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# Purification and Characterization of Novel Ribosome Inactivating Proteins, Alpha- and Beta-Pisavins, from Seeds of the Garden Pea *Pisum Sativum*

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**Two ribosome inactivating proteins designated α- and β-pisavins were isolated from seeds of the garden pea *Pisum sativum var. arvense Poir* with a procedure involving affinity chromatography on Affi-gel Blue gel, immobilized metal ion affinity chromatography on Iminodiacetic acid-agarose, cation exchange chromatography on Resource-S, and gel filtration on Superose 12. α- and β-pisavins are nonglycoproteins with a molecular weight of 20.5 kDa and 18.7 kDa respectively. The sequences of the fist sixty N-terminal amino acids of α- and β-pisavins were identical. In isoelectric focusing these two proteins merged into one band with a pI greater than 9.3. Inhibition of protein synthesis by a rabbit reticulocyte lysate system was achieved at an $IC_{50}$ of approximately 0.5 nM. Activity of the proteins toward tRNA was observed. The proteins acted on ribosomal RNA through its RNA N-glycosidase activity to release an Endo's fragment, and converted the conformation of DNA from supercoiled and circular forms into a linear form.** © 1998 Academic Press

***Key Words:*** **ribosome inactivating proteins; garden pea; pisavin.**

Ribosome inactivating proteins (RIPs) constitute a group of proteins capable of inhibiting protein synthesis by attacking the larger subunit of ribosomes. RIPs are divided into two subgroups based on the number of subunits they possess. Type 1 RIPs are made up of a single polypeptide chain with N-glycosidase activity while type II RIPs consist of two polypeptide chains, an RIP chain and a lectin chain, joined by a disulfide bond.

RIPs exhibit a variety of biological activities. RIP-treated ribosomes are no longer functional in protein synthesis (1). The functions of both elongation factors EF-1 and EF-2 are impaired (2). Cleavage of adenine (A-4324 in rat rRNA) interferes with the EF-1-dependent aminoacyl-tRNA binding to ribosomal acceptor site. Formation of EF-2-GDP-ribosome complex is disrupted and EF-2-catalyzed translocation of acceptor site aminoacyl-tRNA to the donor site is inhibited (3). Consequently protein synthesis is inhibited. RIPs may regulate protein synthesis in mature seeds (4) and in senescent or stressed leaves (5).

Under the influence of RIP, depurination of liver RNA occurs corresponding to A4324 of rat liver 28S rRNA in a highly conserved loop-and-stem structure (6). This activity is known as N-glycosidase activity. RIPs release adenine from adenine-containing polynucleotides such as RNA, DNA and polyA and hence are called polynucleotide: adenine glycosidase (7,8). Momorcharins, RIPs from *Momordica charantia,* induce ribonucleolytic cleavage in naked rRNA (9).

Supercoiled double-stranded DNA was converted by low concentrations of RIP into a nicked circular conformation and into a linear form at high concentrations (10,11). Single-stranded DNA is also a substrate for RIPs (12).

RIPs from *Phytolacca americana* and trichosanthin diminish infectivity of plant viruses (13-15). RIPs are normally excluded from the cytoplasm, but they enter cells, inactivate ribosomes leading to cell death and inhibit viral replication when the cell membranes are damaged by pathogens. The cereal seed RIPs inhibit fungal growth (16-17). The toxicity of RIPs to insect predators permits unthreatened seed germination (18).

Ricin with the galactose-binding lectin B-chain removed or blocked to increase specificity has been used in the construction of immunotoxin for cancer chemotherapy (19). Type 1 RIPs including momordin (20), pokeweed antiviral protein (21), gelonin and saporin (22,23) have been used in construction of immunotoxins which are useful in bone marrow purging, exhibit little toxicity to pluripotent progenitor, inhibit cancer cells and find application in preventing and treating graft-versus-host diseases.

Trichosanthin inhibits the replication of human immunodeficiency virus in acutely infected lymphoblas-



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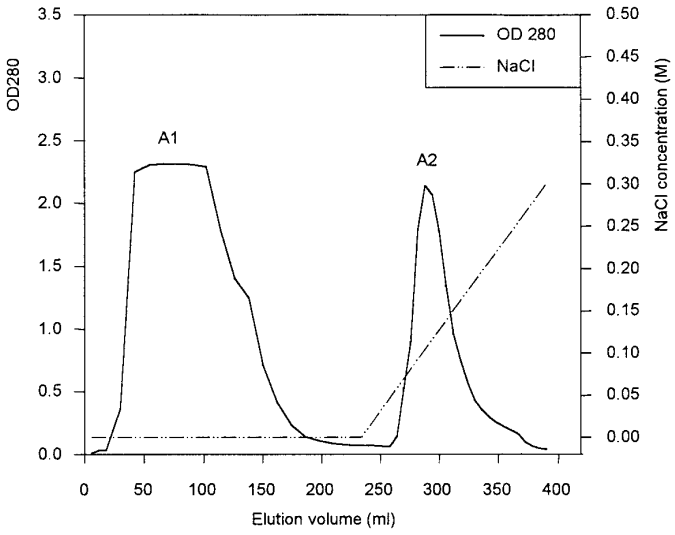


**FIG. 1.** Fractionation of the crude extract on Affi-gel Blue gel column equilibrated with the binding buffer (10 mM Tris-HCl, pH 7.2). The gel was washed with the binding buffer and eluted with a linear gradient of 0–0.3 M NaCl in the same buffer.

