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Preparation and Characterization of Human Recombinant Protein 1/Clara Cell M_r 10 000 Protein

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Summary: Protein 1, which is identical to human Clara cell M_r 10^4 protein, is a homodimeric, low molecular mass protein (M_r 14 000) and an effective inhibitor of phospholipase A_2 activity. We have expressed this protein in *E. coli* and characterized its physiochemical and biological properties. Using a pET expression system, about 1.7 mg of purified recombinant protein 1 was obtained from 250 ml of *E. coli* culture. The amino-terminal sequence of recombinant protein 1 up to the 20th residue was identical to that of native protein 1 except for an extra methionine at the amino-terminus. On reversed-phase HPLC, recombinant protein 1 eluted at the same retention time as native protein 1. The dose-response curves of recombinant protein 1 and native protein 1 in an enzyme-linked immunosorbent assay for protein 1 were identical. Recombinant protein 1 inhibited both porcine pancreas and cobra venom phospholipase A_2 activities. These results indicated that recombinant protein 1 is structurally and biologically identical to native protein 1. We found that recombinant protein 1 also inhibits phosphatidylinositol-specific phospholipase C activity.

Introduction

Human Protein 1, also called Urine Protein 1, is a protein with a relative molecular mass of 14 000 on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It consists of two identical subunits of 70 amino acids joined in anti-parallel fashion by two disulphide bridges (1). We purified this protein from the urine of patients with renal failure and found that it is identical to human Clara cell M_r 10^4 protein (hCC10) (1); it is an effective inhibitor of phospholipase A_2[1]) activity (2) and has been immunohistochemically detected in human endometrium (3) and bronchoalveolar epithelium (4). Protein 1 is also present in prostate epithelium (5) and seminal vesicles (6), which accounts for the higher protein 1 levels in male urine (7, 8). Since the structure, biological functions and tissue distribution of protein 1 are similar to those of rabbit uteroglobin, protein 1 is considered to be the human counterpart of rabbit uteroglobin (9). Besides the inhibition of phospholipase A_2 activity (10), a variety of biological functions have been proposed for rabbit uteroglobin, such as the inhibition of chemotaxis and phagocytosis of neutrophils and monocytes (11), suppression of the maternal immunological reactions against the foetus (12) and masking of male gamete immunogenicity (13). To understand the biological role of this protein, a large amount of homogeneous material is required.

In this study, we describe an efficient production of recombinant protein 1 using the pET expression system. The expressed recombinant protein 1 retained its native structural and biological properties, including inhibitory activity towards phospholipase A_2. Furthermore, our study showed that recombinant protein 1 also inhibited phosphatidylinositol-specific phospholipase C[1]) activity.

Materials and Methods

Plasmid construction

A cDNA coding mature protein 1 was amplified by the polymerase chain reaction (PCR) from cloned protein 1 cDNA in λgt11 (14) using the primers: 5'-CTCCGCTCATATGGAGATCTGCCC-GAGC-3' and 5'-GCTTCGGATCCCTAATTACACAGTGAGC-3'. Amplification with these primers generated a cDNA having *NDe* I and *Bam* HI restriction sites at the 5' and 3' ends, respectively, which facilitated cloning into the pET21a(+) expression vector (Novagen, Madison, WI, USA). PCR was performed with Gene-Amp PCR reagents (Perkin-Elmer Cetus, Norwalk, CT, USA). Thirty cycles were performed. Each consisted of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min and elongation at 72 °C. Amplified mature protein 1 cDNA and the vector pET21a(+) were digested with *Nde* I and *Bam* HI, ligated and transformed into *E. coli* strain BL21(DE3)pLysS competent cells (Novagen) according to the supplier's protocol. The presence of a protein 1 insert in recombinant clones was confirmed by PCR. Direct sequencing was performed using a DNA sequencing kit (Bio-

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[1]) Enzymes:
Phospholipase A_2, EC 3.1.1.4
Phosphatidylinositol-specific phospholipase C, EC 3.1.4.10.

tinylated terminator Sequencing high -Plus-, Toyobo, Osaka, Japan).

