

EXPRESSION OF HUMAN PROTEINASE 3 IN CHINESE HAMSTER OVARY CELLS (CHO-CELLS)

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ABSTRACT

Proteinase 3 (PR3) is a human polymorphonuclear leukocyte serine proteinase and is the main target antigen for antineutrophil cytoplasmic antibodies (ANCA) found in Wegener's granulomatosis (WG). We developed a stable expression system for conformationally intact recombinant PR3 (rPR3) in Chinese hamster ovary cells (CHO-cells). The part of PR3 cDNA that encoded the active form of PR3 was selected by using appropriate primers, and a signal sequence was also added in front of PR3 cDNA. The signal sequence-PR3 (S-PR3) was cloned into the pME18 expression vector and the result product was electroporated into *E. coli* (DH5 α strain). After isolation and purification, the presence of pME18-S-PR3 was confirmed by using appropriate restriction endonuclease and agarose gel electrophoresis. The pME18-S-PR3 was electroporated with CHO-cells and the presence of rPR3 was tested in culture medium after 10 days. There was 12 ng/mL rPR3 in culture medium that had activity and was recognized by ANCA in ELISA.

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INTRODUCTION

The neutral serine protease PR3 is a constituent of the azurophil granules of human neutrophils. PR3 has been identified as the target antigen for ANCA, which circulates in the blood of patients with Wegener's granulomatosis (WG), a specific form of vasculitis.¹⁻⁵ This protease has also been implicated in the pathogenesis of pulmonary emphysema.¹ PR3 is identical to myeloblastin, which has been ascribed a central role in the control of growth and differentiation of leukemic cells.⁴ PR3 is initially identified as a 35 kDa precursor which is converted into the 29 kDa mature protein

after three stages of cleavage.^{4,6} The isolation of human PR3 from blood is very tedious and yields of purified active PR3 are rather low and require large amounts of human blood,^{3,7} so preparing recombinant PR3 in eucaryotic cells is a prerequisite. There are several reports in which PR3 is produced in bacteria, insect cells, yeast or mammalian cell lines, with varying success where often the rPR3 has no enzymatic activity or reactivity with ANCA or both.^{1,3,5,7} In this report we describe the construction and expression of PR3 in CHO-cells. The part of PR3 that encoded the active form of the PR3 molecule was chosen. In addition, a signal sequence was added in front of PR3 cDNA in order for active enzyme to be secreted into the culture medium.

MATERIALS AND METHODS

Ampicillin, kanamycin, N-t-Boc-L-alanine p-nitrophenyl ester and G418 were obtained from Sigma Chemical Company, St. Louis, MO. Restriction endonucleases, T4 DNA

Abbreviations: ANCA, Anti-neutrophil cytoplasmic antibodies; c-ANCA, Cytoplasmic fluorescence pattern ANCA; IIF, Indirect immunofluorescence; PBS, phosphate-buffered saline; RT-PCR, Reverse transcription-polymerase chain reaction; PR3, Proteinase 3; rPR3, Recombinant proteinase 3; WG, Wegener's granulomatosis.

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ligase and DNA purification kit were purchased from Promega Company. PWO-DNA polymerase and all electrophoresis chemicals were from Boehringer Mannheim. Reverse transcriptase, oligo-dT, dNTP, PCR buffer, LB medium (tryptan 1%, yeast extract 0.5%, NaCl 0.5%) and Bacto agar, RPMI 1640 HEPES and fetal calf serum were purchased from Gibco, Paisley, UK. Enhanced chemiluminescence (ECL) system (Amersham, denBos, the Netherlands), nitrocellulose sheets (BA85; Schleicher and Schull, Dassel, Germany) and Qiagen plasmid columns (Diagen GmbH, Dusseldorf, Germany) were other used materials.

