Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 325 (2004) 167–173
BBRC
www.elsevier.com/locate/ybbrc

First report of a glutamine-rich antifungal peptide with immunomodulatory and antiproliferative activities from family Amaryllidaceae

Kin Tak Chu, Tzi Bun Ng*

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China

Received 20 September 2004

Abstract

This represents the first report of purification of a glutamine-rich antifungal peptide from family Amarylliaceace. The peptide, designated as nartazin, was purified from the bulbs of the Chinese daffodil *Narcissus tazetta* var. chinensis by means of ion-exchange chromatography and affinity chromatography. Its molecular mass was 7.1 kDa, as determined by SDS–PAGE and gel filtration. Nartazin stimulated proliferation of mouse splenocytes and bone marrow cells but inhibited proliferation of leukemia L1210 cells. It also inhibited translation in a cell-free rabbit reticulocyte lysate system. The sequence of its first 20 N-terminal residues was characterized by an abundance of glutamine. The peptide possessed antifungal activity on four phytopathogenic fungi. Its activity was retained after incubation with bovine trypsin and chymotrypsin (enzyme: substrate ratio 1:10 w/w) at 37 °C for 1 h but was attenuated after treatment with proteinase K. The data revealed its pronounced resistance to proteolytic digestion.

Keywords: Glutamine-rich; Antifungal; Amaryllidaceae; Immunomodulatory

Plant defense proteins are proteins that protect the host plants from pathogen invasion. Some of them, including ribosome-inactivating proteins [1], chitinases [2], trypsin inhibitors [3] and thaumatin-like proteins [4], β-1,3-glucanases [5], and lectins [6] possess antifungal activity.

Mannose-specific lectins have been isolated from monocots belonging to the families Amaryllidaceae [7], Alliaceae [8], Araceae [9], and Orchidaceae [10]. This kind of defense protein, specifically present in monocotyledonous plants, is classified into one of the superfamilies of evolutionarily related proteins that demonstrate a high extent of homology in protein sequence [11].

Antifungal proteins have been purified from the following monocots: garlic *Allium sativum*, the onion *Allium cepa*, and the chive *Allium tuberosum* [12–14], all belonging to family Alliaceae, and from the lily *Lilium brownii* belonging to family Liliaceae [15]. A ribosome-inactivating protein has been isolated from the iris [16]. Chitinases have been purified from the leek *A. porrum* [17] and the garlic *A. sativum* [18]. However, no antifungal proteins have been isolated from members of family Amaryllidaceae. Mannose-specific lectin is the only defense protein reported from family Amaryllidaceae. Most of the studies on Amaryllidaceae are focused on alkaloids and their biological activities such as acetylcholinesterase-inhibitory effects [19] and antitumor activity [20]. Thus, it would be a worthwhile undertaking to isolate an antifungal protein from the Chinese daffodil *Narcissus tarzetta*, an Amaryllidaceae plant with pharmaceutical values.

Presently, a variety of antifungal proteins are known. It would be interesting to ascertain if daffodil antifungal protein is a novel protein or belongs to any of the

* Corresponding author. Fax: +852 2603 5123.
E-mail address: biochemistry@cuhk.edu.hk (T.B. Ng).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.013

well-known types. Many studies have revealed that some of the plant defense proteins that included lectins [21], trypsin inhibitors [3], and antifungal proteins [14,22] elicited mitogenic response of mouse splenocytes and inhibition of tumor cells. The daffodil antifungal protein isolated in this study was also examined for immunomodulatory and antiproliferative activities.

Materials and methods

Materials. Bulbs of the Chinese daffodil, *Narcissus tazetta* var. chinensis, were purchased from a local vendor. The four species of phytopathogenic fungi examined in this study, namely *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola*, were obtained from Department of Microbiology, China Agricultural University, Beijing, China. The leukemia L1210 cell line was purchased from ATCC. Q-Sepharose, Mono-S column, Superdex 75 column, and methyl-[^{3}H]thymidine and low-molecular-mass marker were purchased from Amersham Biosciences. Affi-gel blue gel and Bradford Reagent were from Bio-Rad. Bovine serum albumin, potato dextrose agar, trypsin, and α-chymotrypsin from bovine pancreas, proteinase K from *Tritirachium album*, agrostin from *Agrostemma githago* seeds, casein, and Con A were products of Sigma. All other chemicals used were of analytical grade.

