Phytochemistry, Vol 28, No 4, pp 1031–1035, 1989
Printed in Great Britain

0031-9422/89 $3.00+0.00

EVIDENCE FOR THE *DE NOVO* SYNTHESIS OF POLY(A) POLYMERASE IN GERMINATED WHEAT EMBRYOS

SUJATA LAKHANI,* ROMA KAPOOR, NEERAJ VERMA and R. C. SACHAR†

Biochemistry and Molecular Biology Laboratory, Department of Botany, University of Delhi, Delhi 110007, India

(*Received in revised form* 30 *August* 1988)

Key Word Index—Wheat embryos; poly(A)$^+$RNA, poly(A) polymerase, *de novo* synthesis, *in vivo* labelling

Abstract—A linear rise in the activity of poly(A) polymerase occurs following germination of excised wheat embryos. A roughly three-fold increase in poly(A) polymerase activity was paralleled by a rise (2.4-fold) in the relative abundance of poly(A)$^+$RNA isolated from 72 hr germinated embryos over that at six hr after imbibition. Administration of cycloheximide (20 μg/ml) to germinated embryos significantly inhibited (80%) poly(A) polymerase activity with a dramatic decline (77%) in the levels of total poly(A)$^+$RNA. This indicated that the relative abundance of poly(A)$^+$RNA is regulated by the modulation of poly(A) polymerase activity. The enhancement of poly(A) polymerase activity following embryo germination is primarily achieved through *de novo* synthesis of the enzyme. This has been conclusively shown by *in vivo* labelling of the newly synthesized total proteins with $^{35}SO_4^{2-}$ in wheat embryos and ultimately recovering the label in the purified preparation of poly(A) polymerase. Fractionation of purified poly(A) polymerase on native polyacrylamide gels revealed a single protein band, thereby establishing the electrophoretic homogeneity of the enzyme preparation. Autoradiography of this gel showed a single radioactive band which corresponded with the protein band of the purified poly(A) polymerase. Further characterization of the purified labelled poly(A) polymerase was obtained by acid hydrolysis of the enzyme protein followed by the chromatographic separation of the ^{35}S labelled amino acids. Autoradiography of the chromatogram revealed the presence of the label in the cysteine residues of poly(A) polymerase. These studies clearly indicated that *de novo* synthesis of poly(A) polymerase indeed occurs during growth of wheat embryos.

INTRODUCTION

Poly(A) polymerase plays an important role in the post-transcriptional polyadenylation of mRNA and hnRNA in germinating seeds [1]. The enzyme catalyses the covalent addition of AMP residues from its substrate ATP to the 3′ terminus of primer RNA [2]. Among plants, poly(A) polymerase activity has been reported in tobacco [3], maize [4], wheat [5] and pea [6]. Two distinct isozymes of poly(A) polymerase have been reported from wheat seedling chloroplasts and nuclear fractions. These isozymes differ in their primer RNA requirement [7].

Phytohormonal regulation of poly(A) polymerase by gibberellic acid (GA_3) has been reported from this laboratory in wheat aleurone layers [8], germinating excised wheat embryos [9] and pea epicotyls [6]. Gibberellic acid also increases the relative abundance of poly(A)$^+$RNA in barley and wheat aleurone layers [10, 11], and in germinated seeds of maize [12], castor bean [13] and hazel [14].

It has been suggested that the GA_3-induced poly(A) polymerase activity is responsible for the increase in the levels of poly(A)$^+$RNA in wheat aleurone layers and excised embryos [8, 9]. Inhibitor data (CHI) has indicated that the GA_3-stimulated poly(A) polymerase activity is dependent on *de novo* protein synthesis. However, blocking of transcription by cordycepin in GA_3-treated aleurone layers and embryos of wheat failed to inhibit poly(A) polymerase activity. This suggested that fresh transcription is not mandatory for the hormone-triggered poly(A) polymerase activity both in wheat aleurone layers [8] and excised embryos [9, 15].

Modulation of poly(A) polymerase activity could also occur by the post-translational structural modification of the enzyme. Rose and Jacob [16, 17] have purified the phosphorylated form of poly(A) polymerase from rat liver and hepatoma cells. The hepatoma enzyme was phosphorylated to a greater extent *in vivo* than the liver enzyme. Phosphorylation of the enzyme resulted in the activation (7-fold) of poly(A) polymerase isolated from the rat liver cells. Phosphorylation did not alter the extent, but augmented the rate of poly(A) synthesis as a result of increased affinity of the enzyme for its polynucleotide primer [16, 17].

