Journal of Peptide Science
J. Peptide Sci. **10**: 103–108 (2004)
Published online 3 September 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.516

JOURNAL OF PEPTIDE SCIENCE

The Trypsin-inhibitory, Immunostimulatory and Antiproliferative Activities of a Napin-like Polypeptide from Chinese Cabbage Seeds

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Received 29 May 2003
Accepted 2 June 2003

Abstract: A heterodimeric 13.8 kDa napin-like polypeptide has previously been isolated from Chinese cabbage (*Brassica parachinensis*) seeds with a procedure involving ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, FPLC-ion exchange chromatography on Mono S and FPLC-gel filtration on Superdex 75. In the present study the *N*-terminal sequence of the 8.8 kDa subunit of the polypeptide (PQGPQQRPPKLLQQQTNEEHE) was found to have pronounced homology to napins, albumins and trypsin inhibitors, but demonstrated little similarity to the 5 kDa subunit. The polypeptide stimulated nitrite production by mouse peritoneal macrophages and reduced the viability of leukaemia (L1210) cells. It inhibited trypsin with a higher potency than it inhibited chymotrypsin, but was devoid of ribonuclease and antifungal activities. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: napin; cabbage; *Brassica parachinensis*; seeds; translation-inhibitory activity

INTRODUCTION

Napins are storage proteins which are 1 : 1 disulfide-linked complexes of a 4.5 kDa small subunit and a 10 kDa large subunit [1]. The most extensively studied napins are those from the oilseed rape *Brassica napus* [32]. The nitrogen storage function of napin is consistent with its abundance of amides and arginine residues [3–6]. Napins demonstrate trypsin-inhibiting activity but the subunits are inactive. However, both napin and its subunits play a role as calmodulin antagonists and as substrates for plant calcium-dependent protein kinases, since calmodulin and its small subunit exhibit similar α-helix-hinge-α-helix motifs [7,8]. Napins can inhibit calmodulin-dependent myosin light-chain kinase [1,7,8]. Napins may also elicit an antifungal action [2].

A napin-like polypeptide, composed of a small (5 kDa) subunit and a large (8.8 kDa) subunit, has been isolated from *Brassica parachinensis* seeds. The subunits exhibit only small differences in molecular mass from those of rapeseed napin. The 5 kDa subunit of *B. parachinensis* napin-like polypeptide manifests a striking resemblance in *N*-terminal sequence to those of napins from various other species [9]. The *N*-terminal sequences of the large and small subunits of napin are similar in *Sinapis alba* napin [10] but different in *B. napus* napin [7,8].

The napin-like polypeptide from *Brassica parachinensis* seeds demonstrates the ability to inhibit translation in a cell-free rabbit reticulocyte lysate system [9]. Its activity is relatively stable in the pH range 6–11 and in the temperature range 10°–50 °C [9]. Kohlrabi seeds [7,8], radish seeds [11] and *Arabidopsis thaliana* [12] produce multiple napins. It is

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Contract/grant sponsor: Research Grants Council of Hong kong.

noteworthy that only one napin-like polypeptide can be purified from *B. parachinensis* seeds [9].

The intent of the present investigation was to examine the isolated *B. parachinensis* napin-like polypeptide for trypsin-inhibitory activity which has been reported for other napins [7,8], and for other unexplored activities including immunomodulatory, antiproliferative, antifungal and ribonuclease activities which are found in other seed proteins such as antifungal proteins, lectins, ribosome inactivating proteins, trypsin inhibitors and ribonucleases [13–22].

