CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

The chemicals used in the experiments were obtained from the following sources :

L-arginine hydrochloride, boric acid, cupric sulphate, diaminoethane tetraacetic acid (EDTA), 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), indane-trione-hydrate (ninhydrin), magnesium sulphate, manganese sulphate, manganese chloride, potassium dihydrogen phosphate, potassium hydroxide, potassium iodide, potassium nitrate, sodium dihydrogen phosphate, sodium chloride, sodium potassium tartarate, sucrose, sodium tungstate, trisodium phosphate and Tris from British Drug Houses, England.

Bovine serum albumin, carbamyl phosphate, cycloheximide, DEAE-sephadex A-50, 5-fluorouracil, kinetin, 2-mercaptoethanol, DL-ornithine hydrochloride, putrescine dihydrochloride and Triton X-100 from Sigma Chemical Co., U.S.A.

Acetic acid, calcium chloride, diacetyl monoxime, 2-oxoglutaric acid, nicotinic acid, pyridoxine hydrochloride, pyridoxal phosphate, thiamine hydrochloride and trichloro-acetic acid (TCA) from E. Merck and Co., Germany.

2-Chloroethanol, ortho aminobenzaldehyde, spermidine trihydrochloride and spermine tetrahydrochloride from Fluka & Co., Switzerland. Ammonium sulphate, ethyl alcohol, lithium

sulphate, sodium carbonate, sodium bicarbonate, sodium hydroxide from Sarabhai M. Chemicals, India. Ferric chloride, sodium molybdate and zinc chloride from Riedel De Haen AG Seelze Hannover, Germany. Aluminium chloride and ferric sulphate from Albright and Wilson Co., London. L-citrulline from Nutritional Biochemical Corporation, U.S.A. Ethrel from Agromore Ltd., India. Gibberellic acid from Polfa Kunto, Poland and DEAE-cellulose from Brown and Company, Berlin.

N-Carbamyl putrescine was a gift from Dr. T.A. Smith, Long Ashton Research Station, University of Bristol, Long Ashton, Bristol (England).

Plant material

Groundnut (Arachis hypogea L.) seeds were obtained from a local farm in bulk and stored. Seeds used for different developmental stages were also obtained from the same farm.

Collection and separation of seeds into various stages of development

The seeds were removed from the pods and separated into different groups based on the size of the seed. 30-40 seeds were taken in each group and the average fresh weight of the seed was determined. The cotyledons and embryos were separated and a portion of these was used for dry weight determination. The remaining portion was used for determination of the enzymes and protein content.

The samples were collected and analysed several times during the development and maturation of seeds over a period of 2-3 months. The data were then grouped into nine different stages on the basis of the average fresh weight per seed as shown below :

Stage	Average fresh weight/seed (mg)
1	1 - 10
2	11 - 50
3	51 - 100
4	101 - 200
5	201 - 300
6	301 - 400
7	401 - 500
8	501 - 600
9	601 - 800

The developmental stages have been reported in the literature in two different ways, i.e. either as the dry weight/seed or the days after flowering. The days after flowering is not considered a good criterion because the rate of seed development may vary within the same pod, among pods on the same plant and with the time of the year. Since the present studies have been carried out on cotyledon and the embryo separately, it was preferred to represent the stages on

the basis of their fresh weight rather than the dry weight/seed because the pattern of changes in the dry weight was found to differ in the two parts of the seed.

Storage

The pods after harvest were brought to the laboratory and stored at room temperature for a period of one year.

Germination of seeds

Seeds, after surface sterilization with lysol and washing with distilled water, were soaked in beakers for 16 hours in water or other test materials as specified. The seeds were then kept for germination at 24°C in light in Petri dishes on moist filter papers. The time when seeds were kept in Petri dishes, after soaking for 16 hours, was considered as zero hour of germination.

Cultivation of embryo

The embryos were cultivated on a liquid medium as described by Murashige and Skoog (1962). The medium was compounded fresh from the stock solutions (given in Table 1) and diluted so as to yield the specific composition. The pH of the medium was adjusted to 5.7 with potassium hydroxide, 10 ml of the medium was distributed to each Erlenmeyer flask of 100 ml capacity containing a pad of Whatman No. 1 filter paper. The flasks were tightly plugged with cotton wrapped

Table 1 : Composition of the culture medium.