Purification of antifungal protein. Three kilograms of bulbs of *Narcissus tazetta* var. chinensis was homogenized in 6 L of 10 mM Tris–HCl buffer (pH 7.3). After centrifugation of the homogenate at 13,000 rpm for 30 min at 4 °C, proteins in the collected supernatant were precipitated by addition of ammonium sulfate to 70% saturation. It was again centrifuged, the supernatant was discarded, and the pellet was reconstituted in 10 mM Tris–HCl buffer (pH 7.3) before it was applied to a 5 × 10 cm Q-Sepharose (Amersham Biosciences) column. The column had previously been equilibrated and was then eluted with 10 mM Tris–HCl buffer (pH 7.3) at a flow rate of 5.3 ml/min. After unadsorbed proteins had been collected by eluting the column with the same buffer, adsorbed proteins were collected by adding 1 M NaCl to the eluting buffer. The unadsorbed fraction (Q1) was immediately chromatographed on an Affi-gel blue gel column (column size: 2 × 18 cm, flow rate: 3.5 ml/min) in 10 mM Tris–HCl buffer (pH 7.3). Unadsorbed proteins were eluted with the same buffer while adsorbed proteins were eluted using the same buffer containing 1.5 M NaCl. The adsorbed fraction (BG2) was dialyzed against 10 mM NH_4OAc buffer (pH 4.5) prior to ion exchange chromatography on a Mono-S column (column size: 0.5 × 5 cm, flow rate: 1 ml/min) in 20 mM NH_4OAc buffer (pH 5.4) by fast protein liquid chromatography (FPLC) using an AKTA Purifier (Amersham Biosciences). A single major adsorbed peak containing the purified *Narcissus* antifungal protein was obtained. It was designated nartazin.

Amino acid sequence analysis. The N-terminal amino acid sequence of nartazin was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with a high performance liquid chromatography system [23].

Assay of protein concentration. Protein concentrations were determined using the Bradford Reagent (Bio-Rad). Bovine serum albumin was used as protein standard.

Assay of antifungal activity. The assay for antifungal activity toward various fungal species was carried out in 60 × 15 mm petri plates containing 5 ml potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (10 μl) of nartazin was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [4].

To determine the antifungal activity quantitatively, three doses of nartazin were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly, and poured into three separate petri dishes of 60 mm diameter. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was added. Buffer only without nartazin served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth was determined in terms of percentage reduction in the area of the mycelial colony.

Determination of molecular mass. The molecular mass of nartazin was determined by Tricine–SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and by gel filtration on an FPLC-Superdex 75 10/300 GL column. Tricine–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli and Favre [24]. After electrophoresis the gel was stained with Coomassie brilliant blue. The molecular mass of nartazin was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences. Gel filtration was conducted using an FPLC-Superdex 75 10/300 GL column which had been calibrated with molecular mass standards from Amersham Biosciences (column size, 1 × 30 cm; flow rate, 0.5 ml/min; and eluting buffer, 0.15 M NH_4HCO_3, pH 8.9).

Proteinase digestion of nartazin. An aliquot of 100 μg nartazin was incubated with 10 μg protease at 37 °C for 1 h. Three kinds of proteases, namely trypsin, chymotrypsin, and proteinase K, were used to digest nartazin using an enzyme to substrate ratio of 1:10 (w:w). At the end of the incubation, the reaction mixture was examined for antifungal activity. The dose of proteases used was proved to be devoid of antifungal activity.

Assay of trypsin and chymotrypsin inhibitory activity. An amount of 50 μg nartazin was incubated with 50 μg trypsin or chymotrypsin in 100 μl of 50 mM Tris–HCl buffer (pH 8.0) containing 200 mM calcium chloride for 5 min at 25 °C. Residual trypsin or chymotrypsin was determined by adding 300 μl of 1% casein substrate at 37 °C. The reaction was terminated by adding 1 ml of chilled 5% trichloroacetic acid after incubation for 1 h at 37 °C. The reaction mixture was centrifuged for 20 min at 10,000 rpm. The absorbance of the clear supernatant was determined at 280 nm [25].

Assay for cell-free translation-inhibitory activity. Nartazin was tested in this assay in view of the report that the chive chitinase-like protein demonstrated such activity [14]. An assay based on the rabbit reticulocyte lysate system was used [23]. The isolated peptide (various doses in a volume of 10 μl) was added to 10 μl of a radioactive mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM creatine phosphate, and 1 μCi [4,5-^{3}H]leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 mM hemin and 5 μl creatine kinase. Incubation proceeded at 37 °C for 30 min before addition of 330 μl of 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 2 ml scintillant and counted in a Packard Tri-Carb 2900TR low-activity liquid scintillation counter. Agrostin, a type 1 ribosome-inactivating protein (RIP) isolated from the seeds of *Agrostemma githago*, was used as a positive control.

$$\%\ \text{inhibition} = \frac{\text{CPM (nartazin)} - \text{CPM (NSB)}}{\text{CPM (negative control)} - \text{CPM (NSB)}} \times 100\%,$$

where NSB is the non-specific binding and CPM is the radioactivity in counts per minute.

