PURIFICATION OF ARGINASE FROM EMBRYONIC AXES OF SOYBEAN (*Glycine max* L.) Merr.

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Abstract

In this research, arginase was extracted from embryonic axes of soybean by using $MnCl_2$ salt solution. Arginase (L-arginine amidinohydrolase, (EC 3.5.3.1) is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of arginine to ornithine and urea. The arginase activity was determined spectrophotometrically by measuring the urea liberated. In diacetylmonoxime method for urea determination, the wavelength of maximum absorption of the colored product was found at 480 nm. The arginase activity was defined as micromole of urea liberated per minute per milileter of enzyme solution. The variation of arginase activity with growth day of embryonic axes of soybean was studied. The maximum arginase activity was found after five day of germination. In the determination of protein content is Biuret method, the wavelength of maximum absorption of copper protein complex was found at 550 nm. After chromatographic separation with Sephadex G-200 gel, the specific activity the relative purity of the enzyme increased about (15) folds from crude to final purification step. The homogeneity of the purified arginase was confirmed by non SDS-PAGE as a single band. In this research effect of metal ions (K^+, Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}) on arginase activities were studied. The Mn^{2+} ion showed the largest activating effect on arginase activities. The presence of manganese ion is essential to get the stable structure of arginase enzyme proteins.

Keywords : arginase, arginine, urea, embryonic axes of soybean, non SDS-PAGE

Introduction

Soybean is known as the "golden bean" of the 20th century (Steech, 1949). Though, soybean is a legume crop, yet it is widely used as oilseed. It is now the second largest oilseed in India after groundnut. It grows in varied agro-climatic conditions. It has emerged as one of the important commercial crop in many countries. Due to its worldwide popularity, the international trade of soybean is spread globally. Several countries such as Japan, China, Indonesia, Philippines, and European countries are importing soybean to

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supplement their domestic requirement for human consumption and cattle feed. Soybean has great potential as an exceptionally nutritive and very rich protein food. It can supply the much needed protein to human diets, because it contains above 40 percent protein of superior quality and all the essential amino acids particularly tryptophan and lysine, similar to cow's milk and animal proteins. Soybean also contains about20 per cent oil with an important fatty acid, lecithin and vitamins A and D. The four percent mineral salts of soybean are fairly rich in phosphorus and calcium. Soybean contains arginase, amylase, urease, lipoxidase, lipase, peroxidase, glucosidase, carboxylase, catalase, ascorbicase, allantoinase, phytase and uricase. It is a good source of arginase. Soybean arginase is employed as an analytical reagent for the estimation of urea in physiological fluids.

Application of arginase enzyme in clinical analysis

Build up of ammonia due to arginase deficiency (Figure 1) is shown. Arginase deficiency is an inherited disorder that causes the amino acid arginine (a building block of proteins) and ammonia to accumulate gradually in the blood. Ammonia, which is formed when proteins are broken down in the body, is toxic if levels become too high. The nervous system is especially sensitive to the effects of excess ammonia. Arginase deficiency belongs to a class of genetic diseases called urea cycle disorders. The urea cycle is a sequence of reactions that occurs in liver cells. This cycle processes excess nitrogen, generated when protein is used by the body, to make a compound called urea (Crombez and Cederbaum, 2005). If urea is not excreted from the body, various health problems will occur. Therefore, arginase is an essential enzyme for the human body which maintains ammonia level.

