Isolation and characterization of a lectin with potentially exploitable activities from caper (Capparis spinosa) seeds

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Synopsis

A dimeric 62-kDa lectin exhibiting a novel N-terminal amino acid sequence was purified from caper (Capparis spinosa) seeds. The purification protocol involved anion-exchange chromatography, cation-exchange chromatography and, finally, gel filtration by FPLC on Superdex 75. Approx. 100-fold purification was achieved. The haemagglutinating activity of the lectin, which was stable in the pH range 1–12 and up to 40°C, could be inhibited by D(+) galactose, α-lactose, raffinose and rhamnose at 1 mM concentration, by 25 mM L(+)-arabinose and by 100 mM D(+)GlcN (glucosamine). The lectin potently inhibited HIV-1 reverse transcriptase with an IC50 of 0.28 μM and proliferation of both hepatoma HepG2 and breast cancer MCF-7 cells with an IC50 of approx. 2 μM. It induced apoptosis in HepG2 and MCF-7 cells. It manifested a weaker mitogenic activity on mouse splenocytes than ConA (concanavalin A). It inhibited mycelial growth in Valsa mali with an IC50 of 18 μM.

Key words: antifungal activity, antiproliferative activity, Capparis spinosa, HIV-1 reverse transcriptase inhibitory activity, lectin

INTRODUCTION

Capparis spinosa is a plant belonging to the family Capparaceae. Various parts of this plant have been shown to have biological activity. Its bud extract inhibits the replication of herpes simplex virus type 2 and up-regulates the expression of pro-inflammatory cytokines including interleukin-12, interferon-γ and tumour necrosis factor-α [1]. The antioxidant principles in the buds have been identified to be flavanols and hydroxycinnamic acid [2]. p-Methoxybenzoic acid has been identified as an antihepatotoxic component in the methanol-soluble fraction of the aqueous extract [3]. The aqueous extract demonstrated hypolipidaemic [4] and antihyperglycaemic [5] activities. Other activities comprised antiviral, immunomodulatory [1], chondrocyte protective [6], antiallergic, antihistaminic [7], antifungal [8], anti-Leishmania [9] and antimicrobial [10] activities, and also an inhibitory effect on fibroblast proliferation and type 1 collagen production in progressive systemic sclerosis [11].

An indication of the possible presence of a lectin in C. spinosa is found in the demonstration of the ability of specific carbohydrates to inhibit the effect of C. spinosa in agglutinating and killing parasites [9]. However, the lectin has not been purified. In view of the dearth of information on proteinaceous constituents of C. spinosa, the present study was undertaken to isolate C. spinosa lectin and compare its characteristics with known lectins.

Lectins are carbohydrate-binding proteins present in a diversity of organisms including humans, vertebrate and invertebrate animals, plants [12], fungi [13] and bacteria [14]. Based on their carbohydrate-binding specificity, they can be divided into (i) mannose binding, (ii) glucose and mannose binding, (iii) GlcNAc (N-acetylglucosamine) binding, (iv) galactose binding, (v) GalN (N-galactosamine) binding, (vi) sialic acid binding, (vii) fucose binding etc. They display a host of biological activities such as antitumour [13], immunomodulatory [14], insecticidal [15], antifungal [16], antiviral [17] and antibacterial [18] activities. The aim of the present study was to examine C. spinosa lectin for some exploitable biological activities.

Abbreviations used: ConA, concanavalin A; GlcN, glucosamine; SP-Sepharose, sulfopropyl-Sepharose.

