Purification of a ribosome-inactivating protein with antioxidation and root developer potencies from *Celosia plumosa*

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Abstract Considering *Celosia plumosa* as a potent antiviral plant, the attempt was made to determine, purify and characterize its proteinaceous antiviral elements against tobacco mosaic virus hypersensitive response on *Nicotiana glutinosa*. By using 60% ammonium sulphate-precipitation, FPLC-based anion and cation-exchange chromatography in 10 and 50 mM NaCl, size-exclusion chromatography in 50 mM NaCl and SDS–PAGE 10%, a 25 kD antiviral protein with ribosome-inactivating/28S rRNase ability was purified from the leaves of *C. plumosa* at vegetative growth stage. The purified protein showed FRAP-based antioxidant activity in vitro and caused 1.7-fold and 1.4-fold increases in the growth rate of root system upon carborundum-based application on the root growth medium of *N. glutinosa*. The present work reports an antiviral protein with ribosome-inactivating, antioxidation and root developer potencies in *C. plumosa* as an edible or ornamental plant that may be useful in health and agricultural biotechnology in the future.

Keywords Antioxidant · Antiviral · *Celosia plumosa* · Ribosome inactivating · Root growth

Abbreviations

FRAP Ferric reducing antioxidant power
AVP Antiviral proteins
RIP Ribosome-inactivating proteins
TMV Tobacco mosaic virus

BME Beta mercaptoethanol
PVP Polyvinylpyrrolidone
FPLC Fast performance liquid chromatography
SDS Sodium dodecyl sulphate
EGTA Ethylene glycol tetraacetic acid
DTT Dithiothreitol
BSA Bovine serum albumin

Introduction

Plants antiviral proteins (AVP) belong to a class of enzymatic ribosome-inactivating proteins (RIP) that have been isolated and characterized from different tissues of a large number of higher plants covering approximately 17 families and 30 species. They are site-specific rRNA N-glycosidases (EC number 3.2.2.22) that specifically remove a universally conserved adenine residue from the sarcin loop of the large ribosomal RNA in both prokaryotic and eukaryotic cells, resulting in the inhibition of the host cell protein biosynthetic apparatus (reviewed by Barbieri et al. 1993; Girbes et al. 2004; Stripe and Batteli 2006; Shu et al. 2009; Schrot et al. 2015). They have also been isolated from different fungi, algae and bacteria (Schrot et al. 2015).

Complex biological roles have been described earlier for ribosome-inactivating antiviral proteins in different organisms. They have been mostly linked to antiviral, antifungal and insecticidal defense mechanisms (Hong et al. 1996; Iglesias et al. 2005). Recent understandings of these proteins enhanced their diverse applications in plant protection against pathogen attacks as well as in therapeutics and medicine in human beings (Stripe and Batteli 2006; Puri et al. 2009; Sobiya and Jannet 2013). Ribosome-inactivating antiviral proteins impart very high level of