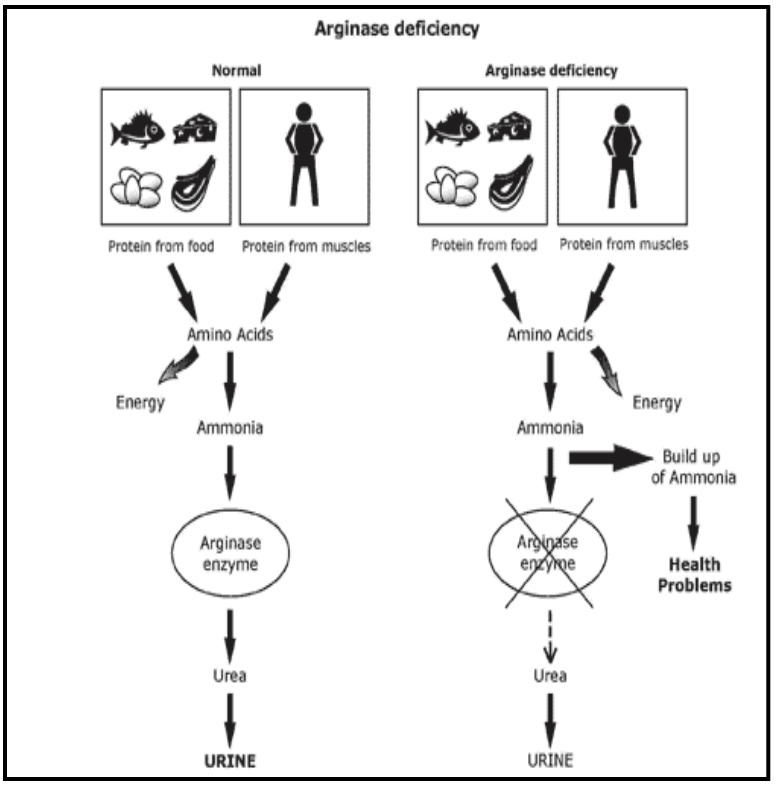

Figure 1: Build up of ammonia due to arginase deficiency

Aim

The aim of this work is to study isolation, purification and characterization of arginase from embryonic axes of soybean.

Materials and Method

In this research, soybean sample was purchased from Bilin Township, Mon State. Soybean (100 g) was wet with water and kept at room temperature. After 1 day germination, embryonic axes of soybean (10 g) were taken and mixed with 25 mL solution mixture of 50 mM phosphate buffer pH 7.5 containing 1 mM $MnCl_2$ and 10 % glycerol. The mixture was blended in a blender for 10 min at room temperature, and then filtered by using gauze (Kang and Cho, 1990). The filtrate was centrifuged at 3000 rpm for 15 min. Finally crude enzyme extract was obtained. Similarly, same procedure was

carried out after 2, 3, 4, 5, 6 and 7 days of germination. The prepared enzyme solutions were kept in refrigerator at 4 °C. The arginase activity was determined spectrophotometrically by measuring the urea liberated at 480 nm. The maximum arginase activity was found after 5 days of germination (Table 1). The wavelength of maximum absorption of copper-protein complex was determined by Biuret method. The wavelength of maximum absorption of copper-protein complex was found at 550 nm. Crude enzyme (1 mL) was puried on a Sephadex G-200 gel filtration column previously equilibrated with the same buffer. The flow rate was adjusted to 2.5 mL per 5 min. A 2.5 mL fraction was collected in each test tube. After collection, the protein content of each test tube was checked by measuring the absorbance at 280 nm by using a UV-visible spectrophotometer. The enzyme in each tube was also measured for arginase activity. The fraction that had the highest arginase activity was pooled. The pooled arginase fraction was measured for protein content by the Biuret method. In this research, determination of molecular weight of the purified arginase was carried out by using non SDS-PAGE electrophoresis technique in European Molecular biology laboratory, Heidel berg Germany. Before electrophoresis, purification of the crude arginase was carried out by Sephacry S-200 gel and Sepharose 6B gel chromatographic techniques. The effect of metal ions (K^{+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}) on arginase activities was determined by UV-visible sepectrophotometric technique.

Results and Discussion

Extraction of Arginase from Embryonic Axes of Soybean at Maximum Arginase Activity

