

## Fip-*vvo*, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*

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A new fungal immunomodulatory protein (Fip) has been purified from the edible mushroom, *Volvariella volvacea*, and designated Fip-*vvo*. Analysis of the purified protein by SDS/PAGE followed by Coomassie Blue staining demonstrated that Fip-*vvo* is a single polypeptide with an apparent molecular mass of 15 kDa. Periodic acid/Schiff staining showed that this single polypeptide lacks carbohydrates. Using an *in vitro* bioassay measuring blast-formation stimulatory activity, Fip-*vvo* was shown to stimulate the maximum proliferation of human peripheral blood lymphocytes at a concentration of 5 µg/ml. Fip-*vvo* was capable of agglutinating rat red blood cells. Neither haemagglutination nor mitogenic activities were inhibited by mono- or dimeric sugars. *In vivo*, repeat administration of Fip-*vvo* greatly reduced the production of BSA-induced Arthus reaction in mice, whereas

little effect was observed on the prevention of systemic anaphylaxis reactions. The selectively enhanced transcriptional expression of interleukin (IL)-2, IL-4, interferon-γ, tumour necrosis factor-α, lymphotoxin and IL-2 receptor by Fip-*vvo* was also demonstrated by reverse transcriptase-PCR. This finding suggests that Fip-*vvo* exerts its immunomodulatory effects via cytokine regulation. In addition, the complete amino acid sequence of Fip-*vvo* was obtained by direct protein sequencing. This protein consists of 112 amino acid residues with a blocked N-terminal end and has a calculated molecular mass of 12 667 Da not including the N-terminal blocking group. By gel filtration analysis, Fip-*vvo* exhibited a molecular mass of 26 kDa for the native molecules in PBS. This result indicates that native Fip-*vvo* is most likely a non-covalently associated homodimeric molecule.

### INTRODUCTION

Modulation of the immune system by various agents may have potential for the management of certain infections, autoimmune diseases, graft rejection, as well as neoplastic diseases. Many proteins purified from plant seeds, for example concanavalin A, phytohaemagglutinin (PHA), wheat germ agglutinin and pokeweed mitogen, bind to specific carbohydrate residues on the lymphocyte membrane and induce a cascade of events leading to cell activation, including proliferation, production of lymphokines and differentiation. A group of fungal proteins defined by amino acid sequence similarity and their actions on immunological responses are classified into a distinct family, fungal immunomodulatory protein (Fip) [1,2]. The molecular masses of Fips were estimated to be approx. 15 kDa by SDS/PAGE and their major biological activities resembled the mitogenic activity of lectins towards mouse spleen cells and human peripheral blood lymphocytes (PBL). LZ-8, a well characterized Fip isolated from the mycelia of *Ganoderma lucidum* [1], showed the optimal blast-formation stimulatory activity towards mouse spleen cells at a concentration of 3.13 µg/ml, while another Fip, Fip-*five*, isolated from the fruit bodies of *Flammulina velutipes* [2], stimulated the maximum proliferation of human PBL at 100 µg/ml. Fips showed various degrees of haemagglutination activity of the red blood cells of different species. Fip-*five* was able to agglutinate human red blood cells, whereas LZ-8 exerted its haemagglutination activity on sheep red blood cells. No aggregation was observed between human red blood cells in the presence of LZ-8.

The immunomodulatory activities of Fips were explored by

investigating their effects on systemic anaphylaxis and Arthus reaction in mice. Treatment of mice with Fips efficiently suppressed BSA-induced systemic anaphylaxis. Evidence that Fips suppress antibody production came from the result that the proportion of Arthus reaction-positive mice was reduced to 40% by LZ-8. Co-incubation of mouse spleen cells or human PBL with Fips resulted in enhanced production of cytokines including interleukin (IL)-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) when analysed by ELISA or reverse transcriptase (RT)-PCR [1,3]. In addition, LZ-8 was able to prevent the occurrence of autoimmune diabetes in young female non-obese diabetes mice [4] and to delay the rejection process of transplanted allogeneic pancreatic rat islets and allografted mouse skin [5].

Fip-*five* and LZ-8 consist of 114 and 110 amino acid residues respectively [1,6]. Alignment of the two Fips shows 63.6% identity. In the present study, a new Fip, Fip-*vvo*, was purified from the fruit bodies of *Volvariella volvacea*, and its biochemical characteristics and biological activities were studied. To further characterize this protein, the complete amino acid sequence of Fip-*vvo* was determined and compared with that of other fungal immunomodulatory proteins.

### EXPERIMENTAL

#### Materials

Grass mushroom, *V. volvacea*, was obtained from the local market. DE-52 cellulose and CM-52 cellulose were purchased from Whatman (Maidstone, Kent, U.K.). Ficoll-Paque, Mono S

Abbreviations used: Fip-*vvo*, fungal immunomodulatory protein isolated from *Volvariella volvacea*; PHA, phytohaemagglutinin; IL, interleukin; RT-PCR, reverse transcriptase-PCR; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; MMLV, Moloney murine leukaemia virus; LT, lymphotoxin; IL-2R, IL-2 receptor; PBL, peripheral blood lymphocytes.

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HR 5/5 column and Superose 12 HR 10/30 column were the products of Pharmacia (Uppsala, Sweden). Chymotrypsin was purchased from Sigma (St. Louis, MO, U.S.A.), and trypsin was from Worthington (Freehold, NJ, U.S.A.). Lysyl endopeptidase and thermolysin were obtained from Wako (Osaka, Japan). Moloney murine leukaemia virus (MMLV) reverse transcriptase was purchased from Clontech (Palo Alto, CA, U.S.A.). *Taq* DNA polymerase was purchased from Promega (Madison, WI, U.S.A.). Chemicals for sequence analysis were obtained from Applied Biosystems Inc. (Foster City, CA, U.S.A.). All other chemicals were of analytical grade.