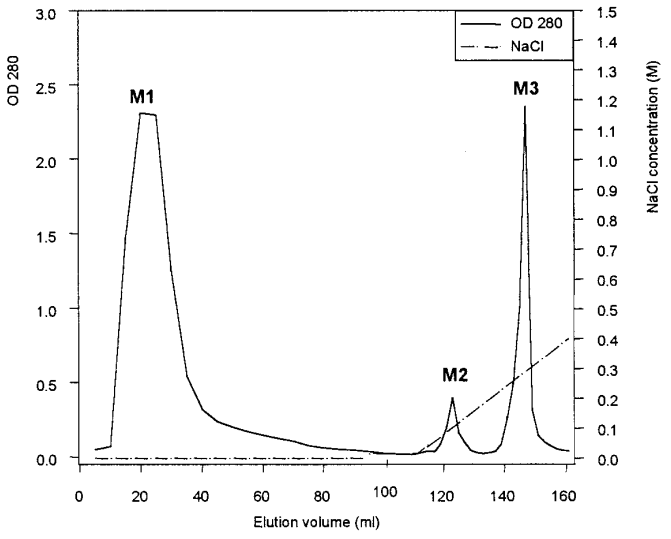


**FIG. 2.** Elution profile of the Iminodiacetic acid-agarose column. After chromatography on Affi-gel Blue gel, the resulting active fraction A2 was applied to Iminodiacetic acid-agarose.

toid cells or chronically infected macrophages (24). Pokeweed antiviral protein shows selective toxicity toward cells infected with poliovirus (25), influenza virus, herpes simplex virus (26,27) and human immunodeficiency virus (28).

The vast majority of RIPs are isolated from flowering plants, and in particular from the families of Cucurbitaceae and Caryophyllaceae, Hitherto no RIPs have been reported from the family of Papilionaceae. We describe herein the isolation and characterization of two closely related RIPs from the seeds of the garden pea *Pisum sativum*.

## MATERIALS AND METHODS

Dried ripe seeds of garden pea (*Pisum sativum var. arvense Poir*) purchased from a local supplier were soaked in 20 mM Tris-HCl buffer (PH 7.2) at 4°C for 4 h. The seeds were blended in 2 volumes of chilled buffer. The slurry formed was stirred at 4°C for 2 h and then passed through cheesecloth. Following centrifugation, proteins in the supernatant were precipitated by addition of 1 volume chilled acetone with gentle stirring on ice. After centrifugation the pellet obtained was dissolved in distilled water, dialyzed against distilled water at 4°C, and lyophilized. The resulting crude powder was dissolved in 20 mM Tris-HCl buffer (pH 7.2) and passed through a 0.22 $\mu$m syringe filter to remove particulates. The clear filtrate was applied on a column of Affi-gel Blue gel pre-equilibrated with the aforementioned buffer. After unadsorbed materials had been eluted, adsorbed proteins were eluted with a linear gradient of 0-0.3 M NaCl in Tris-HCl buffer. The fraction with N-glycosidase activity was concentrated by ultrafiltration and dialyzed against 10 mM Tris-HCl, 2 mM $Mg^{2+}$ (pH 7.2) before being applied to an Iminodiacetic acid-agarose column pre-equilibrated with the same buffer. After unadsorbed proteins had been washed off, adsorbed materials were eluted with a linear gradient of 0-0.4 M NaCl. The active fraction with N-glycosidase activity was applied to a Resource S column which was then eluted with a linear gradient of 0-0.4 M NaCl in the buffer. The fraction with N-glycosidase activity was concentrated and applied to a Superose 12 HR 10/30 column which was eluted with 20 mM Tris-HCl/0.1 M NaCl (pH 7.2). The garden pea RIPs could also be isolated by (i) affinity chromatography on Affi-gel Blue gel and elution of adsorbed active material with 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 7.2), (ii) ion exchange chromatography on DEAE-cellulose and elution of unadsorbed active material with 10 mM Tris-HCl buffer (pH 7.2) and then (iii) ion exchange chromatography on CM-Sepharose and elution of adsorbed material with a 0-1 M NaCl gradient in 10 mM Tris-HCl buffer (pH 7.2). The active material came off the column in 0.65 M NaCl.