Stock solutions	Constituent	gm/litre	Volume taken for 1 litre of medium(ml)
A	NH_4NO_3	82.5	20
B	KNO_3	95.0	20
C	H_3BO_3	1.24	5
	KH_2PO_4	34.00	
	KI	0.166	
	$Na_2MoO_4.2H_2O$	0.050	
	$CoCl_2.6H_2O$	0.005	
D	$CaCl_2.2H_2O$	88.0	5
E	$MgSO_4.7H_2O$	74.0	5
	$MnSO_4.4H_2O$	4.46	
	$ZnSO_4.7H_2O$	1.72	
	$CuSO_4.5H_2O$	0.005	
F	Na_2 EDTA	7.45	5
	$FeSO_4.7H_2O$	5.57	
G	Thiamine HCl	0.20	5
	Nicotinic acid	0.20	
	Pyridoxine	0.20	
	Glycine	0.20	
	Sucrose		20 g
	Myo-inositol		0.100 g

in guaze cloth and the medium was sterilized by autoclaving at 15 lb/sq.inch for 15 minutes. Chloroethanol and cyclohexiimide, when included in the medium, were separately sterilized by filtering through bacterial filter under sterile conditions and then added to the sterilized medium under sterile conditions. Embryos were obtained from dry seeds, which were surface sterilized with lysol and washed thoroughly with distilled water. Four embryos were then transferred to the filter paper pad in the flask under sterile conditions and allowed to grow at 24°C in light.

Preparation of calcium phosphate gel

Calcium phosphate gel was prepared according to the method described by Keilin and Hartree (1938). 150 ml calcium chloride solution (132 g $CaCl_2.6H_2O$/l) was diluted to about 1600 ml with distilled water and shaken with 150 ml trisodium phosphate solution (150 g of $Na_3PO_4.12H_2O$/l). The mixture was brought to pH 7.4 with dilute acetic acid and the precipitate washed three or four times by decantation with large volume of water (15-20 l). The precipitate was finally washed with distilled water and allowed to stand for about a month. After removing the clear water layer, the gel was shaken and the dry weight per ml was determined and found to be 25 mg/ml.

Preparation of alumina Cγ gel

Alumina Cγ gel was prepared according to the method described by Willstätter and Kraut (1923). 300 g of ammonium sulphate was dissolved in 6.5 l of water, heated at 60°C and 420 ml of 20% (w/v) ammonia was added. The fluid remained slightly alkaline during the subsequent precipitation. Hot solution of 500 g aluminium sulphate in 1 l solution was added with constant stirring. Stirring was continued for 15 min after addition, keeping the temperature to about 60°C. The mixture was diluted to 40 l and the precipitate was allowed to settle and decanted. The precipitate was washed repeatedly by decantation. To fourth wash-water 80 ml of 20% ammonia was added. After 12 to 20 washings, the water remained opalescent. After this point is reached precipitate was washed twice again and allowed to stand several weeks to convert the C form into C form. The dry weight per ml was determined and found to be 28 mg/ml.

Preparation of hydrindantin

Hydrindantin was prepared according to the method described by Duggan (1957).

Ninhydrin (80 g) was dissolved in 2 l of water, and ascorbic acid solution (80 g/100 ml) was added to it with stirring at 90°C. The crystallised material was cooled under

running tap water for 1 hr. The crystals were filtered, washed with water, dried in vacuum protected from light and stored in brown bottle.

Estimation of ornithine

Ornithine was estimated according to the method of Ratner and Rochovansky (1956). To 1.0 ml sample was added 1.0 ml of warm ninhydrin reagent (250 mg of ninhydrin + 37.6 mg of hydrindantin dissolved in 4.0 ml of 6M H_3PO_4 and 6.0 ml of glacial acetic acid by heating at 50-60°C) followed by 1.5 ml of acetic acid. The tubes were kept in boiling water bath for 30 min, cooled and 2.5 ml of glacial acetic acid was added. The colour was read at 540 nm in a Klett Colorimeter against a standard ornithine sample treated in the same manner.

Estimation of citrulline and N-carbamyl putrescine

Citrulline and N-carbamyl putrescine were estimated according to the method of Archibald (1944). To 4.0 ml of sample 2.0 ml of a mixture of H_2SO_4.H_3PO_4(1:3) was added followed by 0.25 ml of 3% diacetyl monoxime solution with shaking. The tubes were kept in boiling water bath for 15 min in dark. After cooling the tubes the colour was read at 470 nm in a Klett Colorimeter.

Determination of dry weight

Dry weight of cotyledon and embryo was determined by drying a known weight of fresh tissue at 60°C till constant weight.

Estimation of protein

Protein was determined by the method of Lowry et al (1951).

Preparation of enzyme extract

At specified periods cotyledons and embryos were separated and a 10% (w/v) extract of the tissue was prepared by grinding in a chilled pestle-mortar kept over crushed ice using 5 mM phosphate buffer, pH 7. The extract, after passing through two layers of cheese cloth, was used for enzyme assay.