So far, the enhancement of poly(A) polymerase following germination of wheat embryos was shown to be dependent on *de novo* protein synthesis. This was based on the circumstantial evidence obtained by inhibitor studies. We now provide a more conclusive proof for the *de novo* synthesis of poly(A) polymerase following germination of excised wheat embryos. The enzyme induction was paralleled by an increase in the total population of poly(A)$^+$RNA. Repression of the newly synthesized poly(A) polymerase by a translation inhibitor (CHI) simultaneously lowered the levels of total poly(A)$^+$RNA.

* Present address: ICGEB, NII Campus, New Delhi-67, India.
† Author to whom correspondence should be addressed.

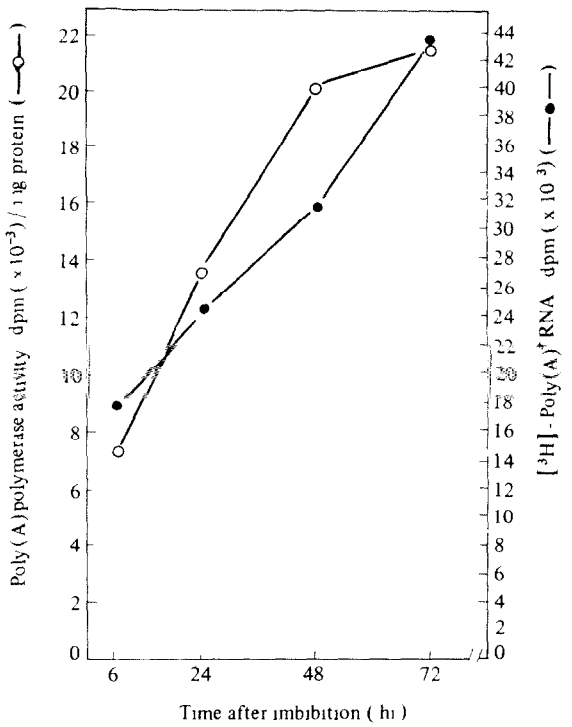

Fig 1 Time course study depicting the rise in the levels of poly(A)$^+$RNA and poly(A) polymerase activity at different times after wheat embryo imbibition The *in vivo* ^{3}H-labelled RNA was isolated from germinated embryos Each sample of total RNA, equivalent in radioactivity (1.5×10^5 dpm), was fractionated by affinity chromatography on poly(U) cellulose In a parallel set of embryos, the activity of poly(A) polymerase was determined at different stages after embryo germination in the *G-25 fraction* as described in [15]

RESULTS

Time course studies revealed a parallel rise in the level of ^{3}H-labelled poly(A)$^+$ RNA (2 4-fold) and poly(A) polymerase activity (3-fold) in germinated (72 hr) excised wheat embryos over that at 6 hr after imbibition (Fig. 1). Earlier, we reported inhibition of poly(A) polymerase activity following administration of cycloheximide (CHI 20 μg/ml) and amino acid analogues (1 mM each) to germinated wheat embryos This indicated the requirement of *de novo* protein synthesis for the rise in activity of poly(A) polymerase [15] We have also observed a significant decrease (77%) in the relative abundance of ^{32}P-labelled poly(A)$^+$RNA in CHI-treated embryos (48 hr) Thus, it seems that the enhancement of poly(A) polymerase activity may be responsible for the increased levels of poly(A)$^+$RNA *in vivo*

In an attempt to obtain conclusive evidence for the *de novo* synthesis of poly(A) polymerase in germinated excised wheat embryos, we adopted a strategy of labelling the proteins with $^{35}SO_4^{2-}$ *in vivo* Thereafter, we purified poly(A) polymerase to electrophoretic homogeneity and assayed it for the presence of the label in the sulphur-containing amino acids of the enzyme. For this purpose, excised wheat embryos were germinated in Nitsch's liquid basal medium supplemented with $^{35}SO_4^{2-}$ Various steps for the purification of poly(A) polymerase are depicted in

Fig 2 PAGE of poly(A) polymerase The ^{35}S-labelled poly(A) polymerase, purified by affinity chromatography on ATP-Sepharose, was electrophoresed on native polyacrylamide gels (7 5%) The position of the protein was localized by staining with silver nitrate a, Purified poly(A) polymerase, b, autoradiogram of a