MATERIALS AND METHODS

Isolation Procedure

The isolation of a napin-like polypeptide from the seeds of green-stalked Chinese cabbage (*Brassica parachinensis* cv green-stalked) has been described [9]. Briefly, the seeds were soaked, homogenized and the supernatant was then applied to a column of DEAE-cellulose (Sigma) (5 × 25 cm). The unadsorbed fraction containing translation-inhibitory activity was eluted with 10 mM Tris-HCl buffer (pH 7.4). It was directly chromatographed on an Affi-gel blue gel (Bio-Rad) column (2.5 × 15 cm). After removal of unadsorbed proteins devoid of translation-inhibitory activity, adsorbed proteins were eluted using a linear NaCl concentration (0–2 M) gradient in 10 mM Tris-HCl buffer (pH 7.4). The second adsorbed peak containing translation-inhibitory activity was then dialysed before ion exchange chromatography on a 1 ml Mono S column (Amersham Biosciences) using fast protein liquid chromatography (FPLC). After removal of unadsorbed proteins devoid of translation-inhibitory activity, absorbed proteins were eluted with two consecutive linear concentration gradients (0–0.3 M NaCl and 0.3–1 M NaCl) in 10 mM NH_4OAc (pH 5.5). The peak eluted with the first salt gradient, which contained translation-inhibitory activity, was further purified by FPLC-gel filtration on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 20 mM NH_4HCO_3 buffer (pH 9.4). The first eluted peak represented purified napin-like polypeptide.

Amino Acid Sequence Analysis

The *N*-terminal amino acid sequence of the 8.8 kDa subunit of the napin-like polypeptide was analysed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system [20]. The *N*-terminal sequence of the 5 kDa subunit has already been reported [9].

Assay for Macrophage Stimulating Activity

The assay for the ability to stimulate the production of nitrite ions by mouse macrophages was conducted as described by Ye and Ng [23]. Peritoneal macrophages were collected 3 days after being elicited by thioglycolate. The cells were washed, counted and resuspended in DMEM medium without phenol red, 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells (2×10^5 cells/well/0.2 ml) were allowed to adhere onto the surface of the wells of a 96-well culture plate for 1 h before incubation with the napin-like polypeptide for 24 h. The amount of NO_2^- in the culture medium was determined by the colorimetric method using $NaNO_2$ as a standard. In the assay, a 100 μl aliquot of cell-free culture medium from each culture well was reacted with 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4–0.1% naphthaleneethylenediamine dihydrochloride) for 10 min before the absorbance was read at 540 nm using a microplate reader (Bio-Rad 355).

Assay for Antifungal Activity

The assay for antifungal activity toward *Botrytis cinerea, Mycosphaerella arachidicola, Physalospora piricola* and *Fusarium oxysporum* was carried out in 100 × 15 mm Petri plates containing 10 ml of potato dextrose agar. These fungal species have been shown to be sensitive to a variety of antifungal proteins. 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 (6 μl containing 10 μg) of a solution of napin-like polypeptide was added to a disk. The plates were incubated at 23°C for 72 h until mycelial growth had enveloped the disks containing the control and had formed zones of inhibition around the disks containing samples with antifungal activity [13].

Assay of Inhibitory Activity on Tumour Cell Lines

The leukaemia L1210 cell line was suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. One hundred microlitres of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different

amounts of the napin-like polypeptide in 100 µl complete RPMI medium were then added to the wells and incubated for 72 h. After 72 h, 20 µl of 5 mg/ml MTT in phosphate buffered saline was spiked into each well and the plates were incubated for 4 h. The well plates were then centrifuged at 2500 rpm for 5 min. The supernatant was carefully removed and 150 µl of dimethyl sulphoxide was added in each well to dissolve the (MTT) formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was then measured by a microplate reader. The inhibitory activity reflects a summation of inhibitory effects on proliferation and viability of tumour cells.

Assay for Ribonuclease Activity

The activity of RNase toward tRNA was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Fong *et al.* [22]. The RNase was incubated with 200 µg of tRNA in 150 µg 100 mM MES (pH 6.0) at 37 °C for 1 h. The reaction was terminated by introduction of 350 µl of ice-cold 3.4% perchloric acid. After leaving on ice for 15 min, the sample was centrifuged (15 000 × **g**, 15 min) at 4 °C. The OD_{260} of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD_{260} of 1 unit per min in the acid-soluble fraction per ml of reaction mixture under the specified condition.

Assay for Trypsin-inhibitory and Chymotrypsin-inhibitory Activities

The napin-like polypeptide was incubated with 25 µg trypsin or chymotrypsin (Sigma) in 100 µl of 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM $CaCl_2$ for 5 min at 25 °C. Residual trypsin or chymotrypsin activity was determined by adding 300 µl of a 1% casein solution (Sigma). The reaction was terminated by adding 1 ml of cold 5% trichloroacetic acid after incubation for 15 min at 25 °C. The reaction mixture was centrifuged for 20 min at 10 000 rpm. The absorbance of the clear supernatant, which reflected the amount of casein fragments, was determined at 280 nm [24].