### Purification of Fip-*vvo*

All purification steps were carried out at 4 °C. Fresh fruit bodies of *V. volvacea* (3 kg) were homogenized with 3 litres of 5% (v/v) acetic acid in the presence of 0.1% (v/v) 2-mercaptoethanol. The homogenates were centrifuged at 10000 g for 20 min in a Beckman JA-14 rotor and soluble proteins in the supernatant were precipitated by addition of ammonium sulphate to 95% saturation. After stirring for 1 h, the precipitates were collected by centrifugation at 20000 g in a Beckman JA-20 rotor for 30 min. The pellets were dialysed against 5 litres of 10 mM sodium phosphate buffer, pH 8.2, for 48 h with four changes of dialysis solution. The dialysate was first fractionated on a Whatman DE-52 cellulose column (2.5 cm × 20 cm) which was pre-equilibrated with 10 mM sodium phosphate buffer, pH 8.2. The activity was monitored by blast-formation stimulatory activity and haemagglutination test, and the flow-through fractions were found to be active. The active fractions were pooled and applied to a Whatman CM-52 cellulose column (2.5 cm × 20 cm) that was previously equilibrated with 10 mM sodium acetate, pH 4.8. The column was first washed with 200 ml of equilibrium buffer (10 mM sodium acetate, pH 4.8), then eluted with a linear gradient of NaCl (0–0.25 M over 1 litre) in 10 mM sodium acetate, pH 4.8. The active fractions eluted from the CM-52 cellulose column were combined and further purified on a Mono S HR 5/5 column that was pre-equilibrated with 10 mM sodium acetate, pH 5.2. After washing the column with 7 ml of the same buffer, Fip-*vvo* was eluted with 15 ml of 0–0.075 M NaCl in 10 mM sodium acetate, pH 5.2.

### Haemagglutination assay

Haemagglutination activity was measured as described previously [7]. Fresh blood was obtained from male animals housed in a specific pyrogen-free room. Blood for monitoring haem-

agglutination activity was from Wistar rats. After washing the whole blood with PBS three times, blood cells were collected by centrifugation at 300 g for 10 min and then suspended to 1.5% (v/v) with PBS. Various fractions (0.1 ml) from each purification step and 1.5% blood (0.1 ml) were placed in 96-well U-bottom microtitre plates and incubated at room temperature. The plates were examined for haemagglutination after 2 h.

### SDS/PAGE

Active fractions isolated from each purification step were subjected to SDS/PAGE analysis for detection of the purity. SDS/PAGE (12.5%, w/v) was performed on a Bio-Rad mini protein II gel apparatus according to the method of Laemmli [8]. The gels were visualized by staining with Coomassie Brilliant Blue R-250 or by periodic acid/Schiff technique to detect the carbohydrate content [9].

### Blast-formation stimulatory activity assay

Heparinized human peripheral blood was centrifuged over a Ficoll–Paque isopycnic gradient to isolate lymphocytes. The cells were resuspended in RPMI 1640 medium supplemented with 15% (v/v) fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, 10 mmol/l Hepes, pH 7.3, and 200 mmol/l L-glutamate [10] and the cell number was adjusted to  $5 \times 10^6$  cells/ml. The cell suspension was seeded in a 96-well microtitre plate (0.1 ml,  $5 \times 10^5$  cells/well) and various concentrations of Fip-*vvo*, PHA or Fip-*vvo* and PHA in 0.1 ml of PBS were added to the wells. After incubation of the cells at 37 °C under 5% CO<sub>2</sub> in air for 42 h, 10 µl of [<sup>3</sup>H]thymidine (0.25 µCi, Amersham) was added and the cells were further incubated for 6 h under the same conditions. The cells were harvested with an automated cell harvester onto a glass filter, and the radioactivity of each sample was determined with a Beckman model LS 250 scintillation counter. Each reported value was the mean of triplicate samples.

### RNA extraction and RT-PCR

Three Balb/c mice (5–8 weeks old) were killed by cervical dislocation and the spleens were removed by sterile technique. Spleen cells were isolated by centrifugation over an Isopaque–Ficoll mixture and then resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, 10 mmol/l Hepes, pH 7.3, and 200 mmol/l L-glutamate, at  $5 \times 10^6$  cells/60-mm Petri dish. The cultures were treated with various concentrations of Fip-*vvo* at

**Table 1** Summary of purification of Fip-*vvo*

nd, not determined.

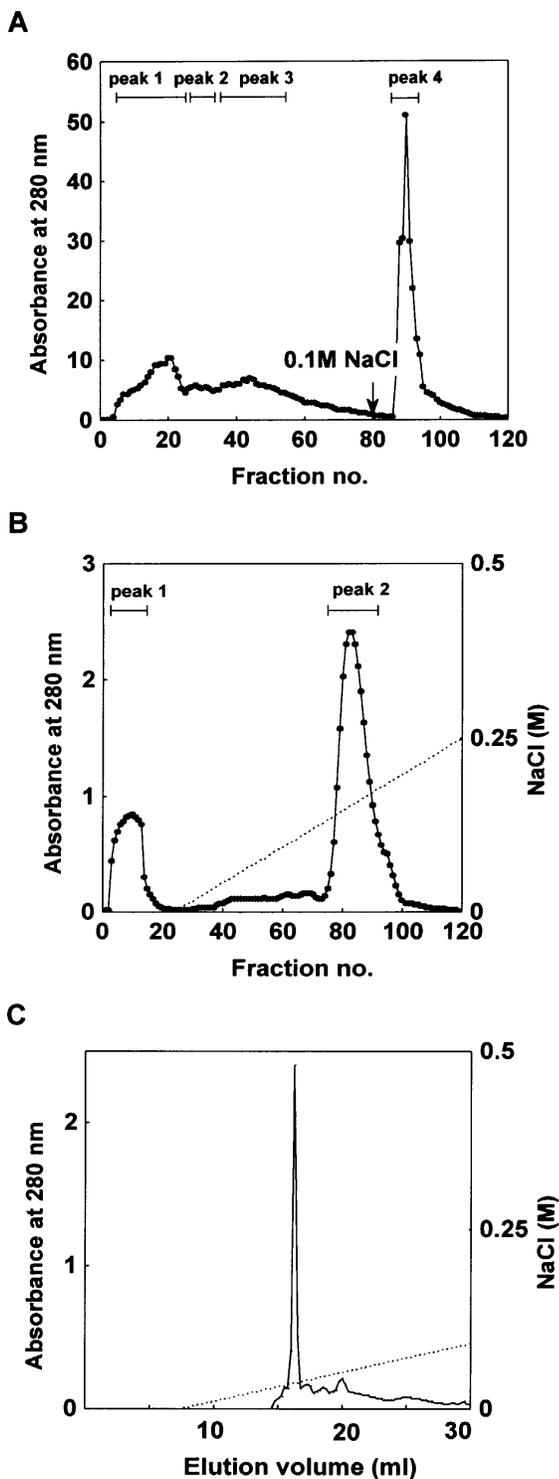
Purification step	Total protein (mg)	Specific activity (units/mg) <sup>a</sup>	Relative specific activity	Total activity (units)	Yield (%)
Ammonium sulphate	7140	nd (nd)	nd (nd)	nd (nd)	nd (nd)
DE-52 cellulose	994	238 (1.26 × 10 <sup>6</sup> ) <sup>b</sup>	1.0 (1.0)	2.36 × 10 <sup>5</sup> (1.25 × 10 <sup>9</sup> ) <sup>c</sup>	100 (100)
CM-52 cellulose	162	1923 (5.25 × 10 <sup>6</sup> )	8.0 (4.2)	3.12 × 10 <sup>5</sup> (8.50 × 10 <sup>8</sup> )	132 (68)
FPLC (Mono S)	115	1923 (5.80 × 10 <sup>6</sup> )	8.0 (4.6)	2.21 × 10 <sup>5</sup> (6.67 × 10 <sup>8</sup> )	94 (53)

<sup>a</sup> Calculated as the inverse of the minimum concentration producing a positive reaction in the haemagglutination assay.

