TISSUE DISTRIBUTION AND BIOCHEMICAL CHARACTERIZATION OF BETA-CYANOALANINE SYNTHASE FROM COMMON BEAN SEEDS (PHASEOLUS VULGARIS)

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ABSTRACT

Cyanide is produced throughout a plant's life cycle, its production increases during certain developmental stages such as seed germination, seedling elongation, fruit ripening and senescence. Beta-cyanoalanine synthase is the most important cyanide metabolizing enzyme found in plants. This work is aimed at studying the tissue distribution, partial purification and some physicochemical properties of beta-cyanoalanine synthase from bean seeds (Phaseolus vulgaris). Beta-cyanoalanine synthase was isolated and partially purified using a combination of ammonium sulphate precipitation, desalting on Sephade X G-10 and gel filtration chromatography on Sephacryl S-200 column. The biochemical characteristics of the enzyme were investigated. Results obtained from this work showed that beta-cyanoalanine synthase is more concentrated in the seeds (20.53 nmol/H S/mg) when compared to the cotyledons (10.08 nmol/H S/mg) and the seed coats (5.82 nmol/H S/mg). The partially purified enzyme showed a specific activity of 26.77 nmol/H S/mg and an apparent molecular weight of about 60,000Da, Kc values for cyanide and L-cysteine of 0.741 mM and 1.724 mM respectively. The V max value obtained for cyanide was 25.00 nmol/H S/ml/minwhile that of L-cysteine was 666.67nmol/H S/ml/min. The enzyme showed an optimum temperature of 40℃ and optimum pH of 10.0. Studies on the effect of chloride salt indicated that NaCl and MnCl had strong inhibitory effect on the enzyme; NH Cl had slight negative effect while KCl and ZnCl activated the enzyme dose dependently. This study showed the presence of beta-cyanoalanine synthase in bean seeds which is believed to function in the detoxification of cyanide produced in its tissues especially during germination.

Key words: beta-cyanoalanine, synthase, bean seeds, tissues, cyanide, seed coat

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INTRODUCTION

Beta-Cyanoalanine synthase (β-CAS; EC 4.4.1.9) catalyzes the conversion of cyanide and cysteine to beta-cyanoalanine. The activity of this enzyme has been detected in a broad spectrum of species among bacteria (McAdam and Knowles, 1984), plants (Maruyama et al., 1998; Han et al., 2007), and insects (Meyers and Ahmad, 1991; Ogunlabi and Agboola, 2007). In insects, β-CAS activity is located principally in mitochondria, which are the main target for cyanide toxicity, and plays a pivotal role in cyanide detoxification (Meyers and Ahmad, 1991). In plants, ethylene synthesis by 1-aminoctylopropane-1-carboxylic acid oxidase results in the production of cyanide (Peiser et al., 1984). Nevertheless, cyanide does not accumulate in non-cyanogenic plants, even in tissues producing ethylene at a very high rate, because of the involvement of β-CAS in cyanide fixation (Yip and Yang, 1988). β-CAS is widely distributed in higher plants including; cabbage, lima bean (Miller and Conn, 1980), tobacco (Liang, 2003), β-CAS appears broadly distributed across the angiosperms as the activity of the enzyme has been detected in numerous plant species. The main physiological role of β-CAS in higher plants has been suggested to be for cyanide (CN) assimilation and detoxification (Warrilow and Hawkesford, 1998; Yu et al., 2012), contribution to various activities, such as plant growth and development (Esashi et al., 1996, Maruyama et al., 1996; Orace et al., 2008, Yu et al., 2012) and...
programmed cell death and stress management (Samuilov et al., 2002). β-CAS primarily metabolizes ethylene-associated cyanide and activity of the pathway increases concomitantly with increases in ethylene synthesis to provide cyanide homeostasis (Takahashi et al., 2006). Several studies have demonstrated that the synthesis of amino acids resulting from cyanide detoxification by the β-CAS also fulfils a fundamental biological role during seed germination (Esashi et al., 1996; Maruyama et al., 1997; Selmar et al., 1988). Esashi et al. (1991) reported that prior to and during seed germination, there is increased evolution of endogenous hydrogen cyanide (HCN) as the cyanogetic nitrogen is liberated. Cyanide is therefore thought to be involved in breaking seed dormancy and promoting germination (Bogatek and Lewak, 1991; Esashi et al., 1996; Maruyama et al., 1996; Oracz et al., 2008).