Expression in *E. coli*

Clones were grown for 3 hours at 37 °C in 5 ml of Luria-Bertani medium containing 50 mg/l of ampicillin (resistance conferred by pET21a(+)) and 34 mg/l of chloramphenicol (resistance conferred by pLys). Cells were then collected by centrifugation (3000 *g*, 5 min, 4 °C), resuspended in 250 ml of fresh Luria-Bertani medium containing the same dose of antibiotics and cultured at 37 °C to an absorbance at 650 nm of 1.2. Protein synthesis was initiated by adding 1 mmol/l isopropyl-β-*D*-thiogalactopyranoside. After 30 min, 200 mg/l rifampicin were added to inhibit *E. coli* RNA polymerase. The incubation was continued for 90 min to allow the preferential expression of protein 1 via the rifampicin-resistant T7 RNA polymerase which is produced by BL21(DE3)pLysS *E. coli* in the presence of isopropyl-β-*D*-thiogalactopyranoside.

Purification of recombinant protein 1

Cells expressing recombinant protein 1 were collected by centrifugation (3000, 10 min, 4 °C) and stored at −20 °C until use. Thawed cells were resuspended in 50 ml of 30 mmol/l Tris-HCl (pH 8.0) containing 30 mmol/l NaCl, sonicated for 5 min on ice at 50 W using an ultrasonic disruptor (Tomy, Tokyo, Japan) and centrifuged (20000 *g*, 30 min, 4 °C). The supernatant was applied to an anti-protein 1 monoclonal antibody-conjugated Sepharose 4B (Pharmacia, Uppsala, Sweden) column (2.5 cm × 15 cm I.D.). Bound fractions rich in recombinant protein 1 were eluted with 0.2 mol/l glycine-HCl (pH 2.5), then purified by high performance liquid chromatography (HPLC) using a reversed-phase column (μBondasphere 5 μm C4-300A, 150 mm × 3.9 mm I.D., Waters, Milford, MA, USA). Immunoaffinity chromatography and HPLC were performed as described (1). Amino-terminal sequences of purified recombinant protein 1 were determined with a gas-phase amino acid sequencer model PPSQ-10 (Shimazu, Kyoto, Japan) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay for protein 1

The immunochemical behaviour of recombinant protein 1 was investigated by means of a sandwich-type enzyme-linked immunosorbent assay (ELISA) using an anti-protein 1 monoclonal antibody (1) and a rabbit anti-protein 1 polyclonal antibody (DAKO, Copenhagen, Denmark) as described (1, 15).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting analysis

SDS-PAGE was performed according to *Schagger & von Jagow* (16). Proteins separated by SDS-PAGE were electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), then stained using the anti-protein 1 monoclonal antibody as described (1).

Assay of phospholipase A_2 activity

To examine the effect of recombinant protein 1 on phospholipase A_2 activity, phospholipase A_2 was preincubated at 37 °C for 5 min with serial dilutions of recombinant protein 1 or vehicle. Phospholipase A_2 activity was then analysed according to *Natori* et al. (17) with a slight modification. Briefly, the assay mixture (100 μl) contained 100 mmol/l Tris-HCl (pH 9.0), 4 mmol/l $CaCl_2$, 1.1 g/l fatty acid-free bovine serum albumin, 2 μmol/l 1-stearoyl, 2-[1-^{14}C]arachidonyl phopshatidylcholine (1.85−2.2 GBq/mmol, Amersham, Buckinghamshire, UK), 0−2 μmol/l recombinant protein 1 and 200 μmol/l porcine pancreas phospholipase A_2 (Boehringer Mannheim, Indianapolis, IN, USA) or 100 pmol/l cobra (*Naja naja*) venom phopsholipase A_2 (Sigma, St. Louis, MO, USA). The reaction was started by adding aliquots of the enzyme-recombinant protein 1 mixture to the radioactive substrate. After a 20 min incubation at 37 °C, the reaction was stopped by adding 500 μl of *Dole*'s reagent (18) and heating to 60 °C for 2 min. The free fatty acid released into the organic solvent was extracted by adding of 200 μl of H_2O and 300 μl of *n*-heptane. After vortex mixing, the radioactivity in the *n*-heptane phase was measured in a liquid-scintillation counter and the inhibition rate of phospholipase A_2 activity was calculated.