<sup>b,c</sup> Values in parentheses indicate the blast-formation stimulatory activity using human PBL.

<sup>b</sup> c.p.m./mg of protein at the maximum uptake of [<sup>3</sup>H]thymidine.

<sup>c</sup> c.p.m.



**Figure 1** Ion-exchange chromatography purification of Fip-*vvo*

(A) Elution profile of Fip-*vvo* from a DE-52 cellulose column. (B) Elution profile of Fip-*vvo* from a CM-52 cellulose column. (C) Mono S column chromatography of Fip-*vvo*. Conditions are described in the Experimental section. Fractions of 10 ml/tube were collected (A, B) and monitored by absorbance at 280 nm. The active fractions were detected by haemagglutination and blast-formation stimulatory assay.

37 °C under 5% CO<sub>2</sub> in air for 4 h. Total cellular RNAs were isolated by the acid guanidinium/thiocyanate/phenol/chloroform method [11]. Random hexamer-primed cDNAs were

prepared in a 20  $\mu$ l volume from 1  $\mu$ g of total RNA using MMLV reverse transcriptase under conditions recommended by the manufacturer. The primers used to amplify the cDNA fragments of specific cytokines for analysis of gene expression were: IL-1 $\alpha$  forward, 5'-CTCTAGAGCACCATGCTACAGAC-3', IL-1 $\alpha$  reverse 5'-TGGAAATCCAGGGGAAACACTG-3', IL-2 forward 5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAG-3', IL-2 reverse 5'-GACAGAAGGCTATCCATCTCCTCAGAAAGTCC-3', IL-3 forward 5'-GAAGTGGATCCTGAGACAGATACG-3', IL-3 reverse 5'-GACCATGGGCCATGAGGAACATTC-3', IL-4 forward 5'-ATGGGTCTCAACCC-CAGCTAGT-3', IL-4 reverse 5'-GCTCTTTAGGCTTTCCAGGAAGTC-3', IL-5 forward 5'-ATGACTGTGCCTCTGTGCC-TGGAGC-3', IL-5 reverse 5'-CTGTTTTTCTGGAGTAACTGGGG-3', IL-6 forward 5'-TGGAGTCACAGAAGGAG-TGGCTAAG-3', IL-6 reverse 5'-TCTGACCACAGTGAGGATGTCCAC-3', IFN- $\gamma$  forward 5'-TGAACGCTACACACTGCATCTTGG-3', IFN- $\gamma$  reverse 5'-CGACTCCTTTCCGTT-CCTGAG-3', TNF- $\alpha$  forward 5'-ATGAGCACAGAAAGCATGATCCGC-3', TNF- $\alpha$  reverse 5'-CCAAAGTAGACCTGCCCGACTC-3', lymphotoxin (LT) forward 5'-TGGCTGGGAACAGGGGAAGGTTGAC-3', LT reverse 5'-CGTGCTTTCT-TCTAGAACCCTTGG-3', IL-2 receptor (IL-2R) forward 5'-ACTGTGAATGCAAGAGAGGTTTCCG-3', IL-2R reverse 5'-AGCAGGACCTCTCTGTAGAGCCTTG-3' [12–21]. For internal control,  $\beta$ -actin was employed by using the primers: forward 5'-GTGGGCCGCTCTAGGCACCAA-3', and reverse 5'-CTCTTTGATGTCACGCACGATTTTC-3' [22]. PCR was carried out in a 50  $\mu$ l mixture (20 mM Tris/HCl, pH 8.3, 2.5 mM MgCl<sub>2</sub>, 25 mM KCl, 0.05% Tween 20, 100  $\mu$ g/ml nuclease-free BSA, 200  $\mu$ M each dNTP, 1  $\mu$ l of first-strand cDNA template, 10 pmol of each primer, and 2 units of *Taq* DNA polymerase), and 30 cycles of exponential amplification were performed. The cycling parameters were as follows: 94 °C for 1 min (5 min before the first cycle), 60 °C for 1 min (65 °C for IL-2 primers), 72 °C for 2 min, with an additional 7 min final extension at 72 °C. The amplified products were analysed by 1.5% (w/v) agarose gel electrophoresis.

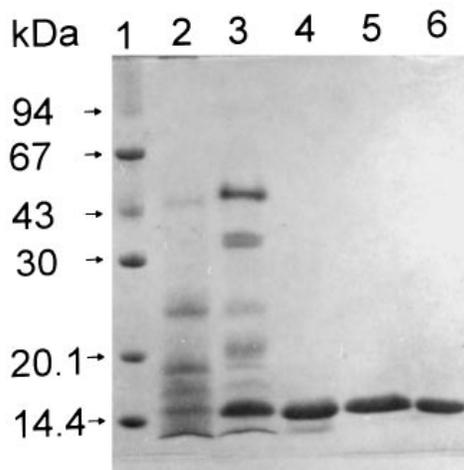
### Immunomodulatory activity assay

#### Systemic anaphylaxis reaction

Male Balb/c mice (5 weeks old, body weight 17–20 g) were sensitized by intraperitoneal or subcutaneous injection with 1 mg of BSA in 0.2 ml of aluminium hydroxide suspension (15 mg/ml) at day 0 and were shocked by intravenous injection with 1 mg of BSA in 0.2 ml of PBS at day 17. For negative control, 1 mg of ovalbumin in 0.2 ml of PBS was injected instead of BSA at the time of the shocking injection. To examine the immunomodulatory activity of Fip-*vvo*, mice in the experimental group were intraperitoneally injected with 7.5 mg of Fip-*vvo*/kg body weight twice a week for a total of seven times including two injections before BSA sensitization. In experiment 2, mice in one experimental group received the shocking injection combined with 150  $\mu$ g of Fip-*vvo* and 1 mg of BSA in 0.2 ml of PBS at day 17. The systemic anaphylaxis reaction was observed within 30 min after the shocking injection and rated in the following way: positive reaction, mouse died or rendered stationary for at least 1 min; negative reaction, no changes were observed and movement remained normal.