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resistance to viruses and are being used as one of the best antiviral strategies against a wide spectrum of plant viruses such as tobacco mosaic virus (TMV), sunnhemp rosette virus (SRP), potato virus X (PVX), citrus ring spot virus (CRSV) (Peumans et al. 2001; Pandit et al. 2013; Sobiya and Jannet 2013). They not only exhibit antiviral activities towards different plants viruses but also they show inhibitory activity against numerous animal and human viruses such as human immunodeficiency virus (HIV), human simplex virus (HSV), polio, influenza and hepatitis B viruses (Uckun et al. 2003; He et al. 2008; Pizzo and Antimo 2016). Nowadays, plant ribosome-inactivating antiviral proteins have attracted a lot of attention in biomedical research towards immunotoxicity (Battelli et al. 1996), abortifacient (Yeung et al. 1988) and bioactive properties including antiviral, antifungal, antibacterial, antioxidant and antitumor (Parikh and Tumer 2004; Stripe and Batteli 2006; Shu et al. 2009; Puri et al. 2009). On the other hand, a toxic group of these proteins could be utilized as biological weapons and defense elements (Knight 1979; Pizzo and Antimo 2016). To date, the genes and the proteins of various AVP are available as strong biotechnological tools in different industries. Among different plants, *Celosia* species are known as potentially AVP/RIP containing plants. They belong to the family of *Amaranthaceae* and mostly planted as ornamental and pharmaceutical edible plants in Africa, South America, India and some other parts of Asia (National Research Council 2006). The red plumed and crested plant “*Celosia cristata*” (commonly known as crested cocks comb) can be potentially estimated as decorative and medicinal plant species. It is often used for the treatment of hematemesis, abnormal uterine bleeding, hematochezia, hemorrhoidal bleeding, leukorrhea, chronic dysentery with persistent diarrhoea, redness of the eye and dizziness (Kirtikar and Basu 1935; Surse et al. 2014). Several biological activities like antioxidant, antiviral, anti-aging and anthelmintic actions are attributed to this plant species (Pyo et al. 2008; Woo et al. 2011; Rubini et al. 2012). Its leaves contain two glycoproteins, namely ‘CCP-25’ and ‘CCP-27’ with ribosome-inactivating antiviral/antioxidant ability (Balasubrahmanyam et al. 2000; Gholizadeh and Kapoor 2004; Gholizadeh et al. 2005).

Considering all these together, to increase our knowledge about the *Celosia* plants, we attempted to purify and characterize the antiviral elements of *C. plumosa*. Another objective of the present work was to investigate and contribute to the novel research challenges of AVP/RIP including the antioxidant and root growth effects of the purified protein.

Materials and methods

Materials

Plant material including *Celosia plumosa* as an ornamentally planted type of *Celosia* was collected from different places of Iran and selected as test for antiviral protein extraction and purification. The seeds of *Nicotiana glutinosa* were obtained from Iran Plant Protection Institute by Dr Reza Pourrahim. The leaves of these plants were utilized for the local lesion assay and antiviral activity test. TMV-inoculated leaves of *Nicotiana tabacum* were provided by Dr Reza Pourrahim from Iran Plant Protection Institute.

Crude protein extraction

Crude protein was extracted from the fresh leaves of *C. plumosa* test plants at vegetative stage, washed sequentially and dried at room temperature. According to Balasubrahmanyam research group (Balasubrahmanyam et al. 2000), about 40 g of dried leaf materials were homogenized with 7-volumes of extraction buffer (consisting of 0.1 mM sodium acetate (pH 5.2), 12 mM β -ME and 20 mg per 250 mL PVP) in a warning blender. The obtained slurry was filtered through the muslin cloth and centrifuged at $12,000\times g$ for 15 min by using refrigerated centrifuge 3K30 (Sigma, Germany). The clear supernatant was collected for the antiviral assessment test.

Virus inoculum preparation

Virus inoculum was prepared by homogenizing the 15 g of TMV-infected leaves of *N. tabacum* with 50 mL of 20 mM sodium–potassium phosphate buffer (pH 7.0) in a sterilized condition. The obtained pulp was squeezed through the two layers of muslin cloth and the filtrate was centrifuged at $12,000\times g$ for 10 min by using refrigerated centrifuge 3K30 (Sigma, Germany). After three times dilution with distilled water, the clear supernatant was used as virus inoculum, so as to produce countable number of lesions on the test plant leaves.

Antiviral test

For antiviral test assay, *N. glutinosa* was selected as host plant. Test plants with the same height, age and vigor were considered for the experiments. The concentrated volume of crude protein extract (containing 50 $\mu\text{g/mL}$ total protein) of *C. plumosa* was uniformly applied on the host plants leaves. To apply the equal amounts of total protein on host leaves, the concentration of the total protein of each

extract was measured by Bradford method using bovine serum albumin (BSA) as standard solutions (Stoscheck 1990). The absorbance of the solutions was determined at 595 nm by using UV-1800 UV/VIS spectrophotometer (Rayleigh, China). As control sample, host plant leaves were only treated with buffer and virus inoculum. After 1 h, the protein treated leaves were washed, gently blotted and dried. The leaves were sprinkled with carborundum powder 600 mesh and inoculated with virus inoculum. After inoculation, the leaves were washed and observed for the development of lesions after 3–4 days of inoculation (Fig. 1).