In the present research, the arginase enzyme was isolated from embryonic axes of soybean sample obtained from Bilin Township, Mon State (Figure 2) and was partially purified by solid ammonium sulphate precipitation method. Then, arginase activity was determined by spectrophotometric method. Ammonium sulphate precipitation method was chosen for salt fractionations because of its high solubility in water, lack of toxicity, cheapness, lack of harmful effects on enzyme activity. This phenomenon of protein precipitation in the presence of excess salt is known as salting out. Many types of salts have been employed to effect protein separation and purification through salting out. Of these salts ammonium

sulphate has been the most widely used chemical because it has high solubility and relatively inexpensive. Because enzymes are proteins, enzyme purification can be carried by following the same set of procedure as these for protein, except that some attention must be paid to the consideration of permanent loss of activity due to denaturation under observed conditions. In this research, the embryonic axes of soybean were suspended in phosphate buffer (pH 7.5). The suspension was filtered through four layers of gauze and centrifuged at 3000 rpm for 15 min. After centrifugation, solid ammonium sulphate was added to the supernatant to give 40 % saturation. After addition of ammonium sulphate to 60 % saturation, the crude enzyme protein precipitate was obtained. Solid ammonium sulphate was slowly added to the crude extract under stirring to obtain the desired enzyme. The procedure of salting out of a protein is well known (Seaman, 1963). Since ammonium sulphate has little effect on enzyme activity and in some cases stability of the enzymes, it is useful as the salt of choice in most case. Thus, it was employed in the present work.

Table 1: Variation of arginase activity with growth day of embryonic axes of soybean

Duration of growth (day)	Absorbance at 480 nm	Activity ($\mu mol\ min^{-1}\ mL^{-1}$)
1	0.209	0.183
2	0.228	0.200
3	0.286	0.250
4	0.344	0.301
5	0.402	0.352
6	0.360	0.315
7	0.313	0.274

Figure 2: Photograph of fifth day germination of soybean (*Glycine max* L.) Merr.

Purification of Arginase from Embryonic Axes of Soybean

In this research, arginase activity of crude enzyme solution was determined spectrophotometrically by using diacetylmonoxime method (Varley and Gowenlock, 1980).

The arginase activity by the measurement of urea from arginine is determined by this method. The purification step involves gel filtration on Sephadex G-200 Gel filtration that separates proteins with difference in molecular size. Sephadex types (G10 – G200) are available in different particle size grades, *viz.*, superfine, fine medium and coarse (Wiseman, 1985). Each Sephadex G- type has a different molecular weight over which molecules can be fractionated. Sephadex G-200, superfine is a new kind of gel filtration medium which combines a highly porous gel structure with excellent chemical and physical stability (Azardan *et al*., 2003). In the present research, Sephadex G-200 was used in glass column 2.0 cm x 25 cm, that will fractionate proteins in the molecular weight range of 60,000 to 400,000. Sephadex G-200 was used and its parameters are bead diameter 40-120 µm. The chromatogram of arginase on Sephadex G-200 gel is shown (Table 2 and Figure 3). The protein content of eluate was checked spectrophotometically at 280 nm and the enzyme activity was determined by diacetylmonoxime

method. The fractions of highest activity (43-52 fractions) were pooled and precipitated with ammonium sulphate. The relative purity of the enzyme increased about 15 folds over crude extract (Table 3). The amount of enzyme in an extract may be expressed in terms of enzyme unit EU (Martin, 1993). The most widely used unit of enzyme is defined as one micromole of substrate reacting or product produced per minute under specified conditions. In this work the arginase activity was defined as micromole of urea liberated from arginine substrate per minute of enzyme solution. The EU of arginase from embryonic axes of soybean was determined as 2.153 EU per gram of embryonic axes of soybean.

Table 2: Changes of Protein Content (280 nm) and Arginase Activity with Fraction Numbers

Fraction Number	Absorbance of Protein Content at 280 nm	Arginase activity (μmol min^{-1} mL^{-1})
1	0.075	0.020
3	0.082	0.039
5	0.102	0.041
7	0.138	0.490
9	0.120	0.055
11	0.078	0.028
13	0.094	0.026
15	0.133	0.056
17	0.152	0.060
19	0.120	0.032
21	0.068	0.039
23	0.082	0.014
25	0.083	0.022
27	0.144	0.041
29	0.066	0.029
31	0.133	0.039
33	0.057	0.260
35	0.162	0.053
37	0.217	0.075
39	0.351	0.079
41	0.188	0.063
43	0.310	0.056