The protease activity remaining after treatment of protease with *B. parachinensis* napin-like polypeptide was equal to the absorbance in the presence of napin-like polypeptide divided by the absorbance in the absence of napin-like polypeptide × 100%.

RESULTS

The 8.8 kDa subunit of *B. parachinensis* napin-like polypeptide displayed little similarity to the 5 kDa subunit. However, there was a remarkable similarity or it was even identical to white mustard allergen, napins, wheat glutenin, the larger chain of antifungal 25 albumin from *Raphanus sativus* and a trypsin inhibitor (Table 1).

The napin-like polypeptide from *B. parachinensis* manifested a dose-dependent inhibition of trypsin and chymotrypsin when the molar ratio of napin-like polypeptide to protease was increased from 1 : 1 to 50 : 1 (Figure 1).

The napin-like polypeptide augmented nitrite production from murine peritoneal macrophages when the concentration of the polypeptide was increased from 0.1 µM to 1 µM. The positive control

Table 1 Comparison of *N*-terminal Sequence of *Brassica parachinensis* Napin with Related Proteins

	Residue no.	Sequence	Residue no.	Total no. of amino acids
B. parachinensis napin (8.8 kDa subunit)	1	PQGPQQRPPLLQQQTNEEHE	20	
B. parachinensis napin 5 kDa subunit)	1	PAGPFRIPKKRKKEE	15	
Allergen sin a I (white mustard)	40	PQGPQQRPPLLQQ	52	145
Napin (Swedish turnip)	40	PQGPQQRPPLLQQ	52	124
Napin (*B. napus*)	38	PAGPFRIPKCRK	49	124
Glutenin (*Triticum aestivum*)	58	PQGPQQTPPVLQQ	70	386
Antifungal 2S albumin larger chain (*Raphanus sativus*)	1	PQGPQQRPPLLQQ	13	20
Trypsin inhibitor (*Sinapis arvensis*)	1	PQGPQQRPPLLQQ	13	91

Residue 1 and residue 20 in *B. parachinensis* napin (8.8 kDa subunit) refer to P and E respectively. Identical corresponding amino acids are underlined.

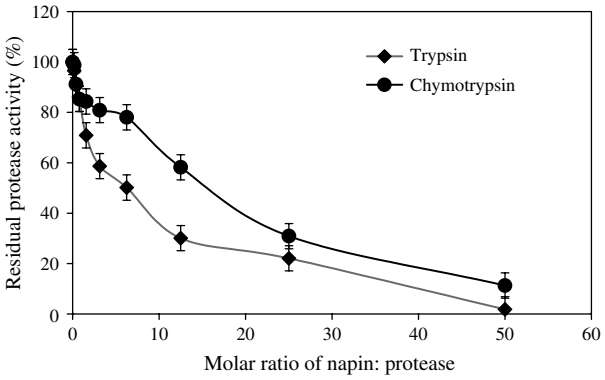

Figure 1 Inhibitory activity of *Brassica parachinensis* napin-like polypeptide on trypsin and chymotrypsin (data represent means ± SD, $n = 3$).

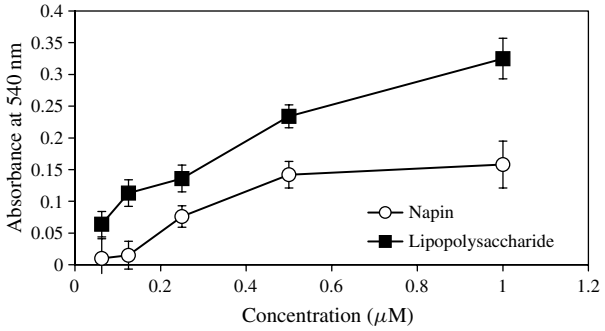

Figure 2 Stimulatory effect of napin, compared with that of the positive control lipopolysaccharide, on nitrite production by murine peritoneal macrophages (data represent means ± SD, $n = 3$). Absorbance at 540 nm reflects the amount of nitrite.

lipopolysaccharide increased in a similar fashion (Figure 2).

The viability of leukaemia cells (L1210) was reduced dose-dependently in response to the napin-like polypeptide (Table 2).

