Phytochemistry, Vol. 26, No. 12, pp. 3201–3205, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00
© 1987 Pergamon Journals Ltd.

TEMPORAL REGULATION IN THE SYNTHESIS OF CONCANAVALIN A AND α-MANNOSIDASE IN THE SEEDS OF *CANAVALIA ENSIFORMIS*

MALLIKA RAYCHAUDHURI, KRISHNAKALI NIYOGI and MANORANJAN SINGH

Biochemical Engineering Division, Indian Institute of Chemical Biology, 4 Raja S C Mullick Road, Calcutta 700032, India

(*Received* 6 *February* 1987)

Key Word Index—*Canavalia ensiformis*; Leguminosae; jackbean; Con A; α-mannosidase; temporal regulation; developmental pattern.

Abstract—The enzyme, α-mannosidase and the lectin, concanavalin A, both of which interact with α-D-mannosides, are present in substantial amounts in the mature seeds of *Canavalia ensiformis*. The changes in the levels of these two proteins and their mRNA have been followed throughout seed development. Although both proteins start appearing in the seeds at day 24 after pod formation, there is a difference in the developmental patterns. While the increase in the activity of α-mannosidase is gradual and continues up until about day 44 followed by a slow phase till the desiccation stage, Con A after a lag phase which lasts to about day 30 shows a logarithmic increase up to about the 36th day followed by a plateau thereafter upto the desiccation stage. The highest amounts of functional mRNA for these two proteins are found at the early stages of seed development, well ahead of the period of highest protein deposition, thereby indicating that post-translational modifications of these proteins are slow and distinct from those of other legumes.

INTRODUCTION

Mature seeds are rich in proteins and other reserve materials like lipids and carbohydrates. The total protein fraction of the seeds is made up of many individual species which can be broadly classified into two groups: the heterogeneous metabolic and structural proteins, such as enzymes, and ribosomal, chromosomal and membrane proteins and the less heterogeneous fraction made up of storage proteins and lectins [1].

Among the legume species, the patterns of seed protein synthesis are remarkably similar during seed development although the total time taken for the seed to mature depends upon the species and to some extent on the environmental conditions. In the developing embryo, there is essentially no synthesis of reserve materials during the period of rapid cell-division. The biosynthesis and the deposition of lectins as well as other storage materials take place during the following growth phase of the seeds. Very few proteins are synthesized in the final desiccation phase [2–4].

In order to study the developmental processes operating in jackbean seeds, the synthesis and accumulation of α-mannosidase (EC 3.2.1.24), an α-D-mannopyranomanno: hydrolase, and concanavalin A (Con A), a lectin which binds to α-D-mannosides and α-D-glucosides, have been investigated as the reference gene products, representing the two classes of seed proteins described earlier. Due to the abundance of lectins in mature seeds and also the developmental specificity of their synthesis, studies on the biosynthesis of these proteins are of great interest for understanding the regulation of gene expression in higher plants. Moreover, since both Con A and α-mannosidase interact with the same sugar moiety, i.e. α-D-mannoside, the pattern of their synthesis and accumulation during seed maturation, may have some physiological significance.

The present studies describe the temporal relationship of synthesis and accumulation of Con A and α-mannosidase in the developing seeds of *Canavalia ensiformis* (L.) D.C. In this paper, we identify more precisely when these two proteins and their mRNAs begin to accumulate during seed development and also demonstrate that the synthesis and accumulation of both these proteins occur in the mid-maturation stage of the jackbean seeds.

RESULTS

Levels of Con A, α-mannosidase and total protein in developing jackbean seeds

The lectin, as determined by hemagglutination (Hgg) assays, is detectable in low amounts at day 22–26 after pod formation (dpf), increasing slowly up to 30 dpf, followed by a sharp rise until it reaches a maximum at about 36 dpf and thereafter it remains constant (Fig. 1).

The α-mannosidase activity is also detectable as early as 22 dpf; but the developmental profile is different from that of the lectin as the enzyme activity increases slowly, reaching essentially the maximum level at about 44 dpf. Moreover, there is a continuous, but rather slow rise even during the desiccation stage. On the other hand, the total protein content of the seed follows a completely different pattern as it increases very slowly up to 48 dpf and highest accumulation occurs during desiccation (Fig. 1).