Determination of arginase (E.C. 3.5.3.1) activity

Arginase was estimated according to the method of Ramley and Bernlohr (1966). The assay system consisted of 50 µmol carbonate-bicarbonate buffer, pH 10.2; 10 µmol arginine; 10 µmol mercaptoethanol; 0.1 µmol $MnCl_2$ and enzyme (0.2 ml) in a total volume of 3 ml. In case of purified enzymes the assay system was same except that 50 µmol of carbonate-bicarbonate buffer, pH 9.5 and 0.1 ml of the enzyme was added in a total volume of 2.5 ml. After incubation at 37°C for 1 hr, the reaction was terminated by adding 0.5 ml of 10% TCA and centrifuged to remove the proteins. The ornithine, in the centrifuged supernatant, was estimated as described above.

Determination of ornithine keto-acid aminotransferase (OKAT) (E.C. 2.6.1.13) activity.

OKAT activity was determined according to the method of Peraino and Pitot (1963). The assay system consisted of 50 µmol Tris-HCl buffer, pH 8.5; 10 µmol ornithine; 10 µmol α-ketoglutarate; 0.1 µmol pyridoxal phosphate and enzyme (0.5 ml) in a total volume of 4 ml. After incubation at 37°C for 1 hr, the reaction was terminated by adding 0.5 ml of 10% TCA followed by 0.1 ml (10 mg/ml) o-aminobenzaldehyde. The coloured complex, after removal of protein by centrifugation, was read at 430 nm (E_o=2.7 x 10^3/mol/cm) (Strecker, 1965).

Determination of ornithine carbamyl transferase (OCT) (E.C. 2.1.3.3) activity

OCT activity was determined according to the method of Bernett and Cohen (1957). The assay system consisted of 100 µmol glycine-NaOH buffer, pH 10.0; 5 µmol ornithine; 5 µmol carbamyl phosphate and enzyme (0,2 ml) in a total volume of 4 ml. After incubation at 37°C for 1 hr, the reaction was terminated by adding 0.5 ml of 10% TCA. The citrulline formed was estimated as described above.

Determination of putrescine carbamyl transferase (PCT) activity

PCT was estimated like OCT except that 5 µmol of putrescine substituted ornithine and 0.5 ml enzyme was used. N-carbamyl putrescine formed was determined as described above.

Enzyme unit and specific activity

The enzyme unit is defined as the amount of enzyme required to form one µmol of product per hour under the assay conditions.

Specific activity is defined as enzyme units per mg protein.

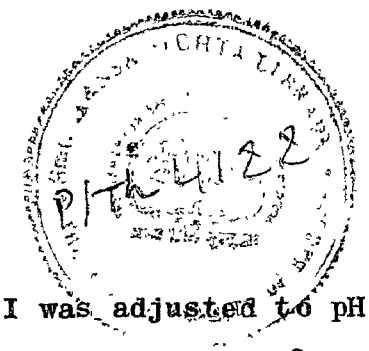
Purification of ornithine carbamyl transferase and putrescine carbamyl transferase from groundnut cotyledons

Step - I

30 g of cotyledons from dry groundnut seeds were homogenized in a chilled pestle-mortar kept on crushed ice using 5 mM phosphate buffer, pH 7. The extract was passed through two layers of cheese cloth, volume made upto 300 ml and then centrifuged at 10,000 g for 30 min.

Step- II

To the supernatant obtained from step I, $(NH_4)_2SO_4$ (17.6 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was discarded and to the supernatant $(NH_4)_2SO_4$ (27.3 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was collected and suspended in 5 mM phosphate buffer, pH 7 and dialysed against 1 mM phosphate buffer pH 7 for 16 hr.



Step - III

The fraction obtained from step - II was adjusted to pH 5.2 with chilled 0.1M acetic acid, allowed to stand at 4°C for 10 min and then centrifuged at 10,000 g for 10 min. The supernatant obtained was adjusted to pH 7 with chilled 0.1M KOH.

Step - IV

The fraction obtained from step - III was mixed with alumina C_7 gel (28mg/ml dry weight)in a gel : enzyme volume ratio of 1:2. After stirring for 30 min, it was centrifuged at 3500 g for 5 min. The adsorbed enzyme from the gel residue was eluted with 50 mM phosphate buffer, pH 7. The eluted enzyme was dialysed against 1 mM phosphate buffer, pH 7 for 16 hr.

Step - V

The fraction from step - IV was applied to a 45 x 1 cm column of DEAE-cellulose equilibrated with 5 mM phosphate buffer, pH 7. The column was washed with equal vol of 100 mM NaCl and eluted with 200 mM NaCl. The enzyme thus purified was stored at 5-10°C.

Purification of arginase from groundnut cotyledons

Step - I

21 g of cotyledons from 4 day germinated groundnut seeds were homogenized in a chilled pestle-mortar kept over crushed

ice using 0.25% Triton X-100 in 5 mM phosphate buffer, pH 7. The extract was passed through two layers of cheese cloth, volume made upto 210 ml and then centrifuged at 10,000 g for 30 min.