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE).* Electrophoresis was performed according to the method of Laemmli and Favre (29). Both reducing SDS-PAGE in the presence of $\beta$-mercaptoethanol and non-reducing SDS-PAGE in the absence of

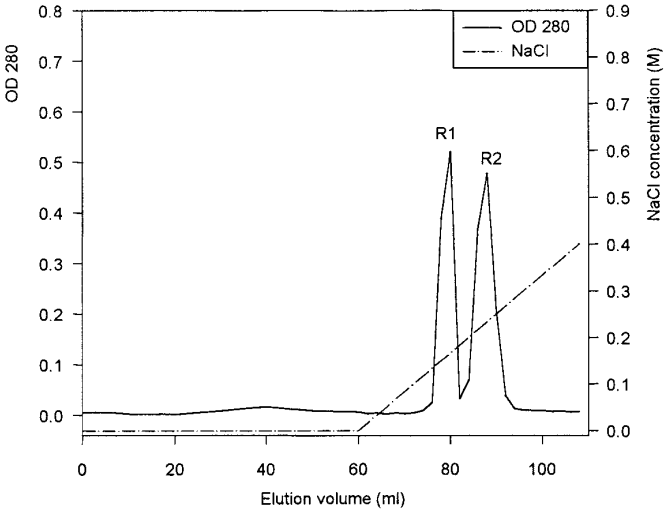


**FIG. 3.** Chromatography of M3 on a Resource-S cation exchange column. Unbound proteins were eluted with the binding buffer and adsorbed proteins with a 0–0.4 M NaCl linear gradient.

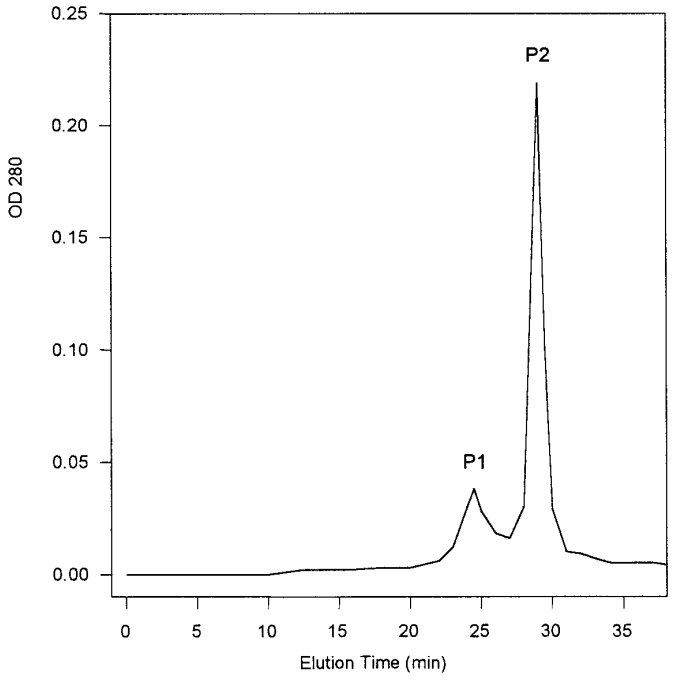


**FIG. 4.** Chromatogram on Superose 12 HR. 200 $\mu$l of R2 were loaded onto the column. P1 and P2 were eluted at the 24th and 29th min., respectively.

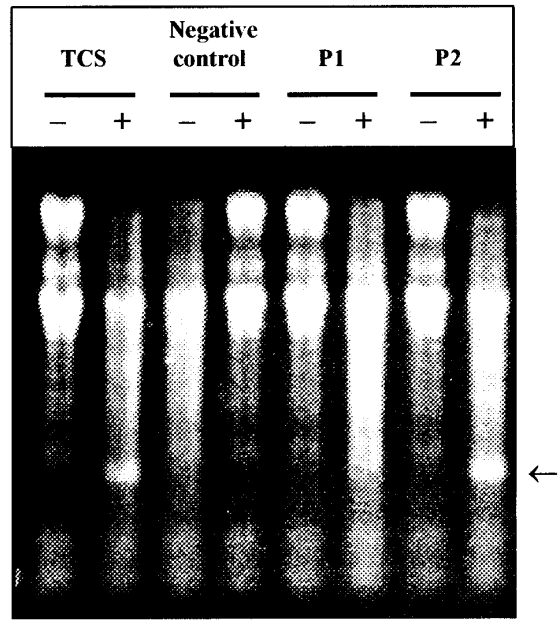


**FIG. 5.** Assay of the fractions from Superose 12 for N-glycosidase activity. 0.04 mg/ml of P1 and P2 were reacted with rabbit reticulocyte lysate. The rRNA was extracted and treated (+) or not treated (−) with aniline and analysed by 1.2 % formamide gel electrophoresis. The arrow denotes the position of rRNA fragments (Endo's band) resulting from aniline-catalysed hydrolysis. TCS (Trichosanthin) was taken as a positive control.