Assay of phospholipase C activity

Recombinant phosphatidylinositol-specific phospholipase C-δ1 was purified to homogeneity as described (19). Phosphatidylinositol 4,5-bisphosphate phospholipase C activity of the enzyme in the presence or absence of 2 μmol/l recombinant protein 1 was measured according to *Smrcka* et al. (20). Briefly, the assay mixture (50 μl) contained 0.25 pmol/l of purified recombinant phosphatidylinositol-specific phospholipase C-δ1, various concentrations (12.5−200 μmol/l) of phosphatidylinositol 4,5-bisphosphate containing ^{3}H-labelled isotype (370 GBq/mmol, NEN Dupont, Wilmington, DE, USA), phosphatidylethanolamine (Sigma) at a 10-fold higher concentration than that of phosphatidylinositol 4,5-bisphosphate, 20 mmol/l Hepes-NaOH (pH 7.2), 0.8 g/l sodium cholate, 1 mmol/l dithiothreitol, 2 mmol/l EGTA, 0.2 mmol/l EDTA, 30 mmol/l KCl, 2 mmol/l $CaCl_2$ and 2 μmol/l recombinant protein 1 or vehicle. The mixture was incubated for 2 min at 37 °C, then the reaction terminated by transfer to 0 °C. Water-soluble hydrolysis products were extracted into 1 ml of chloroform/methanol/HCl (100 + 100 + 0.6) and 0.3 ml of 1 mol/l HCl containing 5 mmol/l EGTA. After vortex mixing the sample, the radioactivity of the water phase was measured in a liquid-scintillation counter and the initial rates of hydrolysis were calculated.

Results and Discussion

Expression and purification of recombinant protein 1 in *E. coli*

Direct DNA sequencing of the protein 1 insert from the selected clones verified that none of them was contaminated with PCR-induced sequence errors. Recombinant protein 1 expression reached a maximum 2 hours after induction by isopropyl-β-*D*-thiogalactopyranoside. The use of rifampicin during induction remarkably improved

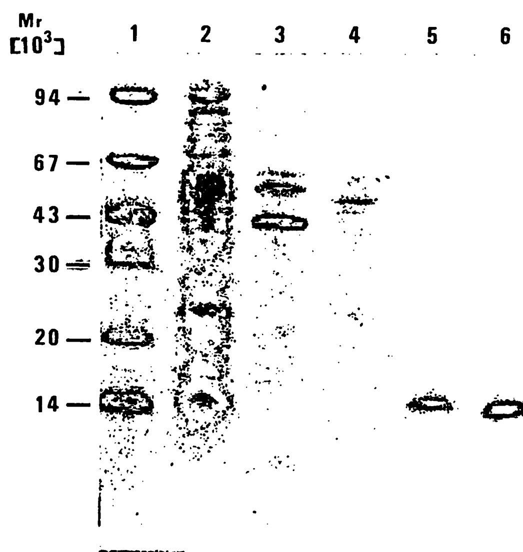

Fig. 1 SDS-PAGE of fractions at each purification step. Samples were resolved by electrophoresis under non-reducing conditions.
Lane 1, molecular mass markers;
lane 2, soluble fraction of *E. coli* lysates;
lane 3, insoluble fraction of *E. coli* lysates;
lane 4, pass-through fraction from anti-protein 1 immunoaffinity gel;
lane 5, bound fraction to anti-protein 1 immunoaffinity gel;
lane 6, purified recombinant protein 1.