Fraction Number	**Absorbance of Protein Content at 280 nm**	**Arginase activity (µmol min^{-1} mL^{-1})**
45	0.555	0.206
47	0.697	0.231
49	0.510	0.185
51	0.310	0.077
53	0.255	0.062
55	0.315	0.091
57	0.397	0.085
59	0.299	0.082
61	0.352	0.071
63	0.295	0.062
65	0.251	0.058
67	0.232	0.055
69	0.222	0.052
71	0.219	0.047
73	0.210	0.040
75	0.205	0.044
77	0.200	0.042
79	0.195	0.039
81	0.185	0.035
83	0.167	0.030
85	0.131	0.031
87	0.120	0.032
89	0.112	0.029
91	0.095	0.027
93	0.084	0.023
95	0.077	0.018
97	0.065	0.015
99	0.055	0.012

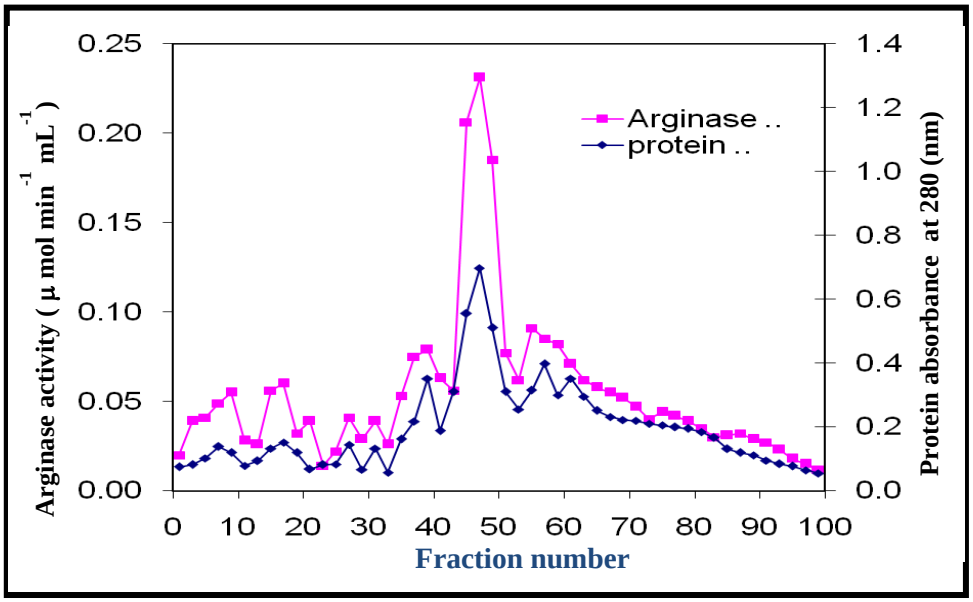

Figure 3: Arginase activity and protein absorbance of separated fractions by gel filtration chromatography

Table 3: Arginase activities, protein contents and specific activities of the enzyme solution at different purification steps

No	Main Steps Purification	Protein Content (mg/mL)	Arginase Activity (unit)	Specific Activity (unit/mg)	Degree of purity (fold)
1.	Crude extract	6.78	1.63	0.09	1
2.	After purification with 40% ammonium sulphate	4.80	0.48	0.10	1.09
3.	After purification with 60% ammonium sulphate	3.30	0.38	0.1	1.25
4.	After passing the Sephardex G-200 column	0.16	0.23	1.43	15.89