Phaseolus vulgaris, also known as the common bean, is an herbaceous annual plant grown worldwide for its edible dry seeds; it is a highly variable species that has a long history of cultivation. Low cyanide concentrations have been reported for various species of bean ranging from 0.04 mg/100g (Chaudhary and Sharma 2013; Difo, 2014; Onwuamanam et al., 2014) to about 56.30 mg/100g (Effiong and Umore, 2011). β-cyanoalanine synthase is one of the most important variable species that has a long history of biosynthesis of amino acids resulting in cyanide homeostasis (Takahashi et al., 2008). This was kept for 1 hour with occasional stirring until all the salt had dissolved completely in the supernatant. The mixture was left in the fridge for about 12 hr, followed by centrifugation at 15,000 rpm for 30 min at 4 °C. The supernatant was discarded and precipitate was collected and resuspended in a small amount of 50 mM Tris buffer, pH 10.

Desalting by on Sephadex G-10
Five grams (5 g) of Sephadex G-10 resin was swollen overnight and then rinsed several times with eluting buffer until equilibration was achieved. This was followed by the packing of the resin into a 1.5 x 20 cm column. 20 ml of the ammonium sulphate precipitate sample was layered on the column. The column was eluted with 100 ml of 50 mM Tris buffer, pH 10. Fractions of 5 ml were collected from the column at a rate of 25 ml per hour. The protein was monitored spectrophotometrically at 280 nm. The fractions were also assayed for β-CAS activity. The active fractions were pooled and immediately dialysed against several changes of 50 % glycerol to concentrate the enzyme.

Determination of effect of temperature and pH on the partially purified Phaseolus vulgaris β-CAS
The effect of temperature was determined by incubating the reaction mixtures and the enzyme at temperatures ranging from 30-80 °C for 5 min before the assay was conducted while the thermal stability of Phaseolus vulgaris β-CAS was studied using the method of Ogunlabi and Agboola (2007) at 30°C and 40°C. The effect of pH on β-CAS activity was studied using several buffers at 10 mM concentrations that ranged from pH 3.0 to 12. The β-CAS activity was routinely determined using the method of Ogunlabi and Agboola (2007).

Effect of Salts on the Phaseolus vulgaris β-CAS
The effect of chloride salts on P vulgaris β-CAS
Purification of \( \beta \)-CAS from Bean seeds (Phaseolus vulgaris)

The summary of the various purification procedures is shown in Table 1. The specific activity of the crude enzyme was 9.46 nmol/H \( \text{S} \)/mg of protein, the value was almost tripled to 26.77 nmol/H \( \text{S} \)/mg of protein after gel filtration on sephadex G-200. The elution profile of the gel filtration on sephadex G-200 showed that \( \beta \)-CAS from Bean seeds has two isoforms (\( \beta \)-CAS A and B) with the B isoform having smaller specific activity (6.05 nmol/H \( \text{S} \)/mg) compared to the A Isoform (26.77 nmol/H \( \text{S} \)/mg) as shown in Fig. 3.

Characterization of \( \beta \)-CAS from bean seeds (Phaseolus vulgaris)

The native molecular weight of \( \beta \)-CAS from Bean seeds (Phaseolus vulgaris) was estimated to be 66 kDa as shown in Fig. 4. The Km and V max of \( \beta \)-CAS from Bean seeds (Phaseolus vulgaris) estimated by Lineweaver-Burk double reciprocal plot for cyanide and L-cysteine were 0.741 mM and 1.724 mM respectively and 25.00 nmol/H \( \text{S} \)/min and 666.67 nmol/H \( \text{S} \)/min respectively as shown in Fig. 5 and 6. The enzyme had its maximum activity at a temperature of 40°C and it was stable at this temperature for 30 minutes after which the activity drops to about 50%, this is as shown in Fig. 7 and 8. The optimum pH obtained for \( \beta \)-CAS from Bean seeds (Phaseolus vulgaris) was 10.0 (Fig. 9).

Exposure of \( \beta \)-CAS from Bean seeds (Phaseolus vulgaris) to some chloride salt showed that NaCl and MnCl, had strong inhibitory effect on the enzyme. NHCl had slight negative effect while KC1 and ZnCl, activated the enzyme.