Table 1. The purified poly(A) polymerase (1299-fold) revealed a single protein band on native polyacrylamide gels (7 5%), thereby proving the electrophoretic homogeneity of the enzyme preparation (Fig 2) The purified enzyme occurs as a single molecular form with a M_r of 65 000 (established by molecular sieving on Sephacryl S-200) Autoradiographic analysis of the labelled enzyme, fractionated on polyacrylamide gels, revealed a single radioactive band which co-migrated with the protein band of unlabelled poly(A) polymerase stained with silver nitrate (Fig 2)

The labelled, purified poly(A) polymerase was next examined for the presence of ^{35}S in the sulphur-containing amino acid residues of the enzyme protein molecule The labelled ATP-Sepharose fraction (2–4 mM ATP eluate) was hydrolysed with hydrochloric acid (6 M) at 110° for 4 hr The acid hydrolysate was subjected to paper chromatography and the sulphur-containing amino acids, cysteine and methionine, localized with ninhydrin reagent Autoradiography of the chromatogram revealed the presence of ^{35}S-label in the cysteine residue of poly(A) polymerase (Fig 3a, b) No label, could, however, be detected in the methionine Thus, the detection of [^{35}S]-label in the purified enzyme proved that the enhancement

Table 1. Purification of wheat embryo poly(A) polymerase

Purification step	Total protein (mg)	Poly(A) polymerase activity (dpm/mg protein)	Purification (fold)	^{35}S-Label incorporated (10^{-6} dpm/mg protein)
Crude extract	2900	28 350	1	2 14
Ammonium sulphate fraction	912	108 250	4	2.08
DE-52 fraction	202	369 000	13	2.96
ATP-Sepharose fraction	2.5	36 830 000	1299	0 65

Poly(A) polymerase was purified from germinated (48 hr) excised wheat embryos The embryos were germinated on Nitsch's liquid basal medium supplemented with $^{35}SO_4^{2-}$ Radioactivity was determined by acid precipitation of an aliquot at each step of purification. Poly(A) polymerase was assayed in embryos germinated in medium devoid of radioactivity.