The inhibitory activity of the test protein extract was determined by counting the local lesions and calculated in terms of percentage inhibition using the following formula according to previous report (Balasubrahmanyam et al. 2000):

$$\% \text{inhibition} = [C - T/C] \times 100$$

where, C is the average number of lesions in control plant and T is the average number of lesions in protein extract treated plants.

Fig. 1 Antiviral test and SDS-PAGE analysis. Upper panel: The antiviral ability of the size-exclusion chromatography product was tested on *N. glutinosa* leaves treated with TMV. Lower panel: SDS-PAGE analysis of size-exclusion chromatography product on 10% polyacrylamide gel. M protein marker, P purified product

Ammonium sulphate precipitation

The ammonium sulphate precipitation of protein extract was carried out at 25, 60, 80, 100 and 120% salt saturations. The obtained protein containing sample at each step was centrifuged at $12,000 \times g$ for 10 min and the supernatant was used for the next precipitation step. The pellets were resuspended in extraction buffer (consisted of 20 mM sodium phosphate, pH 6.2 and 10 mM NaCl) and dialyzed against the same buffer to remove the extra salts using EDTA-treated membranes. The clear sample after dialysis and removal of the salts were tested for antiviral activity. Data presented on the table are related to the extraction process of 40 g of dried starting material.

Anion-exchange chromatography

The active protein fraction obtained from the ammonium sulphate precipitation step was concentrated by freeze dryer. The concentrated protein sample was loaded onto BioRad anion exchanger column using FPLC system and washed with extraction buffer containing 20 mM sodium phosphate, pH 6.2 and 10 mM NaCl. The unabsorbed fraction of protein sample was collected and subjected for antiviral test assay.

Cation-exchange chromatography

The active unadsorbed fraction of anion exchanging step was concentrated by freeze dryer. The concentrated protein sample was loaded onto BioRad cation exchanger column by using FPLC system and washed with extraction buffer (consisted of 20 mM sodium acetate, pH 5.2, 0.01% sodium azide and 50 mM NaCl). The eluted protein fraction was tested for antiviral activity.

Size-exclusion chromatography

The pooled protein fraction of cation exchanging step was concentrated by freeze dryer and loaded onto Sepharose-12 column using filtration buffer consisting of 20 mM sodium acetate, pH 5.2, 0.01% sodium azide and 25, 50, 75, 100 and 125 mM of discontinues gradient of NaCl. Fractions falling within the protein peak at 280 nm were collected and tested for antiviral activity. Data presented in the table are related to the extraction process of 40 g of dried starting material.

SDS-PAGE analysis

SDS-containing polyacrylamide gel electrophoresis was performed in a 10% separating gel according to Laemmli (1970). The concentrated protein sample after 3 min

boiling was loaded and run in 100 V and then 150 V for 2 h. The protein bands were visualized on the gel by using Coomassie Brilliant Blue. The molecular weight of the observed protein was calculated with comparing its size to the protein markers.

Polysome isolation and treatment

For polysome isolation, the leaves of *N. tabacum* were homogenized in five volumes of buffer (containing 200 mM Tris-Cl (pH 8.9), 200 mM KCl, 35 mM MgCl₂, 0.6 M Sorbitol, 12.5 mM EGTA and 15 mM DTT) and centrifuged at 10,000×g and then 30,000×g. After miracloth filtration and Triton-X100 addition, the solution was centrifuged at 160,000×g using 1.5 M sucrose in a buffer consisting of 40 mM Tris-Cl (pH 8.9), 10 mM KCl, 1.5 mM MgCl₂, 5 mM Sucrose, 5 mM EGTA and 5 mM DTT. The pellet resolved in buffer containing 10 mM Tris-Cl (pH 7.6), 25 mM KCl, 5 mM MgCl₂ and centrifuged at 8000×g. The obtained polysome pellet was used for antiviral protein treatment. The reaction mixture included freezed polysome plus 10 µg of purified antiviral protein in 100 µL of a buffer containing Tris-Cl (pH. 7.6), 25 mM KCl, 5 mM MgCl₂ was incubated at 37 °C.