Molecular Weight of Purified Arginase Enzyme

The molecular weight of a protein can be determined by a variety of methods (Murry, 1993). The most accurate molecular weights are those obtained from sequence a procedure hardly suitable in routine studies. Usually, molecular weights are obtained by methods involving the analytical ultracentrifuge method and other methods: gel filtration, osmotic pressure, light scattering, electron microscopy, etc., have been employed. Molecular weights are best determined when both the band of interest and the standards appear as sharp, narrow bands so that there is no mistake as to where to measure the migration distance (Hames, 1981). In this work, proteins from the pharmacies molecular weight calibration kit: Thyroglobulin (667,500) Ferritin (439,000), Urease trimer (230,000), β-Glactosidase (139,000) and Fructose-6-phosphate kinase (66,500) were used for molecular weight determination by non SDS-PAGE. The homogeneity of the purified arginase was confirmed by non sodium dodecyl sulphate polyacrylamide gel electrophoresis (non SDS-PAGE). The use of polyacrylamide gel electrophoresis for determining protein molecular weight has become a routine laboratory technique (Pharmacia Inc, 1987). The molecular weight of a protein under investigation was determined by comparing its electrophoresis mobility with that of protein standards of known molecular weights (Barman, 1969). The purified arginase enzyme showed a single band on non-SDS-PAGE where the molecular weight of purified arginase was located near urease trimer, the standard protein (mol.wt. 240,000) after the final purification (Table 4 and Figure 4). An estimated molecular weight of purified arginase from soybean sample was found as 237,137 Dalton from the log of known marker proteins *vs.* R_f values for non SDS-PAGE (Figure 5).

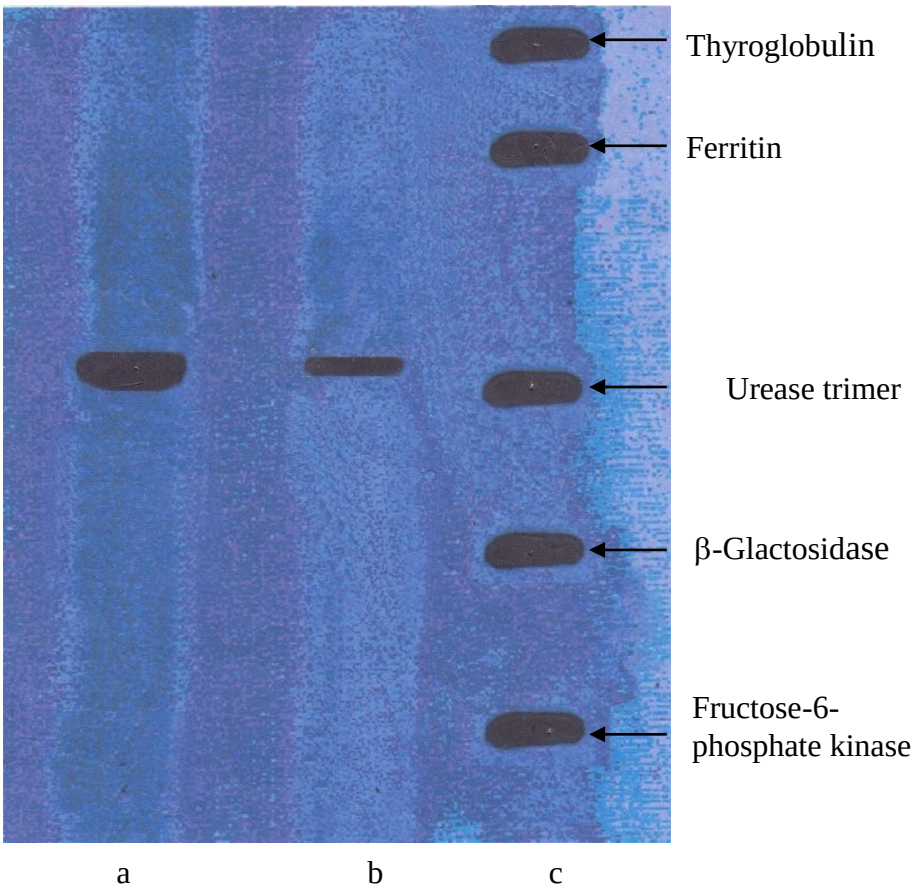

Figure 4: Photograph of non sodium dodecyl sulphate polyacrylamide gel electrophoresis

Lane a purified arginase fraction obtained after sephacryl S-200 gel chromatography

Lane b purified arginase fraction obtained after successive purifications by sephacryl S-200 and sepharose 6B

Lane c high molecular weight maker proteins

Table 4: Relationship between log of Molecular Weight of High Molecular Weight Marker Proteins and Relative Mobility (R_f) values Obtained from Non SDS-PAGE

No.	Standard marker proteins	MW (Dalton)	Log of MW	R_f
1	Thyroglobulin	667,500	5.82	0.05
2	Ferritin	439,000	5.64	0.18
3	Urease trimer	230,000	5.36	0.46
4	β-Glactosidase	139,000	5.19	0.65
5	Fructose-6-phosphate kinase	66,500	4.82	0.86

The R_f value of arginase was found to be 0.44 so that the molecular weight was determined to be 237, 137 Dalton.