#### Arthus reaction

Twenty male Balb/c mice (body weight 17–20 g) were sensitized with 1 mg of BSA in 0.2 ml of aluminium hydroxide suspension



**Figure 2** SDS/PAGE analysis of Fip-*vvo*

Fip-*vvo* purified by ion-exchange chromatography was analysed by SDS/12.5% PAGE. Lane 1, molecular mass markers: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactoalbumin (14.4 kDa); lane 2, crude extract; lane 3, active fractions from DE-52 cellulose column chromatography; lane 4, active fractions from CM-52 cellulose column chromatography; lane 5, Fip-*vvo*; lane 6, Fip-*vvo* reduced with 2-mercaptoethanol. The gel was stained with Coomassie Brilliant Blue R-250.

(15 mg/ml) at day 0 and day 7 subcutaneously. Ten mice were treated with Fip-*vvo* (7.5 mg/kg body weight; intraperitoneally) were administered seven times, at days -6, -3, 0, 3, 6, 9 and 12. At day 14, 20  $\mu$ l of BSA (0.5 mg/ml in PBS) was injected intradermally into the left footpad of each mouse and 20  $\mu$ l of PBS was injected into each right footpad as a control. The thicknesses of footpads were measured at least six times with a digital-type measure at 2 h, 1 day, 2 days and 3 days after injection of BSA and PBS. After discarding the extreme values, the difference between left and right footpads was calculated by a statistical method. The mice with positive reaction were defined as those differing at  $P < 0.05$ .

#### Amino acid analysis

Protein concentration was determined by the bicinchoninic acid method [23]. Samples for amino acid analysis were hydrolysed with 6 M HCl for 24, 48 and 72 h at 110 °C. After removal of HCl, the hydrolysate was dissolved in Beckman Na-S buffer, pH 2.0, and applied to an automatic Beckman 6300 system amino acid analyser. Values for threonine and serine were corrected for hydrolysis loss by extrapolating to zero time relative to alanine. Tryptophan content was measured from spectroscopic absorbance at 280 and 288 nm in 6 M guanidine/HCl by the method of Edelhoch [24].

#### Enzymic cleavage of Fip-*vvo*

For proteolytic peptide mapping, approx. 500  $\mu$ g of Fip-*vvo* was dried in a Speed-Vac and digested with trypsin, lysyl-C endopeptidase, chymotrypsin and thermolysin. The enzyme/substrate ratio was 1:50. Tryptic, chymotryptic or thermolytic digestion was carried out in 50 mM ammonium bicarbonate, pH 8.0, at 37 °C for 18 h, and lysyl-C endopeptidase digestion was performed in 50 mM Tris/HCl, pH 9.0, at 37 °C for 18 h [25].

**Table 2** Amino acid composition of Fip-*vvo*

Thr and Ser values were corrected for hydrolysis loss by extrapolating to zero time hydrolysis. Half-Cys determined from cysteic acid in performic acid-oxidized protein. Trp value was determined from spectroscopic absorbance at 280 and 288 nm in 6 M guanidine/HCl. Fip-*vvo* data are normalized to 112 total residues (parentheses).

Amino acid	Fip- <i>vvo</i> (residues/molecule)
Asx	16.4 (16)
Thr	7.8 (8)
Ser	6.7 (7)
Glx	10.2 (10)
Pro	5.2 (5)
Gly	8.2 (8)
Ala	6.1 (6)
Half-Cys	0.0 (0)
Val	11.1 (11)
Met	0.0 (0)
Ile	5.9 (6)
Leu	9.1 (9)
Tyr	9.7 (10)
Phe	3.8 (4)
His	0.0 (0)
Lys	6.9 (7)
Arg	2.7 (3)
Trp	1.9 (2)
Total residues	112

#### Fractionation of peptides and sequence analysis

The peptides generated by various enzymic digestions were fractionated by reversed-phase HPLC on a Beckman C-18 column (4.6 mm  $\times$  10 cm) or Vydac C-18 column (4.6 mm  $\times$  25 cm) using a linear gradient of 10–65% (v/v) acetonitrile containing 0.07% (v/v) trifluoroacetic acid over 30 min at a flow rate of 1 ml/min. Some peaks were further purified on a Beckman C-8 column (4.6 mm  $\times$  25 cm). The peptides were collected by monitoring at 215 nm and dried under vacuum, then the purified peptides were subjected to amino acid sequence analysis using an Applied Biosystems 470A protein sequencer.

## RESULTS AND DISCUSSION

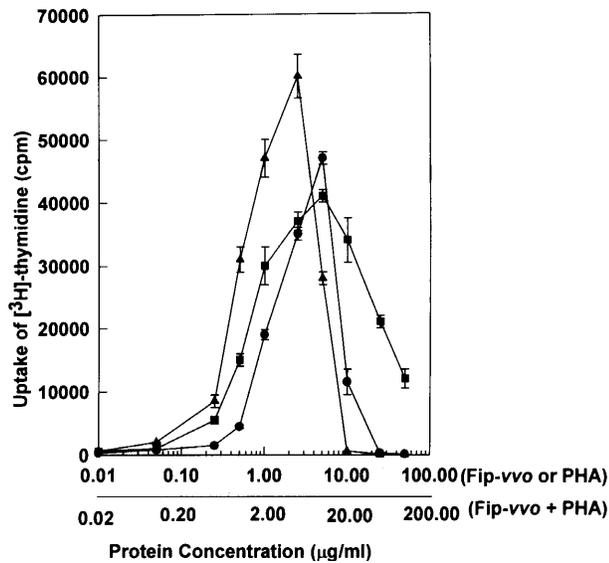
#### Purification of Fip-*vvo*

##### Assay of Fip-*vvo*

The assay procedure for Fip-*vvo* was described in the Experimental section and used in the chromatographic purification of the protein. The assay was not applicable to crude preparations of *V. voluacea* such as the ammonium sulphate fraction (Table 1), which contained substances causing cell lysis in the haemagglutination and the blast-formation stimulatory assay.

##### Purification of Fip-*vvo*

The purification procedure for Fip-*vvo* consisted of ammonium sulphate precipitation, column chromatography on DE-52 cellulose, CM-52 cellulose, and Mono S. Elution profiles of proteins are shown in Figure 1. DE-52 cellulose column chromatography gave four elution peaks absorbing at 280 nm. The first three peaks were eluted by 10 mM sodium phosphate buffer, pH 8.2, and the fourth peak was eluted by 0.1 M NaCl in the same buffer. Highest specific activity was observed in peak 1 by haemagglut-



**Figure 3** Stimulatory effects of Fip-*vvo* on proliferation of human PBL

Human PBL were cultured for 42 h with various indicated doses of Fip-*vvo* (●), PHA (■) and Fip-*vvo* + PHA (▲) and pulsed with tritiated thymidine for the last 6 h as indicated in the Experimental section. Each point, expressed in c.p.m./well, is the mean of triplicate cultures.