Ribosomal RNA N-glycosidase test

Polysomes were treated with phenol–chloroform–isoamyl alcohol Mixture (25:24:1) and precipitated with 75% ethanol. 1 M aniline was added to the RNA pellet and incubated at 50 °C for 2 min. The aniline treated RNA was precipitated with 75% ethanol and electrophoresed on 4.5% polyacrylamide gel containing 8 M urea in TBE running buffer. Aniline non-treated sample was used as control and compared with test sample observed on the gel by ethidium bromide dye under UV light.

Antioxidant activity test

The total antioxidant activity of purified RIP was determined using ferric reducing antioxidant power (FRAP) assay (Benzie and Strain 1996). For this, to 100 µL of purified RIP (100 µM), 3 mL of reagent was added and reaction mixture incubated at 37 °C for 4 min. Absorbance of sample was determined at 593 nm relative to reagent blank. Antioxidant potential of sample was determined against a standard curve of ferrous sulphate (100–1000 µM). Ascorbic acid (100 µM) served as a standard antioxidant. BSA served as negative control. FRAP values were calculated as follows: FRAP value (µmol L⁻¹) = (A₅₉₃ of test sample/A₅₉₃ of standard) × FRAP value (µmol L⁻¹) of standard. Values were

expressed as µmol L⁻¹ Fe II. FRAP values were also analyzed by time-course experiments during 30 min.

Root growth effect analysis

For this purpose, the seeds of *N. glutinosa* after 0.05% sodium hypochlorite sterilization were grown up to seedling stage in a vermiculite containing plate under laboratory natural light and moisture conditions. The uniformly grown seedlings were separately transferred into 1 l container with 1 L of Hoagland growth solution, containing 1 mg of purified ribosome-inactivating protein along with 5 mg of carborundum powder 600 mesh. Seedlings were supported on the surfaces by aluminum foils and solutions were aerated using narrow glass tubes. They were allowed to grow for a week without renewing the growth solutions. The root growth of the hydroponic seedlings were observed and comparatively analyzed after 7 days at the same time. The plant growth was assessed based on seminal root lengths and dried weights of detached roots. The purified protein and carborundum powder non-treated samples were considered as controls.

The effect of purified protein on the root growth of test plant seedlings were also analyzed by concentration-dependent experiment. For this, the uniformly grown seedlings were separately transferred into 1 l of Hoagland solutions containing 0, 0.5, 1, 1.5 and 2 mg L⁻¹ of purified ribosome-inactivating protein along with 5 mg of carborundum powder 600 mesh. The growth of the detached test roots was analyzed similar as described above.

Statistics

All tests were analyzed using three replicates. Data points on the graphs represented as the mean ± SD values of independent tests with three replicates. The presented differences between the mean values of independent tests were determined by analysis of variance (ANOVA) at $P \leq 0.05$. The values followed by different Latin letters in each assay on the graphs were statistically different at $P \leq 0.05$.

Results and discussion

Purification of ribosome-inactivating antiviral protein

Our recent antiviral analysis indicated that *C. plumosa* leaves have antiviral ability and their crude protein extract was able to inhibit TMV-induced hypersensitive responses on *N. glutinosa* leaves. Partial biophysical characterization of the leaf extracts and identification of their temperature

Table 1 Test for antiviral ability with different ammonium sulphate fractionation

(NH ₄) ₂ SO ₄ (%)	25	60	80	100	120
Test	–	+	+	–	–

Table 2 Test for antiviral ability with size-exclusion chromatography

NaCl (mM)	25	50	75	100	125
Test	–	+	–	–	–

resistance to about 50 °C revealed that *C. plumosa* antiviral elements might be proteinaceous in nature. Accordingly, proteinaceous-based isolation and purification methods were suggested for their further processing and characterization (Gholizadeh and Pourrahim 2017).

