



# A haemagglutinin from the medicinal fungus *Cordyceps militaris*

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## Synopsis

There are only a few reports on agglutinins from ascomycete and medicinal fungi. An HA (haemagglutinin), with an N-terminal amino acid sequence different from those of known lectins, was isolated in the present study from dried fruiting bodies of the medicinal ascomycete fungus *Cordyceps militaris*. The purification protocol consisted of affinity chromatography, ion-exchange chromatography and gel filtration. The haemagglutinating activity of the HA could not be inhibited by simple sugars or heparin, and was stable over the pH range 2–13 and up to 60°C. Chemical modification of tryptophan and tyrosine residues had no effect. The HA exhibited some antiproliferative activity towards hepatoma (HepG2) cells and inhibited HIV-1 reverse transcriptase (IC<sub>50</sub> = 10 µM). However, it did not exhibit antifungal activity, mitogenic activity towards splenocytes, nitric oxide-inducing activity towards macrophages or RNase activity. The results of the present study add to the meagre information pertaining to agglutinins from ascomycete and medicinal mushrooms. It is revealed in this study that *C. militaris* HA differs from other ascomycete mushroom HAs in a variety of biochemical characteristics.

**Key words:** *Cordyceps militaris*, fungus, HIV-1, haemagglutinin (HA), isolation, tumour

## INTRODUCTION

Lectins/HAs (haemagglutinins) are proteins with carbohydrate-binding specificity. They are of non-immunogenic origin and do not catalyse reactions with their binding activity. They are produced by a diversity of organisms including animals [1–3], plants [4–6], fungi [7–9], bacteria [10,11] and viruses [12]. They have attracted the attention of some researchers in view of their potentially exploitable activities including antiproliferative [7,13–15], immunoenhancing [16], antifungal [17] and antiviral [7,18,19] activities.

Fungal lectins are attracting the attention of many scientists because of their different carbohydrate-binding specificities [7,20,21], molecular masses [7], numbers of subunits [9,20,22] and complete or N-terminal amino acid sequences [21–23]. Fungal lectins may manifest mitogenic activity toward splenocytes [9,22] and antiproliferative activity towards tumour cells [22].

*Cordyceps militaris* and *Cordyceps sinensis* are renowned medicinal fungi with a multitude of pharmacological activities [9,24,25]. To date, very few proteins have been purified from

*C. militaris* and *C. sinensis*. The intent of the present study was to isolate an HA from the fruiting bodies of *C. militaris* and to compare it with the HA reported earlier from the same species [9]. We present evidence herein that the HA isolated in this study differs from the lectin reported earlier. The isolated HA was assayed for a number of activities in view of the report of only mitogenic activity of *C. militaris* HA by Jung et al. [9].

## MATERIALS AND METHODS

### Materials

Dried *C. militaris* (100 g) collected from Mainland China were homogenized in liquid nitrogen with a pestle. The homogenized powder was dissolved in distilled water and centrifuged. To the resulting supernatant, NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 9.4) was added until a final concentration of 20 mM was attained. The sample was loaded on to an affinity column (2.5 cm × 16 cm; Affi-gel blue gel; Bio-Rad). Then the unadsorbed fraction was subjected to anion-exchange chromatography on a 2.5 × 16 cm column

**Abbreviations used:** FBS, fetal bovine serum; HA, haemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NAI, N-acetylimidazole.

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of Q-Sepharose (GE Healthcare). After the removal of unadsorbed proteins, adsorbed proteins were eluted with 1 M NaCl in 20 mM  $\text{NH}_4\text{HCO}_3$  buffer (pH 9.4). The adsorbed fraction was dialysed and concentrated by using an Amicon filter device (10 kDa cutoff). Then the dialysed and concentrated sample was applied on a Mono Q column and eluted using an AKTA Purifier FPLC System (GE Healthcare). After the unadsorbed fraction had been eluted, the adsorbed protein was desorbed by using two linear NaCl concentration gradients (0–0.2 M and 0.2–1 M) in 20 mM  $\text{NH}_4\text{HCO}_3$  buffer (pH 9.4). The fraction containing haemagglutinating activity from the Mono Q column was concentrated with an Amicon filter device and subjected to a final purification on a Superdex 75 column in 20 mM  $\text{NH}_4\text{HCO}_3$  buffer (pH 9.4). The single peak eluted represented purified HA.