$\beta$-mercaptoethanol were carried out. Proteins in the gel were stained using either the Coomassie blue staining procedure or the silver staining procedure.

*Isoelectric focusing.* In order to determine the isoelectric point, the procedure of Edelstein (30) was followed.

*Detection of glycoproteins.* Glycoproteins were detected by staining with periodic acid-Schiff agent.

*Determination of N-terminal amino acid sequence.* Proteins were subjected to SDS-PAGE using 15% separating gel. Blotting was performed at 16 V for 30 min using a polyvinylidene fluoride membrane and a semi-dry transfer cell (Bio-Rad USA). After the transfer the membrane was stained and destained and then washed extensively with Milli-Q water. The stained band was cut out and air-dried. Amino acid sequencing was conducted with an HP G-1000A Edman degradation unit and an HP 1000 HPLC system.

*Assay for cell-free translation-inhibitory activity.* Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system (31) was used. The test sample (10 $\mu$l) was added to 10 $\mu$l of hot mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine and 1 $\mu$Ci-[4, 5-$^3$H] leucine ) and 30 $\mu$l working rabbit reticulocyte lysate containing 0.1 $\mu$M hemin and 5 $\mu$l creatine kinase. Incubation proceeded at 37°C for 30 min before addition of 330 $\mu$l 1 M NaOH and 1.2% $H_2O_2$. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckmam liquid scintillation counter.

*Assay for N-glycosidase activity.* The assay was conducted as detailed by Endo et al. (32) and Fong et al. (33). The test sample was

**TABLE 1**

Summary of Purification of RIP from Garden Pea

| Fraction | Protein (mg[a]) | IC50 (U[b]) | Specific activity (U/mg × $10^3$) | Total activity (U × $10^3$) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 625 | 0.4 | 0.0025 | 1.565 | 100 |
| Affi-gel Blue gel (A2) | 204 | 0.26 | 0.004 | 0.816 | 52.1 |
| Iminodiacetic acid-agarose (M3) | 21 | 0.035 | 0.028 | 0.59 | 37.7 |
| Resource-S (R2) | 5 | 0.01 | 0.1 | 0.5 | 31.9 |
| Superose 12 (P2) | 0.02 | $1 \times 10^{-5}$ | 10 | 0.4 | 25.6 |

[a] Protein obtained from 100g starting material.
[b] $IC_{50}$ is expressed in U. One U is defined as the concentration of protein (mg/ml) inhibiting protein synthesis by 50%.

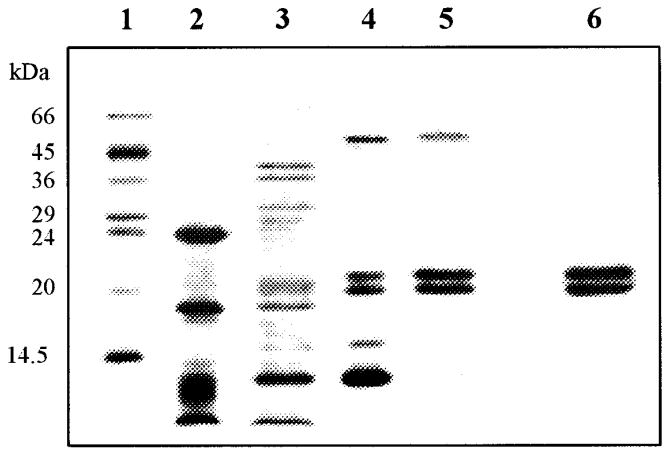


**FIG. 6.** Purity of the proteins at different stages of purification shown by SDS–PAGE. Lane 1: molecular weight standards; lane 2: crude extract; lane 3: A2 (from Affi-gel Blue); lane 4: M3 (from Iminiodiacetic acid-agarose); lane 5:R2 (from Resource-S); lane 6: P2 (from Superose 12).

incubated at 37°C with rabbit reticulocyte lysate in 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6, for 30 min. The reaction was terminated by addition of SDS to a final concentration of 0.5%. Total rRNA was extracted with phenol/chloroform, recovered by ethanol precipitation, and allowed to react with 1 M aniline/0.8 M acetic acid (pH 4.5) for 3 min at 60°C prior to electrophoresis in 1.2% agarose gel containing 50% formamide for 1.5 h at a constant voltage of 50 V. The gel was stained in ethidium bromide (0.5 mg/ml) and destained with distilled water. The RNA bands were visualized on a UV transilluminator and photographed with Polaroid 667 instant film.

