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 β-cyanoalanine synthase from bean seeds (*Phaseolus vulgaris*). β-cyanolalanine synthase was isolated and partially purified using a combination of ammonium sulphate precipitation, desalting on Sephadex 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 β-cyanolalanine synthase is more concentrated in the seeds (20.53 nmol/H_2S/mg) when compared to the cotyledons (10.08 nmol/H_2S/mg) and the seed coats (5.82 nmol/H_2S/mg). The partially purified enzyme showed a specific activity of 26.77 nmol/H_2S/mg and an apparent molecular weight of about 60,000Da, K_m 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_2S/ml/minwhile that of L-cysteine was 666.67nmol/H_2S/ml/min. The enzyme showed an optimum temperature of 40°C and optimum pH of 10.0.Studies on the effect of chloride salt indicated that NaCl and $MnCl_2$ had strong inhibitory effect on the enzyme; NH_4Cl had slight negative effect while KCl and $ZnCl_2$ activated the enzyme dose dependently.This study showed the presence of β-cyanoalanine synthase in bean seeds which is believed to function in the detoxification of cyanide produced in its tissues especially during germination.

Key words: β-cyanolalanine, 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-aminocyclopropane-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; Oracz *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 cyanogenic 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 beans 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 0.04mg/100g(Chaudhary and Sharma2013;Difo, 2014; Onwuamanam *et al.,* 2014) to about 56.30mg/100g *(*Effiong and Umoren, *2011).* β-cyanoalanine synthase is one of the most important cyanide metabolizing enzymes found in plants, it also plays a significant role in germination.Hence, *Phaseolus vulgaris* seeds known to contain some amount of cyanide were investigated for β-CAS activity, the tissue distribution and biochemical properties of this enzyme was also studied. This work will provide information on the possible roles of for β-CAS in germinating *Phaseolus vulgaris* seeds and also provide possible information on its biochemical and kinetic properties.

Materials and Methods

Materials/Reagents

All chemicals and reagents used for this work were of analytical grades and were obtained from either Sigma chemical company, St. Louis, U.S. A. or British Drug House BDH, Poole, England. Apparently healthy bean seeds were purchased in Ikenne Market, identified and authenticated in the Department of Botany, Obafemi Awolowo University, Ile-Ife

Germination of bean seeds (*Phaseolus vulgaris)* and isolation of β-cyanoalanine synthase

The bean seeds used were steeped for 24 hours and thereafter grown with periodic moistening for six days. A portion of the germinating seeds were withdrawn at the 1st, 3rd and 6th days of growth for analysis. The seed coats and cotyledon were separated from the seeds and weighed, carefully homogenized using mortar and pestle in 2.5 volumes of 0.1M Tris-HCl buffer pH 8.0and then centrifuged at 10000 x g for 30 minutes using a Sorvall super speed RC2-B cold centrifuge. The supernatants were decanted and used as the crude enzyme. The tissue distribution of β-CAS was done by assaying for the enzyme in the seeds, cotyledons and seed coats, the activity of the enzyme in these tissues were used as the concentration of the enzyme in the various tissues.

β-cyanoalanine synthase Assay

The enzyme assay was done using little modification of the methods of both Hendrickson and Conn (1969) and Yip and Yang (1988). Assay was performed in a 10 ml serum bottle with a rubber cork. The assay mixture contained 1 ml of the substrate solution (25 mM L-cysteine and 25 Mm NaCN in 0.1 M Tris–HCl buffer, pH 8.5) and 1 ml of 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 of the supernatant was read at 650 nm. The amount of hydrogen sulphide produced was calculated using the correlation by Hendrickson and Conn (1969) in which A_{650} of 1.0 is equivalent to 0.5 nmol of hydrogen sulphide 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.

Determination of Protein Concentration

The protein concentration of the samples was determined according to Bradford (1976) using BSA as the standard protein.

Enzyme Purification

Ammonium Sulphate Fractionation

The crude enzyme obtained above was brought to 70 % ammonium sulphate saturation (436 g/L) by the slow addition of solid ammonium sulphate as described by Ezima *et al.* (2007). 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 mMTris buffer, pH 10.

Desalting by on Sephadex G-10

Five grams (5 g) of Sephadex G-10 resin was swollen overnight and then rinsed severally 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 50mMTris buffer, pH 10. Fractions of 5 ml were collected from the column at a rate of 25 ml per hour. Protein was monitored spectrophotometrically at 280nm. 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.