### Assay of haemagglutinating activity

In the assay for haemagglutinating activity, a serial 2-fold dilution of the HA solution in microtitre U-plates (50  $\mu\text{l}$ ) was mixed with 50  $\mu\text{l}$  of a 2% suspension of rabbit erythrocytes in PBS (pH 7.2) at 20°C. The results were read after approx. 1 h, when erythrocytes in the blank had fully sedimented and formed a dot at the bottom of the well. The haemagglutination titre, defined as the reciprocal of the highest dilution exhibiting haemagglutination, was reckoned as one haemagglutination unit. Specific activity is the number of haemagglutination units per mg of protein.

The tests to investigate inhibition of HA-induced haemagglutination by various carbohydrates, including fucose, galactose, galactosamine, galacturonic acid, polygalacturonic acid, glucose, glucosamine, glucuronic acid, lactose, mannosamine, mannose, melibiose, raffinose, rhamnose, xylose and heparin, were performed in a manner analogous to the haemagglutination test. Serial 2-fold dilutions of sugar samples were prepared in PBS. All of the dilutions were mixed with an equal volume (25  $\mu\text{l}$ ) of a solution of the HA with 16 haemagglutination units. The mixture was allowed to stand for 30 min at room temperature (24°C), and then mixed with 50  $\mu\text{l}$  of a 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture, which completely inhibited 16 haemagglutination units of the HA preparation, was calculated.

The effects of temperature, NaOH solution and HCl solution on haemagglutinating activity of the HA were examined as described previously [26]. Briefly, for the temperature stability assay, a solution of *C. militaris* agglutinin was incubated at the following temperatures for 30 min: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C. The reaction tubes were then put on ice and an assay of haemagglutinating activity was then carried out. For the pH stability test, a solution of *C. militaris* agglutinin was incubated at various pH values for 30 min: pH 0–14. The reaction mixtures were neutralized and an assay of haemagglutinating activity was carried out.

### Molecular mass and N-terminal sequence determination

The purified HA was subjected to SDS/PAGE for molecular mass determination by the method of Nielsen and Reynolds [27]. Gel filtration on an FPLC-Superdex 75 column, which had been calib-

rated with molecular mass markers (GE Healthcare), was conducted to determine the molecular mass. The N-terminal sequence of the HA was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

### Chemical modification of tryptophan residues

Tryptophan modification was carried out in 0.1 M sodium acetate buffer (pH 6.0), by adding different concentrations of NBS (*N*-bromosuccinimide). The modification reaction was done in a spectrophotometer cuvette on an HA sample at 1 mg/ml concentration. Modification was monitored by following the changes in the attenuation (*D*) at 280 nm. The reagent was then removed by using a centrifugal filter device (Amicon), and the haemagglutinating activity was determined [28].

### Chemical modification of tyrosine residues

The phenoxy side chains of tyrosine residues were modified using NAI (*N*-acetylimidazole). Briefly, an HA solution in PBS (1 mg/ml concentration) was incubated with different concentrations of NAI (0.1–10 mM) for 1 h at room temperature. The reagent was then removed by using a centrifugal filter device (Amicon) and the sample was tested for haemagglutinating activity [28].

### Assay of mitogenic activity

The assay of mitogenic activity was performed as previously described [4]. Splenocytes were isolated from Balb/c mice. The cells were diluted with RPMI 1640 medium containing 10% (v/v) FBS (fetal bovine serum) and then seeded ( $2 \times 10^6$  cells/0.2 ml per well) in 96-well microplates. The HA was then added at various concentrations. Cells cultured in the absence of the HA served as a negative control. The cells were incubated at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  for 24 h. The cells were viable after 24 h. During the last 6 h, the cells in each well were pulsed with 0.5  $\mu\text{Ci}$  of [ $^3\text{H}$ -methyl]thymidine (specific radioactivity: 5  $\mu\text{Ci}/\text{mmol}$ ; GE Healthcare) in 10  $\mu\text{l}$  and were then harvested on to a glass fibre filter using a cell harvester. The radioactivity was determined using a Beckman scintillation counter. The proliferative (mitogenic) response was expressed as mean c.p.m.