*Activity toward tRNA.* The test sample was incubated with 200 $\mu$g tRNA (Sigma) in 150 $\mu$l sodium acetate buffer (pH 5.5) at 37°C for 1 h before the reaction was stopped by addition of 300 $\mu$l ice-cold 3.4% perchloric acid. The mixture was allowed to stand on ice for 15 min before centrifugation at 15000 g for 15 min at 4°C. The absorbance of the supernatant was measured at 260 nm after suitable dilution. One unit of RNase activity is defined as the amount of enzyme which produces an absorbance increase of one per hour at OD 260 nm under the specified conditions.

*Activity toward DNA.* The assay for DNase activity was performed as reported by Go et al. (34). The substrate, M13 mp 18 DNA (200 $\mu$g), was allowed to react with test sample in 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.5) at 37°C. The reaction was stopped by addition of agarose gel loading buffer. Electrophoresis was carried out in Tris-borate-EDTA buffer (pH 8) at a constant voltage of 60 V. After staining of the gel in ethidium bromide (0.5 mg/ml) and destaining in water, the DNA bands were visualized on UV transilluminator and photographed with Polaroid 667 instant film.

## RESULTS

The garden pea crude extract was fractionated on Affigel Blue gel into an unadsorbed peak A1 and an adsorbed peak A2 (Fig. 1). N-glycosidase activity resided in A2. When A2 was applied on the Iminodiacetic acid-agarose column a large unadsorbed peak M1, a small adsorbed peak M2 eluted at approximately 0.1 M NaCl and a sharp adsorbed peak M3 eluted at about 0.25 M NaCl resulted (Fig. 2). N-glycosidase activity was detected only in M3. M3 was fractionated by ion exchange chromatography on Resource S into two closely spaced adsorbed peaks R1 and R2 (Fig. 3). The latter peak possessed N-glycosidase activity. Gel filtration by fast protein liquid chromatography on Superose 12 yielded a small peak P1 and a sharp peak P2 (Fig. 4). P2 contained two bands with a molecular weight around 20 kDa and it possessed N-glycosidase activity (Fig. 5). The yields and specific activities of the various

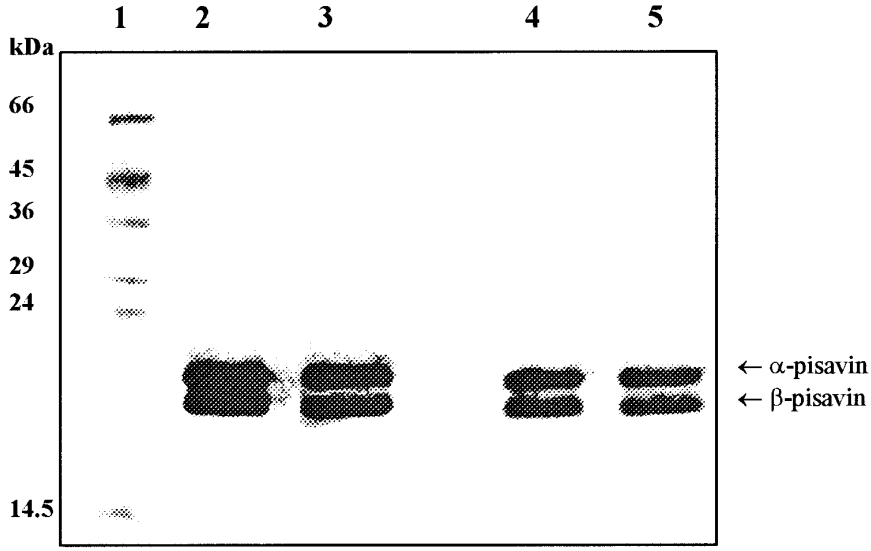


**FIG. 7.** Reducing and non-reducing SDS–PAGE following silver staining. Lane 1: molecular weight standards; lane 2: pisavin, $\beta$-mercaptoethanol present in sample loading buffer, mixture had not been boiled; lane 3: pisavin, $\beta$-mercaptoethanol present in sample loading buffer, mixture had been boiled; lane 4: pisavin, $\beta$-mercaptoethanol not present in sample loading buffer, mixture had not been boiled; lane 5: pisavin, $\beta$-mercaptoethanol not present in sample loading buffer, mixture had been boiled.