In order to correlate the Hgg activities with Con A, inhibition by α-methyl-D-mannoside has been carried out. It was found that the latter can completely inhibit the Hgg activities of the extracts prepared from the seeds of various developmental stages. The minimum concentration of the sugar required for complete inhibition of the same Hgg units of the extracts of mid-maturation stage (36–40 dpf) and desiccation stage seeds are comparable to

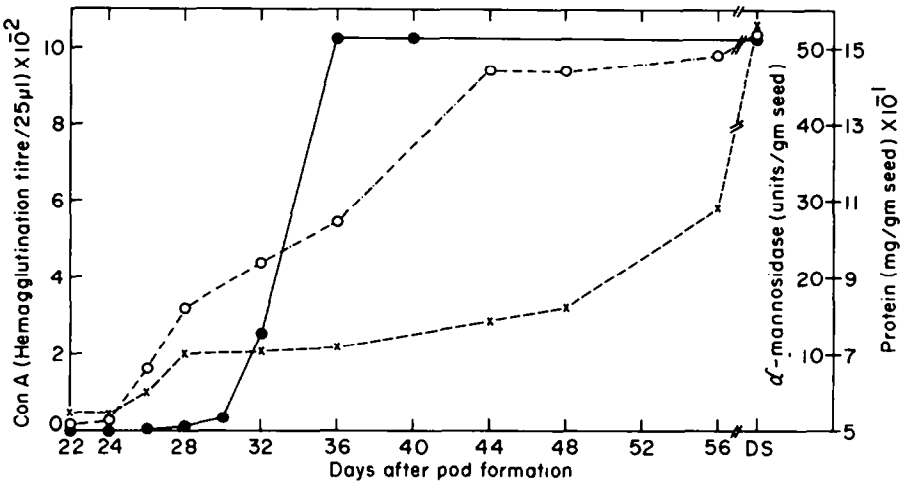

Fig. 1. Hemagglutinating and α-mannosidase activities of developing jackbean seeds. ●——● Hgg activity; O---O, α-mannosidase activity (1 unit = amount of enzyme that liberates 1 µmol of *p*-nitrophenol/min at 37°); ×---×, protein; DS, desiccation stage.

Table 1. Inhibition of hemagglutination by α-methyl-D-mannoside

Sample	Hgg dose of seed extract (as µg of Con A)	Minimum concentration of α-methyl-D-mannoside required for complete inhibition of Hgg activity (mM)
Early stage (24-26 dpf)	0.016	0.32
Middle stage (36–40 dpf)	0.163	1.3
Dry seed	0.163	1.3
Standard Con A	0.166	1.3

For inhibition of Hgg activity, serially diluted α-methyl-D-mannoside was first incubated with a definite Hgg dose of seed extracts (as determined earlier); then further incubated with 2% rabbit erythrocyte suspension as described in the text.

that for purified Con A, whereas for the early stage seeds, it is found to be considerably lower (Table 1).

Protein profile of developing jackbean seeds by SDS-PAGE

Developmental changes in the accumulation of jackbean seed proteins are shown in Fig. 2. Con A, as revealed by its subunit of M_r 26000, starts appearing at about 28 dpf, accumulates to a considerable extent at about 36 dpf and the maximum level is reached in the dry seeds.

The developmental profile of α-mannosidase, which is composed of two non-identical subunits of M_r 64000 and 44000 [5], is also presented in Fig. 2. The appearance of the larger subunit is detectable as early as 20 dpf and the intensity of the protein band increases with development, being highest in the dry seeds, whereas the smaller subunit cannot be resolved by SDS-PAGE from canavalin, the major storage protein of this seed.

The level of translatable mRNA for Con A and α-mannosidase in the developing jackbean seeds

To determine the levels of functional mRNA of Con A and α-mannosidase in the developing jackbean seeds, total RNA was prepared from the seeds of four different stages, i.e. early stage (24–26 dpf), early mid-maturation stage (30–32 dpf), mid-maturation stage (36–38 dpf) and early desiccation stage (above 50 dpf). The average yield was 1 mg/g of fresh tissue and the ratio of A_{260} to A_{280} was in the range of 1.9–1.95.

