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Enzymic synthesis of *sym*-homospermidine in *Lathyrus sativus* (grass pea) seedlings

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An enzyme catalysing the synthesis of *sym*-homospermidine from putrescine and NAD^+ with concomitant liberation of NH_3 was purified 100-fold from *Lathyrus sativus* (grass pea) seedlings by affinity chromatography on Blue Sepharose. This thiol enzyme had an apparent mol.wt. of 75000 and exhibited Michelis–Menten kinetics with K_m 3.0mM for putrescine. The same enzyme activity could also be demonstrated in the crude extracts of sandal (*Santalum album*) leaves, but with a specific activity 15-fold greater than that in *L. sativus* seedlings.

sym-Homospermidine (1,9-diamino-5-azanonane) first shown to occur in the free state in large amounts in sandal (*Santalum album*) leaves (Kuttan *et al.*, 1971), was subsequently detected in some algae (Kneifel, 1977; Rolle *et al.*, 1977), in photosynthetic (Tait, 1979), thermophilic (Oshima, 1978) and symbiotic (Smith, 1977) bacteria, and, more recently, in several amphibia (Hamana & Matsuzaki, 1979). Despite the varied natural occurrence of this unusual polyamine, available information on the mode of its biosynthesis is very meagre. Previously Kuttan & Radhakrishnan (1972) suggested that the amine might derive all its carbon and nitrogen atoms from putrescine, and proposed a reaction sequence wherein the Schiff base formed between putrescine and its oxidation product, 4-aminobutyraldehyde, is converted by subsequent reduction into *sym*-homospermidine. Recent studies with *Rhodopseudomonas viridis* (Tait, 1979) have shown, in addition, the involvement of nicotinamide nucleotides in the proposed biosynthetic reactions.

During our studies on putrescine metabolism in *Lathyrus sativus* seedlings (Ramakrishna & Adiga, 1974*a*; Suresh *et al.*, 1976; Srivenugopal & Adiga, 1980), it was intriguing to find that synthesis *de novo* of a new polyamine occurred in substantial amounts in the undialysed crude extracts while either diamine oxidase [amine oxidase (copper-containing), EC 1.4.3.6] (Suresh *et al.*, 1976) or putrescine carbamoyltransferase (EC 2.1.3.6) (Srivenugopal & Adiga, 1980) were assayed with putrescine as the sole exogenous amine substrate. After large-scale purification, this polyamine could be clearly distinguished from both spermidine and spermine and conclusively identified as *sym*-homospermidine by various physicochemical criteria. In the present paper we describe the partial purification by affinity chromatography of the enzyme catalysing the synthesis of *sym*-homospermidine in the plant and show that the enzymic mechanism shares several characteristics with that proposed for the microbial system (Tait, 1979). Evidence is also provided for the participation of a similar enzymic reaction in its biogenesis in sandal leaves as well.

Materials and methods

sym-Homospermidine was a kind gift from Professor A. N. Radhakrishnan, University of Hyderabad, Hyderabad, India, and was also isolated from sandal leaves as described previously (Kuttan & Radhakrishnan, 1972). [^{14}C]Putrescine dihydrochloride (55mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Amine hydrochlorides were from Sigma Chemical Co., St. Louis, MO, U.S.A., and purified before use. Blue Sepharose and Sephacryl S-200 were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of Analytical grade.

The source, germination conditions of *L. sativus* seeds and initial steps of enzyme purification were reported previously (Suresh *et al.*, 1976). Aliphatic amines were purified (Inoue & Mizutani, 1973) and resolved by circular paper chromatography (Ramakrishna & Adiga, 1973) with methoxyethanol/propionic acid/water (14:3:3, by vol.) as solvent system (Herbst *et al.*, 1958). Two-dimensional chromatography (Kuttan *et al.*, 1971; Hamana & Matsuzaki, 1979), dansylation followed by t.l.c. (Smith, 1977) and paper electrophoresis using 0.03M-sodium citrate buffer, pH 4.5 (Herbst *et al.*, 1958) were also employed for this purpose. Protein was determined by the method of Lowry *et al.* (1951),

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with bovine serum albumin as standard. I.r. spectra of *sym*-homospermidine trihydrochloride (KBr pellet) was measured with Perkin–Elmer i.r. spectrophotometer in the wavelength range 600–3300 cm^{-1}.

Enzyme assay

The homospermidine synthase was assayed in a reaction mixture (1.0 ml) containing Tris/HCl, pH 8.4 (50 mM), dithiothreitol (2 mM), KCl (10 mM), putrescine (5 mM) or labelled putrescine (0.5 mM; 0.1 μCi), NAD^+ (1 mM) and the enzyme protein (0.1–1.5 mg). After terminating the reaction with 0.1 ml of 20% (w/v) $HClO_4$, the amines were purified on a column of Dowex-50 resin (H^+ form) (Inoue & Mizutani, 1973) and separated by paper chromatography or electrophoresis and the product quantified either by ninhydrin reaction (Rosen, 1957) or measuring the radioactivity by liquid-scintillation spectrometry (Suresh & Adiga, 1977). The enzyme activity was also determined by collecting the NH_3 liberated by diffusion (Suresh *et al.*, 1976) and by quantifying it with phenol/hypochlorite reagent (Schwartz, 1971).