**Table 3** Effects of Fip-*vvo* on the production of active systemic anaphylaxis in mice

OA, ovalbumin; s.c., subcutaneous; i.p., intraperitoneal; i.v., intravenous. S/T is the number with anaphylactic symptoms/total number of mice. D/T is the number of anaphylactic deaths/total number of mice.

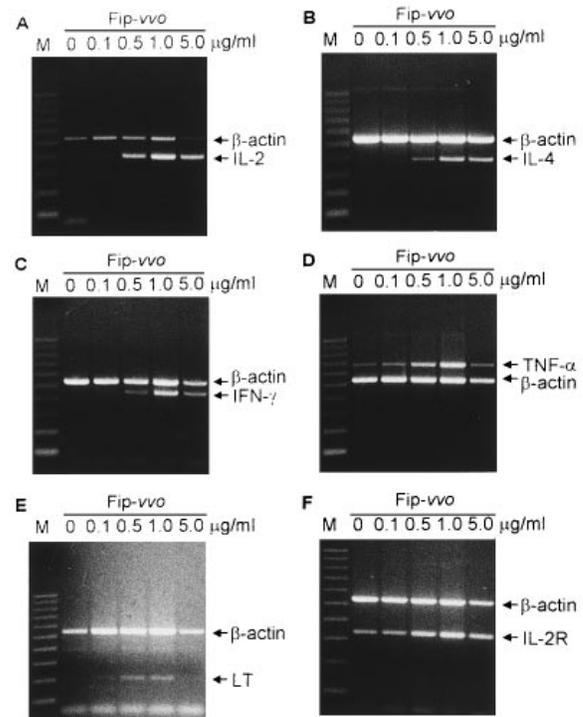
Experiment no.	Sensitizing injection	Shocking injection	Fip- <i>vvo</i> treatment	Result	
				S/T	D/T
1	BSA (s.c.)	BSA (i.v.)	—	10/10	1/10
	BSA (s.c.)	OA (i.v.)	—	0/10	0/10
	BSA (s.c.)	BSA (i.v.)	+	7/10	1/10
2	BSA (i.p.)	BSA (i.v.)	—	10/10	2/10
	BSA (i.p.)	BSA + Fip- <i>vvo</i> (i.v.)	—	9/10	0/10
	BSA (i.p.)	BSA (i.v.)	+	6/10	1/10

ination and blast-formation stimulatory activity assay. The active fractions were collected for further purification by CM-52 cellulose column chromatography and two major absorption peaks were obtained. The second peak eluted with a linear gradient of NaCl (0–0.25 M in 10 mM sodium acetate, pH 4.8) exhibited the haemagglutination and mitogenic activities, and showed minor contamination of other proteins by SDS/PAGE analysis. Using Mono-S cation exchange FPLC, Fip-*vvo* was eluted with a linear gradient of NaCl (0–0.075 M) in a total volume of 15 ml of 10 mM sodium acetate, pH 5.2. After three chromatographic steps, 115 mg of Fip-*vvo* was normally obtained from 3 kg of fresh fruit bodies. As the specific activity could not be quantified in the ammonium sulphate fraction, the yield of Fip-*vvo* in the first step could not be calculated (Table 1). In the haemagglutination assay using rat red blood cells, the fraction purified from CM-52 cellulose column chromatography showed higher total activity than that isolated from DE-52 column

**Table 4** Inhibitory effects of Fip-*vvo* on Arthus reaction in mice

Data are given as the percentage of mice with positive reaction at the indicated times after footpad injection.

Treatment	Positive reaction (%)			
	2 h	1 day	2 day	3 day
PBS	100	10	0	0
Fip- <i>vvo</i>	40	0	0	0



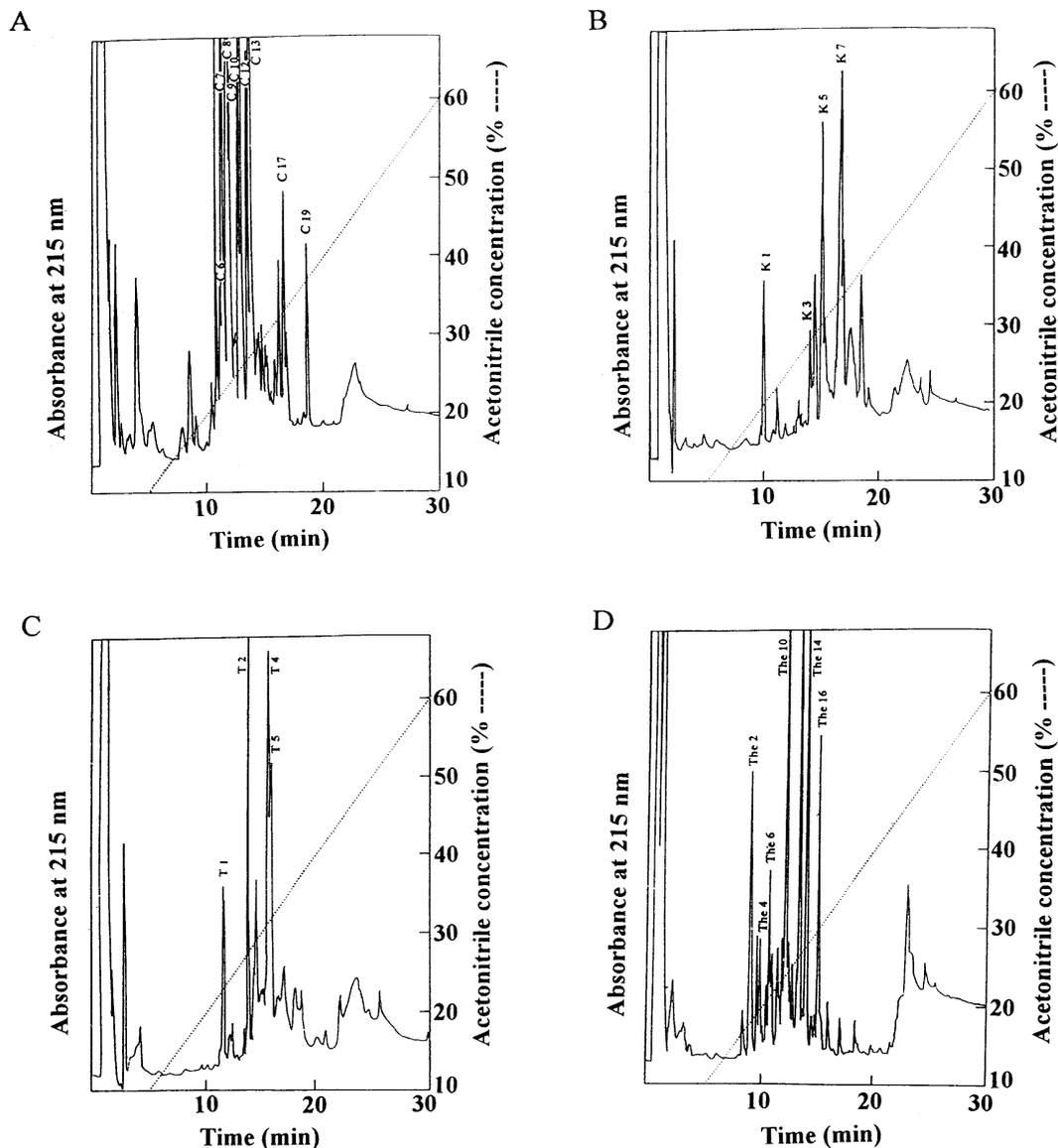
**Figure 4** Effects of Fip-*vvo* on the expression of (A) IL-2, (B) IL-4, (C) IFN- $\gamma$ , (D) TNF- $\alpha$ , (E) LT and (F) IL-2R genes in mouse spleen lymphocytes