Construction of the cDNA

The U937 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (Δ FCS). The total RNA of U937 was isolated using RNAzol TM B according to the manufacturer's protocol. The poly A mRNA was selected using oligo dT and reverse transcribed using reverse transcriptase with the buffers provided by the manufacturer and incubated for 1 hour at 42°C. The following primers based on the published nucleotide sequence of PR3⁶ and Scd89 nucleotide sequence⁸ were synthesized (by Gibco BRL custom primers):

- 1) 5'-TAGTACACCGGTATCGTGGCGGGCAGGAGGCG-3'
- 2) 5'-TCGTATCTAGATCAGCGCAGCGTGAACGGATCCA-3'
- 3) 5'-CCCAAGCTTCACTCATTAGGCACCCAGGCT-3'
- 4) 5'-TCATGGATCCACCGGTGCTGAATCCTCTGGCCAGACA-3'

The cDNA prepared in the above way was used for the PCR amplification of PR3 cDNA with 1,2 primers in a 40 μ L reaction mixture using 0.5 U of PWO-DNA polymerase, 250 nmol dNTPs, 5 pmol primers for 35 cycles (95°C for 25", 60°C for 35" and 72°C for 2"). The PCR amplification of cDNA for making signal sequences was performed on pUC18-Scd89 with 3 and 4 primers.

Vector construction

A) pUK21-S-PR3

The PCR products of 3,4 primers were separated on agarose gel and a PCR product of 220 bp was purified by promega purification DNA kit. The DNA and pUK21 vector were digested with the restriction enzymes HindIII and BamHI and were ligated by using T4 DNA ligase (22°C, 3h) after agarose gel electrophoresis and purification by promega purification DNA kit. The ligation mixture of pUK21-signal sequence (pUK21-S) was electroporated ($V=2.5$ kV, $C=25$ μ F, $R=200\Omega$) with competent *E. coli* (DH5 α strain) and plated on LB agar plates containing 50 μ g/mL kanamycin. After 24 h incubation at 37°C, colonies were picked and incubated overnight in LB medium with 50 μ g/mL kanamycin and plasmid DNA was purified using Qiagen plasmid columns according to the manufacturer's protocol.

The presence of pUK21-S was tested by electrophoresis and using appropriate restriction enzymes. The PCR product of 1,2 primers was separated by promega purification DNA kit. The pUK21-S vector and the purified PR3 cDNA were digested by AgeI and XbaI restriction enzymes and ligated by T4 DNA ligase (22°C for 3h). The ligation mixture electro-transformed into the DH5 α and was cultured and purified as explained for pUK21-S. Confirmation of identity of the S-PR3 insert was by digestion using appropriate restriction enzymes.

B) pME18-S-PR3 expression vector

pUK21-S-PR3 and pME18 expression vector were digested with the restriction enzymes EcoRI/SpeI and after electrophoresis on agarose gel (1%), S-PR3 and pME18 were separated and purified using promega purification DNA kit. The DNA was ligated (22°C for 3h) into the pME18 vector. After ligation, the plasmid was amplified in DH5 α and the bacteria plated, cultured on LB agar medium with 50 μ g/mL ampicillin for 24h at 37°C. Colonies were picked and cultured in 4 mL LB medium containing 50 μ g/mL ampicillin at 37°C for 16h. The plasmid DNA was purified using Qiagen plasmid columns and the presence of S-PR3 was confirmed by digestion using appropriate restriction endonucleases.