The *in vitro* translation of the total RNA was carried out by a wheat germ lysate and the translation product was immunoprecipitated with rabbit anti-Con A and anti-α-mannosidase sera. The specificities of the antisera were verified by immunoelectrophoresis (Fig. 3) and it was found that neither anti-Con A nor anti-α-mannosidase sera showed any cross-reactivity with α-mannosidase and Con A, respectively.

The high incorporation of labelled amino acids as revealed by TCA precipitable counts shows the efficient template activities of all the RNA preparations, the maximum efficiency being observed with the preparation from the mid-maturation stage seeds. However, the immunoprecipitable count for Con A is maximum at 24–26 dpf, with a sharp fall thereafter and it comprises of about 12% of the total translation product. In the case of α-mannosidase, the maximum immunoprecipitable count is obtained at early mid-maturation stage followed by a gradual decline thereafter (Table 2).

DISCUSSION

The biosynthesis and deposition of Con A and α-mannosidase follow definite patterns during the course of jackbean development. In the case of Con A, there is an initial lag phase followed by a logarithmic phase which lasts until about 36 dpf, whereas there is no pronounced lag for α-mannosidase and the rise is more or less gradual till it reaches an essentially high level. Nevertheless, in spite of these minor differences in the developmental patterns, maximal or near maximal accumulation of both

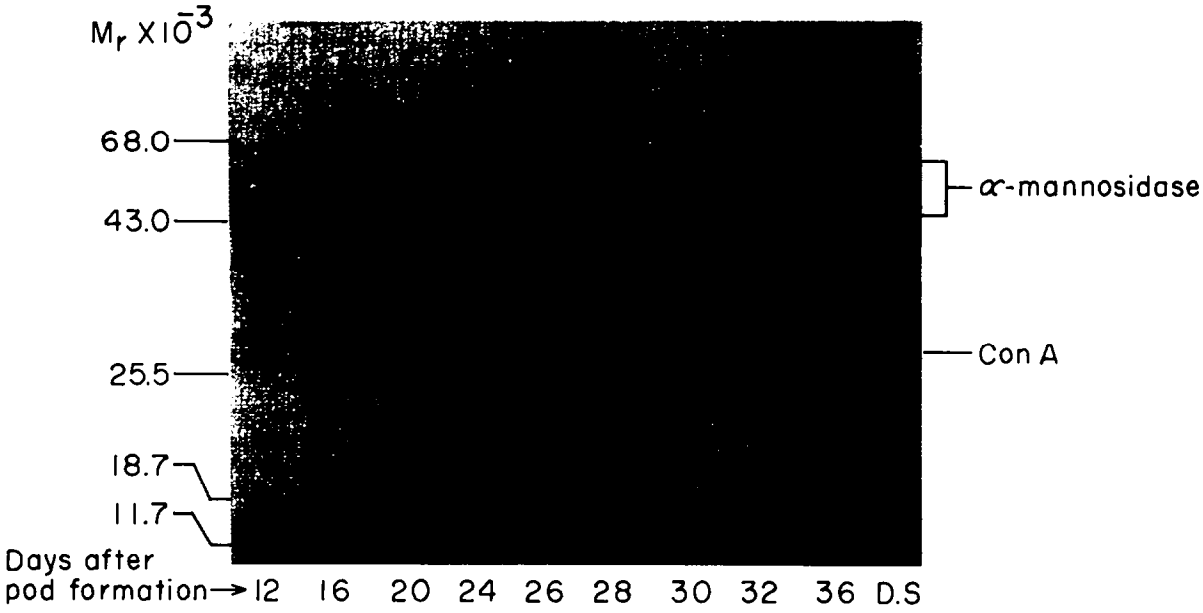

Fig. 2. Protein profile of developing jackbean seeds by SDS–PAGE. Crude extracts of the developing cotyledons were prepared as described in the text. 50 μl samples, representing the extracts from equivalent weights of seeds at various stages of development, were analysed by SDS-PAGE (12% acrylamide). Proteins were stained with Coomassie Blue. The numbers in the left margin represent the M_r of various standard protein markers.