Gel filtration on Sephadex G-200 column chromatography

The purification of the enzyme by gel filtration was done according to the method of Elias *et al* (1997) with little modifications. Twenty grams (20 g) of Sephadex G-200 resin was swollen overnight and equilibrated with 50mMTris buffer, pH 10. This was followed by the packing of the resin into a 2.5 x 40 cm column. 20 ml of the post CM-Sephadex sample was layered on the column and eluted with 200 ml of 50 mM Tris buffer, pH 10). Fractions of 5 ml were collected from the column at a rate of 10 ml per hour. Protein content was monitored spectrophotometrically at 280nm. 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 apparent molecular weight on Sephacryl S-200 gel filtration chromatography column

The apparent molecular weight of the native enzyme was determined by gel filtration on Sephacryl S-200 Column (1.5 x 70 cm) using the method of Andrews (1964). 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).

Determination of the kinetic parameters for *β-CAS P. vulgaris*

The kinetic parameters (apparent K_m and V_{max}) were determined from the double reciprocal plots as described by Lineweaver-Burk (1934). The Michaelis-Menten constant for sodium cyanide (NaCN) was determined by the variation of the concentration of NaCN between 1.0-2.5 mM while the concentration of L-cysteine was held constant at 10 mM while that for L-cysteine was determined by varying the concentration of L-cysteine between 1.0 and 2.5 mM while NaCN was held constant at 10 mM.

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 5minute 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

was studied using the method of Ezima and Agboola (2007) with little modifications at final concentrations of 1.0, 2.5 and 5.0 mM of each salt. The salts used were NaCl, $MnCl_2$, NH_4Cl, KCl and $ZnCl_2$. The experimental control had no salts and its activity was regarded as100%. All measurements were made relative to the control.

Results

Distribution of *β-CAS in Bean seeds (Phaseolus vulgaris)*

The results of the tissue distribution of β-CASin the seeds of *Phaseolus vulgaris* and the distribution of the enzyme during different days of germination are given in Fig. 1 and 2. The result showed that β-CAS is more concentrated in the seed than the cotyledon and the seed coat.

Purification of β-CASfrom 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_2S /mg of protein, the value was almost tripled to 26.77 nmol/H_2S/mg of protein after gel filtration on sephadex G-200. The elution profile of the gel filtration on sephadex G-200 showed that *β-CAS from Bean seeds has two isoforms (β-CAS A and B) with the B isoform having smaller specific activity(6.05* nmol/H_2S/mg*) compared to the A Isoform (*26.77 nmol/H_2S /mg*) as shown in Fig. 3.*

Characterization of β-CASfrom bean seeds (Phaseolus vulgaris)

The native molecular weight of *β-CAS from Bean seeds (Phaseolus vulgaris)*was estimated to be 66 kDa as shown in Fig. 4. The Km and V max *of β-*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 25.00nmol/H_2S /min and 666.67nmol/H_2S /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 30minutes after which the activity drops to about 50%, this is as shown in Fig. 7 and 8. The optimum pH obtained for *β-CAS from Bean seeds (Phaseolus vulgaris*) was 10.0 (Fig.9). Exposure of *β-CAS from Bean seeds (Phaseolus vulgaris*) to some chloride salt showed that NaCl and $MnCl_2$ had strong inhibitory effect on the enzyme,NH_4Cl had slight negative effect while KCl and $ZnCl_2$ activated the enzyme.

Tables and figures

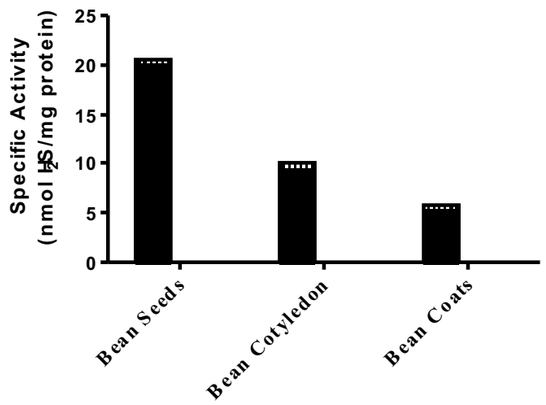


Fig. 1:Tissue distribution of β-cyanoalanine synthase in germinating bean seeds (*Phaseolus vulgaris*)