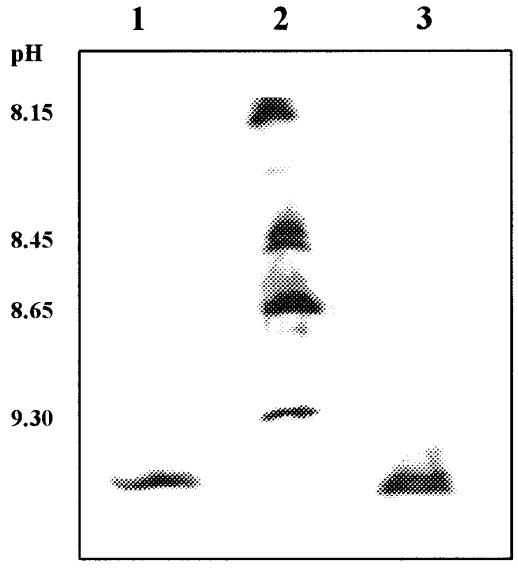


**FIG. 8.** Isoelectric focusing of pisavin. Lane 1: pisavin; lane 2: pI markers (Pharmacia); lane 3: Trichosanthin.

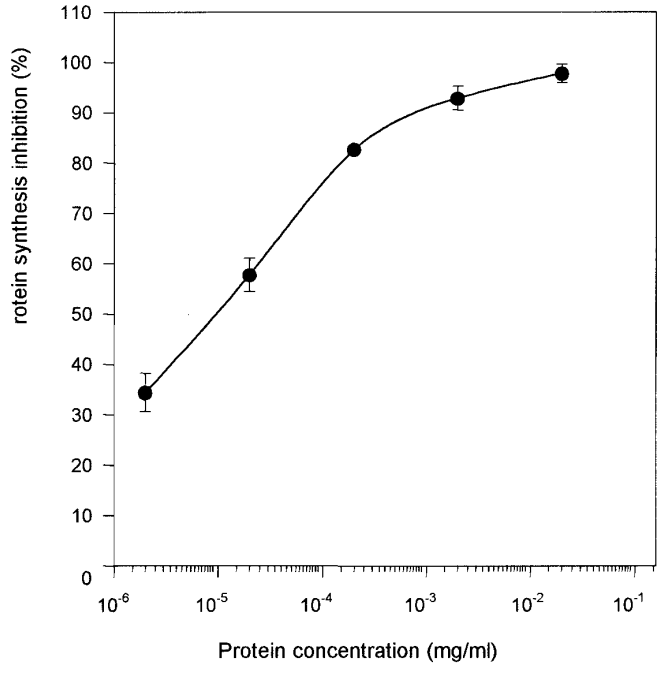


**FIG. 9.** Effect of pisavin on protein synthesis carried out by rabbit reticulocyte lysate system.

chromatographic fractions are presented in Table 1. The patterns of the chromatographic fractions in SDS-PAGE are shown in Fig. 6.

The molecular weights of the two components in P2 were calculated by linear regression from the molecular weight calibration curve, to be 20.5 kDa and 18.7 kDa respectively. The same picture was seen whether or not $\beta$-mercaptoethanol was present and whether or not the sample had been boiled prior to electrophoresis (Fig. 7).

The two aforementioned bands in P2 could not be further separated by altering the chromatographic conditions e.g. by making the salt gradient used for elution shallower, or by using other types of chromatographic media such as ConA-Sepharose and CM-Sepharose.

P2 was designated pisavin. The MW 20.5 kDa and 18.7 kDa components in P2 were designated $\alpha$- and $\beta$-pisavin respectively. They could not be separated by isoelectric focusing. They had a pI greater than 9.3 (Fig. 8), very similar to that of trichosanthin, another ribsome inactivating protein, and out of the range of pI standards.

In the staining test for glycoproteins using periodic acid-Schiff reagent, the nonglycoprotein RIP trichosanthin (20 $\mu$g) did not stain while the glycoprotein RIP $\alpha$-momorcharin (20 $\mu$g) did. Of the molecular weight standards, only ovalbumin stained. Alpha- and beta-pisavins did not give a positive reaction when tested at 42 $\mu$g pisavin per lane.

Pisavin inhibited protein synthesis in a rabbit reticulocyte lysate system with an $IC_{50}$ of 0.5 nM (Fig. 9). N-glycosidase activity was observable at 30 nM but was undetectable at 3 nM. The concentration of rRNA used in the assay was about 38 nM. Thus pisavin was capable of acting on an equimolar concentration of tRNA.

Preincubation of M13 mp18 DNA with pisavin yielded a linear form of DNA which exhibited a mobility in agarose gel electrophoresis in between those of supercoiled and circular DNA (Fig. 10). The linearized DNA was absent from the negative control, and from another control with the RIP $\alpha$-momorcharin added and the reaction terminated at time zero.

Pisavin acted on yeast tRNA with a ribonucleolytic activity about the same as that of $\alpha$-momorcharin. The activity of pisavin was 18 units/mg while that of $\alpha$-momorcharin was 13.5 units/mg, 1 unit being defined as an absorbance increase of one at 260 nm in 1 hour.