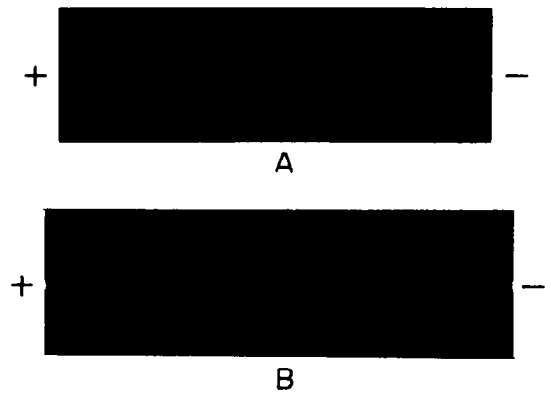

Fig. 3. Specificity of rabbit anti-Con A and anti-α-mannosidase sera. Immunoelectrophoresis was carried out in 1.5% agarose containing 0.5 M α-methyl-D-mannoside. (A). Well a, purified Con A (10 μg); well c, crude extract (50 μg); groove b, anti-Con A serum. (B). Well x, purified α-mannosidase (15 μg), well z, crude extract (100 μg); groove y, anti-α-mannosidase serum.

the proteins occurs in the mid-maturation stage of seed development, ranging approximately between 30 and 44 dpf, after the rapid cell division stage. These findings are consistent with the present concept of developmental regulation of seed reserve proteins [6, 7].

It has been shown earlier by employing Con A-specific antiserum that the Hgg activities associated with developing jackbean seeds are due to Con A and/or a Con A-like lectin (CLL) [8]. The present finding of the inhibition of Hgg activity by α-methyl-D-mannoside further substantiates this conclusion. Therefore, the apparent correlation between the Hgg activity profile and the relative amounts of two polypeptides of M_r 28 500 and 26 000 is to be expected since these polypeptides are the subunits of CLL and Con A respectively [8]. In the early stage (22–24 dpf) seeds, the Con A subunit is not detectable and the low Hgg activity is accounted for by CLL, as revealed by the presence of the polypeptide of M_r 28 500. The latter also constitutes one of the major polypeptides of the mid-maturation stage seeds while it is virtually absent in the desiccated seeds and at this stage, the mature Con A becomes one of the major proteins of the seed.

SDS-PAGE analysis also reveals the developmental changes of the larger subunit of α-mannosidase and the increasing intensity of this polypeptide (M_r 64 000) correlates with the enzyme activity profile. However, the smaller subunit (M_r 44000) is not fully resolved from canavalin (M_r 43000), the major storage protein of jackbean.

The accumulation of mRNA for these proteins, however, does not occur in the same manner since the highest amount of functional mRNA of Con A is found in the early stage seeds when the M_r 26 000 subunit of Con A is still undetectable, even though the lectin activity is positively identified. There is a sharp fall in the level of Con A mRNA as the seed develops and it remains low up to the early desiccation phase. In the case of α-mannosidase, the maximum accumulation of translatable mRNA occurs in the early mid-maturation stage (30–32 dpf) though there is no significant change in the mRNA content following this period. Hence in the case of both the proteins, the highest concentrations of mRNA are found at a stage which is well ahead of the period of the highest amount of protein accumulation. This is in contrast to the findings in the case of phytohemagglutinin (PHA) of *Phaseolus vulgaris* [9] and pea lectin [10, 11],

Table 2. Level of functional mRNA for Con A and α-mannosidase in developing jackbean seeds

Stages (dpf)	Amount of RNA added (μg)	TCA precipitable count (cpm × 10^{-5})	Immunoprecipitable count (cpm × 10^{-4})	
			Con A	α-Mannosidase
Early stage (24–26)	30.0	3.4	4.0	1.9
Early mid-maturation stage (30–32)	30.0	4.1	2.2	2.4
Mid-maturation stage (36–40)	30.0	5.1	1.7	1.8
Early desiccation stage (above 50)	30.0	2.5	1.8	1.5

Total RNA was isolated from four different stages of cotyledons and translated in wheat germ *in vitro* translation system as described in the text. Protein-bound radioactivity was determined by TCA-precipitation of an aliquot of the translation mixture and the radioactivity in the immunoprecipitates was determined for a portion of the SDS-solubilized protein in a liquid scintillation counter.

where the period of maximum lectin synthesis during seed formation coincides with that of highest lectin mRNA accumulation. This apparent variation in the kinetics of the appearance of mRNA and mature proteins in different developing seeds, seems to indicate that post-translational processing events in case of Con A and α-mannosidase are slow and distinct from those of PHA and pea lectin.