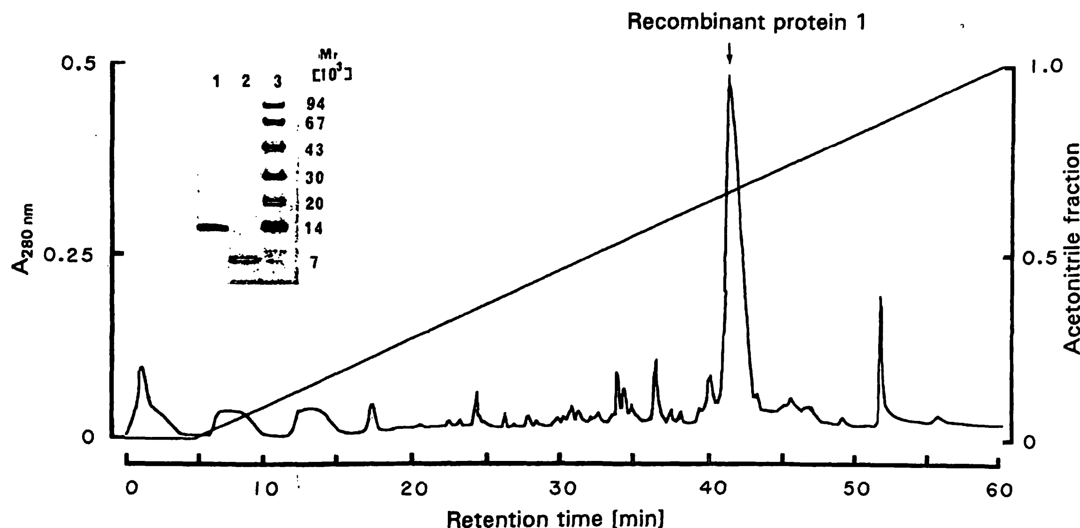

Fig. 2 Chromatogram of reversed-phase HPLC. The position of the recombinant protein 1-rich fraction is indicated by an arrow. Insert: SDS-PAGE of purified protein 1. Purified recombinant protein 1 (lanes 1 and 2) and molecular mass markers (lane 3) were resolved by electrophoresis either under non-reducing (lane 1) or reducing (lanes 2 and 3) conditions.

the expression efficiency. About 3 mg of recombinant protein 1 were expressed in 250 ml of culture. Immunoblotting showed that recombinant protein 1 was totally dimeric in *E. coli* lysates (data not shown), suggesting that synthesized recombinant protein 1 subunits were immediately dimerized with interchain disulphide bridges in *E. coli.*

Most recombinant protein 1 was recovered in the soluble fractions of *E. coli* lysates, which simplified the purification procedure. The anti-protein 1 immunoaffinity chromatography alone removed most of contaminating proteins derived from *E. coli* as judged by SDS-PAGE (fig. 1, lane 5). Recombinat protein 1 was further purified to homogeneity by reversed-phase HPLC (fig. 2). Finally, about 1.7 mg of purified recombinant protein 1 were obtained from 250 ml of *E. coli* culture. According to our published findings (1), over 10 l of pathological urine were necessary to obtain an equivalent quantity. Recombinant protein 1 was efficiently purified without the previous necessary laborious and time-consuming procedures.

Physicochemical characterization of recombinant protein 1

SDS-PAGE analysis showed that recombinant protein 1 was purified to homogeneity with a relative molecular mass of 14 000 and 7000 under non-reducing and reducing conditions, respectively (fig. 2, insert). Recombinant protein 1 also reacted with an anti-protein 1 monoclonal antibody on immunoblotting (data not shown). On reversed-phase HPLC, recombinant protein 1 eluted at the same retention time (42 min) as native protein 1 (fig. 2 and l. c. (1)). This indicated that the hydrophobicity of recombinant protein 1, which is affected by the tertiary structure of the protein, is very similar to that of native protein 1. The amino-terminal sequence of purified recombinant protein 1 was read up to the 20th residue and proved identical to that of native protein 1 except for an additional methionine at the amino-terminus of recombinant protein 1. On ELISA for protein 1, serial dilutions of recombinant protein 1 fitted the dose-response curve of native protein 1 within the actual measurement range, indicating that there was no difference in immunochemical behaviour between the two (fig. 3). All of the above findings indicated that recombinant protein 1 and native protein 1 are structurally identical, save for the presence of an extra methionine at the amino-termunus of recombinant protein 1.

Effect on phospholipase A_2 and phospholipase C

Recombinant protein 1 inhibited both porcine pancreas and cobra venom phospholipase A_2 activity in a dose-dependent manner (fig. 4), indicating that this recombinant protein retained its important biological activities. It has also been reported that protein 1 binds not only to

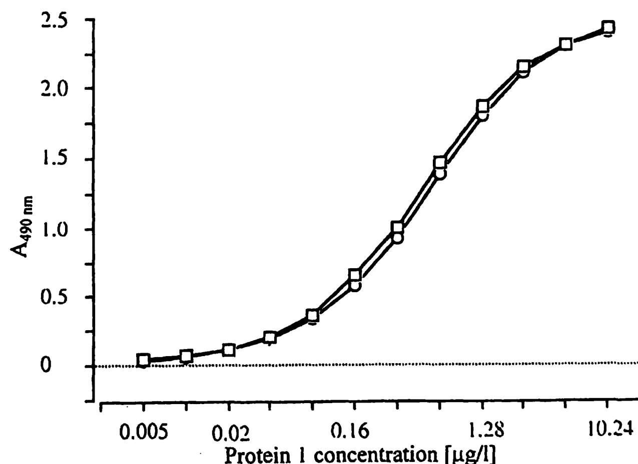

Fig. 3 Dose-response curves of recombinant protein 1 (squares) and native protein 1 (circles) on ELISA.