### Assay of nitric oxide-inducing activity

Assay of nitric oxide production by murine peritoneal macrophages was conducted as described by Wong and Ng [5]. Macrophages were collected from the peritoneal cavity of mice after an intraperitoneal injection of 3% thioglycolate medium. The cells were washed and resuspended in RPMI 1640 medium, 10% FBS, 100 i.u./ml penicillin and 100 mg/ml streptomycin. Cells ( $2 \times 10^5$  cells per well) were seeded on to a 96-well culture plate for 1 h before incubation with the isolated HA for 24 h. The amount of nitric oxide in the culture medium was determined by the colorimetric method using sodium nitrite as a standard. In the assay, a 100  $\mu\text{l}$  aliquot of cell-free culture medium from each culture well was allowed to react with 50  $\mu\text{l}$  of Griess reagent (1% sulfanilamide in 5%  $\text{H}_3\text{PO}_4/0.1\%$  naphthalene-ethylenediamine

dihydrochloride) for 10 min before the attenuation was read at 540 nm using a microplate reader. Lipopolysaccharide was used as a positive control in this assay.

### Assay of antiproliferative activity towards hepatoma cells

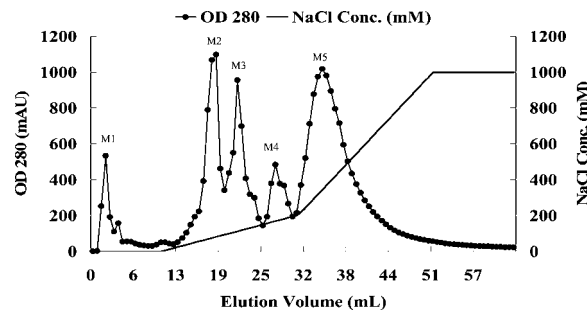
The antiproliferative activity of the purified HA was determined as follows. The cell lines HepG2 (hepatoma) and WRL68 (embryonic liver cells) were purchased from A.T.C.C. (Manassas, VA, U.S.A.). The various cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, 100 mg/l streptomycin and 100 i.u./ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells ( $1 \times 10^4$ ) in their exponential growth phase were seeded on to each well of a 96-well culture plate and incubated for 3 h before the addition of the HA. Incubation was carried out for another 24 or 48 h. After 24 or 48 h, 20 µl of a 5 mg/ml solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] in PBS was spiked into each well, and the plates were incubated for 4 h. The plates were then centrifuged at 324 g for 5 min. The supernatant was carefully removed and 150 µl of DMSO was added in each well to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the attenuation was measured at 590 nm with a microplate reader [29].

### Assay for ability to inhibit HIV-1 reverse transcriptase activity

The assay was carried out as detailed in [30] using a non-radioactive ELISA kit (Boehringer Mannheim). The detection and quantification of synthesized DNA as a parameter to reverse transcriptase activity follows a sandwich ELISA protocol. Biotin-labelled DNA binds to the surface of microtitre plate modules precoated with streptavidin. An antibody to digoxigenin, conjugated with peroxidase (anti-DIG-POD), binds to the digoxigenin-labelled DNA. The peroxidase substrate is then added. The peroxidase enzyme catalyses substrate cleavage to produce a coloured reaction product. The attenuation of the samples at 405 nm is directly correlated with the level of reverse transcriptase activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the test protein was calculated as percentage inhibition as compared with a control without the test protein.