Furthermore, the present studies provide a more precise time scale of synthesis and deposition of these two proteins and it is expected to be the basis of more detailed studies of temporal regulatory processes operating during the process of seed development and maturation.

EXPERIMENTAL

Materials. Trizma, α-methyl-D-mannoside, *p*-nitrophenyl-D-α-mannoside, SDS, *N,N,N',N'*-tetramethylethylene diamine (TEMED), bis-acrylamide, β-mercaptoethanol (β-MET), Coomassie Blue R 250, Bromophenol Blue, sodium deoxycholate, Triton X 100, guanidine hydrochloride, dithiothreitol (DTT), 2,5-diphenyloxazole (PPO) and 1,4-bis-5-phenyl-2-oxazolyl benzene (POPOP) were purchased from Sigma. Phenylmethyl sulphonylfluoride (PMSF) and pancreatic RNase (EC 3.1.27.5) were from Boehringer-Mannheim, Gmbh. Micrococcal nuclease-treated wheat germ lysate as an *in vitro* translation kit was supplied by BRL, U.S.A. Protein A-Sepharose was from Pharmacia, Sweden. [^{35}S]-methionine (800 Ci/mmol) was supplied by Amersham, U.K. Complete and incomplete Freund's adjuvants were obtained from Difco Chemicals, U.S.A. and acrylamide and agarose were purchased from SRL, India. Protein A-suspension was prepared from *Staphylococcus aureus* (Cowan I strain) according to the method of Kessler [12]. Other chemicals were analytical grade reagents.

Plant material. Jackbean plants were grown in the garden of the institute and fresh seeds were collected from the green pods. Developmental stages of pods were determined by tagging the pods immediately after their formation. This stage was taken as day zero of development.

Preparation of seed extracts. Seeds of different developmental stages were peeled off and extracts were prepared in 10 vols of 10 mM Tris–HCl, pH 7.5. For the experiments with Con A, the extraction buffer was supplemented with 1 M NaCl and 1 mM PMSF.

Seeds were homogenized in a Sorvall Omni-mixer immersed in ice-water for 2 min at a time with 1 min intervals in between for a total of 10 min. The homogenates were then centrifuged at 20 000 *g* for 30 min and the clear supernatant fluid was collected.

Determination of protein. Protein was determined by the method of ref. [13].

Hemagglutination and its inhibition by α-mannoside. Hemagglutinating activity of the seed extract was determined [14] by incubating 25 μl of the serially diluted extract with 25 μl of 2% fresh rabbit erythrocyte suspension in saline as described in detail previously [8]. For the inhibition of hemagglutination by α-methyl-D-mannoside, assays were carried out essentially according to the method of ref. [14].

Assay of α-mannosidase activity. The assay method is based on [15] using *p*-nitrophenyl α-D-mannoside as the substrate. The liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm.

SDS-PAGE analysis of the seed proteins. Seed extracts of different stages were analysed by SDS–PAGE according to ref. [16] and stained with Coomassie Blue.

Preparation of antibody and immunoelectrophoresis tests. Antibodies against purified Con A and α-mannosidase were raised in rabbits following the same protocol as described previously [8]. Immunoelectrophoresis [17] was carried out in 1.5% agarose containing 0.5 M α-methyl-D-mannoside, using both purified proteins and crude seed extracts.

Preparation of total RNA from the seed. Total RNA was prepared from the seeds of different developmental stages, by the guanidinium chloride method of ref. [18]. In order to minimize RNase contamination the following precautions were taken: all the glasswares were baked at 250° for 4–6 hr; autoclaved glass-distilled H_2O was used for preparing solns and most of the solns except those containing Tris were treated with 0.1% diethyl-pyrocarbonate (Sigma Chemicals) for at least 12 hr and then autoclaved; gloves were worn throughout the experiment.