Gene expression was analysed by RT-PCR. The equivalent of 1  $\mu$ g of total RNA extracted from the mouse spleen cells incubated with various concentrations of Fip-*vvo* was reverse transcribed and amplified under the conditions specified. The amplified fragments and  $\beta$ -actin-specific fragments are shown as visualized by ethidium bromide staining. M, bp DNA (100–1000 bp).

chromatography. However, the assay measuring blast-formation stimulatory activity of human PBL gave a purification of 4.2-fold after CM-52 ion-exchange chromatography and a final recovery of 53%. From these results it was suggested that the DE-52 column chromatography purified fraction contained substances that were capable of inhibiting the haemagglutination activity.

#### Homogeneity of Fip-*vvo*

Following each purification step, SDS/PAGE was used to examine the homogeneity of Fip-*vvo*. Purified Fip-*vvo* migrated as a single band to the position corresponding to that of the 15-kDa polypeptide (Figure 2). By staining the polyacrylamide gel with periodic acid/Schiff reagent, Fip-*vvo* showed negative



**Figure 5** Reversed-phase HPLC of (A) chymotrypsin digest of Fip-*vvo*, (B) lysyl endopeptidase digest of Fip-*vvo*, (C) trypsin digest of Fip-*vvo*, (D) thermolysin digest of Fip-*vvo* on a Beckman C-18 column

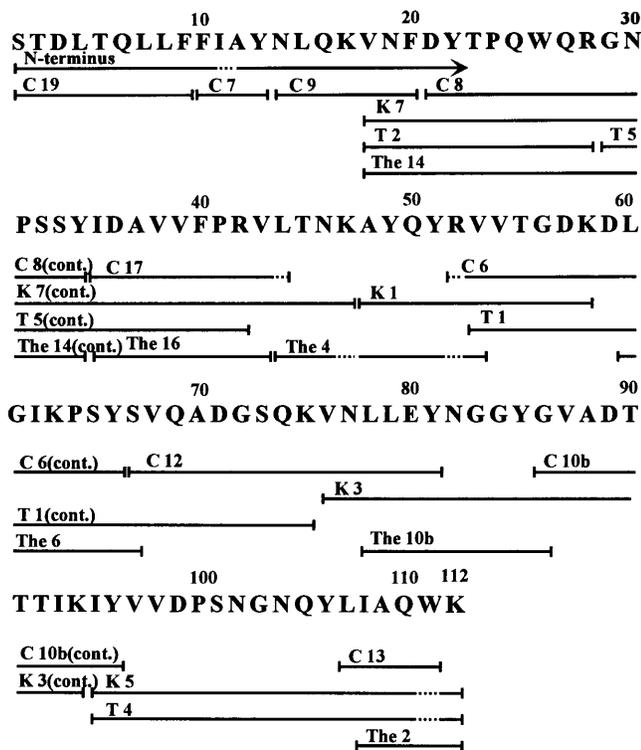
The details are described in the Experimental section.

staining. These observations indicated that Fip-*vvo* is a single protein with very low or no carbohydrate content. The amino acid composition of Fip-*vvo* is presented in Table 2. Half-cysteine, methionine, histidine and hydroxyproline were not detected. The results of amino acid analysis reveal that Fip-*vvo* comprises large amounts of asparagine or aspartic acid. In addition, Fip-*vvo* has a higher number of lysine residues than of arginine residues, which is one of the properties of Fips.

#### Blast-formation stimulatory activity assay

Preliminary experiments were performed on human PBL using different doses of Fip-*vvo*. At 1–5  $\mu\text{g}/\text{ml}$ , weak thymidine uptake was elicited by Fip-*vvo* after 24 h incubation; however, a substantial response was observed at 48 h. Thus for the blast-

formation stimulatory activity assay, a 48-h incubation period was used. As shown in Figure 3, Fip-*vvo* induced the proliferation of human PBL in a dose-dependent manner. The concentration of Fip-*vvo* giving the optimal thymidine uptake was 5  $\mu\text{g}/\text{ml}$ , which was similar to the optimal concentration of PHA. When Fip-*vvo* and PHA were incubated together, the maximum uptake of [ $^3\text{H}$ ]thymidine was shifted to 5  $\mu\text{g}/\text{ml}$  total mitogens or 2.5  $\mu\text{g}/\text{ml}$  each. Analysis of the proliferation curve showed that Fip-*vvo* and PHA act synergistically on human PBL. It is well known that PHA is a polyclonal activator of T-cells. The synergistic effect on uptake of [ $^3\text{H}$ ]thymidine induced by Fip-*vvo* and PHA implies that the target cells of Fip-*vvo* may be similar to those of PHA. Incubation of isolated non-B- and non-T-cells with Fip-*vvo* showed that Fip-*vvo* merely activates the proliferation of non-B-cells. The proliferative response induced by Fip-*vvo* was similar to that induced by LZ-8 and Fip-*five* reported



**Figure 6** Complete amino acid sequence of Fip-*vvo*

Solid lines and arrows indicate the amino acid residues determined by Edman degradation and the numbering of the peptides corresponds to the numbering of the proteolytic peptides shown in Figure 5. Dotted lines represent amino acid residues established by amino acid composition analysis. 'C', 'K', 'T' and 'The' are the designations of chymotryptic, lysyl endopeptidase, tryptic and thermolysin peptides respectively.

previously; however, Fip-*vvo* was 20-fold more potent than Fip-*ve* on lymphocyte activation.