### Assay of antifungal activity

The assay of HA for antifungal activity towards *Botrytis cinerea*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, *Helminthosporium turcicum*, *Helminthosporium maydis*, *Valsa mali*, *Rhizoctonia solani* and *Fusarium oxysporum* was carried out in 90 mm × 15 mm Petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper discs (0.625 cm in diameter) were placed at a distance of 0.5 cm from the rim of the mycelial colony. An aliquot of a solution of the HA was added to a disc. The plates were incubated at 25°C for 20–48 h (depending on growth rates of the fungi) until mycelial growth had enveloped discs containing the control and had



**Figure 1** FPLC on a Mono Q column previously equilibrated with 20 mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 9.4) at a flow rate of 2 ml/min

The adsorbed fraction was eluted with one breakpoint linear gradient of 0–0.2 M NaCl, followed by 0.2–1 M NaCl in the same buffer as shown by the slanting lines across the chromatogram. Only the fraction labelled M4 showed haemagglutinating activity. mAU, milli-absorbance units; OD 280, absorbance at 280 nm.

formed crescents of inhibition around discs containing samples with antifungal activity [29].

### Assay of RNase activity

Yeast tRNA (Sigma) was used as a substrate. The HA was incubated with 0.2 µg of tRNA in 135 µl of 100 mM sodium phosphate buffer (pH 7.5) for 15 min. Then, 350 µl of ice-cold 3.7% HClO<sub>4</sub> was added to terminate the reaction. After standing on ice for 15 min, the reaction mixture was centrifuged and the attenuation of the supernatant was read at 260 nm. One unit of RNase activity is defined as the amount of enzyme that produces an absorbance increase of one per min in the acid-soluble supernatant per ml of reaction mixture under specified conditions [31].

## RESULTS

Haemagglutinating activity of the crude fungal extract was concentrated in the fraction unadsorbed on Affi-gel blue gel. The fraction adsorbed on Affi-gel blue gel was devoid of haemagglutinating activity (results not shown). When the active fraction was chromatographed on Mono Q, it was resolved into a small unadsorbed fraction M1 and several adsorbed fractions M2–M5. Haemagglutinating activity resided in the smallest adsorbed fraction M4 (Figure 1). This fraction was further purified on Superdex 75 to yield a single 30 kDa fraction with haemagglutinating activity (Figure 2). The purified HA appeared as a single band with a molecular mass of 30 kDa in SDS/PAGE (Figure 3) and a single 30 kDa peak on rechromatography on Superdex 75 (results not shown). Its N-terminal sequence bore no similarity to any reported lectin/HA, but showed some resemblance to proteins unrelated to lectins/HAs (Table 1).

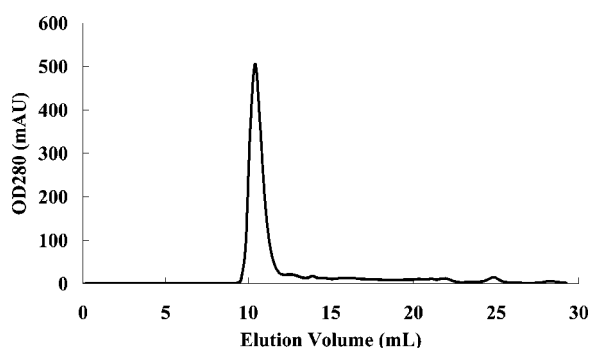
Haemagglutinating activity of the isolated HA could not be inhibited by any of the following sugars at 250 mM concentration: fucose, galactose, galactosamine, galacturonic acid, polygalacturonic acid, glucose, glucosamine, glucuronic acid, lactose, mannosamine, mannose, melibiose, raffinose, rhamnose, xylose

**Table 1 Comparison of N-terminal amino acid sequence of *C. militaris* HA with those of other proteins from a PubMed BLAST search**

Identical amino acid residues are underlined.

Sample	Residue number	Partial sequence	Residue number
<i>C. militaris</i> HA	1	NSTD <u>I</u> SLNHG	10
RNA polymerase $\sigma$ factor ( <i>Psychromonas ingrahamii</i> 37)	8	NSTD <u>I</u> TLNN	16
Mannosidase 2, $\alpha$ 2 ( <i>Rattus norvegicus</i> )	1145	NSTD <u>I</u> SL	1151
N-glycan processing $\alpha$ -mannosidase IIx ( <i>Mus musculus</i> )	1133	NSTD <u>I</u> SL	1139
$\gamma$ -Glutamyl carboxylase ( <i>Ciona intestinalis</i> )	557	NSTD <u>V</u> SL	564
Carboxylesterase ( <i>Drosophila pseudoobscura</i> )	421	NSTE <u>I</u> SLDLHRKHG	434
Heat-shock protein 105 kDa ( <i>Gallus gallus</i> )	369	NSTD <u>I</u> PLN	376
<i>C. militaris</i> lectin from Jung et al. [9]	1	SYDADXQRVXNDKGIXND*	18

\*'X' indicates that the residue could not be identified.