<table>
<thead>
<tr>
<th>Table 1: Summary of the purification processes for ( \beta )-cyanoalanine synthase from bean seeds (Phaseolus vulgaris)</th>
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<tr>
<td>Volume (ml)</td>
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<tr>
<td>Crude enzyme</td>
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<td>(NH4)(2)SO(4) Precipitate</td>
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<td>Deastring on Sephadex G-10</td>
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<td>Gel filtration on Sephadex G-200</td>
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A represents the enzyme that eluted as the major peak
B represents the enzyme that eluted as the minor peak
Figure 3: Elution profile of the gel chromatography of bean seed β-cyanoalanine synthase on Sephadex G-200 column (1.0 x 40 cm). Elution was carried out using 50 mMTris-HCl buffer, pH 10. Protein contents were determined by measuring the absorbance at 280nm. Fractions containing enzyme activity were pooled and dialysed against 50% glycerol.

Figure 4: Calibration curve for the determination of native molecular weight of bean seed β-cyanoalanine synthase. The following protein standards were used at 5 mg/ml each, BSA (66 kDa), lysozyme (13 kDa), egg albumin (44 kDa) and g-Globulin (150 kDa). The void volume of the column was determined using blue dextran (2 mg/ml).

Fig. 5. Lineweaver-Burk plot for the determination of the kinetic parameters (apparent $K_m$ and $V_{max}$) for cyanide of *P. vulgaris* β-CAS. The Michaelis-Menten constant for cyanide was determined by the variation of the concentration of sodium cyanide (NaCN) between 1.0-2.5 mM while the concentration of L-cysteine was held constant at 10 mM.

Fig. 6. Lineweaver-Burk plot for the determination of the kinetic parameters (apparent $K_m$ and $V_{max}$) for L-cysteine of *P. vulgaris* β-CAS. The Michaelis-Menten constant for L-cysteine was determined by varying the concentration of L-cysteine between 1.0 and 2.5 mM while sodium cyanide (NaCN) was held constant at 10 mM.
Effect of temperature on \( P. \) vulgaris \( \beta \)-CAS. The enzyme was assayed between 30 to 80 °C.

Fig. 8. Thermal stability studies of \( P. \) vulgaris \( \beta \)-CAS. The enzyme was assayed at 30 and 40 °C for one hour.

Fig. 9. Effect of pH on \( P. \) vulgaris \( \beta \)-CAS. The enzyme was assayed between pH 3.0 to 12.

Fig. 10. Effect of chloride salts on \( P. \) vulgaris \( \beta \)-CAS. The salts used were \( \text{NaCl}, \text{MnCl}_2, \text{NH}_4\text{Cl}, \text{KCl} \) and \( \text{ZnCl}_2 \). The experimental control had no salts and its activity was regarded as 100%.
Discussion and Conclusion

Cyanide is produced normally by several biochemical processes in plants (Peisetel et al., 1984), its production in an uninterrupted condition is quite slow (e.g., <10 μM in nature), but the quantity of cyanide being produced differs depending on the species, developmental stage of the plant, and growing conditions (Manning, 1986; Yip and Yang, 1988; Vahala et al., 2003). Cyanide is a potent cytotoxic agent, especially for cytochrome oxidase and other vital metabolic processes such as respiration (Vennesland et al., 1982). Plants detoxify cyanide by assimilating this molecule directly into primary metabolism via the β-cyanoalanine pathway mediated by β-CAS and cysteine synthase. The result showed the presence of β-CAS in the seeds, seed coat, and cotyledons of the germinated bean seeds with the highest concentration in the seeds, the expression of this enzyme remarkably reduced after the third day of growth. This could be as a result of the enzyme having its peak function when there is active growth such as the shoot elongation and production of secondary metabolites after germination. There were further drastic drops after the sixth day (data not shown). This result corroborates the work of Oracz et al. (2008) that cyanide may be involved in breaking seed dormancy and promoting germination, since increase in cyanide increases in β-CAS. The purification of P. vulgaris β-CAS from cassava seeds using a combination of ammonium sulphate precipitation and gel filtration on Sephadex G-200 yielded two isoenzymes (major and minor peaks) of P. vulgaris seeds β-CAS, β-CASA and β-CAS-B with specific activities of 26.77 and 1.86 nmolH₂S/mg respectively (Table 1). Maruyama et al. 1996 reported three isofoms for cocklebur seeds cystolic β-CAS, Phaseolus vulgaris β-CAS A was used for the further biochemical characterization studies. The specific activity of β-CAS was lower than that of β-CAS in blue lupine seedlings and shoots reported as 33.8 nmolH₂S/mg (Hendrickson and Conn, 1969) and 43.5 nmolH₂S/mg (Akopyan et al., 1975) but higher than those of apple seed with a specific activity of 12.0 nmolH₂S/mg (Han, et al., 2007) and rice seed 13.5 nmolH₂S/mg (Lai, 2007). Higher activities has been reported for cyanogetic plants; Maruyama et al. (1996) reported a specific activity for purified cocklebur seed β-CAS of 90.3 nmolH₂S/mg while Elias et al. (1997), reported specific activity of 108, 105 and 150 nmolH₂S/mg for β-CAS from cassava leaf, rind and tuher respectively.