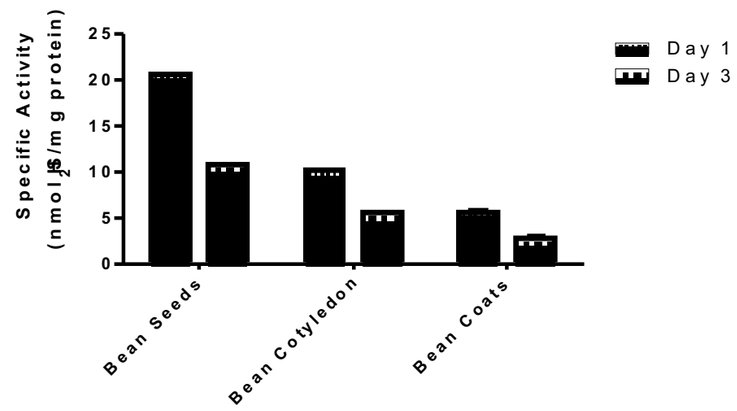


Fig. 2: Distribution of β-cyanoalanine synthase in germinating bean seeds (*Phaseolus vulgaris*) during germination.

Table 1: Summary of the purification processes for β-cyanoalanine synthase from bean seeds (*Phaseolus vulgaris)*

	Volume (ml)	Activity (nmol/H_2S/ ml/min)	Protein (mg/ml)	Total Activity (nmol/H_2S)	Total Protein (mg)	Specific Activity (nmol/H_2S/mg)	Yield (%)	Purification Fold
Crude enzyme	250.00	54.00	5.71	13500.00	1426.48	9.46	100.00	1.00
$(NH4)_2SO4$ Precipitate	125.00	75.00	6.77	9375.00	845.59	11.09	59.60	1.17
Desalting on Sephadex G-10	75.00	157.50	11.06	11812.50	829.41	14.24	58.14	1.50
Gel filtration on Sephadex G-200	[A]60.00	167.50	6.26	10050.00	375.41	26.77	26.31	2.83
	[B]20.00	12.42	2.05	248.40	41.00	6.05		

A represents the enzyme that eluted as the major peak
B represents the enzyme that eluted as the minor peak

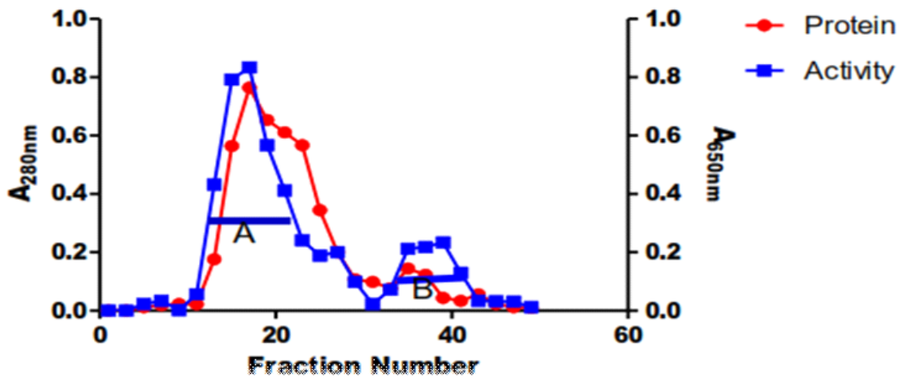


Figure 3: Elution profile of the gel chromatography of bean seed β-cyanoalanine synthase on Sephadex G-200 column(1.0 x 40 cm). Elution was carried out using 50 mMTris-HCl buffer, pH 10. Protein contents were determined by measuring the absorbance at 280nm. Fractions containing enzyme activity were pooled and dialysed against50% glycerol.