Mitogenic activity of murine splenocytes and bone marrow cells. Nartazin was tested in this assay since some antifungal proteins such as chrysancorin demonstrate activity in this assay [22]. Four Balb/c mice

(20–25 g) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 2×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 U penicillin/ml, and 100 μg streptomycin/ml. The cells (2×10^5 cells/100 μl/well) were seeded into a 96-well culture plate and serial concentrations of nartazin in 100 μl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h, 10 μl methyl-[^{3}H]thymidine (0.25 μCi, Amersham Biosciences) was added, and the cells were incubated for a further 12 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Packard Tri-Carb 2900TR low-activity liquid scintillation counter. Con A was used as a positive control. All reported values are means of triplicate samples [22]. Assay for mitogenic activity of nartazin on murine bone marrow cells was conducted using a similar procedure. The cells were collected from femurs.

Anti-proliferative activity on L1210 leukemia cells. Nartazin was tested in this assay because some antifungal proteins exhibit this activity [14]. Leukemia (L1210) cells were seeded at a density of 2×10^4 cells/100 μl in 96-well plates for 48 and 72 h, at 37 °C, in an atmosphere of 5% CO_2. Serial concentrations of the isolated peptide (100 μl/well) were added. At 24 h before the end of the incubation, 10 μl methyl-[^{3}H]thymidine (0.25 μCi, Amersham Biosciences) was added to each well. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Packard Tri-Carb 2900TR low-activity liquid scintillation counter. All reported values are means of triplicate samples [14].

Results

Antifungal activity was precipitated by 70% saturated ammonium sulfate. The unadsorbed peak (Q1) demonstrated antifungal activity whereas the adsorbed peak (Q2) lacked antifungal activity. Q1 was subsequently applied to an Affi-gel blue gel column, and fractionated into an unadsorbed peak (BG1) without antifungal activity and an adsorbed peak (BG2) with antifungal activity. The active fraction BG2 was finally purified by means of ion exchange chromatography on an FPLC-Mono-S column (Fig. 1). The major adsorbed peak (MS1) obtained was a purified protein as it appeared as a single band in Tricine–SDS–PAGE and a single peak upon gel filtration on a Superdex 75 column (Fig. 2). It was designated as nartazin. Its molecular mass as determined by SDS–PAGE and gel filtration was 7.1 kDa. The sequence of its first 20 N-terminal residues was GNNNLTSSQQQQIQFILQQI. There was some similarity to low-molecular-mass glutenin and gliadin from wheat and auxin-responsive factor from *Arabidopsis thaliana* (Table 1). The yields of the various chromatographic fractions throughout the purification procedure from 3 kg bulbs of daffodil were 1967 mg crude extract, 1292 mg ammonium sulfate precipitate, 590 mg fraction Q1, 180 mg fraction BG2, 27 mg fraction MS1, and 22 mg peak MS from Mono-S representing purified nartazin.

Nartazin inhibited mycelial growth in the four phytopathogenic fungi examined (Fig. 3). Its antifungal activity was retained after treatment up to 60 °C for 5 min

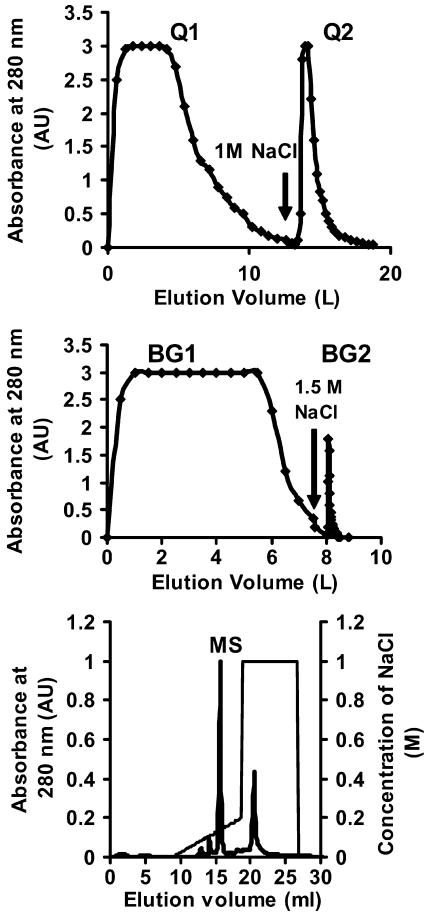

Fig. 1. Purification of nartazin. Anion-exchange chromatography of ammonium sulfate precipitate of crude extract on a Q-Sepharose column (Top). Affinity chromatography of unadsorbed fraction Q1 from Q-Sepharose on an Affi-gel blue gel column (Center). Cation-exchange chromatography of adsorbed fraction BG2 from Affi-gel blue gel on an FPLC-Mono-S column (Bottom).

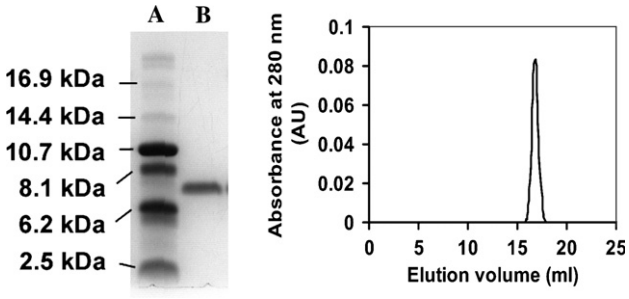

Fig. 2. Determination of molecular mass by Tricine–SDS–PAGE and by gel filtration on an FPLC Superdex 75 column. The molecular mass of nartazin shown in lane B was 7.1 kDa as it exhibited a single band at the same position that lay half way in between the 6.2 and 8.1 kDa marker bands in lane A (Left). Gel filtration of fraction MS from the Mono-S column on an FPLC-Superdex 75 10/300 GL column (Right).

