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AN ALKALINE PHOSPHATASE FROM FLOWERS OF CALOTROPIS PROCERA

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Calotropis procera is a perennial shrub that grows in many parts of Asia and Africa. It requires little or no water for survival and is very suitable for arid or desert regions.

Two varieties of the plant have been widely discussed in the literature, *Calotropis procera* and *Calotropis gignatia*.[1] The former is the smaller of the two and is found widely in and around Karachi. A number of medicinal and therapeutic properties have been ascribed to the various parts of this plant but remain to be proven scientifically.[2] Earlier investigators reported the presence of compounds with digitalis-like properties in the aqueous extract of *Calotropis procera*.[3,4] From the latex of the plant several steroid glycosides with pharmacological properties have been reported by Hesse *et al.*, and their structure has been studied.[5–7] Pant and Srivastava have separated two proteins from the latex by the use of electrophoresis,[8] while several proteins have recently been isolated from the same source.[9,10] Isolation of a polysacharide from the green leaves of the plant has been reported recently from these Laboratories.[11]

In the present report we describe the isolation and some properties of an alkaline phosphatase from the flowers of this plant.

Materials and Methods

Substrate. A solution of *p*-nitrophenyl phosphate (0.011M) in water was employed as the substarate. The stock substrate solution was divided into several aliquots, which were stored frozen until used.

Enzyme. One unit of the enzyme is defined as the amount catalyzing the formation of 1 μ-mole of *p*-nitrophenol at 37°C under standard assay conditions. The enzyme used in the experiments was either freshly extracted from the flowers prior to the assay or, in some cases, had been kept frozen for no more than few days.

Protein Estimation. Protein was determined by the method of Lowry *et al.*[12] using bovine casein as standard for the calibration graph.

Extraction of the Enzyme. Fresh flowers from the plant, *Calotropis procera*, growing in the vicinity of PCSIR Laboratories, Karachi, were collected (January–June) and processed the same day, as follows :

The flowers were washed with water in order to remove the adhering dust particles. Thereafter, 250 g lots were suspended in cold buffer (50 ml) in a precooled homogenizer and homogenization carried out at maximum speed for 15 min. The coarse homogenate was centrifuged and the supernatant saved. The foregoing extraction was followed using three buffers : 0.02M phosphate, (pH 6.9); 0.02M tris-HCl buffer (pH 8.0); and 0.02M glycine–NaOH (pH 9.0).

Gel Filtration. The clear extract obtained as above were used for the evaluation of phosphatase activity as well as for chromatography on a column of Sephadex G-150 (44×3.5 cm).

Before the column preparation, Sephadex resins (fine grade, Pharmacia, Uppsala, Sweden) were allowed to swell completely in water over a period of 48 hr. The finer particles were removed by decantation several times. The column was allowed to equilibrate completely with the appropriate buffer prior to the application of the crude enzyme extract. The solution was carried out with the same buffer used for the initial extraction of the enzyme. A total of 40 fractions (5 ml) were collected and each was monitored for absorption at 280 nm and alkaline phosphatase activity.

Assay of Alkaline Phosphatase. Alkaline phosphatase activity was assayed according to the method described by Lowry *et al.*[13] with slight modification.

An aliquot (0.2 ml) of the enzyme extract was added to *p*-nitrophenyl phosphate solution (1 ml, 0.011M) made up to a final volume of 2.6 ml with the appropriate buffer. The control solutions contained, instead of the enzyme, equivalent amounts of heat-denatured enzyme and all the other reagents.

The test and control solutions were incubated at 37°C for 30 min, following which they were transferred to an ice-bath and 0.25N NaOH (0.25 ml) was rapidly added. The solutions were thoroughly mixed and the absorbance measured at 410 nm. The amount of *p*-nitrophenol liberated was determined from a straight-line calibration graph which was prepared by measuring the absorbance of solutions of known concentrations. Phosphatase activity was determined at pH 6.9, 8.0 and 9.0 using buffers 0.02M phosphate/tris-HCl/glycine–NaOH.

Results and Discussions

In order to determine optimum conditions for the extraction of phosphatase from *Calotropis procera*, three different buffers of pH 6.9, 8.0 and 9.0 were employed and the extracts investigated for their enzyme activity.