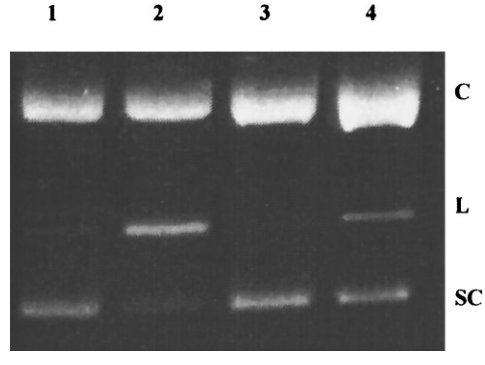


**FIG. 10.** Activity of pisavin on M13mp18 DNA using $\alpha$-momorcharin as a positive control. Lane 1: 200 ng $\alpha$-momorcharin, reaction stopped at time 0; lane 2: 200 ng $\alpha$-momorcharin; lane 3: negative control; lane 4: 200 ng pisavin. For lanes 2 to 4, reaction was stopped after an incubation period of 12 hours. C: circular; L: linear; and SC: supercoiled forms of DNA.

**TABLE 2**

Comparison of N-Terminal Amino Acid Sequences of Miraculin and α- and β-Pisavins

| | |
|---|---|
| Miraculin | D S A P N P V L D I D G E K L R T G T N Y Y • I • V P V L R D H |
| α- and β-Pisavin | A P E P V L D V S G K K L I T G V K Y Y F I L • P V I R G K |
| Miraculin | G G G L T V S A T T P • • • • • N • G T F V • C P P R V V Q T R |
| α- and β-Pisavin | G G G L • N V A • N V N N L N S N ? P T H P L Y • • • V V Q E K |
| Miraculin | K E V D H |
| α- and β-Pisavin | L ? V W N |

*Note.* Identical residues are underlined. ? = could not be determined with certainty.

Table 2 presents a comparison of the N-terminal amino acid sequences of α-pisavin, β-pisavin and miraculin, a taste modifying protein from the miracle fruit *Richadella_dulcifera*. Many sites of identity were detected between the first 36 amino acids of miraculin and the first 34 amino acids in α- and β-pisavins.

The N-terminal amino acid sequences of α-pisavin and β-pisavin are presented in comparison with those of type 1 and type 2 RIPs in Table 3. There are 15 amino acids which are invariant out of the first 60 N-terminal residues examined in the RIPs αMMC, TCS, abrin A chain and ricin A chain. There were 9 amino acids in pisavin which were identical to these invariant amino acids. Alignment of the N terminal sequences of pisavin and abrin A chain in order to maximize identity revealed 19 identical residues out of the 60 examined (Table 4). From Table 5, it can be seen that a higher degree of similarity existed between pisavin and miraculin (47% identity). The resemblance between pisavin and the reported RIPs was less striking (15-28% identity). There is also 18-30% identity between the type 1 and A chains of type 2 RIPs examined. The similarity among the type 1 RIPs and also that among the A chains of type 2 RIPs are higher (over 50% identity).

**TABLE 3**

Comparison of N-Terminal Amino Acid Sequences of Pisavin and Other Ribosome Inactivating Proteins

| | |
|---|---|
| α-MMC (1): | DVSFRLS••GADPRSYGMFIKDL•RNALPFREK |
| TCS (1): | DVSFRLS••GATSSSYGVFISNL•RKALPNERK |
| Abrin A (4): | PIKFSTE••GATSQSYKQFIEAL•RERLRGGL• |
| Ricin A (8): | IINFTTA••GATVQSYTNFIRAV•RGRLTTGAD |
| Pisavin (7): | DVSGKKLITGV••KYY••FILPVIRGKGGGLN• |
| α-MMC (31): | V•YNIP•LL••L•PSVSGAGRYLLMHL•F•NYDG |
| TCS (31): | L•YDIP•LL••R•SSLPGSQRYALIHL•T•NYAD |
| Abrin A (30): | I•HDIP•VLPDP•TTLQERNRYITVEL•S•NS•D |
| Ricin A (31): | VRHDIP•VLPNR•VGLPINQRFILVEL•S•NHAE |
| Pisavin (29): | ••VANVNNLNSN?PTHPLYVVQEK••L?VWN |

*Note.* α-MMC (1) and α-MMC (31) refer to D and V being respectively the 1st and 31st residues in α-momorcharin.rcs=trichosanthin. Identical residues are underlined. ? = could not be determined with certainty.

## DISCUSSION

In the present investigation two new RIPs were purified from garden pea which belongs to family Papilionaceae from which no RIP has been reported. A series of chromatographic methods was employed in the purification scheme, including affinity chromatography on Affi-gel Blue gel, immobilized metal ion affinity chromatography (IMAC) on Iminodiacetic acid-agarose, cation exchange chromatography on Resource-S and gel filtration on Superose 12 HR 10/30. IMAC has not previously been used for isolation of RIPs but was found to be useful in the present study. The rest of the aforementioned chromatographic techniques have been utilized in RIP purification scheme. A lot of proteins without N-glycosidase (i.e. RIP) activity was removed from the active fraction by IMAC.