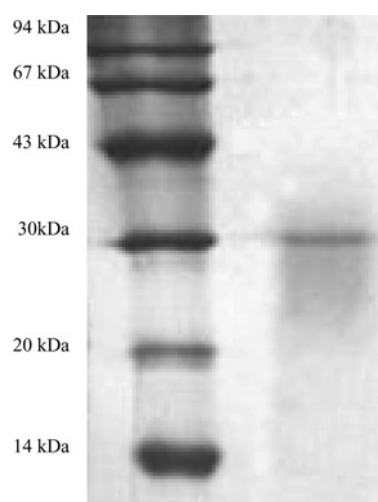
**Figure 2 Gel filtration of peak M4 on a Superdex 75 column in 20 mM  $\text{NH}_4\text{HCO}_3$  buffer (pH 9.4) at a flow rate 0.5 ml/min** mAU, milli-absorbance units; OD 280, absorbance at 280 nm.

and heparin. The haemagglutinating activity was stable in the temperature range 0–60°C. The activity fell by 50% after exposure to 70°C and by approx. 97% at 80°C. Activity was indiscernible at 90 and 100°C. The haemagglutinating activity of the HA of *C. militaris* was not affected over the pH range 2–13 (results not shown). Chemical modification of tryptophan and tyrosine had no effect on the haemagglutinating activity of the HA.

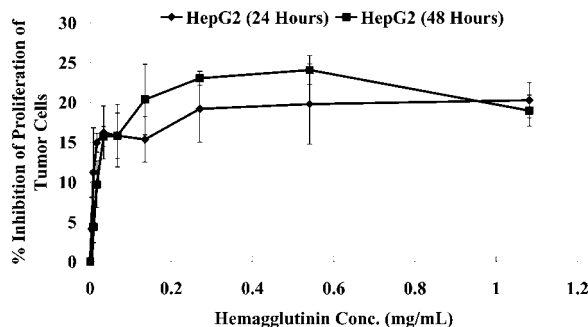
The HA exerted some antiproliferative activity on HepG2 cells (Figure 4). There was only slight antiproliferative activity on embryonic liver cells (Figure 5). There was no mitogenic activity towards mouse splenocytes (Figure 6). The HA was also incapable of stimulating nitric oxide production by mouse macrophages. There was no antifungal activity either (results not shown). However, it inhibited HIV-1 reverse transcriptase with an  $\text{IC}_{50}$  of approx. 10  $\mu\text{M}$  (Figure 7). A comparison of *C. militaris* HA with other ascomycete mushroom HAs is shown in Table 2. Many differences were revealed.

## DISCUSSION

Jung's agglutinin preparation was isolated using gel filtration on Sephadex G-75 followed by affinity chromatography on



**Figure 3 SDS/PAGE of HA purified using a Superdex 75 column** Right-hand lane: a single peak from the Superdex 75 column representing purified HA from *C. militaris*. Left-hand lane: molecular-mass standards (in kDa, GE Healthcare).