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MATERIALS AND METHODS

Purification of lectin
Fresh seeds (580 g) were collected from caper (C. spinosa). They were extracted by homogenizing in distilled water. After centrifugation at 20 000 g for 30 min at 4 °C, Tris/HCl buffer (2 M, pH 7.4) was added to the supernatant until the final concentration of Tris reached 20 mM. The supernatant was then applied on to a 5 cm × 9 cm column of DEAE-cellulose (Sigma). Unadsorbed proteins were eluted with 20 mM Tris/HCl buffer (pH 7.4). Adsorbed proteins were eluted stepwise, first with 0.1 M NaCl and then with 1 M NaCl added to the 20 mM Tris/HCl buffer. The fraction eluted with 1 M NaCl was taken, dialysed and then subjected to chromatography on a 5 cm × 4.5 cm column of SP-Sepharose (sulfopropyl-Sepharose; GE Healthcare). After removal of unadsorbed proteins with 20 mM Tris/HCl buffer (pH 7.4), adsorbed proteins were eluted with 1 M NaCl added to the 20 mM Tris/HCl buffer. The adsorbed fraction was saved and dialysed extensively against water and freeze-dried before chromatography on a 0.34 ml CIM-Q column (BIA Separations) using an AKTA purifier (GE Healthcare). Unadsorbed proteins were removed with 20 mM Tris/HCl buffer. Adsorbed proteins were eluted, first with a linear 0–0.3 M NaCl gradient and then with 1 M NaCl in the 20 mM Tris/HCl buffer (pH 7.4). The fraction that eluted at 0.15–0.25 M NaCl was saved, dialysed and freeze-dried before re-dissolving and chromatography on a Superdex 75 HR10/30 column using an AKTA purifier. The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to indicate the void volume), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), myoglobin (17.6 kDa), RNase A (13.7 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.3 kDa) (GE Healthcare), to determine the molecular mass of the protein. The first fraction collected represented purified lectin.

Molecular mass determination using SDS/PAGE, gel filtration and N-terminal amino acid sequencing
The purified lectin was subjected to SDS/PAGE [19]. Gel filtration on an FPLC Superdex 75 column was conducted to determine the molecular mass of the lectin. The N-terminal sequence of the lectin was determined as described in [12].

Inhibition of lectin-induced haemagglutination by carbohydrates
The haemagglutinating inhibition tests to investigate inhibition of lectin-induced haemagglutination by various carbohydrates were performed as described in [12].

Effects of temperature and pH on lectin-induced haemagglutination
The purified lectin (50 μg/ml) was incubated at various temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C) or buffers of various pH values (pH 1–14) for 15 min. It was then cooled to 4 °C or neutralized to pH 7 respectively immediately before the haemagglutination assay.

Assay of mitogenic activity in mouse splenocytes
The assay was performed as described in [12].

Assay of antifungal activity
The assay for antifungal activity towards the phytopathogenic fungi Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis, Valsa mali and Rhizoctonia solani was carried out as described in [21]. The IC50 value for the antifungal activity of the lectin against V. mali was as described in [21].

Assay of HIV-1 reverse transcriptase inhibitory activity
An assay of the ability of the protein to inhibit HIV-1 reverse transcriptase was carried out by using an ELISA kit from Boehringer Mannheim as described in [12].

Assay of antiproliferative activity
The assay of the antiproliferative activity of the isolated protein was carried out by testing its inhibition on the growth of the human hepatoma HepG2 cells and the human breast cancer MCF-7 cells as described in [12].

Annexin V and propidium iodide staining
The method was modified from [20]. Phosphatidyl serine externalization (loss of membrane asymmetry) was studied by using the annexin V–FITC and propidium iodide staining method. Cells (5 × 105) were plated on to a 6-well culture plate and incubated for 24 h. Then the cells were trypsinized before centrifugation at 2000 g for 4 min. The cells were washed with 1 ml of PBS and then centrifuged at 2000 g for 4 min. Cells were resuspended in 250 μl of binding buffer (0.01 M Hapes, pH 7.4, 140 mM NaCl and 25 mM CaCl2) and then stained with 2.5 μl of annexin V solution (BD Pharmingen) and 0.5 μl of propidium iodide (Sigma) in darkness for 20 min at room temperature. The sample was analysed with an FACSort™ flow cytometer (Becton...
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RESULTS