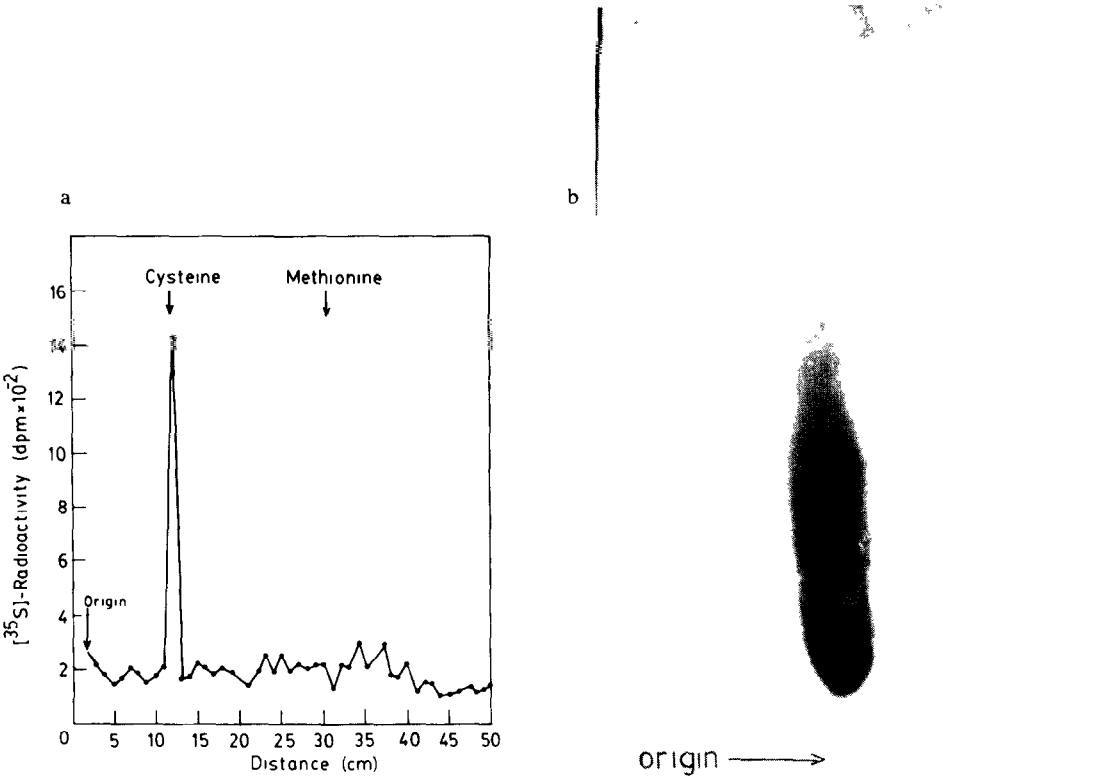

Fig 3. Chromatographic separation of sulphur-containing amino acids of purified poly(A) polymerase acid hydrolysate Purified wheat embryo poly(A) polymerase was hydrolysed in the presence of HCl (6 M) at 110° for 4 hr The amino acids in the hydrolysate were separated by PC using the solvent system, butanol–acetic acid–water (12.3 5) a, The chromatogram was scanned for localization of radioactivity in the sulphur-containing amino acids which co-chromatographed with authentic cysteine and methionine, b, autoradiogram showing ^{35}S-label in the cysteine residue.

of poly(A) polymerase activity, observed following germination of wheat embryos, is predominantly due to *de novo* synthesis of the enzyme.

DISCUSSION

Transcriptional and translational activities are rapidly initiated during early germination of wheat embryos [18, 19]. The post-transcriptional polyadenylation of both conserved and newly transcribed mRNA plays a vital role in the processing of RNA, so that it may become translationally active [1]. The present investigation has revealed that the enhancement of poly(A) polymerase activity in wheat embryos following germination is paralleled by an increase in the relative abundance of poly(A)$^+$RNA In wheat aleurone layers also, GA_3-

stimulated poly(A) polymerase activity was associated with a concomitant increase in the total poly(A)$^+$RNA levels [8, 11] Administration of cycloheximide to wheat embryos not only blocked the activity of poly(A) polymerase [15], but also results in a downward shift in the levels of poly(A)$^+$RNA. Similarly, the auxin-mediated inhibition of poly(A) polymerase was associated with a significant decrease in the levels of poly(A)$^+$RNA in pea epicotyls [6] Thus, it seems logical to conclude that modulation of poly(A) polymerase could be responsible for controlling the levels of poly(A)$^+$RNA

Although poly(A) polymerase has been reported in several plant systems [3–6], its regulation by *de novo* synthesis of the enzyme protein molecule has not been demonstrated by any rigorous method We now provide conclusive evidence for the *de novo* biosynthesis of poly(A) polymerase* enzyme in wheat embryos following germination This has essentially been shown by labelling the total newly synthesized proteins of germinating embryos with $^{35}SO_4^{2-}$ and finally recovering the radioactivity in the purified preparation of poly(A) polymerase The ^{35}S-labelled poly(A) polymerase revealed a single molecular form on acrylamide gels. Autoradiography of the gel showed a co-migrating radioactivity band which unequivocally proved that *de novo* synthesis of poly(A) polymerase occurs following germination of wheat embryos Further characterization of labelled poly(A) polymerase by acid hydrolysis revealed that the enzyme was selectively labelled at the cysteine residues

In mung bean hypocotyls (24 hr), the *de novo* synthesis of poly(A) polymerase has also been proved by labelling the enzyme with $^{35}SO_4^{2}$ The label was recovered both in the cysteine and methionine residues of poly(A) polymerase It appears that the three to four-fold stimulation of poly(A) polymerase in the hypocotyls of germinated mung bean embryos is due to *de novo* synthesis of this enzyme [29, 30]

EXPERIMENTAL

Isolation and culture of excised wheat embryos Dry wheat embryos (*Triticum aestivum* L var Sonalika) were isolated by a slightly modified procedure of [21]

Extraction and isolation of radioactively labelled poly(A)$^+$RNA from germinated excised wheat embryos Excised wheat embryos (0 5 g) were surface-sterilized with $HgCl_2$ soln, washed extensively, and cultured on Nitsch's liquid medium containing chloramphenicol (50 μg/ml) The cultures were maintained at 25° in the dark and transferred into a medium (10 ml) supplemented with [^{3}H]uracil (sp act 6100 mCi/mmol, 20 μCi/ml) and chloramphenicol (50 μg/ml) and placed on a reciprocating shaker for 6 hr at 25 The labelled RNA was extracted at various stages of germination and growth (6, 24, 48 and 72 hr) as well as from embryos grown (48 hr) in medium containing CHI (20 μg/ml), and suspended in Tris HCl buffer (20 mM, pH 7.6), heated at 60° for one min and chilled rapidly The labelled poly(A)$^+$ RNA was purified by affinity chromatography on poly(U) Sepharose as described previously [9] RNA was estimated according to [22]