Of the three buffers tested, extract in 0.02M tris-HCl, pH 8.0, showed the highest specific activity (680 mg protein). In comparison, considerably less activity was exhibited by buffer extracts of pH 6.9 (540/mg) and 9.0 (450/mg). As an enzyme might not be stable at the pH where it shows optimal activity and/or is most easily extractable, the results presented here are to be interpreted with this consideration in mind. Further studies are required

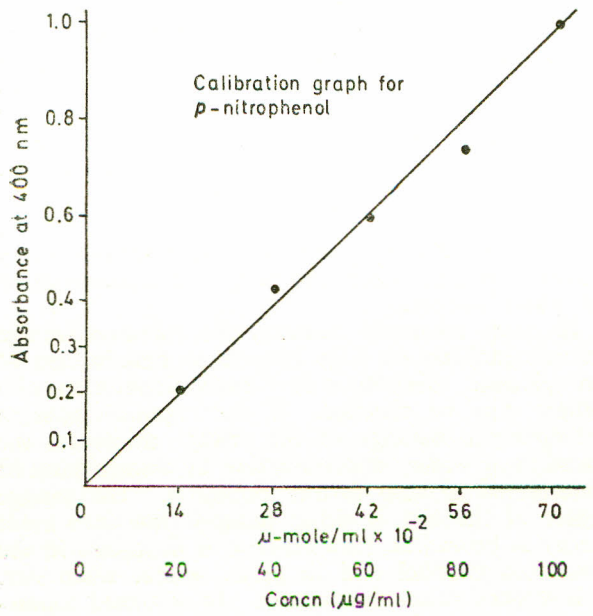

Fig. 1

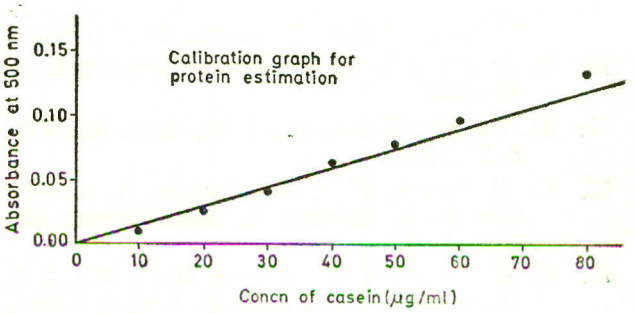

Fig. 2

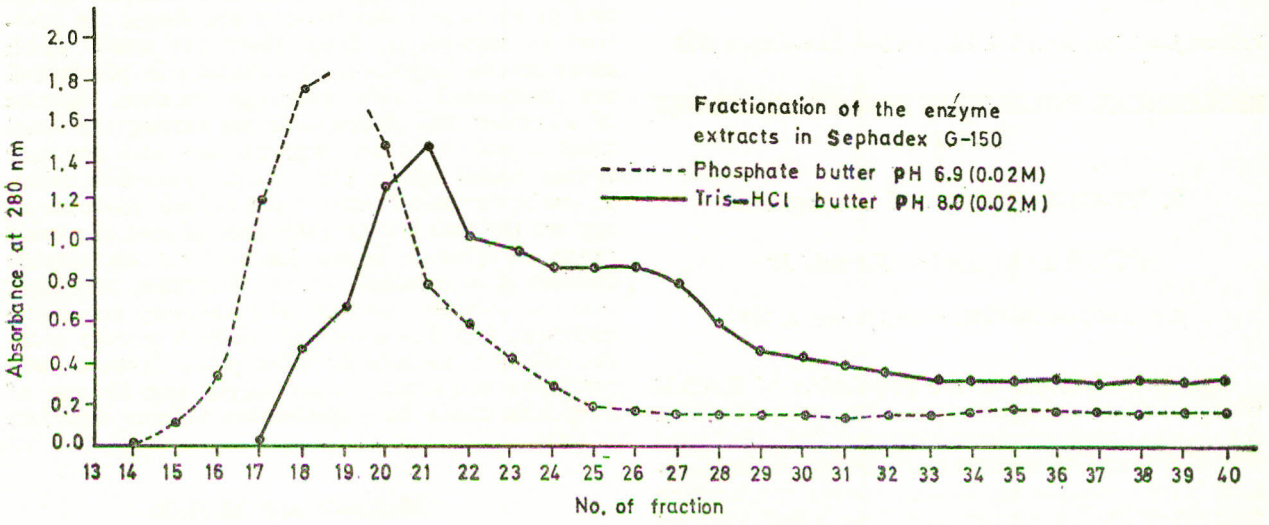

Fig. 3

before the conditions of optimal activity and stability can be clearly distinguished.