In the present work, following the previous suggestion, our attempt was made to purify the antiviral elements of *C. plumosa* by protocols partly on the basis of the purification methods of *C. cristata* antiviral proteins (Balasubrahmanyam et al. 2000; Gholizadeh and Kapoor 2004). Upon stepwise ammonium sulphate fractionation, the antiviral activity was found in two protein precipitates between 60 and 80% (Table 1). However, fraction precipitate of 80% showed more activity than of 60%. The fractions of 25, 100 and 120% did not exhibit antiviral ability.

At the next step, 80% fraction was selected and subjected to FPLC-based anion-exchange chromatography by using 10 mM NaCl containing buffer, pH 6.2. The antiviral activity that came through in the unadsorbed fraction was further separated by FPLC-based cation-exchange chromatography using 50 mM NaCl containing buffer, pH 5.2. The eluted adsorbed protein fraction was found to have considerable antiviral ability. The relevant pooled fraction of cation-exchanging step was screened by size-exclusion chromatography on Sepharose-12 through discontinuous gradient of 25–125 mM NaCl. The results of this screening step indicated that the protein extract eluted with buffer containing 50 mM NaCl have antiviral activity (Table 2).

Table 3 Stepwise-purification of *C. plumosa* antiviral protein

Fraction	Total protein (mg)	Percentage of TMV-inhibition
Crude extract	26.3	34.0 ^a
80% ammonium sulphate	20.0	62.5 ^b
Anion exchanger	10.4	93.0 ^c
Cation exchanger	2.2	96.7 ^d
Size exclusion	1.9	98.8 ^e

Data present the extraction process of 40 g of dried starting material. The values followed by a, b, c, d, and e letters are statistically different

Fig. 2 Stepwise SDS-PAGE analysis of purification. The products of each purification step of *C. plumosa* antiviral protein was subjected to 10% SDS-PAGE and visualized by Coomassie Brilliant Blue. *M* protein marker, *CP* crude protein extract, *ASF* ammonium sulphate fraction, *AEC* anion-exchanger fraction, *CEC* cation-exchanger fraction, *SE* size exclusion chromatography product **Fig. 3** rRNA N-glycosidase test. Antiviral protein-treated (+AVP) and non-treated (–AVP) rRNA containing samples were subjected to glycosidase test and compared for the presence of cleaved fragment

SDS-PAGE analysis of the obtained protein fraction from size-exclusion chromatography revealed that it contains one detectable protein with the molecular weight of

about 25 kD. The antiviral test of the size-exclusion step and the related SDS–PAGE result is shown (Fig. 1). The step-wise purification and SDS–PAGE analysis results are presented in Table 3 and Fig. 2. This step by step presentation once again confirmed the homogenous purification of a 25 kD antiviral protein from *C. plumosa* leaves.

Due to strategic importance of plant antiviral proteins with ribosome-inactivating abilities in different industries and because of potent pharmaceutical applications of *Celosia* plants, the ribosome-inactivating potential of the purified protein was investigated. The ribosome-inactivating N-glycosidase test result showed the presence of a clear extra band on the running gel (Fig. 3). In consistence with the previous obtained data related to the ribosome-inactivating ability of the purified plant antiviral proteins, this obtained extra band might be the cleaved fragment of the large ribosomal RNA (Balasubrahmanyam et al. 2000; Gholizadeh and Kapoor 2004).

The antiviral activity along with ribosome-inactivating ability has been previously studied in various *Amaranthaceae* plants. In the genus of *Amaranthus*, the leaves of *A. tricolor*, *A. viridis* and *A. mangostanus* have been reported to contain one, one and two ribosome-inactivating antiviral proteins with the molecular weights of 27, 29, 28 and 30 kD, respectively. In genus *Beta*, three antiviral proteins with the molecular weights of 27, 29 and 30 kD have been detected. The genus *Spinachia* has been shown to contain two antiviral proteins with the molecular weights of 31 and 36 kD. The genus *Chenopodium* has been reported to have a 30 kD antiviral protein. Besides these, the genus *Alternanthera* and *Achyranthes* have also been shown to possess potent antiviral ability in their leaf extracts (reviewed by Pandit et al. 2013).