Ion-exchange chromatography of the seed extract on DEAE-cellulose produced a very large unadsorbed fraction (D1) and two adsorbed fractions (D2 eluted with 0.1 M NaCl and D3 eluted with 1 M NaCl) of approximately the same size. Haemagglutinating activity resided only in fraction D3. This fraction was separated on SP-Sepharose into a large unadsorbed fraction (P1) devoid of haemagglutinating activity and a smaller adsorbed fraction (P2) with haemagglutinating activity. Fraction P2 was subsequently resolved on CIM-Q into a small unadsorbed fraction (Q1) and several large adsorbed fractions (Q2–Q5). Haemagglutinating activity was confined to an adsorbed fraction Q4 eluted towards the second half of the 0–0.3 M NaCl gradient (Figure 1). This active fraction was subjected to final purification on Superdex 75. Two fractions, S1 and S2, were obtained. Haemagglutinating activity resided in the first fraction (S1), which appeared to be much smaller than the second fraction (S2) (Figure 2). Fraction S1 was re-chromatographed on Superdex 75. It was eluted as a single homogeneous peak with a molecular mass of 62 kDa. It demonstrated a single 31 kDa band on SDS/PAGE (Figure 3). A summary of the purification of the lectin is included in Table 1. The lectin was obtained with a specific activity of 35 000 titres/mg of protein and a 100-fold purification. The N-terminal sequence of the lectin was highly homologous with a partial sequence of ribosomal subunit interface protein from Roseobacter sp. but was distinct from sequences of published lectins (Table 2). Raffinose, α-lactose, rhamnose and D(+)galactose were the most potent in inhibiting haemagglutination induced by the isolated lectin and inhibition was discernible at 1 mM. L(+)arabinose and D(+)GlcN (glucosamine) were inhibitory at 25 and 100 mM respectively. Other sugars tested were inactive at 100 mM. They included D-mannitol, sucrose, GalA (galacturonic acid), xylitol, glucose, D(+)xylose and D-GlcA (glucuronic acid). The haemagglutinating activity of the lectin was stable throughout the pH range 1–12 and was completely destroyed at pH 13 and 14. It was stable in the temperature range 0–40 ºC. The lectin did not form aggregates and remained in
Table 1 Yields from 580 g of fresh C. spinosa seeds and specific haemagglutinating activities at different stages of purification of C. spinosa lectin

<table>
<thead>
<tr>
<th>Column</th>
<th>Chromatographic fraction</th>
<th>Yield (mg of protein)</th>
<th>Haemagglutinating activity</th>
<th>Total (titre)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Crude extract</td>
<td>3000</td>
<td>350</td>
<td>1050000</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>D3</td>
<td>830</td>
<td>1000</td>
<td>830000</td>
<td>79</td>
<td>3</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>P2</td>
<td>330</td>
<td>2400</td>
<td>792000</td>
<td>76</td>
<td>7</td>
</tr>
<tr>
<td>CIM-Q</td>
<td>Q4</td>
<td>80</td>
<td>9000</td>
<td>720000</td>
<td>69</td>
<td>26</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>S2</td>
<td>20</td>
<td>35000</td>
<td>700000</td>
<td>66</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 4 Induction of apoptosis of MCF-7 cells by C. spinosa lectin
MCF-7 cells were incubated with lectin on a 6-well culture plate for 24 h. After washing the lectin-treated/untreated MCF-7 cells with PBS, they were stained with annexin V/propidium iodide and then analysed by flow cytometry. The lower left quadrant shows healthy cells. The upper and lower right quadrants of each plot show annexin V/propidium iodide double-positive cells (i.e. cells undergoing late apoptosis) and annexin V single-positive cells (i.e. cells undergoing early apoptosis) respectively. Results are expressed as the means for triplicate experiments. The level of apoptosis was increased by 34% (i.e. 18.6 ± 26.3 – 6.5 – 4.6%) when the cells were incubated with 30 μM lectin for 24 h.

solution at 100°C. The haemagglutinating activity was reduced to half at 50°C and lost at 60°C (Table 3). The lectin inhibited proliferation of HepG2 and MCF-7 tumour cells with an IC₅₀ of approx. 2 μM. Apoptosis was observed in treated HepG2 (results not shown) and MCF-7 tumour cells (Figure 4). The level of apoptosis was increased by 34% when the cells were incubated with 30 μM lectin for 24 h. The lectin evoked a maximal mitogenic response at a concentration of 1 μM, whereas
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Figure 5 Antifungal activity of \textit{C. spinosa} lectin towards \textit{V. mali}