In vivo labelling of poly(A) polymerase with $^{35}SO_4^{2-}$ Dry excised wheat embryos (30 g) were cultured on a modified Nitsch's liquid medium without sulphate in Petri dishes (15 ml/dish) Chloramphenicol (50 μg/ml) was added as a bactericidal agent The embryos were germinated in the dark at 25° After 18 hr, the imbibed embryos were transferred into Erhlenmeyer flasks containing Nitsch's basal liquid medium supplemented with $^{35}SO_4^{2-}$ (0 33 mCi/ml, 21 ml/flask) and incubated on a reciprocating shaker at 25° at 100 rpm for the next 30 hr

Extraction and purification of wheat poly(A) polymerase The germinated (48 hr) excised wheat embryos were rinsed in sterile dist H_2O, frozen in liquid nitrogen and stored at −60° Poly(A) polymerase was isolated from the germinated embryos grown in the absence and presence of $^{35}SO_4^{2-}$ in the culture medium

Preparation of the crude extract The embryonal tissue (100 g fr wt) was homogenized in Tris- HCl buffer (50 mM, pH 8 0) (1 3, w/v) containing 2-mercaptoethanol (5 mM) and polyvinyl polypyrrolidone (4%, w/v) Acid-washed sand was used as an abrasive The homogenate was filtered through 4 layers of muslin cloth and the residue was re-extracted in the homogenization buffer (1 2, w/v) The filtrates were pooled and centrifuged at 10000 *g* for 10 min at 0-4 An aliquot of the supernatant fraction (crude extract) was dialysed exhaustively against Tris–HCl buffer (20 mM, pH 8 0) containing 2-mercaptoethanol (2 mM) (dialysis buffer) for the assay of poly(A) polymerase activity

Salt fractionation The crude extract was brought to 30% saturation with recrystallized $(NH_4)_2SO_4$ chilled and centrifuged for 15 min at 15000 *g* The supernatant fraction was subsequently brought to 50% saturation, centrifuged as before, and suspended in *ca* 100 ml dialysis buffer containing EDTA (1 mM) The $(NH_4)_2SO_4$ fraction ppt (30–50% satn) was dialysed for the assay of poly(A) polymerase activity

Ion exchange chromatography The $(NH_4)_2SO_4$ ppt (30–50% satn) was further diluted (× 5) and loaded on a DEAE-cellulose (DE-52) column (20 × 3 5 cm) which had been equilibrated with Tris-HCl buffer (10 mM, pH 8 0) containing 2-mercaptoethanol (2 mM), EDTA (1 mM) and NaCl (0 1 M), at a flow rate of 0 5 ml/min After the protein was loaded, the column was washed with 3 bed volumes of the equilibration buffer and finally with the same buffer containing NaCl (0 15 M) Poly(A) polymerase was collected in the unbound fraction and concentrated against sucrose to *ca* one-tenth its original vol An aliquot (DF-52 fraction) was dialysed against the dialysis buffer for the assay of poly(A) polymerase activity

ATP-Sepharose affinity chromatography The *DE-52 fraction* was loaded on an ATP-Sepharose affinity column (9 5 × 2 cm, 50 mg protein) at a flow rate of 1 ml 4 min The affinity column was equilibrated with Tris–HCl buffer (10 mM, pH 7 9) containing 2-mercaptoethanol (2 mM), $MnCl_2$ (2 mM), EDTA (0 25 mM), NH_4Cl (50 mM) glycerol (20%) and bovine serum albumin (2 mg/ml) (equilibration buffer I), followed by the same buffer without bovine serum albumin (equilibration buffer II) ATP was covalently linked to Sepharose CL-4B by the method of ref [23] Poly(A) polymerase was eluted from the affinity column with buffer containing ATP (2–4 mM) according to [24] The fractions were pooled and the ATP was removed from the poly(A) polymerase preparation by selective binding to DF-52 (15 ml column) The wheat embryo poly(A) polymerase was eluted in Tris- HCl buffer (10 mM, pH 8 0) containing 2-mercaptoethanol (2 mM) The enzyme was frozen in liquid nitrogen and stored at −60°

