well as investigating the possible roles other cyanide removing enzymes (rhodanese and mercaptopyruvate transferase) play in this seed.

**Conflict of interest statements:** The authors declare no conflict of interest.

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