(A) 100 μM lectin, (B) 33 μM lectin, (C) 11 μM lectin, (D) 3.7 μM lectin and (E) buffer control.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>N-terminal amino acid sequence of \textit{C. spinosa} lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Residue number</td>
</tr>
<tr>
<td>\textit{C. spinosa} lectin</td>
<td>1</td>
</tr>
<tr>
<td>Ribosomal subunit interface protein, putative (\textit{Roseobacter} sp. MED193)</td>
<td>69</td>
</tr>
</tbody>
</table>

ConA (concanavalin A) did so at 150 nM. The maximal response elicited by the lectin was 75% in magnitude compared with the maximal response to ConA (results not shown). The lectin inhibited HIV-1 reverse transcriptase with an \( IC_{50} \) of 0.28 μM. It inhibited mycelial growth in \textit{V. mali} with an \( IC_{50} \) of 18 μM (Figure 5), but not in \textit{M. arachidicola}, \textit{F. oxysporum}, \textit{H. maydis} and \textit{R. solani} (results not shown).

DISCUSSION

Ion-exchange chromatography and gel filtration were used to purify \textit{C. spinosa} lectin, the first lectin isolated from the family Capparaceae, with a high purification fold (100-fold) compared with 10-fold in the case of French bean lectin [23]. The subunit molecular mass (31 kDa) and dimeric nature of \textit{C. spinosa} lectin were similar to those of many plant lectins [22], but its N-terminal sequence was novel.

Table 3 Haemagglutinating activity of \textit{C. spinosa} lectin at different temperatures and pH values

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Initial haemagglutinating activity (haemagglutinating unit)</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C, 10°C, 20°C, 30°C, 40°C</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>50°C</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>60°C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 1–12</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>pH 13, 14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

It is of great interest that \textit{C. spinosa} lectin can be inhibited by as many as six sugars, including galactose, lactose, arabinose, rhamnose, raffinose and \( \text{D(+)-GlcN} \). French bean lectin cannot be inhibited by simple sugars [23]. Chive lectin can be inhibited by mannose only [24]. Emperor banana lectin can be inhibited by mannose and glucose [12]. \textit{Canavalia gladiata} lectin can be inhibited by mannose, glucose and rhamnose [25]. \textit{Pleurotus citrinopileatus} lectin can be inhibited by \( \text{olp-nitropheny1-\beta-D-glucuronide} \), \( \text{olp-nitropheny1-\beta-D-galactopyranoside} \) and maltose, and a polysaccharide, inulin [13]. \textit{C. spinosa} lectin was marked by pronounced pH stability from pH 1 to 12. The lectin had only moderate thermostability up to 40°C. These characteristics were similar to those of most lectins [23].

To date, several lectins have been reported with antifungal activity. For example, red kidney bean lectin inhibits...
F. oxysporum, Coprinus comatus and R. solani [22]. The spectrum of antifungal activity of C. spinosa lectin (against V. mali, but not others) is also distinct from other antifungal lectins. It exhibited potent antiproliferative activity against hepatoma and breast cancer cells, in keeping with similar demonstrations for other lectins [12]. It is noteworthy that it can induce apoptosis in both HepG2 and MCF-7 tumour cells. Only galectin-9 [26] and mistletoe lectin [27] have shown to induce apoptosis in MCF-7 cells, whereas there are more reports showing that lectins can induce apoptosis in HepG2 cells, e.g. Pouteria torta lectin [28]. C. spinosa lectin potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.28 μM. This activity has been shown by only some lectins with an IC_{50} of 1–35 μM.

The distinctive features of C. spinosa lectin isolated in the present study include (i) a novel N-terminal sequence, (ii) pH stability of haemagglutinating activity, (iii) inhibition of haemagglutinating activity by as many as six sugars and (iv) a greater diversity of biological activities than other lectins comprising mitogenic activity, antifungal activity, highly potent HIV-1 reverse transcriptase activity and antiproliferative activity due to induction of apoptosis.

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REFERENCES