In vitro *translation of mRNA.* This was carried out in wheat germ lysate according to the protocol of the supplier (BRL) which is essentially based on the method of ref. [19]. The reaction mixture consisted of the following in a total vol. of 30 μl: 0.8 A_{260} units of wheat germ lysate (10 μl, in 20 mM HEPES, pH 7.5, 5 mM

magnesium acetate, 100 mM KCl, 5 mM β-MET, 1 mM $CaCl_2$, 2 mM EGTA), 30 mM potassium acetate, 0.1 mM magnesium acetate, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 0.2 mg/ml creatine kinase and 50 μM each of 19 amino acids (minus methionine) along with 10 μCi [^{35}S]-methionine and 30 μg total RNA. The reaction mixtures were incubated at 25° for 60 min.

Immunoprecipitation of translated product and analysis by SDS-PAGE. After the incubation for *in vitro* translation of the RNA, the reaction mixtures were centrifuged at 100000 *g* for 1 hr to remove the ribosomes. The supernatant solns were collected and the pellets were washed carefully with PBS. The combined supernatant soln and the washing was incubated with pre-immune sera for 1 hr at 37° in 20 mM phosphate buffer, pH 7.0, containing 0.3% sodium deoxycholate, 0.6% Triton X-100 and 0.5 M α-methyl-D-mannoside. The reaction mixture was further incubated for 3 hr at 4° followed by incubation with Protein A-sepharose (1 : 1 slurry in PBS) for 1 hr at 37°. After centrifugation, the supernatant fluid was collected and the immunoprecipitation was carried out with anti-Con A or anti-α-mannosidase sera as described above. This method is essentially based on ref. [20].

Acknowledgement—MRC and KN are Senior Research Fellows of the Council of Scientific and Industrial Research, of the Government of India.

REFERENCES

1. Spencer, D. and Higgins, T. J. V. (1982) in *The Molecular Biology of Plant Development* (Smith, N. and Grierson, D., eds) p. 306. Blackwell, Oxford.
2. Millerd, A. (1975) *Annu. Rev. Plant Physiol.* **26**, 53.
3. Dure, L. S. III (1975) *Annu. Rev. Plant Physiol.* **26**, 259.
4. Gatehouse, J. A., Croy, R. R. D. and Boulter, D. (1984) *CRC Critical Review Plant Sci.* Vol. I. 287.
5. Sammour, R. H., Gatehouse, J. A., Gilroy, J. and Boulter, D. (1984) *Planta* **161**, 61.
6. Chlan, C. A. and Dure, L. S. III (1983) *Mol. Cell. Biochem.* **55**, 5.
7. Bowles, D. J., Marcus, S. E., Pappin, D. J. C., Findlay, J. B. C., Eliopoulos, E., Maycox, P. R. and Burgess, J. (1986) *J. Cell Biol.* **102**, 1284.
8. Raychaudhuri, M. and Singh, M. (1986) *Phytochemistry* **25**, 793.
9. Hoffman, L. M., Ma, Y. and Barker, R. F. (1982) *Nucl. Acids Res.* **10**, 7819.
10. Higgins, T. J. V., Chandlar, P. M., Zurawski, G., Button, S. C., and Spencer, D. (1983) *J. Biol. Chem.* **258**, 9544.
11. Higgins, T. J. V., Chrispeels, M. J., Chandlar, P. M. and Spencer, D. (1983) *J. Biol. Chem.* **258**, 9550.
12. Kessler, S. W. (1975) *J. Immunol.* **115**, 1617.
13. Warburg, O. and Christian, W. (1942) *Biochem. Z.* **310**, 384.
14. Sarkar, M., Wu, A. M. and Kabat, E. A. (1981) *Arch. Biochem. Biophys.* **209**, 204.
15. Li, Y. T. (1966) *J. Biol. Chem.* **241**, 1010.
16. Laemmli, U. K. (1970) *Nature* **227**, 680.
17. Graber, P. and Williams, C. A. (1953) *Biochim. Biophys. Acta* **10**, 193.
18. Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinex, K. P., Bina-Stein, M. and Goldberger, R. F. (1977) *J. Biol. Chem.* **252**, 8310.
19. Roberts, B. E. and Peterson, B. M. (1973) *Proc. Natl Acad. Sci., U.S.A.* **70**, 2230.
20. Roberts, L. M. and Lord, J. M. (1981) *Eur. J. Biochem.* **119**, 31.