The apparent molecular weight obtained for P. vulgaris β-CAS was 60 kDa. Previous report on the native molecular weight of β-CAS from several sources such as spinach, blue lupine seedlings and cassava leave fall within 50 to 62 kDa (Hendrickson and Conn, 1969; Hatfeld et al., 2000). Our result falls within these reported values. Lower native molecular weight has been reported from those of β-CAS from rice seeds of 36.5 kDa (Lai, 2007), apple seeds 40.5kDa (Han, 2007). There are established reports that β-CAS exists as homodimers with subunit molecular weights between28 and 35 kDa (Elias et al., 1992; Maruyama et al., 1996; Ikegami et al., 1988a; Hasegawa et al., 1995).

There are several reports on the kinetic properties of β-CAS from various sources. The Vₐ₅₀ value of P. vulgaris β-CAS for cyanide was determined to be 0.79 μM. The Km values of P. vulgaris β-cyanoalanine synthase were obtained by Ikegami et al. (1988a); Ikegami et al. (1988b) and Maruyama et al. (1996) for pea vine, spinach and cocklebur cys 2nd cys 3 with km values of 0.51, 0.73, 0.73 and 0.63 mM respectively. Lower Km values for cyanide has been reported from those of β-CAS from rice seeds of 0.51, 0.73, 0.73 and 0.63 μM (Maruyama et al., 2001), rice 0.27 mM (Lai, 2007) and Spinach 0.1mM (Hatfeld et al., 2000) while Elias et al. (1997) reported high Km values for β-CAS from the leaf, rind and tuher of cassava as 7.0, 8.0 and 5.0mM. The Km values for cysteine of most reported β-CAS from different plant species showed higher values indicating that the enzyme has higher affinity for cyanide than for L-cysteine. Our result showed a km of 1.724 mM for L-cysteine, this result compares well with the works of Ikegami et al. (1996); Maruyama et al. (1996); Elias et al. (1997); Hatfeld et al. (2000); Maruyama et al. (2001); Lai, (2007). P. vulgaris β-CAS gave a Vmax value of 25.00 nmolH₂S/ml/min for cyanide and 666.67 nmolH₂S/5ml/min for L-cysteine. This is indicative of the fact that P. vulgaris β-CAS reaches maximum rate with much smaller amount of cyanide compared to L-cysteine.

P. vulgaris β-CAS exhibited an optimum temperature of 40°C (Fig. 7). This result showed that the enzyme is more heat stable than that obtained from cassava and variegated grasshopper with optimum temperature of 30°C (Sudhakaran et al., 1997, Ogunlabi and Agboola, 2007). Heat stability studies revealed that P. vulgaris β-CAS was relatively stable at 40°C for 30 minutes after which the activity dropped to about 50%. There are few pH optima reported for β-CAS. The result of our work showed P. vulgaris β-CAS has an optimum pH of 10.0. This result is close to that obtained by Hendrickson and Conn, (1969) for blue lupine seedling (pH 9.5) and Ogunlabi and Agboola (2007) for variegated grasshopper (pH 9.0). This result indicated that this enzyme will perform well in alkaline medium. Studies on the effect of chloride salt showed that NaCl and MnCl₂ had strong inhibitory effect on the enzyme; NH₄Cl had slight negative effect while KCl and ZnCl₂ activated the enzyme dose dependently.

The roles of β-CAS in plants have been established, where it is known to play crucial roles during germination, β-CAS is an enzyme required to maintain growth. The presence of this enzyme in virtually all parts of the germinating bean seedlings is indicative of its ubiquitous nature perhaps during active growth. The biochemical properties of this enzyme further highlight the characteristics of P. vulgaris β-CAS. It is recommended that further work is carried out to understand the mechanism of action of this cyanide metabolizing enzyme and the role it plays with other related enzymes such as cysteine synthase, 3-mercaptoproline transferase and rhodanese during germination.

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REFERENCES