The effect of carbohydrates on the blast-formation stimulatory activity of Fip-*vvo* was also examined. In the presence of 0–50 mM galactose, glucose, mannose,  $\alpha$ -methyl-D-mannoside, fucose, fructose, *N*-acetylglucosamine, *N*-acetylgalactosamine, 1-*O*-methyl  $\alpha$ -D-glucopyranoside, lactose or sucrose, no inhibition of lymphocyte proliferation was observed at 5  $\mu$ g/ml Fip-*vvo*.

### Hemagglutination reaction

Using red blood cells from various species, the haemagglutination activity of Fip-*vvo* was examined. Fip-*vvo* was able to agglutinate red blood cells of Wistar rats at concentrations greater than 0.52  $\mu$ g/ml. With red blood cells from Balb/c mice and rabbits, the minimal concentration of Fip-*vvo* required for haemagglutination was 1.1  $\mu$ g/ml and 0.13  $\mu$ g/ml respectively. The aggregation of human red blood cells of all four types (A, B, AB and O) in the presence of Fip-*vvo* was not seen when the concentration was raised to 175  $\mu$ g/ml. This result was consistent with the low haemagglutination activity of LZ-8 towards human red blood cells, whereas Fip-*ve* aggregates human red blood cells efficiently.

The sugar specificity of Fip-*vvo* was determined with a series of sugars (galactose, glucose, mannose,  $\alpha$ -methyl D-mannoside, fructose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosamine, galactosamine, 1-*O*-methyl  $\alpha$ -D-glucopyranoside, 1-*O*-methyl  $\alpha$ -D-galactopyranoside, *N*-acetylneuraminic acid, lactose and sucrose) all at a final concentration of 100 mM

(5 mM for *N*-acetylneuraminic acid). None of the sugars studied was found to have inhibitory effects on the haemagglutination activity using rat red blood cells. Goldstein et al. [26] have defined a lectin as a sugar-binding protein or glycoprotein that agglutinates cells and/or precipitates glycoconjugates. The specificity of a lectin is usually defined in terms of the monosaccharides or oligosaccharides that inhibit lectin-induced agglutination reactions. From the haemagglutination and cell proliferation assays, Fip-*vvo* exhibited the major biological activities of lectins. However, the sugar specificity of Fip-*vvo* could not be determined by using mono- and disaccharides in these experiments. It is known that the monosaccharide residue in a terminal non-reducing position on a glycan is not the only carbohydrate moiety recognized by a lectin [27]. Fip-*vvo* may specifically recognize a more complex oligosaccharide chain on a glycan instead of the monosaccharide at the terminus.

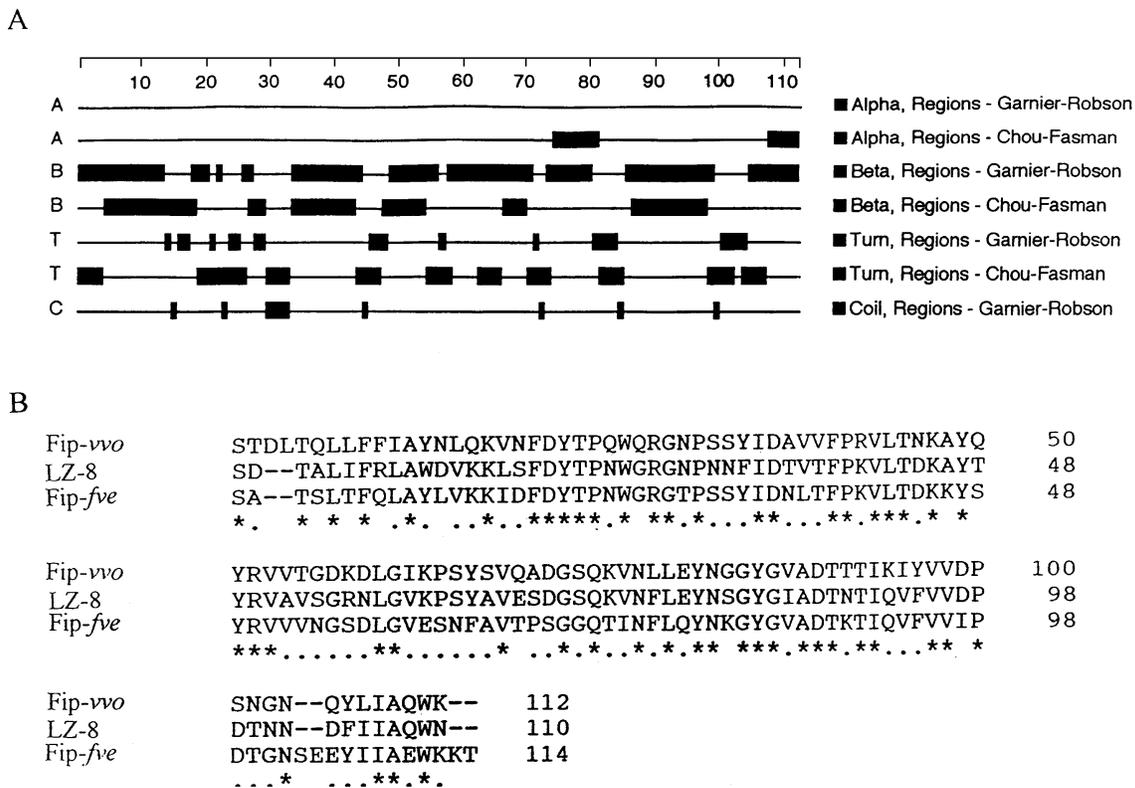
### Immunomodulatory activity assay

The immunomodulatory activity of Fip-*vvo* was explored by examining its effects on systemic anaphylaxis and Arthus reaction in Balb/c mice. Table 3 shows the effects of Fip-*vvo* on the production of systemic anaphylaxis. All the mice in the positive control group sensitized with BSA in aluminium adjuvant subcutaneously (Experiment 1) or intraperitoneally (Experiment 2) at day 0, then shocked with BSA in PBS at day 17, displayed symptoms of systemic anaphylactic shock. As a negative control, BSA in the shocking injection was replaced with ovalbumin and the mice showed no anaphylactic reaction. After administration of Fip-*vvo* seven times, including two injections before BSA sensitization, there were fewer mice with systemic anaphylaxis than in the positive control group both in Experiment 1 (7/10) and in Experiment 2 (6/10). However, when compared with other Fips (Fip-*ve* and LZ-8), Fip-*vvo* exhibited less potency in the prevention of systemic anaphylaxis. The immediate effect of Fip-*vvo* on anaphylactic shock was not seen when 150  $\mu$ g of Fip-*vvo* was injected in combination with BSA in the shocking injection (Experiment 2).