Gel filtration

Gel filtration of the concentrated protein fraction eluted by NAD^+ from the Blue Sepharose affinity step was carried out on a Sephacryl S-200 column (8.5 cm × 71 cm), with potassium phosphate buffer (0.05 M), pH 7.5, as the eluent. The marker proteins used were: catalase (mol.wt. 240000), bovine serum albumin (monomer 68000; dimer 136000), peroxidase (45000) and cytochrome *c* (13500).

Affinity chromatography on Blue Sepharose

Etiolated seedlings of *L. sativus* (5 days old) or young leaves of sandal were homogenized in 0.05 M-potassium phosphate buffer, pH 8.0, containing 2 mM-2-mercaptoethanol. The supernatant, after $MnCl_2$ precipitation to remove nucleoproteins, was clarified (Suresh *et al.*, 1976) and applied at a low flow rate (0.5 ml/min) on to a Blue Sepharose column (2 cm × 10 cm) pre-equilibrated with 0.05 M-potassium phosphate, pH 7.5, containing 2 mM-2-mercaptoethanol. After the column had been washed extensively with this buffer until the A_{280} was < 0.05, the enzyme was eluted with 5 mM-NAD^+ in the equilibration buffer.

Results and discussion

Partial purification of the enzyme and product characterization

On the basis of the preliminary observation that the enzymic synthesis of *sym*-homospermidine from putrescine by the dialysed crude extracts of *L. sativus* requires the obligatory participation of exogenous NAD^+, the enzyme purification was attempted by employing Blue Sepharose as the group-specific affinity matrix. Table 1 summarizes the protocol used to achieve 100-fold purification of the enzyme with 78% recovery. The finding that substantial purification resulted from specific elution of the enzyme from the affinity matrix with 5 mM-NAD^+ clearly supports the involvement of the nicotinamide nucleotide in the polyamine synthesis. The unequivocal identity of the polyamine produced with *sym*-homospermidine was clearly established from its behaviour in two-dimensional paper chromatography (Kuttan *et al.*, 1971; Hamana & Matsuzaki 1979), its mobility on t.l.c. after dansylation (Smith, 1977) and during high-voltage electrophoresis on paper (Herbst *et al.*, 1958). Furthermore the i.r. spectrum of the purified amine was also identical with (Kuttan *et al.*, 1971), and hence superimposable on, that obtained by using the authentic *sym*-homospermidine from sandal leaves (results not shown).

Non-involvement of diamine oxidase in the biosynthetic reaction

According to the sequence of reactions proposed for the biosynthesis of *sym*-homospermidine in *Rps. viridis* (Tait, 1979), the first step involves NAD^+-dependent oxidation of putrescine to yield 4-aminobutyraldehyde and NH_3, followed by the condensation of the aminoaldehyde with another molecule of putrescine to give rise to a Schiff base, which in turn is reduced to *sym*-homospermidine by the NADH produced in the first step. Since the diamine oxidase of *L. sativus* (Suresh *et al.*, 1976) acting on putrescine also produces the corresponding aminoaldehyde and NH_3, the possible involvement of the diamine oxidase-type reaction in *sym*-homospermidine biogenesis was considered. However the following observations clearly distinguish between the two

Table 1. *Partial purification of homospermidine synthase from L. sativus seedlings*

Assay conditions of the reaction were as described in the text. A unit of enzyme activity corresponds to 1 μmol of *sym*-homospermidine formed/h per mg of protein.

Step	Total protein (mg)	Total activity (units)	Sp. activity (units/mg of protein)	Purification (fold)	Recovery (%)
Crude extract	765	11.5	0.015	1.00	100
$MnCl_2$ supernatant	653	10.3	0.016	1.06	85
Specific elution from Blue Sepharose with NAD^+	6	9.0	1.50	100	78

enzyme activities: (*a*) the diamine oxidase was excluded from the affinity matrix, since there was no requirement of nicotinamide nucleotides for activity (Suresh *et al.*, 1976); (*b*) unlike the diamine oxidase, the enzyme system catalysing the biogenesis of *sym*-homospermidine was inhibited by thiol blockers but not by semicarbazide and did not produce H_2O_2 (see Table 3 below).