(Fig. 4). The percentage inhibition of fungal growth by nartazin was 87.0% against *Botrytis cinerea*, 62.1% against *Physalospora piricola*, 55.6% against *Fusarium*

Table 1
Comparison of N-terminal sequence of nartazin with other plant proteins

	Position of residue	N-terminal sequence	Position of residue	Identity (%)	Length
Narcissus tazetta var. chinensis	1	GNNNLTSSQQQQIQFILQQI	20		
Auxin-responsive factor (ARF7) [*A. thaliana*] (NP_851047)	546	-NNNQSQSQQQQ-Q-LLQQ-	561	66	1150
Low-molecular-weight glutenin subunit group 3 type II [*Triticum aestivum*] (BAB78747)	62	-------SQQQQIPFVHPSILQQ	77	68	238
α-Gliadin storage protein [*Triticum aestivum*] (AAA96523)	126	-------QQQQFQQILQQI	140	83	288
Antifungal protein from family Liliaceae					
Lilin		PRGRERYEYEAVRVRVQEAE			
Antifungal proteins from family Alliaceae					
Allivin		DTFSDAGSFL			
CHITAS1		QQCGSQAGGAL			
CHITAS2		QQCGSQGSGAL			
APC-Dr		EQCGRQAGGAL			
APC-D		EQCGRQA			
APC-F		EQCGRQAGGAL			
Ace-AMP1		QNICPRVNRIV			
ATC		EQHGSQAGGAL			

Residues identical to corresponding residues in nartazin are underlined. Accession numbers of sequences from BLAST are in brackets. Sequences of lilin from *Lilium brownii* are from [15]. *Allium sativum* chitinases CHITAS1 and CHITAS2 are from [18], those of *Allium porrum* chitinase isoforms APC-Dr, -D, and -F are from [17], that of *Allium cepa* antimicrobial protein Ace-AMP1 is from [27], that of allivin is from [12], and that of *Allium tuberosum* chitinase (ATC) is from [14].

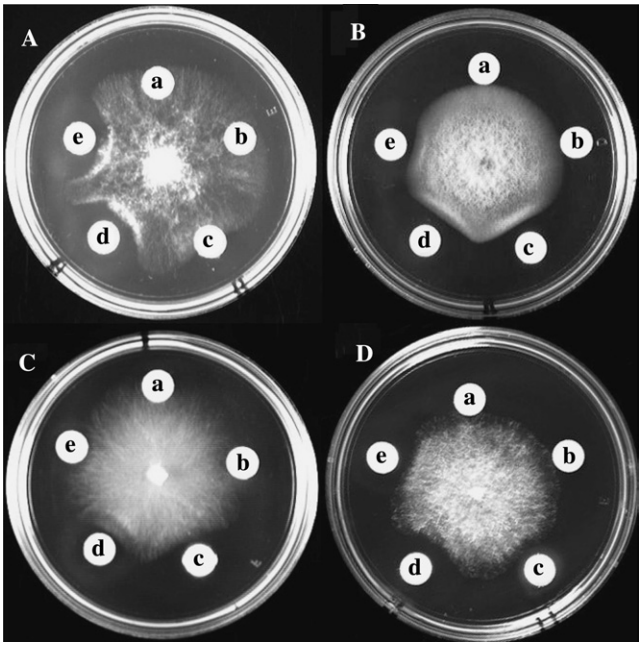

Fig. 3. Antifungal activity of nartazin. The fungi tested were: (A) *Botrytis cinerea*, (B) *Mycosphaerella arachidicola*, (C) *Fusarium oxysporum*, and (D) *Physalospora piricola*. The samples applied to the paper disks were as follows. (a) 10 mM Tris–HCl buffer (pH 7.3) as control. (b) One hundred micrograms of bovine serum albumin as negative control. (c) One hundred micrograms of thaumatin-like protein from chestnut as positive control. (d) Twenty-five micrograms of nartazin. (e) One hundred micrograms of nartazin.