Transfection, selection, amplification and expression in CHO-cells

The CHO-cells were grown in RPMI 1640 medium supplemented with 10% Δ FCS. The pME18-S-PR3 expression vector (20 μ g) was transfected into the CHO-cells (1×10^7) by electroporation ($V=260$ V, $C=1050$ μ F, $R=\infty$). Nevertheless, pME18 contained a neomycin resistant gene, and 500 μ g/mL G418 (a neomycin analog) was added to the RPMI 1640 supplemented with 10% Δ FCS after all cells were attached to the bottom of bottles. Half of the cell population died after 6 days. Bottles were washed and fresh medium containing 500 μ g/mL G418 was added. A solid-phase sandwich ELISA as described by Berger et al.⁹ was used to determine the amount of the rPR3 secreted into the culture medium. In these experiments, 96-well plates (Nunc, Maxisorp, Roskilde, Denmark) were coated with an optimal concentration of polyclonal rabbit IgG anti-PR3 [production and purification described elsewhere¹⁰], diluted in coating buffer (74 mM NaHCO₃, 26 mM Na₂CO₃, pH=9.6) for 2 hours at 37°C. After each incubation step, plates were washed three times with 10 mM PBS containing 0.05% Tween-20 and all further dilutions were prepared in 10 mM PBS containing 0.05% Tween-20 and 2% casein. Following coating, 100 μ L blocking buffer (10 mM PBS, 0.05% Tween-20, 2% casein) was added to each well and was incubated for 30 min. at 37°C. Then two-fold serial dilutions of the PR3 sample were added and tested against serial dilutions of the PR3 standard (148.5 ng/mL- 1.16 ng/

mL). Bound PR3 was detected using digoxigenin (DIG)-conjugated polyclonal rabbit IgG anti-PR3 and subsequently horseradish peroxidase (HRP)-conjugated sheep F(ab)2 fragments anti-DIG. Finally, the ELISA was developed with 2,2-azinobis-3-ethyl benzothiazoline-G-sulphonic acid (ABTS), containing H_2O_2 (0.0005%), and the absorbance was measured at 415 nm.

Western blotting

Protein samples were prepared for gel electrophoresis by denaturation in a non-reducing sample buffer. Separation was done in 10% SDS gels using the mini protean II system (BioRad Veenendaal, the Netherlands). After electrophoresis, proteins were electroblotted onto nitrocellulose sheets. ECL system and polyclonal antibodies were used for the detection of rPR3 and native PR3.

RESULTS

The manufacturing of vectors are described in the *Materials and Methods* section. The signal sequence PCR product (Fig. 1a) was inserted into the pUK21 vector and after that PR3 PCR product (Fig. 1a) was inserted into the pUK21-S vector. Vectors were tested by using appropriate restriction endonuclease and electrophoresis in order to confirm the presence of signal sequence and PR3 cDNA (Fig. 1b-c). The results showed that signal sequence cDNA and PR3 cDNA had been successfully introduced into the vectors and they had correct length. pME18-SPR3 was sent to Eurogenetec Company (Belgium) in order to be sequenced. Data showed that a mutation had taken place, A instead of G base, in PR3 cDNA, that replaces the amino acid isoleucine instead of valine in rPR3.

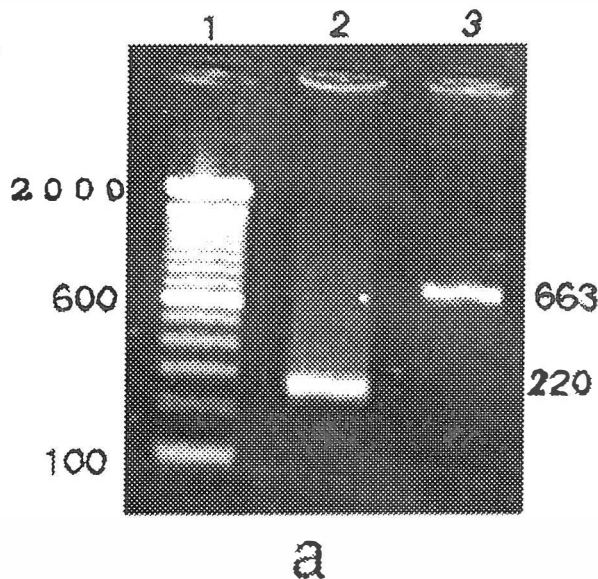


Fig. 1. a) 1= Size marker 100-2000 b.p, 2= Signal sequence PCR product, 3= PR3 PCR product.