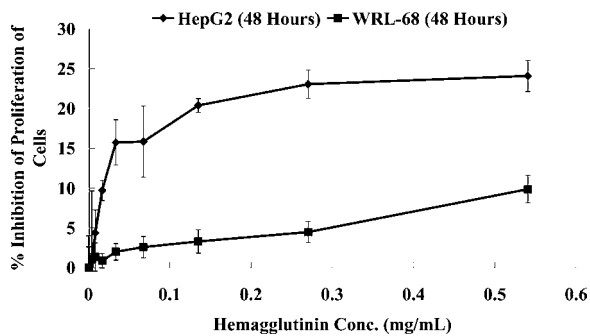
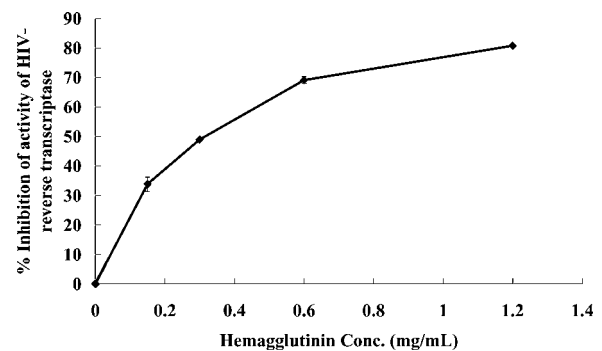
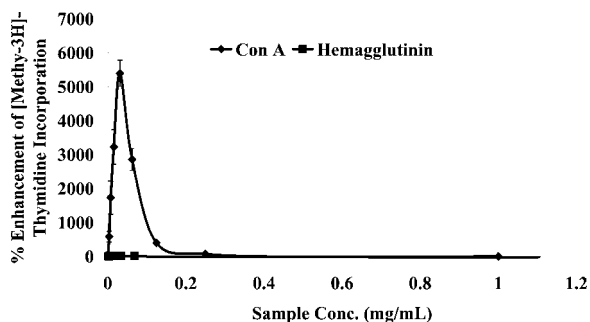
**Figure 4 Antiproliferative activity of *C. militaris* HA towards liver tumour (HepG2) cells after treatment for 24 and 48 h** Results are means  $\pm$  S.D. ( $n = 3$ ).

fetuin-agarose. An approx. 16-fold purification was achieved [9]. The present preparation was obtained by employing affinity chromatography on Affi-gel blue gel, FPLC-ion-exchange

**Table 2 Comparison of characteristics of lectins/HAs from ascomycete mushrooms**

N.D., not determined; N.I., haemagglutinating activity not inhibited by simple sugars and heparin.

Characteristic	<i>X. hypoxylon</i> [45]	<i>S. sclerotiorum</i> [43]	<i>Cib. camelliae</i> [42]	<i>M. chateri</i> [45]	<i>C. militaris</i> (present study)
N-terminal sequence	SSAHNTLGGG	VFLGVGTYEI	VFTGRGTYDI	PYEFLYGGKE	NSTDISLNHG
Molecular mass (kDa)	28.8	16	34	40	30
Number of subunits	2	1	2, 3 or 4	1	1
Sugar specificity	Xylose	GalNAc/Gal	GalNAc	Fucose and starch	N.I.
Thermal stability (°C) of haemagglutinating activity	0–35	N.D.	0–40	N.D.	0–60
HIV-1 reverse transcriptase-inhibitory activity IC <sub>50</sub> (μM)	N.D.	N.D.	N.D.	N.D.	10
Antitumour activity	M1 and HepG2 cells	N.D.	N.D.	N.D.	HepG2 cells
Mitogenic activity towards splenocytes	Anti-mitogenic	N.D.	N.D.	N.D.	Without mitogenic activity
pH stability of haemagglutinating activity	N.D.	N.D.	N.D.	N.D.	pH 2–13

**Figure 5 Antiproliferative activity of *C. militaris* HA towards liver tumour (HepG2) cells and embryonic liver (WRL-68) cells after treatment for 48 h**Results are means  $\pm$  S.D. ( $n = 3$ ).**Figure 7 Anti-HIV-1 reverse transcriptase activity of *C. militaris* HA**Results are means  $\pm$  S.D. ( $n = 3$ ).**Figure 6 Assay for mitogenic activity using murine splenocytes**Results are means  $\pm$  S.D. ( $n = 3$ ). Con A, concanavalin A.

chromatography on Mono Q and FPLC–gel filtration on Superdex 75. A 31-fold purification ensued.