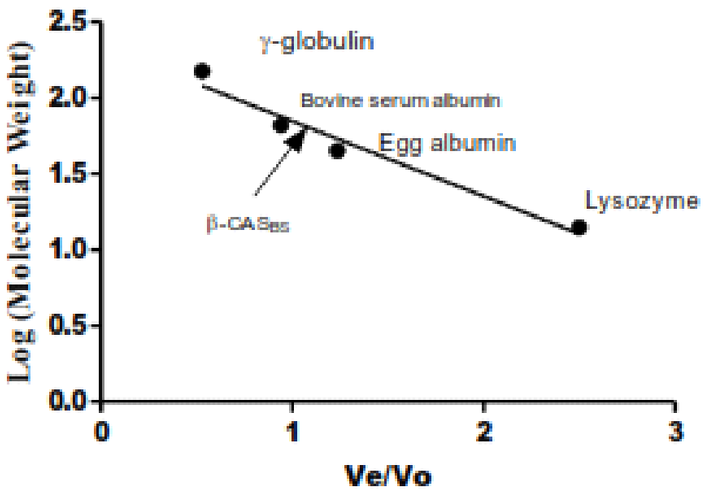


Figure 4: Calibration curve for the determination of native molecular weight of bean seed β-cyanoalanine synthase. The following protein standards were used at 5 mg/ml each, BSA (66 kDa), lysozyme (13 kDa), egg albumin (44 kDa) and g-Globulin (150 kDa). The void volume of the column was determined using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

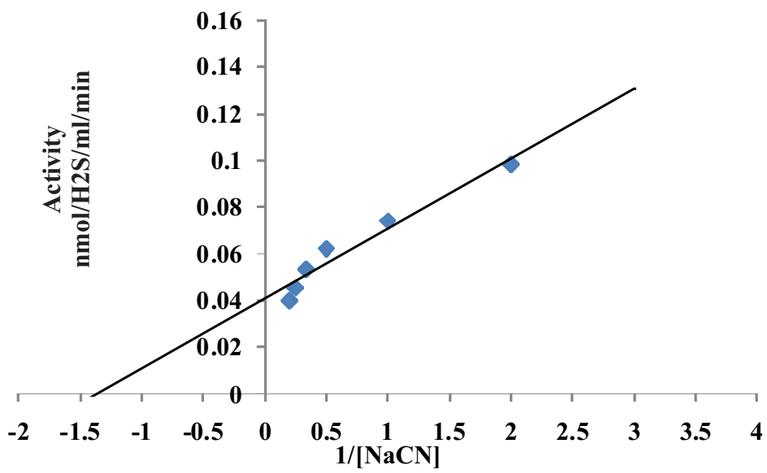


Fig. 5.Lineweaver-Burk plot for the determination of the kinetic parameters (apparent K_m and V_{max}) for cyanide of *P. vulgaris* β-CAS.The Michaelis-Menten constant for cyanide was determined by the variation of the concentration of sodium cyanide (NaCN) between 1.0-2.5 mM while the concentration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

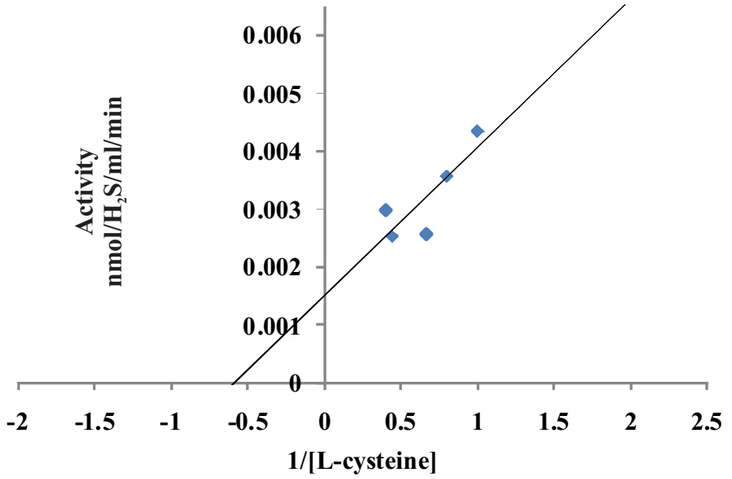
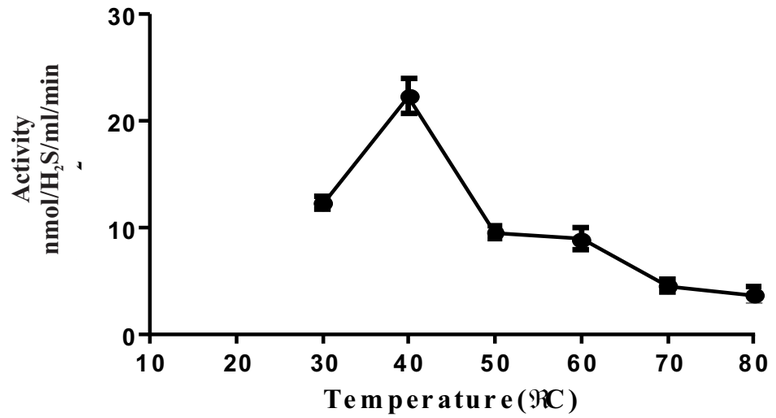