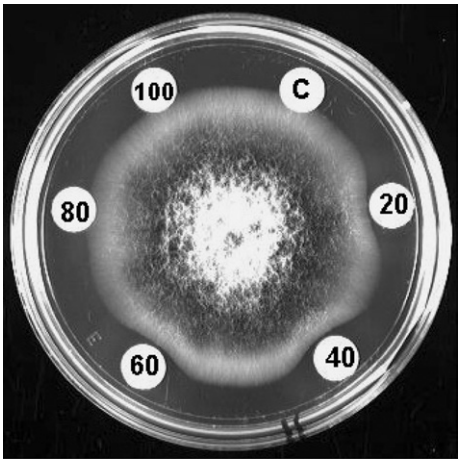

Fig. 4. Thermostability of antifungal activity of nartazin. The thermostability of nartazin was tested by pretreating the protein at different temperatures for 5 min. The antifungal activity of nartazin was preserved after treatment up to 60 °C and nullified when treated at 80 °C or above. The same amount of 25 μg nartazin was added to each respective paper disk (except control disk labeled as C). The numbers 20–100 labeled on the paper disks represent the various temperatures at which the aliquot of nartazin introduced to the disk had been pretreated.

oxysporum, and 36.7% against *Mycosphaerella arachidicola*, when 100 μg of nartazin was mixed in 4 ml potato dextrose agar giving rise to a protein concentration of 3.52 μM). The activity did not diminish after incubation with trypsin and chymotrypsin at room temperature for 1 h but it was abolished by proteinase K (Fig. 5). Nartazin did not inhibit the digestion of casein by trypsin and chymotrypsin, when the ratio of enzyme to nartazin was 1:1, and the duration of incubation was 60 min (data not shown).

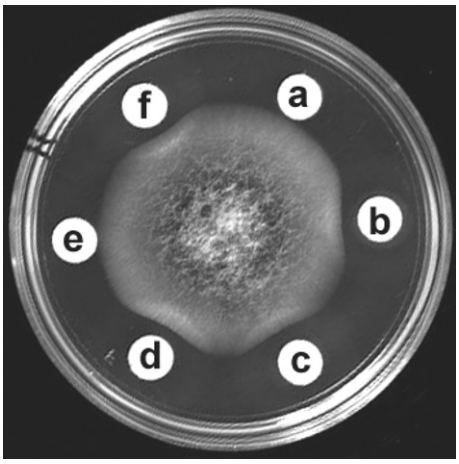

Fig. 5. Digestion of nartazin by three different proteases. Nartazin was digested by trypsin, proteinase K, and chymotrypsin individually. The digests were then applied to the paper disks on the agar plate. The letters a–f labeled on the paper disks represent different kinds of samples added as shown below. All proteases at the concentration used in the digestion were devoid of antifungal activity. (a) Ten micromolar Tris–HCl buffer (pH 7.3) as control. (b) One hundred micrograms of nartazin. (c) One hundred micrograms of thaumatin-like protein from chestnut. (d) One hundred micrograms of nartazin treated with 10 μg trypsin for 1 h. (e) One hundred micrograms of nartazin treated with 10 μg proteinase K for 1 h. (f) One hundred micrograms of nartazin treated with 10 μg chymotrypsin for 1 h.

Nartazin possessed cell-free translation-inhibitory activity in the rabbit reticulocyte lysate system. It manifested 13.2% and 35.5% inhibition at a concentration of 26.7 and 266 μM, respectively. Compared with the positive control, 0.667 and 6.67 μM agrostin inhibited protein synthesis by 54.4% and 72.5%, respectively.

Nartazin stimulated the mitogenic responses of murine splenocytes and bone marrow cells (Table 2). The

Table 2
Proliferation reflected in methyl-[^{3}H]thymidine incorporation of murine splenocytes and bone marrow cells in response to nartazin and Con A as positive control

Concentration (μM)	Methyl-[^{3}H]thymidine uptake by	
	Splenocytes (cpm)	Bone marrow cells (cpm)
Con A		
0	2199 ± 433	1800 ± 134
0.033	13,863 ± 836	3051 ± 424
0.065	16,151 ± 1650	2905 ± 336
0.13	17,631 ± 1455	4829 ± 348
0.26	20,872 ± 1909	5949 ± 792
0.52	15,893 ± 1174	5323 ± 835
Nartazin		
0	2199 ± 406	1800 ± 134
0.19	2572 ± 406	2080 ± 46
0.39	2999 ± 235	2507 ± 312
0.78	3659 ± 406	2881 ± 449
1.56	3963 ± 234	2293 ± 611
3.12	3308 ± 296	2185 ± 391

Results are means ± SEM ($n = 3$).

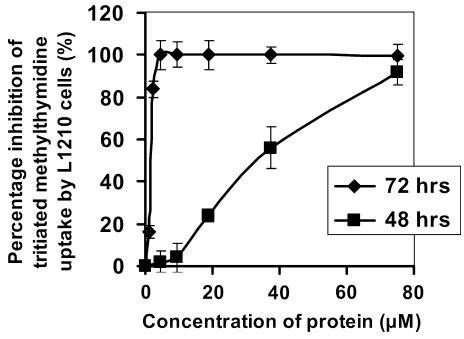

Fig. 6. Antiproliferative activity of nartazin on L1210 leukemia cells. The concentration of nartazin used in this assay did not exert cytotoxic activity on murine spleen cells, macrophages, and bone marrow cells.

maximal response was achieved at 1.56 μM and 0.78 μM nartazin for splenocytes and bone marrow cells, and at 0.26 μM Con A in both cases. The maximal response induced by nartazin was 19.08% and 48.43% of that induced by Con A in splenocytes and bone marrow cells, respectively.