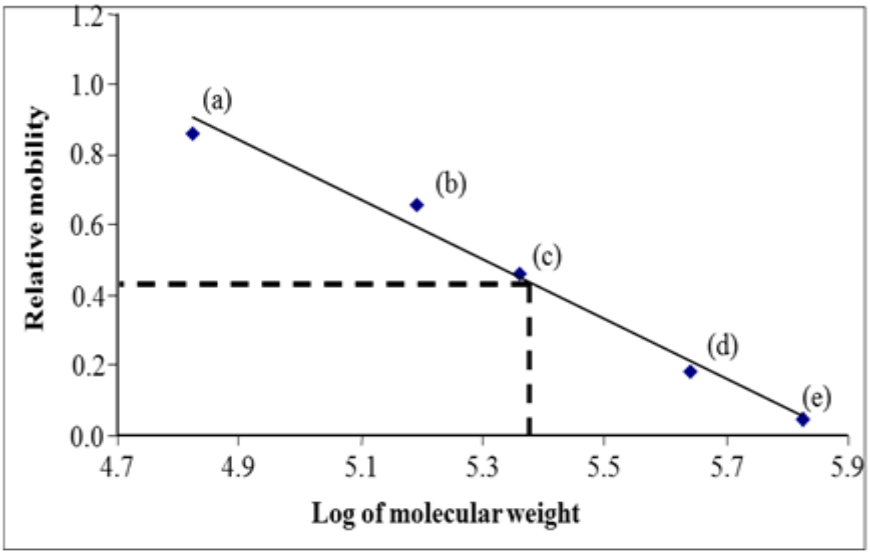

Figure 5: Plot of log of molecular weight marker proteins as a function of relative mobility (R_f) obtained from non SDS-PAGE

(a) Fructose-6-phosphate kinase (b) β-Glactosidase (c) Urease trimer
(d) Ferritin (e) Thyroglobulin

Effects of Mn^{2+}, K^{+}, Cu^{2+}, Co $^{2+}$, Zn^{2+}, $Fe^{2+}$and Ca^{2+} Ions on Arginase Activity

Enzyme activators are molecules that bind to enzymes and increase their activity. Certain enzymes require inorganic ions as cofactors. These inorganic ions are called activators. The most important metal ion activators are Mn^{2+}, K^{+}, Cu^{2+}, Co $^{2+}$, Zn^{2+}, $Fe^{2+}$and Ca^{2+} (Robert, 2000). The function of the metal ion is to stabilize the tertiary and quaternary structures and in some cases the metal ion provides an important reactive group at the catalytic site. For some enzymes, it appears that a preliminary combination of substrate molecule with metal ion to form substrate-metal complexes is necessary prior to the substrate can be bound to the enzyme and then broken down. In this work, the effects of metal ions (Mn^{2+}, K^{+}, Cu^{2+}, Co $^{2+}$, Zn^{2+}, Fe^{2+} and Ca^{2+}) on arginase activity were studied. The Mn^{2+}, K^{+}, Co $^{2+}$ and Ca^{2+} ions behaved as activators, whereas Zn^{2+}, Fe^{2+} and Cu^{2+} ions showed inhibitory effects on arginase activity (Table 5 and Figure 6). In general, salt solution of the presence of alkali and alkaline earth metals can increase the stability of the native structure of the enzyme protein. Consequently, this can lead to increase the enzyme activity. The activating effect of Mn^{2+} ion on arginase activity was related with the arginase structure. Arginase is a manganese containing metalloenzyme in which manganese acts as a cofactor as well as activator in almost all of the reported arginase (Dabir *et al*., 2005).