Fig. 6.Lineweaver-Burk plot for the determination of the kinetic parameters (apparent K_m and V_{max}) for L-cysteine of *P. vulgaris* β-CAS, The Michaelis-Menten constant for for L-cysteine was determined by varying the concentration of L-cysteine between 1.0 and 2.5 mMwhile sodium cyanide (NaCN) was held constant at 10 mM.



Effect of temperature on *P. vulgaris* β-CAS. The enzyme was assayed between 30 to 80 °C

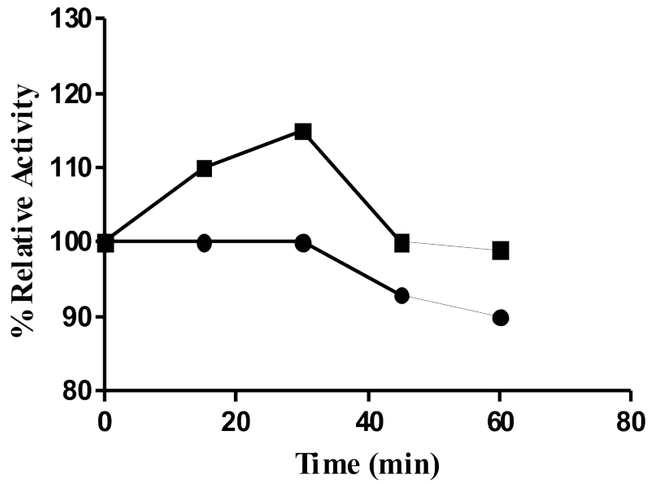


Fig. 8. Thermal stability studies of *P. vulgaris* β-CAS. The enzyme was assayed at 30 and 40 °C for one hour.

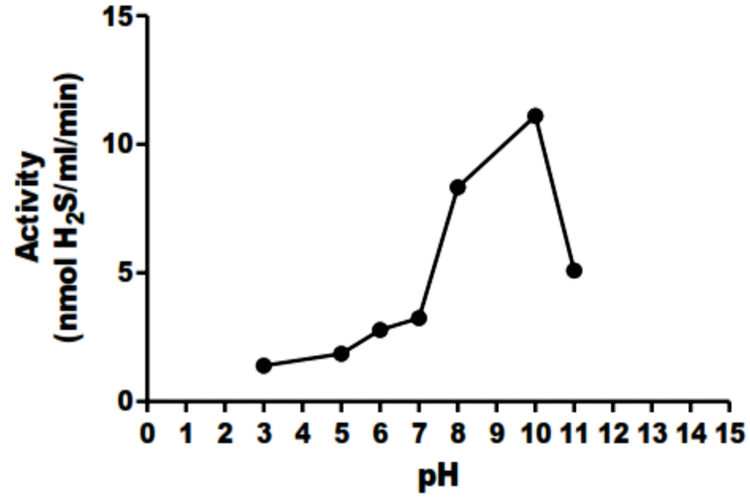


Fig. 9.Effect of pH on *P. vulgaris* β-CAS. The enzyme was assayed between pH 3.0 to 12.