In the genus *Celosia*, two growth stage-dependent antiviral proteins (namely CCP-25 and CCP-27) with ribosome-inactivating ability have already been purified from *cristata* species (Balasubrahmanyam et al. 2000; Gholizadeh and Kapoor 2004; Gholizadeh et al. 2004, 2005). Besides *C. cristata*, *C. plumosa* is also known as one of the ornamentally planted *Celosia* species in most of the countries. This plant species is usually used as decorative or edible plant. As a first report, our recent antiviral screening assessment data revealed that the leaves of *C. plumosa* plants contain potent antiviral ability (Gholizadeh and Pourrahim 2017). Following this research, in the present work, the homogeneity of *C. plumosa* virus inhibitor was demonstrated. The molecular weight of *C. plumosa* antiviral protein was found to be similar to that of *C. cristata* and others already characterized from different plant species such as *Dianthus caryophyllus*, *Phytolacca americana* and so on (Stripe et al. 1981; Irvin 1995; Pandit et al. 2013; Schrot et al. 2015). Thereby, it is predicted that *C. plumosa* virus inhibitor may be structurally similar to

others. According to the previous data, *C. plumosa* leaves contain virus inhibitory elements at both vegetative and flowering growth stages. These antiviral elements were reported to be similar at their partial biophysical properties (Gholizadeh and Pourrahim 2017). Thereby, the pre-flowering and flowering stage dependent viral inhibitors of *C. plumosa* leaves are predicted to have the similar structural basis that needs to be more investigated.

Similar to other plant antiviral proteins, *C. plumosa* inhibitor was tested and suggested to belong to the ribosome-inactivating proteins which have been well recognized as defense elements against broad spectrum of plant and human viruses (Sobiya and Jannet 2013). In addition to antiviral ability, their recent developments on chimeric immunotoxins and nanomaterials open up different possibilities to their use in novel therapeutic approaches in human diseases (Pizzo and Antimo 2016). The current finding is the first indication of ribosome-inactivating antiviral potency in *C. plumosa* plant. This may generally guarantee the health of the people who eat this plant in most of the countries. On the other hand, it may help us to understand more about its specific and novel pharmaceutical applications in the future.

Antioxidation property of purified RIP

Following ribosome-inactivating ability of purified viral inhibitor, the next attempt was made to evaluate its antioxidation capacity, if any, by using the FRAP test assay. Analysis of the obtained data revealed that the test RIP is an active antioxidant protein. In comparison to ascorbic acid as a standard antioxidant, its activity was found to be about 1.9-fold higher (Table 4). The time course study of antioxidant assay showed a steady increase in absorbance at 593 nm in time, indicating a prolonged antioxidation capacity of test RIP (Fig. 4).

The antioxidant power of plant antiviral proteins had been firstly reported by our research team in relation to *C. cristata* ribosome-inactivating virus inhibitor (Gholizadeh et al. 2004). The present investigation reports *C. plumosa* ribosome-inactivating protein as a second antioxidant RIP. This may generally suggest the complexity and

Table 4 Antioxidant ability of *C. plumosa* RIP

Sample	FRAP value ($\mu\text{mol L}^{-1}$ Fe II)	Relative activity
Ascorbic acid	117 $\pm$ 1.9	1.0
Purified RIP	221.7 $\pm$ 2.4	1.9
BSA	ND	ND

Data presented as the mean values $\pm$ SD

ND not detected

Fig. 4 FRAP reaction kinetics test. Absorbance values at 593 nm were measured after reagent: sample mixing and considered as FRAP indicators. Dark columns represent ascorbic acid as positive control and light columns presents the test RIP values

multifunctionality of plant RIP in nature. The ribosome-inactivating and antioxidant potencies of antiviral proteins are interesting. They both are proposed to be the protective tools against viral infection and the subsequent oxidative damages in plant system.

Oxidative damages usually occur under various stress conditions or diseases in different organisms. Protection against these damages is an important strategy that is mostly executed by antioxidant compounds including proteins. Following report of *C. plumosa* RIP as second antioxidant RIP, plant ribosome-inactivating proteins can be categorized as antioxidant proteins. Due to the therapeutic uses of plant ribosome-inactivating proteins, their antioxidant potencies may increase their medicament power. *C. Plumosa* is propagated as edible or ornamental plant in most countries. Thereby, our present results not only help the health of the people who eat this plant usually, but also indicate its application in medical therapy.