Like the RIPs α- and β-momorcharins from bitter gourd (*Momordica charantia*) seeds, α- and β-pisavins were adsorbed on Affi-gel Blue gel (33). The pisavins also resembled most other RIPs (35) in that they were adsorbed on cationic exchangers such as Resource-S and CM-Sepharose and unadsorbed on anionic exchangers such as DEAE-cellulose. The garden pea RIPs demonstrated a pI similar to that of trichosanthin.

Two RIPs with very close molecular weights, 20.5 kDa and 18.7 kDa, designated α- and β-pisavin respectively, were isolated from garden pea seeds. Type 1 RIPs reported to date mostly have a molecular weight in the range 25 kDa-30 kDa. Although small RIPs with a molecular weight of approximately 10 kDa have re-

**TABLE 4**

Comparison of N-Terminal Amino Acid Sequences of Abrin A Chain and Pisavin

| | |
|---|---|
| Abrin A: | •••••QDRP•IK•FSTEGATSQSYKQFIEA•LR |
| Pisavin: | APEPVLD•VSGKKLIT•GV••KYY••FILPVIR |
| Abrin A: | ERLRGGLIHDIP•VLPD••PTT•L•••QER |
| Pisavin: | •GKGGGLNVANVNNLNSN?PTHPLYVVQEK |
| Abrin A: | NRYITVEL•S•N |
| Pisavin: | •••••••L?VWN |

*Note.* Identical residues are underlined. ? = could not be determined with certainty.

**TABLE 5**

Sequence Similarity between Pisavin and Ribosome Inactivating Proteins and between Pisavin and Miraculin

| Proteins compared | % identity in sequence examined (no. of identical residues) |
|---|---|
| Pisavin-α-MMC | 20% (12) |
| Pisavin-TCS | 20% (12) |
| Pisavin-Abrin A | 31% (19) |
| Pisavin-Ricin A | 18% (11) |
| Pisavin-Miraculin | 47% (28) |
| α-MMC-Abrin A | 30% (18) |
| α-MMC-Ricin A | 28% (17) |
| α-MMC-TCS | 57% (34) |
| TCS-Abrin A | 37% (22) |
| RCS-Ricin A | 38% (23) |
| Abrin A-Ricin A | 51% (31) |

*Note.* The sequence examined was the first 60 N-terminal amino residues. The sequences of the proteins compared were aligned to maximize similarity. α-MMC, α-momorcharin; TCS, trichosanthin.

cently been reported (36), a molecular weight of circa 20 kDa has not so far been found for RIPs.

Alpha-and beta-pisavins merged into a single band in isoelectric focusing, indicating that they have the same or very close pI values. The sequences of the first 60 N-terminal amino acids in the 2 proteins are identical, suggesting that α- and β-pisavins are closely related proteins. The difference of 2 kDa in molecular weight between them might be due to deletion of a sequence of amino acids from the C-terminal of the larger protein, α-pisavin in view of the observation that neither of them is glycosylated.

The pisavins are unique in their sequence similarity to both miraculin and RIPs, with the former occurring to a greater extent. Remarkable resemblance (about 65% identity) exists between pisavins and miraculin, a taste-modifying protein from *Richadella dulcifica* (37,38), in the first 34 to 36 N-terminal amino acid residues. There is a reduced extent of similarity (approximately 25% identity) between the proteins in the next 25 residues. Although pisavins resemble RIPs in amino acid sequence to a lesser degree, the similarity is still considerable. Pisavins possess 8 out of the 15 invariant residues identified in the RIPs α-momorcharin, trichosanthin, abrin A chain and ricin A chain. Some 20 to 30% identity in amino acid sequence between pisavins and these RIPs is observed. It deserves mention that a similar extent of sequence identity exists between type 1 RIPs (α-momorcharin and trichosanthin) and the A chains of type 2 RIPs (abrin and ricin).

In spite of sequence similarity, pisavins differ from miraculin in a number of aspects. Pisavins are single-chained while miraculin is composed of 2 subunits. Pisavins are nonglycoproteins while miraculin is glycosylated. The molecular weight of miraculin (28 kDa) is appreciably larger than those of α- and β- pisavins.

The $IC_{50}$ of pisavin in the cell-free translation inhibition assay is within the range of $IC_{50}$ values reported for RIPs. The enzymatic properties of pisavin are also typical of RIPs. Pisavin possesses RNA N-glycosidase activity, DNase activity and ribonuclease activity on tRNA.

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