Step - II

To the supernatant obtained from step - I, $MnCl_2$ (1×10^{-4} mM) was added and the enzyme was then heated at 60°C for 10 min, cooled to 0°C and centrifuged at 10,000 g for 30 min.

Step - III

The supernatant obtained from step - II was applied to a 20 x 2.5 cm column of DEAE-cellulose equilibrated with 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$. The column was washed with equal vol of 100 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$ and eluted with 300 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$.

Step - IV

The enzyme from step - III was mixed with alumina C_γ gel (28 mg/ml dry weight) in a gel to enzyme vol ratio of 1:3. The mixture was stirred for 30 min and centrifuged at 3500g for 5 min. The enzyme from gel residue was eluted with 200 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$. 5.0 ml of this fraction was dialysed for

16 hr against 1 mM phosphate buffer, pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$ for checking the activity. The remaining portion was processed further.

Step - V

To the eluted enzyme from step - IV, $(NH_4)_2SO_4$ (11.4 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was discarded and $(NH_4)_2SO_4$ (18.9 g/100 ml) was again added to the supernatant with stirring. After standing for 30 min at 4°C, it was centrifuged at 10,000 g for 30 min. The residue was collected and suspended in 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and dialysed against 1 mM phosphate buffer pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$ for 16 hr.

Step - VI

The fraction from step - V was applied to a 30 cm column of DEAE-Sephadex A-50 equilibrated with 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol. The column was washed with equal vol of 100 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$ and eluted with 300 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$.

Step - VII

The fraction obtained from step - VI was mixed with calcium phosphate gel (25 mg/ml dry weight) in a gel : enzyme

volume ratio of 1:4. After stirring for 30 min, it was centrifuged at 3500 g for 5 min. The adsorbed enzyme from the gel residue was eluted with 200 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and 1 x 10^{-4} mM $MnCl_2$. 5 ml of this fraction was dialysed for 16 hr against 1 mM phosphate buffer pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$ for checking the activity. The remaining portion was processed further.

Step - VIII

To the eluate obtained from step - VII, $(NH_4)_2SO_4$ (31.3 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was suspended in 5 mM phosphate buffer pH 7 containing 10 mM mercaptoethanol and dialysed for 16 hr against 1 mM phosphate buffer, pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$. The enzyme thus purified was stored at 5-10°C.

Purification of arginase from groundnut embryos

Step - I

10 g of embryos from 2 day germinated groundnut seeds were homogenized in a chilled pestle mortar kept over crushed ice using 0.25% Triton X-100 in 10 mM mercaptoethanol. The extract was passed through two layers of cheese cloth, volume made upto 100 ml and centrifuged at 10,000 g for 30 min.

Step - II

To the supernatant obtained from step - I, $(NH_4)_2SO_4$ (11.4 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was discarded and to the supematant $(NH_4)_2SO_4$ (18.9 g/100 ml) was again added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was collected and suspended in 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and dialysed against 1 mM phosphate buffer containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$ for 16 hr.

Step - III

The dialysed fraction from step - II was applied to a 12 x 2.5 cm column of DEAE-cellulose equilibrated with 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol. It was washed with equal vol of 100 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$ and eluted with 300 mM NaCl containing 10 mM mercaptoethanol and 1×10^{-4} mM $MnCl_2$.

Step - IV

The fraction from step - III was mixed with alumina C_γ gel (28 mg/ml dry weight) in a gel to enzyme volume ratio of 1:4. After stirring for 30 min, it was centrifuged at 3500 g for 5 min. The adsorbed enzyme from the gel residue was eluted with 200 mM phosphate buffer, pH 7 containing 10 mM

mercaptoethanol and 1 x 10^{-4} mM $MnCl_2$. 5 ml of this fraction was dialysed for 16 hr against 1 mM phosphate buffer, pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$ for checking the activity. The remaining portion was processed further.

Step - V

To the eluate obtained from step - IV, $(NH_4)_2SO_4$ (31.3 g/100 ml) was added with stirring. It was centrifuged at 10,000 g for 30 min after standing for 30 min at 4°C. The residue was suspended in 5 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and dialysed for 16 hr. against 1 mM phosphate buffer, pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$.

Step - VI

The enzyme obtained from step - V was mixed with calcium phosphate gel (25 mg/ml dry weight) in a gel : enzyme volume ratio of 1:5. After stirring for 30 min, it was centrifuged at 3500 g for 5 min. The adsorbed enzyme from the gel residue was eluted with 200 mM phosphate buffer, pH 7 containing 10 mM mercaptoethanol and 1 x 10^{-4} mM $MnCl_2$. The eluate was dialysed for 16 hr against 1 mM phosphate buffer, pH 7 containing 1 mM mercaptoethanol and 0.1 mM $MnCl_2$. The enzyme thus purified was stored at 5-10°C.