The effect of Fip-*vvo* on Arthus reaction is shown in Table 4. All the mice in the control group displayed positive footpad reactions within 2 h. However, only 40% displayed positive footpad reactions to BSA for mice administered with Fip-*vvo* twice a week. Statistical analysis showed that the comparison of two proportions of positive reactions was significant ( $P < 0.05$ ). The footpad thicknesses were also measured at 2 and 3 days; no difference was observed between the control and the Fip-*vvo*-treated groups. This ensured that the swelling of the footpad was not caused by a cell-mediated delayed-type reaction.

The Arthus reaction is a dermal inflammatory response caused by the reaction of precipitating antibody with antigen placed in the skin. The deposition of immune complex results in activation of complement and subsequent neutrophil infiltration. Despite it not being clear how Fip-*vvo* exerts its effect on reduction of Arthus reaction, the immunomodulatory action of Fip-*vvo* is evident from this study.

The immunomodulatory activities of Fip-*vvo* *in vitro* were investigated by examining the induction of cytokine gene expression. Mouse spleen cells were cultured with various concentrations of Fip-*vvo* for 4 h and the total RNAs were prepared for subsequent RT-PCR analysis. After converting the RNAs to single strand cDNA, the transcripts of the genes encoding IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, TNF- $\alpha$ , IFN- $\gamma$ , LT and IL-2R were analysed by PCR using specific primers described in the Experimental section. As shown in Figure 4, Fip-*vvo* markedly enhanced the expression of IL-2, IL-4, TNF- $\alpha$ , IFN- $\gamma$ , LT and



**Figure 7** (A) Predictions of secondary structure using the methods of Garnier et al. and Chou and Fasman and (B) alignment of amino acid sequence of Fip-*vvo* with sequences of Fip-*fve* [2] and LZ-8 [6]

Amino acid identities are marked by asterisks, while similarities are represented by dots.

IL-2R, but not IL-1, IL-3, IL-5 or IL-6, in a dose-dependent fashion. It is clear that cytokines are made by different cells in response to their activation. Mouse T helper (Th1) cells produce IL-2, IFN- $\gamma$  and LT, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Some other proteins including IL-3 and TNF- $\alpha$  are secreted both by Th1 and Th2 cells. The characteristic cytokines of Th1 and Th2 cells are often mutually exclusively expressed [28]. Fip-*vvo* induced most Th1-specific cytokines (IL-2, IFN- $\gamma$  and LT) and one Th2-specific cytokine (IL-4) within 4 h in mouse spleen cells. This result indicates that Fip-*vvo* principally acts on Th1 (or type 1) cells and to a lesser extent on Th2 (or type 2) cells in the early event of activation. Compared with Fip-*fve*, Fip-*vvo* induced the expression of IL-4 in addition to the expression of IFN- $\gamma$  and IL-2. It is known that IL-4 acts on B-cells to induce activation and differentiation, leading in particular to the production of IgE. The lower effect of Fip-*vvo* on the prevention of systemic anaphylaxis may be attributed to the elevated expression of IL-4.

#### Complete amino acid sequence of Fip-*vvo*

The complete primary structure of Fip-*vvo* was determined by sequencing the peptides generated by chymotrypsin, lysyl endopeptidase, trypsin and thermolysin digestion. After enzymic cleavage the resulting peptides were purified by reversed-phase HPLC and subsequently analysed by automated protein sequencing (Figure 5). C10 (Figure 5A) and The10 (Figure 5D) were further purified on a Beckman C-8 column. The N-terminal

end of native Fip-*vvo* was blocked. By treating native Fip-*vvo* or N-terminal peptide (C-19) with 100% trifluoroacetic acid at 70 °C for 30 min, the N-terminal blocking group was removed and the deblocked products were then subjected to amino acid sequencing. Figure 6 shows the complete amino acid sequence of Fip-*vvo* together with sequencing strategy. This protein is composed of 112 amino acid residues and its calculated molecular mass is 12667 Da not including the N-terminal blocking residue. To identify the N-terminal blocking acyl group, the molecular mass of native Fip-*vvo* was measured by matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry in a Bruker Biflex system and a molecule mass  $m/z$  12711 was obtained. This mass is 44 Da greater than the theoretical mass calculated from the sequence data. It is most likely that the N-terminal blocking group is an acetyl group with a molecular mass of 43 Da. By gel filtration FPLC on a Superose 12 column, Fip-*vvo* exhibited a molecular mass of 26 kDa for the native molecules in PBS. This result indicates that native Fip-*vvo* is most likely a non-covalently associated homodimeric protein that functions divalently (or multivalently) in cell aggregation and activation.

Analysis of the primary structure of Fip-*vvo* reveals that there is no consensus sequence (Asn-Xaa-Ser or Thr) as an attachment site for an Asn-linked oligosaccharide chain, which is compatible with the negative results of periodic acid/Schiff staining of SDS/PAGE. In addition, Fip-*vvo* contains one potential casein kinase II phosphorylation site at Ser<sup>33</sup>-Tyr-Ile-Asp and three potential protein kinase C phosphorylation sites at Thr<sup>45</sup>-Asn-Lys, Ser<sup>73</sup>-Gln-Lys and Thr<sup>92</sup>-Ile-Lys [29,30]. The involvement of

Fip-*vvo* in signal transduction leading to T-cell activation needs to be further investigated.

The circular dichroism spectrum of Fip-*vvo* shows that there is a very low content of  $\alpha$ -helical structure (results not shown). This result is consistent with the secondary structure predictions using the methods of Garnier et al. and Chou and Fasman [31,32] (Figure 7A). Both methods predict that Fip-*vvo* is constructed from  $\beta$ -sheet and turn conformation. The alignment of Fip-*vvo*, LZ-8 and Fip-*five* is shown in Figure 7(B). Amino acid sequences of three Fips show a considerable similarity: there are 60 invariant amino acid residues (53.6%) between Fip-*vvo* and Fip-*five*, 65 invariant amino acid residues (59.1%) between Fip-*vvo* and LZ-8, and 70 invariant amino acid residues (63.6%) between Fip-*five* and LZ-8. Among the three Fips, there are 51 invariant amino acid residues (44%). The phylogenetic tree constructed from sequence similarity indicates that Fip-*five* and LZ-8 are more closely related to each other than to Fip-*vvo*; nevertheless the biological activities of Fip-*vvo* are more similar to LZ-8 than to Fip-*five*. The differences of immunomodulatory activities and sequence similarities among the three Fips provide valuable clues for further investigation into the relationships between the structures and functions of Fips.

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