At a dose of 300 μg, the polypeptide did not exhibit antifungal activity on the three fungal species tested. Neither did it demonstrate ribonuclease activity when tested at 100 μg.

DISCUSSION

The napin-like polypeptide from *B. parachinensis* seeds is highly homologous to napin from *B. napus*. The two subunits differ from each other in molecular mass and amino acid sequence.

It was surmised that the translation-inhibiting activity of the napin-like polypeptide may be linked

Table 2 Effect of *B. parachinensis* Napin-like Polypeptide on Viability of Leukaemia Cells (L1210) (data represent means ± SD, $n = 3$)

Concentration of napin-like polypeptide (μM)	Viability (%)
0	100 ± 1.1
0.31	96.9 ± 1.4
0.62	89.0 ± 1.3
1.25	76.2 ± 2.7
2.5	59.4 ± 2.1
5	34.8 ± 3.0

to the proposed antifungal activity of napins in view of the observation that antifungal proteins and peptides in general exhibit the ability to inhibit translation in the cell-free rabbit reticulocyte lysate system, albeit with only a low potency [13,18,19,25–28]. On the other hand, ribosome inactivating proteins, which may represent storage proteins in some seeds, display a highly potent translation-inhibiting activity [20,29] similar in magnitude to that of *B. parachinensis* napin-like polypeptide [9]. Lectins, another type of seed storage proteins, however, lack translation-inhibiting activity unless they have antifungal activity [14]. However, interestingly, the napin-like polypeptide from *B. parachinensis* seeds is devoid of antifungal activity. Not all biomolecules with translation-inhibitory activity have antifungal activity, e.g. some ribonucleases and ubiquitin-like peptides from mushrooms lack any inhibitory effect on mycelial growth [30–31].

Antifungal proteins from the pinto bean stimulate nitrite production by mouse peritoneal macrophages and also inhibit translation in the rabbit reticulocyte lysate system [23]. A variety of antifungal proteins including the chitinase-like antifungal proteins dolichin from field beans and delandin from rice beans [19,27], chrysancorin from garland chrysanthemum seeds [13], lilin from lily bulbs [25], antifungal peptides from pinto beans and red beans [32], and cyclophilin-like protein from chickpeas [33] possess mitogenic activity toward mouse splenocytes. Ribosome inactivating proteins in general show antimitogenic activity [34]. *B. parachinensis* napin-like polypeptide resembles antifungal proteins and ribosome inactivating proteins in possessing translation-inhibitory as well as immunomodulatory activities. Other seed proteins like lectins [35] and trypsin inhibitors [24] may also show immunomodulatory activities.

The antiproliferative activity of *B. parachinensis* napin-like polypeptide toward a leukaemia cell line is in conformity with similar observations on some antifungal proteins [36], ribosome inactivating proteins [15,37], lectins [35] and trypsin inhibitors [21]. It is well known that protease inhibitors have an antineoplastic action [38].

Proteases have an effect equivalent to translation inhibition on account of their proteolytic action. It may thus seem paradoxical that a protease inhibitor should exhibit translation-inhibitory activity, but the sporamin-type trypsin inhibitor from wampee seeds indeed inhibits translation-inhibitory activity probably due to its antifungal activity [34]. There may be a relationship between the translation-inhibitory activity of a polypeptide like napin and its proposed defensive role although it lacks antifungal activity. The translation inhibiting activity of *B. parachinensis* napin is stable over half of the pH scale, from pH 6 to pH 11 but is stable only up to 50 °C.

Ribonucleases display inhibitory activity in the rabbit reticulocyte system [28,29]. However, the napin-like polypeptide lacks ribonuclease activity. Antifungal activity is indiscernible in the napin-like polypeptide although other molecules with translation-inhibitory activity including ribosome inactivating proteins [37,39], antifungal proteins [18,19,26,27] and ribonucleases [40,41] have been reported to possess antifungal activity.

In summary, the napin-like polypeptide from *B. parachinensis* seeds is similar to *B. napus* napin [7,8] in the possession of two structurally dissimilar subunits. It is endowed with some potentially exploitable biological activities.

Acknowledgement

We thank the Research Grants Council of Hong Kong for the award of an earmarked grant and Miss Fion Yung for the excellent secretarial assistance.

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