Nartazin inhibited methyl-[^{3}H]thymidine uptake by L1210 mouse leukemia cells in a dose-dependent and a time-dependent manner (Fig. 6). No cytotoxic activity of nartazin towards mouse splenocytes appeared in the concentration range of 0–50 μM used in the assay for anti-proliferative activity toward L1210 cells.

Discussion

The N-terminal sequence of nartazin, which is characterized by an abundance of glutamine residues, is homologous to partial sequences in the cereal storage proteins, glutenin and gliadin from wheat *Triticum aestivum*. However, glutenin and gliadin, with 238 and 288 amino acid residues, have a much larger molecular size than nartazin and have not been shown to possess antifungal activity. Nartazin, which stimulates proliferation of spleen cells and bone marrow cells, also resembles auxin-responsive factor from *A. thaliana* in partial sequence. Auxin response factors are transcription factors that activate and repress the transcription of early auxin-response genes which regulate cell division, extension, and differentiation [26]. Thus, it appears that nartazin is a novel antifungal protein.

It is interesting to note that lectins from Amaryllidaceae, Alliaceae, Araceae, and Liliaceae families exhibit striking structural resemblance [11], while antifungal proteins isolated from members of these different families have a lesser extent of similarity. The latter is evident from a sequence comparison of nartazin, allivin [12], lilin [15], and chitinases, chitinase-like protein, and lipid transfer protein-like peptide from various *Allium* species [14,17,18,27].

The potent antifungal activity of nartazin indicates its role as a defense protein in the daffodil plant. The bulb, a characteristic storage organ of the Amaryllidaceae family, teeming with nutrients, has direct contact with soil which is full of soil-borne pathogens. Thus, the presence of pathogenesis-related proteins in the bulb serves to combat various pathogens in order to support the budding of the plant.

Storage proteins, especially those found in seeds and bulbs, are usually involved in host defense against pathogens by exerting particular activities such as antifungal activity and ribosome-inactivating activity. There are many reports about RIPs found in seeds of various plants, for example, cinnamomin, a storage protein present in the cotyledons of the seeds of the camphor tree, is a type-2 RIP [28] and trichoanguin from the seeds of *Trichosanthes anguina* is classified as a type-1 RIP [29]. However, three type-1 RIP isoforms and one type-2 RIP were found to be major storage proteins in bulbs of *Iris hollandica*, a monocot belonging to family Iridaceae. They all have IC_{50} values of cell-free translation inhibitory activity in a rabbit reticulocyte system below 0.16 nM [16]. Plant antifungal proteins also possess translation-inhibitory activity with IC_{50} values in the micromolar range and are thus less potent than plant ribosome-inactivating proteins with IC_{50} values in the nanomolar range. For nartazin, it inhibits protein synthesis in a rabbit reticulocyte system with a much lower potency than iris RIP. In comparison, antifungal proteins from inner shoots of *A. tuberosum* and bulbs of *Allium sativum* inhibit cell-free translation in a rabbit reticulocyte system with IC_{50} values of 0.8 and 1.6 μM, respectively [12,14]. This indicates that nartazin is a novel antifungal protein and that its antifungal activity is not largely attributed to RIP activity.

Nartazin manifests a broad spectrum of antifungal activity against four different common phytopathogens which are infectious to some important agricultural crops. The 10-kDa peptide from onion seeds, with sequence homology to plant non-specific lipid transfer protein, inhibits 11 species of phytopathogenic fungi [27]. On the contrary, some antifungal peptides with a narrow spectrum of antifungal activity may target selectively on a particular fungus. For instance, ascalin from shallot bulbs, with a molecular mass of 9.5 kDa, inhibits mycelial growth in the fungus *Botrytis cinerea* but not in the fungi *Mycosphaerella arachidicola* and *Fusarium oxysporum* [13]. No antifungal activity has been shown for CHITAS1 and CHITAS2 from *A. sativum* and APC-Dr, -D, and -F from *A. porrum* [17,18]. Loss of the antifungal activity of nartazin is evident after exposure to 80 °C for 5 min. By comparison, chive antifungal protein displays similar thermostability [14].

Nartazin has marked resistance against proteolytic inactivation by trypsin and chymotrypsin but not proteinase K. A large proportion of nartazin was still intact, as revealed in SDS–PAGE, after protease digestion for 1 h. However, it was cleaved and inactivated by proteinase K (data not known). There are two speculations for its resistance to cleavage by trypsin and chymotrypsin. The first one is related to the glutamine-rich amino acid sequence of nartazin. The wheat serpins, which are serine proteinase inhibitors of chymotrypsin-like enzymes, with reactive centers similar to the glutamine-rich repetitive sequences of prolamin, are believed to inhibit insect digestive proteinases that degrade wheat storage proteins [30]. From bulbs of onion classified under family Alliaceae that is phylogenetically close to Amaryllidaceae, three trypsin inhibitors with molecular masses of 7–8 kDa and sequences very different from the N-terminal sequence of nartazin were purified [31]. No inhibitory activity of nartazin is exerted on tryptic and chymotryptic digestion of casein. It suggests that the resistance of nartazin to tryptic digestion is attributed to its protein structure such as its N-terminal sequence.