The high lability of the enzyme was demonstrated when all fractionation attempts on a Sephadex G-150 column resulted into complete loss of the activity. The same results were obtained regardless of the buffer the chromatography was performed in. Although the protein-rich peak was eluted roughly between fractions 16–23, all of these were devoid of the enzyme activity. Similarly, other side fractions eluted from the column and tested soon after elution exhibited no phosphatase activity. Because of non-availability of working facilities at low temperatures, we were unable to pursue the purification work any further. It seems likely that the enzyme is sensitive to heat and is destroyed by heat promoted processes such as denaturation, proteolysis or autolysis. Alternatively, gel-filtration might have resulted in removal of certain metal ions or activators, essential for enzyme activity. The latter possibility remains to be tested.

In conclusion, we have shown the presence of an alkaline phosphatase in the fresh flowers of *Calotropis procera*, which can be extracted with 0.02M tris-HCl buffer, pH 8.0. The enzyme loses all activity on gel filtration.

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BIOLOGICAL POLLUTION (SEWERAGE FUNGI) Part I

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Due to an increase in the population of Karachi without corresponding increase in the sewage facilities, the disposal problems have increased and it is feared that if appropriate measures are not taken the situation would further aggravate. Hardly any work on sewerage microbiology has been done in Pakistan, and thus there is an acute need for such studies. There is also a need for finding out speedy processes for the disposal of wastes in different other forms. The important points to justify these contentions are: (a) in all the advanced countries population is increasing and consequently regular studies are conducted regarding speedy disposal of waste by microorganisms to convert it into manure. Disposal through microbes on one hand involves minimal expenditure and on the other hand a lot of air pollution could be avoided ; (b) polluted water is a very common source for enteric bacteria causing frequent human infections, at times in epidemic forms. In addition to this, there are several fungal and bacterial diseases of animals and human beings caused by contaminated drinking water. The problem involving sewage, drinking water contamination and human diseases caused is a complicated one and requires a considerable amount of coordination and hard work to evolve precautionary and remedial measures to improve sanitary conditions and thus reduce many of the human diseases caused by microorganisms from drinking water. Sewage is the chief source of pollution and contamination of water, and the methods used for disposal are important to assure greater public health. These methods are concerned with the disposal of large volumes of waste products in the manner that minimizes stream pollution and danger of contamination of water supply.

In every advanced country, the common sewage microorganisms are fully known, so that in case of an epidemic, this source as a contaminant alongwith others may be checked. If the organism causing an epidemic belongs to the sewage microbes, the location of water contamination by sewage must be determined to stop further pollution. The sewage water of the city is either pumped raw into fields where it turns into manure or it is deposited in the irrigation channel and in plots, where, when dry, it is scroned as sewage manure. In advanced countries raw sewage is treated to make it inert, digested in the absence of air, reducing the bulk and facilitating drying up. This means sewage manure which we get contains the eggs and larvae of different worms and parasites and also bacteria and fungi. In addition to saprophytic fungi there are many which cause several important root diseases in plants and are associated with sewerage manure. Species of *Fusarium* and *Rhizoctonia* are among the fungi most prevalent in sewerage manure and are very potent causal agents for wilting. *Fusarium solani*, *F. semitectum* and *F. oxysporum*, isolated from sewerage are the well known pathogens of root in a wide range of plants. If one knows that the sewage manure is a constant source of certain important root rot diseases one may take necessary preventive measures, and thus avoid the infection or even when the infection has already taken place, curative measures like minimizing water supply and the use of fungicides could be implemented without any loss of time.

Materials and Methods

Several sewage manure samples were collected from different places and nurseries during 1969. For the isolation of fungi, large lumps from these samples were selected, and broken under aseptic conditions for the isolation of fungi by Warcup and streaking techniques.[4] Inoculations of sewage samples were made on the same day. The media used were potato–dextrose agar and Czapek's agar. For each of the sewage sample four petri plates (two with 20 cc P.D.A. and two with 20 cc of Czapek's agar) were used for the Warcup technique and like-wise for the streaking technique. To inhibit the bacteria and fast growing fungi, rose Bengal in the ratio of 1:30,000 was used with these media. A total of thirty-four species belonging to fourteen genera of fungi were isolated. These were purified and identified in due course. The

material was also sent to the Commonwealth Mycological Institute for confirmation. All the fungi included here have been reported for the first time from the sewerage source in Pakistan.

Most of the references included were used for identification purposes and, therefore, the same have not been repeated in the text unless considered necessary.

Aspergillus egyptiacus Mubasher, was never been reported before from Pakistan. For this reason detailed microscopic observations were made on this fungus and a description is provided. The following fungi constitute a portion of the micro-organisms.