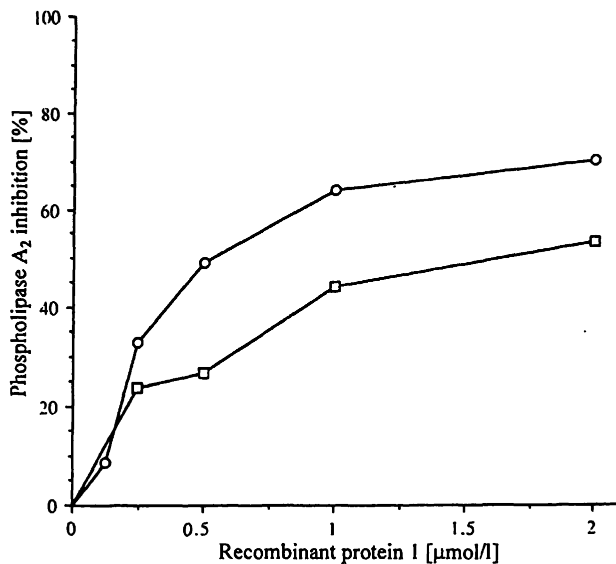

Fig. 4 Dose-dependent inhibition of porcine pancreas (squares) and cobra venom (circles) phospholipase A_2 by recombinant protein 1.

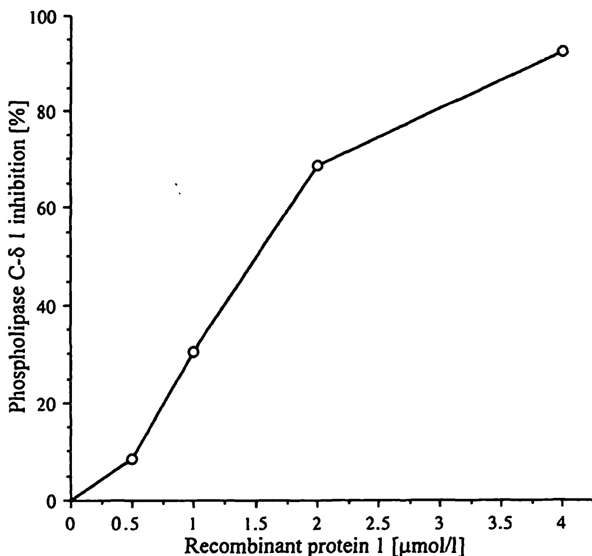

Fig. 5 Dose-dependent inhibition of recombinant phosphatidylinositol-specific phospholipase C-δ1 by recombinant protein 1.

phosphatidylcholine but also to phosphatidylinositol (21). Therefore, we examined whether recombinant protein 1 inhibited phosphatidylinositol-specific phospholipase C activity. Our data revealed that recombinant protein 1 also inhibits recombinant phosphatidylinositol-specific phospholipase C-δ1 in a dose-dependent manner (fig. 5). In the presence of 50 µmol/l phosphatidylinositol 4,5-bisphosphate and 0.25 pmol/l recombinant phosphatidylinositol-specific phospholipase C-δ1, 2 µmol/l of recombinant protein 1 inhibited recombinant phosphatidylinositol-specific phospholipase C-δ1 by 68%. To characterize the inhibition mechanism, we constructed *Lineweaver-Burk* plots. In the presence of 2 µmol/l recombinant protein 1, the K_m value apparently increased whereas the V_{max} value remained unchanged (fig. 6), indicating that the inhibition by recombinant protein 1 is competitive.