Root growth effect of purified RIP

The possible effect of purified ribosome-inactivating protein on the growth rate of plant root was examined through the exogenous application of purified protein and carborundum powder into the hydroponic growth solutions of *N. tabacum* seedlings. Comparison of the dried root weights and lengths of the test plants with those of non-treated controls (purified protein and carborundum powder non-treated samples) showed that the growth rate of root system in treated plants considerably differed from those of controls. Analysis of dried weights data showed that the root growth rate of test plants has 1.7-fold increase in the presence of 1 mg purified ribosome-inactivating protein (Fig. 5). Despite the treated samples, the control plants that were not treated with the purified protein or carborundum

powder did not exhibit root growth effect. This means that carborundum powder might act as tissue abrading material to enter the exogenously applied protein into the root cells.

The effect of the purified protein on the roots length of test plants was analyzed by measuring the length of seminal roots. The results showed that the length of seminal roots of purified protein-treated plants considerably increased as compared to non-treated plants. Data analysis revealed that the length of the seminal roots of treated plants increased 1.4 times as compared to control plants (Fig. 5). Comparison of the data related to the roots weight and length revealed that the root growth effect of ribosome-inactivating protein is more due to its positive effect on coronal root production of test plants.

The effect of purified protein on roots growth of test plants was also examined by concentration dependent experiment. Analysis of the data showed that the growth rate (including weight and length) of the test root is linearly gained as the concentration of the purified ribosome-inactivating protein is increased in the growth solutions (Fig. 5). This experiment result eliminated the possible involvement of the other exogenous elements in the growth process of test plants roots and confirmed the positive root growth effect of the purified protein.

Because of the broad distribution of ribosome-inactivating antiviral proteins, they have been generally accepted to make important contribution to the plants biology including developmental biology (Hartley and Lord 1993; Veronese et al. 2003; Sobiya and Jannet 2013; Pizzo and Antimo 2016). However, their contributions have not been fully understood in details, thus far.

Our recent tissue-dependent gene expression results suggested the potential contribution of the maize ribosome-inactivating protein in development of its root system (Gholizadeh 2016). In this regards, it had been earlier reported that some plants such as *Luffa cylindrica* and *Phytolacca americana* are able to produce ribosome-inactivating proteins in their hairy roots and secrete them as a part of root exudates to the rhizosphere to prevent pathogen infection (Poma et al. 1997; Park et al. 2002, 2004). Considering the previous reports together with the present results, the role of ribosome-inactivating antiviral proteins on the growth and developmental processes of the plant root system could not be negligible. However, we recommend more complementary experiments for the detailed biochemical identification of this biological role in the future attempts. The results of this research may be useful and interesting in plants developmental biology, genetics and engineering. Besides these, RIP may be used as one of the resistance strategy against pathogens in plant biotechnology in the future.

Fig. 5 Root growth effect analysis. The possible effect of purified antiviral protein on the root growth (including root weight and length) of test plants was analyzed by exogenous application of carborundum powder. The root weight (g) or length (mm) of each test sample was divided to the root weight (g) or length (mm) of purified protein non-

treated control sample (–P) and presented as root weight or length rate. –P: purified protein non-treated control sample; –C: carborundum powder non-treated control sample; +P+C: carborundum powder and purified protein-treated test sample

Conclusion

The presence of a 25 kD antiviral protein with ribosome-inactivating ability in the leaf extract of *C. plumosa* plant was concluded as the first ever report. The antioxidation power and the considerable root developer potency of the purified ribosome-inactivating antiviral element were also concluded. Thereby, these results may open up new avenues for multifunctional studies on RIP family. The accumulative potencies of RIP were suggested to signaling for plant ribosome-inactivating proteins as one of the most important contributors in the fields of pharmaceutical, developmental genetics and engineering against pathogen attacks.

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