Table 5: Changes of Arginase Activities with Various Metal Ions

No.	Metal ions (0.01 M)	Arginase activity (µmol min^{-1} mL^{-1})	Degree of activitation (+) or inhibition (-) (%)
1	Without metal	0.238	-
2	K^{+}	0.277	+16.39
3	Ca^{2+}	0.269	+13.03
4	Mn^{2+}	0.453	+90.34
5	Co^{2+}	0.388	+63.03
6	Fe^{2+}	0.091	-61.76
7	Zn^{2+}	0.145	-39.08
8	Cu^{2+}	0.109	-54.20

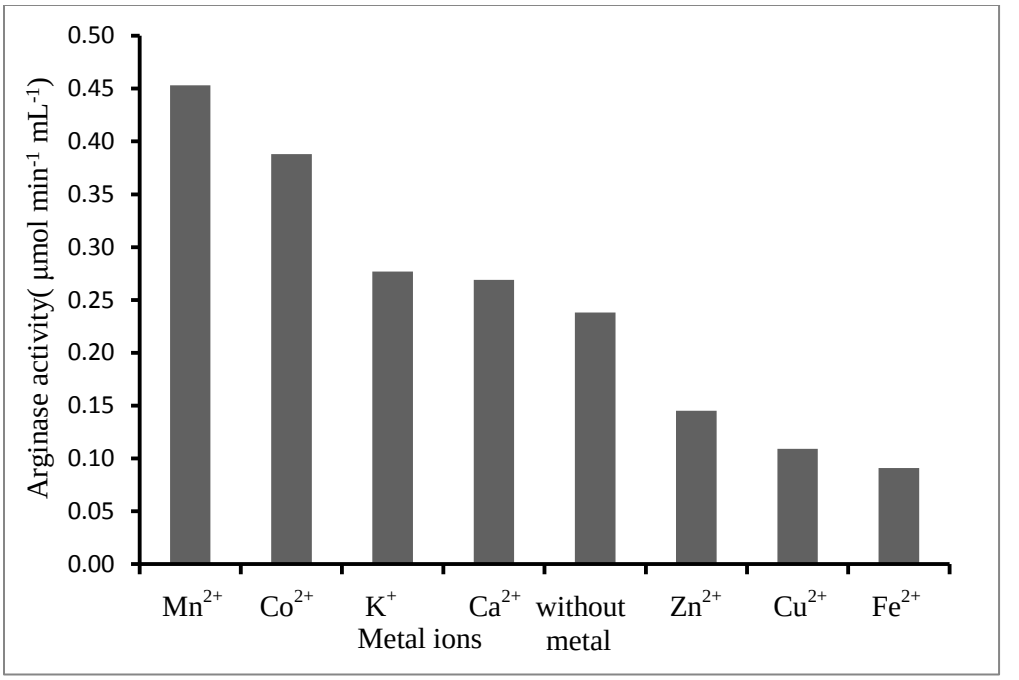

Figure 6: Histogram of arginase activity with respect to various metal ions

Conclusion

In this research, arginase was extracted from embryonic axes of soybean by using $MnCl_2$ salt solution. The arginase activity was determined spectrophotometrically by measuring the urea liberated. The variation of arginase activity with growth day of embryonic axes of soybean was studied. The maximum arginase activity was found at fifth growth day. The crude arginase enzyme solution (170 mL) was obtained from (100 g) of embryonic axes of soybean. After chromatographic separation with Sephadex G-200 gel, the specific activity the relative purity of the enzyme increased about (15) folds from crude to final purification step. The arginase activity was found to be 2.153 EU per gram of embryonic axes of soybean at final purification step. The homogeneity of the purified arginase was confirmed by non SDS-PAGE as a single band. The molecular weight of the purified arginase was determined to be 237,137 Dalton. The Mn^{2+} ion showed the largest activating effect on arginase activities (90.34 % degree of activation). Among the inhibitors (Fe^{2+}, Zn^{2+}, Cu^{2+}), the Fe^{2+} ions give the largest inhibitory effect on

arginase activities (61.76 % degree of inhibition). Degrees of activation were found to be 90.34 and 63.03% for Mn^{2+} and Co^{2+} ions, respectively. Degrees of inhibition were found to be 39.08 and 54.20 % for Zn^{2+} and Cu^{2+} ions, respectively.

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