Two possible mechanisms are considered for the inhibitory effects of recombinant protein 1 on both phospholipases. One is hydrophobic interaction between protein 1 and phospholipid substrates. Recombinant protein 1 may limit phospholipase A_2 and C binding to the lipid substrates. Indeed, *Umland* et al. have shown that phosphatidylcholine and phosphatidylinositol bind to the inside of the large internal hydrophobic cavity of protein 1 (21). The other mechanism is the sequestration of calcium ions by protein 1. Both phospholipase A_2 and recombinant phosphatidylinositol-specific phospholipase C-δ1 require calcium ions for their enzymatic activities. *Andersson* et al. have suggested that protein 1 inhibits phospholipase A_2 activity by binding to calcium ions (22). However, a search for protein sequence motifs using the PROSITE (Univ. of Geneva) (23) and MOTIF (Univ. of Kyoto) data libraries revealed that there was no apparent calcium-binding domain such as an EF-hand motif (24) in the amino-acid sequence of protein 1. Furthermore, our preliminary experiments employing the $^{45}Ca^{2+}$ equilibrium dialysis assay, the $^{45}Ca^{2+}$ overlay assay or the Ca^{2+} gel shift assay (25) have so far given no indication of recombinant protein 1 binding to calcium ions (data not shown).

Inhibition of phospholipases may be the basis for the biological functions of protein 1. As pointed out by other workers, inhibition of phospholipase A_2 may be important in the regulation of inflammatory activity in the lung. In particular the protein is likely to be instrumental in controlling excess inflammatory activity (2, 9, 10). Inhibition of the production and functional activity of cytokines by protein 1 may add to its anti-inflammatory activity (26). The protection against endogenous phospholipases released by pulmonary cells is likely to be

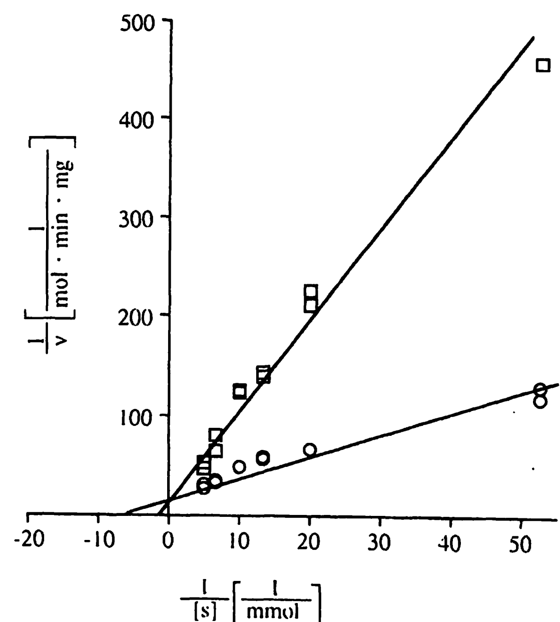

Fig. 6 *Lineweaver-Burk* plot of recombinant phosphatidylinositol-specific phospholipase C-δ1 activity with recombinant protein 1 (squares) and without recombinant protein 1 (circles).

important in chronic disorders, e. g. chronic obstructive airway disease. Inhibition of bacterial phospholipases would provide a defence against the deleterious effects of microbial infections. In addition to down regulating potential run-away inflammatory responses, the inhibition of phospholipase C and A_2 is likely to preserve the functional integrity of small airways be preventing degradation of surfactant. *Enhorning* et al. have shown that surfactant is important in maintaining the patency of small airways (27, 28). They have also demonstrated that degradation of surfactant by phospholipases, in particular phospholipase C, impairs the biological activity of surfactant in the small airways. Small airways generally bear the brunt of microbial infections, e. g. bronchopneumonia, and the collapse of such airways due to degradation of surfactant by bacterial phospholipases can be expected to aggravate the impairment of pulmonary function by pneumonia. Inhibition of phospholipase C may be instrumental in protecting the integrity of small airways by preserving the biological activity of surfactant. Inhibition of phospholipases may also be important in other chronic lung disorders, e. g. asthma.

In conclusion, a large quantity of recombinant protein 1 was obtained, which retained its native structural and biological properties. Using recombinant protein 1, we found that protein 1 inhibited not only phospholipase A_2 but also phospholipase C activity. This recombinant product will greatly facilitate biological and pharmacological studies on protein 1.

Acknowledgements

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