(1) *Aspergillus egyptiacus* Mubasher, PCSIR H. No. 421, I.M.I. No. 176791. Colony fast growing, cottony, fungus pure white on Czapek's agar, reverse colourless in the young colonies, when it becomes old it gradually turns dirty white to light brown. Conidiophores hyaline, straight, smooth, usually 100.0–140.0×2.0–3.0 μ. Vesicle flask-shaped, hyaline. Strigmata in two rows, primary and secondary, hyaline, primary strigmata about 10.0×3.0 μ and secondary about 5.0–6.0×2.5 μ. Conidia hyaline, rough, somewhat globose, 3.0–4.0 μ in dia, attached to the top of secondary strigmata in the form of chains. All the fungi included have been reported for the first time from sewage manure.

(2) *Alternaria alternata* (Fr.) Keissler, PCSIR H. No. 156, I.M.I. No. 152262.

(3) *Aspergillus terreus* Thom, PCSIR H. No. 151, I.M.I. No. 124979.

(4) *A. fumigatus* Thom, PCSIR H. No. 155, I.M.I. No. 128092.

(5) *A. niger* Thom, PCSIR H. No. 153.

(6) *A. nidulans* (Eidam) Wint, PCSIR H. No. 150, I.M.I. No. 152259.

(7) *A. stellatus* Curzi, PCSIR H. No. 294, I.M.I. No. 146289.

(8) *A. ochraceus* Wilhelm, PCSIR H. No. 129, I.M.I. No. 146290.

(9) *A. ustus* Thom and Church, PCSIR H. No. 171, I.M.I. No. 146291.

(10) *A. chivalieri* (Moug.) Thom and Church, PCSIR H. No. 238, I.M.I. No. 133702.

(11) *Cochliobolus lunatus* Nelson and Hassis, PCSIR H. No. 268, I.M.I. No. 152264.

(12) *Drechslera hawaiiensis* (Bugn.) Subram and Jain, PCSIR H. No. 229, I.M.I. No. 152271.

(13) *D. rostrata* (Drechsl.) Richardson and Fraser, PCSIR H. No. 242, I.M.I. No. 152272.

(14) *D. halodes* (Drechsl.) Subram and Jain, PCSIR H. No. 292, I.M.I. No. 14630.

(15) *Fusarium aquaeductum* (Radle and Rab.) Lagerheim, PCSIR H. No. 423, I.M.I. No. 152269.

(16) *F. solani* (Mart.) Sacc., PCSIR H. No. 162, I.M.I. No. 152277.

(17) *F. oxysporum* Schlecht, PCSIR H. No. 299, I.M.I. No. 146294.

(18) *F. semitectum* Berk. and Rav., PCSIR H. No. 170, I.M.I. No. 146299.

(19) *Humicola fuscoatra* Traaen, PCSIR H. No. 176, I.M.I. No. 146302.

(20) *Myrothecium verrucaria* Ditmar e Fr., PCSIR H. No. 295, I.M.I. No. 146293.

(21) *Penicillium chrysogenum* Thom, PCSIR H. No. 237, I.M.I. No. 146306.

(22) *P. citrinum* Thom, PCSIR H. No. 298, I.M.I. No. 146305.

(23) *P. funiculosum* Thom, PCSIR H. No. 168, I.M.I. No. 152273.

(24) *P. crustosum* Thom, PCSIR H. No. 296, I.M.I. No. 146303.

(25) *P. coryophilum* Dierck, PCSIR H. No. 297, I.M.I. No. 152274.

(26) *P. oxalicum* Currie and Thom, PCSIR H. No. 422, I.M.I. No. 176795.

(27) *Scopulariopsis brevicaulis* (Sacc.) Bain, PCSIR H. No. 145, I.M.I. No. 146310.

(28) *Stachybotrys atra* Corda, PCSIR H. No. 230, I.M.I. No. 176785.

(29) *Trichothecium roseum* (Pers.) Link ex Fr., PCSIR H. No. 300, I.M.I. No. 146312.

(30) *Ulocladium botrytis* Preuss, PCSIR H. No. 225, I.M.I. No. 152265.

(31) *U. atrum* Preuss, PCSIR H. No. 424, I.M.I. No. 152267.

(32) *Pleospora infectoria* Fuckel, PCSIR H. No. 271, I.M.I. No. 146286.

(33) *Phoma herbarum* Westend, PCSIR H. No. 425, I.M.I. No. 146309.

(34) *P. eupyrena* Sacc., PCSIR H. No. 293, I.M.I. No. 146308.

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