*C. militaris* HA isolated in the present study has a molecular mass (30 kDa) similar to the *C. militaris* lectin purified by

Jung et al. [9] reported earlier. The haemagglutinating activity of Jung's preparation could not be inhibited by a variety of simple saccharides at 100 mM concentration. The activity of the present preparation could not be reduced by similar sugars at 250 mM concentration. Neither could it be inhibited by the polysaccharide heparin. However, the two proteins have distinctly different N-terminal amino acid sequences. Furthermore, the haemagglutinating activity of the present preparation was stable up to 60°C, whereas the activity of Jung's preparation vanished at temperatures higher than 55°C [9]. Jang's preparation has some mitogenic activity as judged by the increase in  $A_{450}$  [9]. In contrast, the present preparation was devoid of a similar activity. As judged from the aforementioned data, it appears that the present preparation is a protein different from Jung's preparation although there are some similarities between the two proteins.

The haemagglutinating activity of the present agglutinin preparation remained unaltered after chemical modification of tryptophan and tyrosine residues. The findings are in contrast



with those of other studies, which demonstrate that tryptophan and/or tyrosine residues are crucial for haemagglutinating activity [32–34].

The present preparation has been assayed for activities not tested in the case of Jung's preparation, i.e. HIV-1 reverse transcriptase-inhibitory activity, antiproliferative activity towards tumour cells, nitric oxide-inducing activity towards macrophages, antifungal activity and RNase activity. The present preparation is similar to some of the previously reported lectins/HAs in that it demonstrates HIV-1 reverse transcriptase-inhibitory activity [4,5,17,26,35,36]. The inhibitory potency is high compared with other proteinaceous and non-proteinaceous anti-HIV natural products. The mechanism of inhibition is probably protein–protein interaction similar to the inhibition of HIV-1 reverse transcriptase by the homologous retroviral protease [37]. The HA also manifests antiproliferative activity towards hepatoma cells. This finding is consistent with previous observations on antiproliferative and antitumour activities of lectins [5,22,36,38,39]. Some of the previously reported lectins, e.g. banana lectin, induce nitric oxide production from macrophages [5]. However, the present preparation lacks such an activity. It is also devoid of antifungal activity. In fact, only very few lectins/HAs exhibit antifungal activity [17,40]. The antifungal protein bacisubin from *Bacillus subtilis* exhibits haemagglutinating as well as RNase activities [7]. However, *C. militaris* HA does not have RNase activity.

There are only a few reports on the purification of ascomycete mushroom HAs/lectins in the literature including those on *Xylaria hypoxylon*, *Sclerotinia sclerotiorum*, *Ciborinia camelliae* and *Melastiza chateri* [7,41–43]. A comparison of *C. militaris* HA with the aforementioned ascomycete lectins reveals that *C. militaris* HA has a much larger molecular mass and higher thermostability, and, unlike other lectins, it is not inhibited by simple sugar. Information about the pH stability and HIV-1 reverse transcriptase-inhibitory activity of the other ascomycete lectin is not available. Only *X. hypoxylon* lectin and the present lectin preparation have been shown to possess antiproliferative activity.

In summary, the HA obtained in the present study is distinct from fungal lectins/HAs reported earlier in several ways including N-terminal amino acid sequence, pH stability and higher thermostability, and lack of mitogenic activity. The present and Jung's HA preparations are similar in molecular mass and carbohydrate specificity. The difference between the present agglutinin preparation and Jung's preparation probably lies in the different strains of *C. militaris* used, one from China and one from Korea. Previously, lectins with dissimilar characteristics have been obtained from the same mushroom species, e.g. *Flammulina velutipes* [17] and *Ganoderma lucidum* [20,44]. The present agglutinin has been assayed for a variety of activities and found to have antiproliferative activity towards hepatoma cells and HIV-1 reverse transcriptase-inhibitory activity. RNase, antifungal and nitric oxide-inducing activities exhibited by some lectins are lacking in the present HA. Results of the present study add to the meagre information pertaining to agglutinins from ascomycete and medicinal mushrooms.

## FUNDING

This work was supported by the Medicine Panel, The Chinese University of Hong Kong Research Committee.

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Received 13 October 2008/8 December 2008; accepted 18 December 2008

Published as Immediate Publication 18 December 2008, doi 10.1042/BSR20080153