Other properties

The partially purified enzyme exhibited a single pH optimum of 8.4 in Tris/HCl buffers and a temperature optimum of 37°C. Under the assay conditions employed, the reaction was linear up to 60 min and was proportional to the amount of enzyme protein (0.1–0.5 mg). The requirement for NAD^+ was absolute, since even $NADP^+$ could not substitute in the enzyme reaction (Table 2). In terms of the affinity for putrescine, maximal activity was observed with the diamine in the concentration range of 1–5 mM, and a double-reciprocal plot of the data yielded a K_m of 3 mM with respect to putrescine. With regard to substrate specificity, both 1,3-diaminopropane and cadaverine were inactive, as revealed by the absence of both NH_3 liberation and production of corresponding polyamines. The marked inhibition obtained with *N*-ethylmaleimide suggests that the enzyme is of a thiol nature (Table 3). The possibility that multiple enzyme proteins acting in concert accomplish the proposed sequence of reactions appears unlikely in view of the observation that gel filtration on a Sephacryl S-200 column yielded a single peak of enzyme activity with an apparent mol. wt. of 75000. This is in accordance with the findings of Tait (1979) with the *Rps. viridis* enzyme. The partially purified homospermidine synthase of *L. sativus* was stable at 4°C for at least 2 weeks in the presence of 1 mM-NAD^+, which has a good stabilizing influence.

Table 2. *Requirements for the homospermidine synthase reaction*

Assay conditions and other procedures have been detailed in the Materials and methods section. *sym*-Homospermidine formed was quantified by ninhydrin colour reaction as well as by measurement of associated radioactivity when [^{14}C]-putrescine was used as the substrate.

Component omitted	*sym*-Homospermidine formed (μmol)
None	0.40
NAD^+	0.0
NAD^+ replaced by $NADP^+$	0.05
KCl	0.18
Boiled enzyme preparation used	0.0

Table 3. *Effect of different compounds on L. sativus homospermidine synthase activity*

The enzyme was assayed as outlined in the Materials and methods section.

Compound tested (mM)	Inhibition (%)
N-ethylmaleimide (0.5)	40
Semicarbazide (1)	0
Cadaverine (1)	5
Spermidine (1)	30
1,3-Diaminopropane (1)	10

Homospermidine synthase in sandal leaves

In view of the experience gained with *L. sativus* seedlings, we decided to examine whether the sandal leaves, with their high concentration (0.5–1.5% dry wt.), of this polyamine (Kuttan *et al.*, 1971), employ a similar enzymic mechanism for its elaboration. In fact the dialysed crude extracts of the leaves efficiently utilized putrescine for *sym*-homospermidine synthesis when assayed under conditions identical with those employed with *L. sativus*. However, in terms of specific activity, the sandal enzyme was relatively more efficient, since in crude extracts it had a specific activity of 0.23 μmol of *sym*-homospermidine formed/h per mg of protein compared with *L. sativus* extracts (0.015 μmol/h per mg of protein).

The data presented above thus clearly demonstrate that an efficient enzyme system, apparently consisting of a single protein and catalysing synthesis *de novo* of *sym*-homospermidine from putrescine can be rapidly purified by affinity chromatography. Mechanistically it appears to function on lines suggested by Kuttan & Radhakrishnan (1972) in sandal leaves and by Tait (1979) in *Rps. viridis*. However it is pertinent to mention that our attempts hitherto to demonstrate the natural occurrence of this unusual polyamine in *L. sativus* have not been successful. It is possible that *sym*-homospermidine has only transient existence in this plant, being converted *in vivo* into other metabolites such as alkaloids, as in *Solanum tripartitum* (Kupchan *et al.*, 1969) and *Senecio isatideus* (ragwort) (Robins & Sweeney, 1979). Alternatively, it is conceivable that spermidine, occurring in substantial amounts in this plant (Ramakrishna & Adiga, 1974*b*) and being a potent inhibitor of the enzyme (Table 3), might have severely curtailed its endogenous accumulation by functioning in a manner analogous with oxaloacetate inhibition of malic enzyme in potato (*Solanum tuberosum*) (Sarawek & Davies, 1976). It is significant that the natural occurrence of this symmetrical polyamine is largely confined to biological systems in which spermidine is either absent (*Rps. viridis*) (Tait, 1979) or present only in

traces, as in sandal leaves (Kuttan & Radhakrishnan, 1972). The question as to whether the enzyme system represents a mere evolutionary vestige [as suggested by McKee (1962) for alkaloid synthesis from diamines in the pea (*Pisum sativum*)] or has any physiological relevance to the plant will remain speculative until the exact biological significance of symmetrical polyamines in general and of *sym*-homospermidine in particular is delineated. Isolation and characterization of several new symmetrical polyamines in recent years, such as norspermine and norspermidine from thermophilic (Oshima, 1975, 1978) and halophilic (Yamamoto *et al.*, 1979) bacteria and from some aquatic invertebrates (Stilway & Walle, 1977; Zappia *et al.*, 1978) and the implication that their functional significance may be related to the ecological habitats of the organisms, are pertinent in this context. On the basis of these assumptions, it is tempting to speculate that *L. sativus*, being a hardy plant (Ganapathy & Dwivedi, 1961; Purseglove, 1968) capable of withstanding nutritional and water stress under drought-like conditions for considerable time, might utilize with advantage the existing enzyme machinery to elaborate *sym*-homospermidine under these adverse climatic conditions as an adaptive mechanism. This discrete possibility requires further study.

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