The immunomodulatory effects of nartazin are noteworthy. As in the case of lilin from lily bulbs, nartazin elicited a mitogenic response from murine splenocytes [15]. However, at the same range of concentrations, nartazin inhibited the proliferation of leukemia cells. The antiproliferative activity of very few antifungal proteins and peptides has been demonstrated. For instance, chive chitinase-like antifungal protein is inhibitory toward breast cancer cells [14]. It is thus noteworthy that nartazin exerts similar activity toward leukemia cells. The ability of nartazin to stimulate proliferation of bone marrow cells, as evidenced by a potentiation in methyl-[^{3}H]thymidine uptake, has not been shown for other antifungal proteins. It reflects an increase in hematopoiesis which is potentially useful in alleviating the reduction in hematopoiesis seen in cancer patients receiving anticancer drugs.

Ascalin, allivin, and lilin, the antifungal proteins from shallot (*Allium ascalonicum*) bulbs, the antifungal protein from round-cloved garlic (*Allium sativum*) bulbs, and lily (*Lilium brownii*) bulbs, respectively, have molecular masses that are several kilodaltons larger than that of nartazin [12,13,15]. However, the chitinases from garlic bulbs and leek (*Allium porrum*) and the chitinase-like antifungal protein from chive (*A. tuberosum*) have a much higher molecular mass of 36 kDa [14].

In summary, a novel glutamine-rich antifungal peptide with stimulatory effects on splenocytes and bone marrow cells and an inhibitory action on leukemia cells has been isolated from Chinese daffodil bulbs. This constitutes the first account of an antifungal peptide from Amaryllidaceous plant. Its N-terminal sequence and molecular size are markedly different from those of the related Alliaceous and Liliaceous antifungal proteins.

References

[1] J.M. Vivanco, B.J. Savary, H.E. Flores, Characterization of two novel type I ribosome-inactivating proteins from the storage roots of the andean crop *Mirabilis expansa*, Plant Physiol. 119 (1999) 1447–1456.

[2] M. Rauscher, A.L. Adam, S. Wirtz, R. Guggenheim, K. Mendgen, H.B. Deising, PR-1 protein inhibits the differentiation of rust infection hyphae in leaves of acquired resistant broad bean, Plant J. 19 (1999) 625–633.

[3] T.B. Ng, S.K. Lam, W.P. Fong, A homodimeric sporamin-type trypsin inhibitor with antiproliferative, HIV reverse transcriptase-inhibitory and antifungal activities from wampee (*Clausena lansium*) seeds, Biol. Chem. 384 (2003) 289–293.

[4] K.T. Chu, T.B. Ng, Isolation of a large thaumatin-like antifungal protein from seeds of the Kweilin chestnut *Castanopsis chinensis*, Biochem. Biophys. Res. Commun. 301 (2003) 364–370.

[5] R. Zareie, D.L. Melanson, P.J. Murphy, Isolation of fungal cell wall degrading proteins from barley *Hordeum vulgare* L. leaves infected with *Rhynchosporium secalis*, Mol. Plant Microbe Interact. 15 (2002) 1031–1039.

[6] J. Ciopraga, O. Gozia, R. Tudor, L. Brezuica, R.J. Doyle, *Fusarium* sp. growth inhibition by wheat germ agglutinin, Biochim. Biophys. Acta 1428 (1999) 424–432.

[7] L.S. Ooi, H. Wang, T.B. Ng, V.E. Ooi, Isolation and characterization of a mannose-binding lectin from leaves of the Chinese daffodil *Narcissus tazetta*, Biochem. Cell Biol. 76 (1998) 601–608.

[8] A. Gupta, R.S. Sandhu, A new high molecular weight agglutinin from garlic (*Allium sativum*), Mol. Cell. Biochem. 166 (1997) 1–9.

[9] J. Singh, J. Singh, S.S. Kamboj, A novel mitogenic and antiproliferative lectin from a wild cobra lily, *Arisaema flavum*, Biochem. Biophys. Res. Commun. 318 (2004) 1057–1065.

[10] E.J. Van Damme, J. Balzarini, K. Smeets, F. Van Leuven, W.J. Peumans, The monomeric and dimeric mannose-binding proteins from the Orchidaceae species *Listera ovata* and *Epipactis helleborine*: sequence homologies and differences in biological activities, Glycoconj. J. 11 (1994) 321–332.