*We have characterized the protein band on the native acrylamide gel to be poly(A) polymerase by (i) observing its polyadenylating activity on the gel [20], and (ii) characterizing its reaction product to be polyadenylate sequences This ruled out the possibility of BSA contamination which is used in the equilibration buffer of ATP-Sepharose affinity column and has almost the same M_r as poly(A) polymerase on SDS-polyacrylamide gels

Determination of radioactivity in the purified poly(A) polymerase. An aliquot of the ^{35}S-labelled poly(A) polymerase preparation was plated on a Whatman 3 MM disc and washed (×3) in chilled trichloracetic acid (5%) The acid was removed from the filter by repeated washings in Et_2O–EtOH (1 1) and Et_2O The filter was dried and radioactivity was determined in a toluene-based scintillation fluid [15]

Assay for poly(A) polymerase activity Poly(A) synthesis was measured essentially as described previously [25] The standard assay included HEPES buffer (20 μmol, pH 8 0), dithiothreitol (1 μmol), $MnCl_2$ (1 μmol), bovine serum albumin (100 μg), poly(A) primer (40 μg), [^{3}H]-ATP (10 μCi/μmol, 4 μCi, 0 4 μmol) in a final vol of 250 μl The concn of the enzyme protein preparation ranged from 500 μg for the crude extracts to 0 5 μg for the purified poly(A) polymerase The assay mixture was incubated at 37° for 30 min and the reaction was terminated by chilling and plating 40 μl on Whatman 3 MM discs The unincorporated [^{3}H]-ATP was removed by extensive washing in chilled trichloroacetic acid (5%) followed by Et_2O–EtOH (1 1) and finally in Et_2O The filters were dried at 60°, and the radioactivity was determinated as described previously [15] Radioactivity resulting from non-specific binding was measured in an assay mixture in which the enzyme preparation had been omitted The reading of this blank was subtracted from the values of each assay

Acrylamide gel electrophoresis of poly(A) polymerase The ATP-Sepharose fractions, representing poly(A) polymerase isolated from unlabelled and *in vivo* ^{35}S-labelled embryos, were fractionated on native polyacrylamide gels (7 5%) according to the method of ref [26] The slab gels were pre-washed in Tris–glycine buffer (100 mM, pH 8 3) for 40 min at 20 mamps/gel at 4–6° The ATP-Sepharose fractions (2–10 μg protein each) were loaded on the gel in sucrose (10%) at 20 mamps/gel After the protein had entered the stacking gel, the current was increased to 40 mamps/gel. Bromophenol Blue was used as a tracking dye After electrophoresis (2 5 hr), the unlabelled poly(A) polymerase was stained with $AgNO_3$ for the localization of the protein band according to ref. [27], and the position of the labelled enzyme was indicated by autoradiography.

Autoradiography of polyacrylamide gels The dried polyacrylamide gels were soaked in toluene for 40 min and then transferred into fresh toluene containing PPO (4 g/l) and POPOP (0 3 g/l) [28] The gels were again dried at 60° for 30 min and exposed to Sakura X-ray films with intensifying screens at −60° for 14 days for the detection of the radioactive bands on the gels.

Acid hydrolysis of the ^{35}S-labelled poly(A) polymerase The ^{35}S-labelled purified poly(A) polymerase was subjected to acid hydrolysis for the identification of ^{35}S-labelled amino acids For this purpose, an aliquot of the purified labelled enzyme was hydrolysed with HCl (6 M) for 4 hr at 110° under N_2. The hydrolysate was applied to Whatman 3 MM for ascending paper chromatography Authentic sulphur-containing amino acids (methionine 30 μg, cysteine 30 μg) were applied as markers Cysteine and methionine were also added as carrier amino acids. PC of the hydrolysate was performed for 18 hr in a solvent system comprising *n*-BuOH–HOAc–H_2O (12.3 5) at 25° The chromatogram was dried completely for the removal of acid The position of the marker amino acids were visualized by spraying with ninhydrin reagent followed by development of the colour at 80° for 10 min The chromatogram was autoradiographed as described above for locating the position of the ^{35}S-labelled amino acids.

Acknowledgements—RK and NV are thankful to CSIR for the award of junior research fellowships Supported by grant from D A E

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