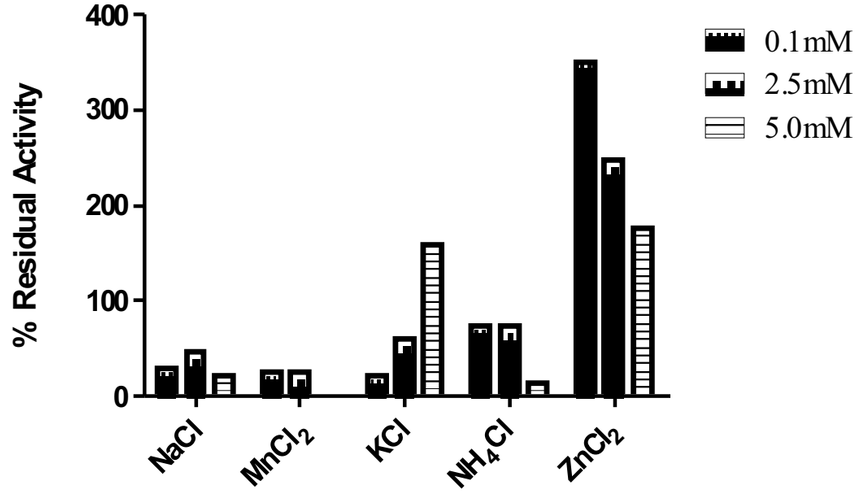


Fig. 10.Effect of chloride salts on*P. vulgaris* β-CAS.The salts used were NaCl, $MnCl_2$, NH_4Cl, KCl and $ZnCl_2$.The experimental control had no salts and its activity was regarded as 100%.

Discussion and Conclusion

Cyanide is produced normally byseveral biochemical processes in plants (Peiser*et al.*.1984),its production inan uninterrupted condition is quite low (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 coxidase 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 hormones necessary for 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 induces increase in *β-CAS*. The purification of *P. vulgaris* seeds β-CAS 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-CAS A and β-CAS B* with specific activities of 26,77 and 1.86 nmolH_2S/mg respectively (Table 1). Maruyama et al 1996 reported three isoforms for cocklebur seeds cytosolic β-CAS.*Phaseolus vulgaris β-CAS A* was used for the further biochemical characterization studies.The specific activity of β-CAS obtained from our study is lower than that of β-CAS in blue lupine seedlings and shoots reported as 33.8 nmolH_2S/mg (Hendrickson and Conn, 1969) and 43.5 nmolH_2S/mg (Akopyan *et al.,*1975) but higher than those of apple seed with a specific activity of 12.0 nmolH_2S/mg(Han, *et al.,*2007) and rice seed 13.5nmolH_2S/mg(Lai, 2007). Higher activities has been reported for cyanogenic plants; Maruyama *et al.,*(1996) reported a specific activity for purified cocklebur seed β-CAS of 90.3 nmolH_2S/mgwhile Elias *et al.,*(1997), reported specific activity of 108, 105 and 150 nmolH_2S/mg for β-CAS from cassava leaf, rind and tuber 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 andConn, 1969; Hatzfeld *et al.,*2000). Our result falls within these reported values. Lower native molecular weight has been reported from those of β-CAS from rice seedsof 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 (Droux *et al*., 1992; Maruyama *et al.,* 1996; Ikegami *et al.,* 1988a, b; Hasegawa *et al.,* 1995).

There are several reports on the kinetic properties of β-CAS from various sources. The K_m value of *P. vulgaris* β-CAS for cyanide was determined to be 0.741 mM, this result compares well with those obtained by Ikegami *et al.,*(1988a); Ikegami *et al.,*(1988b) and Maruyama *et al.,*(1996) for pea vine, spinach and cocklebur cyt 2and cyt 3 with km values of 0.51, 0.73, 0.73 and 0.63 mM respectively. Lower Km values for cyanide has been reported for β-CAS from potatoes 0.134 mM (Maruyama *et al.,*2001), rice 0.27 mM (Lai, 2007) and Spinach 0.1mM (Hatzfeld *et al.,*2000) while Elias *et al.,*(1997) reported high Km values for β-CAS from the leaf, rind and tuber 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.(*1988a); Maruyama *et al.* (1996); Elias *et al.*(1997); Hatzfeld *et al.(*2000); Maruyama *et al,*(2001); Lai, (2007).

P. vulgaris β-CAS gave a Vmax value of 25.00 nmol/H_2S/ml/min for cyanide and 666.67 nmol/H_2S/ml/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 $MnCl_2$ had strong inhibitory effect on the enzyme; NH_4Cl had slight negative effect while KCl and $ZnCl_2$ 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-CASin bean seeds can also be assumed to function in assimilation of cyanide produced during germination,cyanide being an electron transport inhibitor, if allowed to build up may stop ATP synthesis which will undermine germination and eventually kill the seedling, also assimilation of cyanide provides amino acids 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 therefore 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-mercaptosulfotransferase and rhodanese during germination

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