[11] A. Barre, E.J. Van Damme, W.J. Peumans, P. Rouge, Structure–function relationship of monocot mannose-binding lectins, Plant Physiol. 112 (1996) 1531–1540.

[12] H.X. Wang, T.B. Ng, Purification of allivin, a novel antifungal protein from bulbs of the round-cloved garlic, Life Sci. 70 (2001) 357–365.

[13] H.X. Wang, T.B. Ng, Ascalin, a new anti-fungal peptide with human immunodeficiency virus type 1 reverse transcriptase-inhibiting activity from shallot bulbs, Peptides 23 (2002) 1025–1029.

[14] Y.W. Lam, H.X. Wang, T.B. Ng, A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive *Allium tuberosum*, Biochem. Biophys. Res. Commun. 279 (2000) 74–80.

[15] H. Wang, T.B. Ng, Isolation of lilin, a novel arginine- and glutamate-rich protein with potent antifungal and mitogenic activities from lily bulbs, Life Sci. 70 (2002) 1075–1084.

[16] E.J. Van Damme, A. Barre, L. Barbieri, P. Valbonesi, P. Rouge, F. Van Leuven, F. Stirpe, W.J. Peumans, Type 1 ribosome-inactivating proteins are the most abundant proteins in iris (*Iris hollandica* var. Professor Blaauw) bulbs: characterization and molecular cloning, Biochem. J. 324 (1997) 963–970.

[17] R. Vergawen, F. Van Leuven, A. Van Laere, Purification and characterization of strongly chitin-binding chitinases from salicylic acid treated leek (*Allium porrum*), Physiol. Plant 104 (1998) 175–182.

[18] E.J.M. Van Damme, P. Willems, S. Torrekans, F. Van Leuven, W.J. Peumans, Garlic (*Allium sativum*) chitinases: characterization and molecular cloning, Physiol. Plant 87 (1993) 177–186.

[19] E.E. Elgorashi, G.I. Stafford, J. Van Staden, Acetylcholinesterase enzyme inhibitory effects of Amaryllidaceae alkaloids, Planta Med. 70 (2004) 260–262.

[20] D.H. Hua, S. Saha, D.J. Takemoto, Anticancer activities of 2,5,8,9-substituted 6-oxo-1,2,3,4,5,6-hexahydrophenanthridines on multi-drug-resistant phenotype cells, Anticancer Res. 17 (1997) 2435–2441.

[21] Y.W. Lam, T.B. Ng, A monomeric mannose-binding lectin from inner shoots of the edible chive (*Allium tuberosum*), J. Protein Chem. 20 (2001) 361–366.

[22] H. Wang, X.Y. Ye, T.B. Ng, Purification of chrysancorin, a novel antifungal protein with mitogenic activity from garland chrysanthemum seeds, Biol. Chem. 382 (2001) 947–951.

[23] S.S.L. Lam, H.X. Wang, T.B. Ng, Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum sativum*, Biochem. Biophys. Res. Commun. 253 (1998) 135–142.

[24] U.K. Laemmli, M. Favre, Maturation of the head of bacteriophage T4. I. DNA packaging events, J. Mol. Biol. 80 (1973) 575–599.

[25] X.Y. Ye, T.B. Ng, P.F. Rao, A Bowman–Birk-type trypsin-chymotrypsin inhibitor from broad beans, Biochem. Biophys. Res. Commun. 289 (1) (2001) 91–96.

[26] T. Ulmasov, G. Hagen, T.J. Guilfoyle, Activation and repression of transcription by auxin-response factors, Proc. Natl. Acad. Sci. USA 96 (1999) 5844–5849.

[27] B.P. Cammue, K. Thevissen, M. Hendriks, K. Eggermont, I.J. Goderis, P. Proost, J. Van Damme, R.W. Osborn, F. Guerbette, J.C. Kader, A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins, Plant Physiol. 109 (1995) 445–455.

[28] R.S. Liu, G.Q. Wei, Q. Yang, W.J. He, W.Y. Liu, Cinnamomin, a type II ribosome-inactivating protein is a storage protein in the seed of the camphor tree (*Cinnamomum camphora*), Biochem. J. 362 (2002) 659–663.

[29] L.P. Chow, M.H. Chou, C.Y. Ho, C.C. Chuang, F.M. Pan, S.H. Wu, J.Y. Lin, Purification, characterization and molecular cloning of trichoanguin, a novel type I ribosome-inactivating protein from the seeds of *Trichosanthes anguina*, Biochem. J. 338 (1999) 211–219.

[30] H. Ostergaard, S.K. Rasmussen, T.H. Roberts, J. Hejgaard, Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins. Cloning and characterization of five major molecular forms, J. Biol. Chem. 275 (2000) 33272–33279.

[31] M. Deshimaru, A. Watanabe, K. Suematsu, M. Hatano, S. Terada, Purification, amino acid sequence, and cDNA cloning of trypsin inhibitors from onion (*Allium cepa*) L. bulbs, Biosci. Biotechnol. Biochem. 67 (2003) 1653–1659.