Selection

pME18-SPR3 was electroporated into CHO-cells and transfected cells were cultured in RPMI 1640 containing 10% FCS and 500 μ g/mL G418 as described in *Materials and Methods* section. After 10 days the culture medium was tested for the expression of rPR3 by sandwich ELISA using PR3 polyclonal antibody. To obtain monoclonal rPR3 producing cells, they were seeded in three 24 well plates. In 22 wells cells grew, and four clones were positive and produced rPR3. One clone with high product (12 ng/mL) was selected and propagated.

Protease assay

Nevertheless, FCS inhibits some PR3 functions.¹¹ Results showed that FCS inhibited PR3 protease activity and its effect increased with increasing concentrations of FCS, however a concentration of 0.5% FCS in culture medium has minimum inhibitory effects. So transfected CHO-cells were grown in RPMI 1640 containing 10% Δ FCS and when cells filled the flask entirely, new medium supplemented with 0.5% FCS was added. Protease activity was assayed using 100 μ L of the synthetic substrate (1mM) N-t-Boc-alanine p-nitro-phenyl ester (in PB 10 mM, pH= 7.2, 2% DMSO) which was mixed with 100 μ L of culture medium of the transfected CHO-cells or purified native PR3 as control. The rPR3 produced in this way had 80% protease activity compared to native PR3.

Recognition of rPR3 by c-ANCA sera

To determine whether rPR3 expressed by CHO-cells is recognized by c-ANCA, 14 consecutive serum samples that had previously been determined to be c-ANCA positive by standard IIF on neutrophil cytosin preparation¹ and control sera (10 normal) were obtained from the clinical laboratory. For c-ANCA testing, serum samples were tested in duplicates at a dilution of 1: 25 (in PBS 10 mM containing 0.02% Tween-20 and 2% casein) in wells coated with polyclonal anti-PR3. Antigens (rPR3 and native PR3) were added to each well. In control wells no antigens were added (background). Net absorbance values calculated by subtraction of the background value from the value obtained from wells containing captured antigen are reported (Table I).

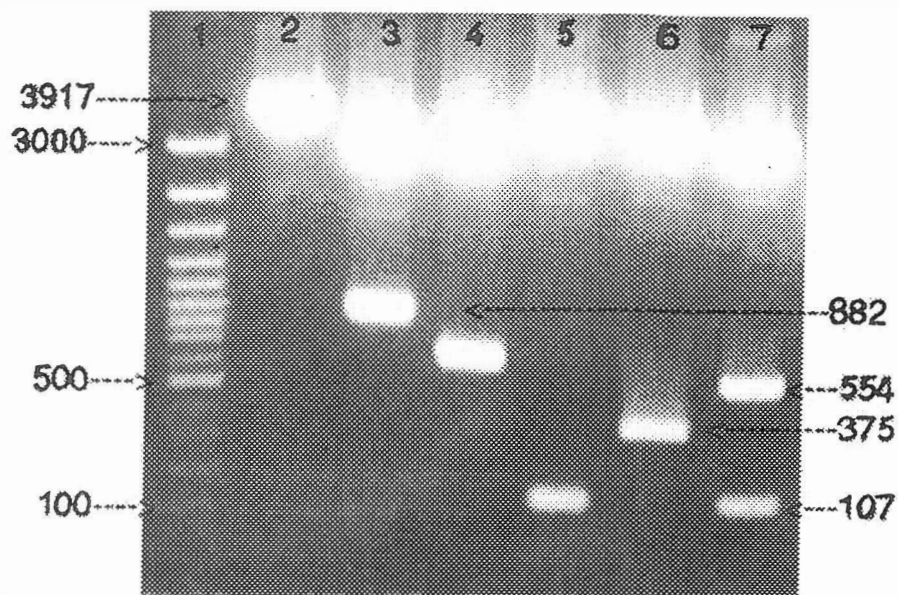
Western blotting

The results of western blotting showed that rPR3 had 3

Table I. Diagnoses and anti-PR3 assay results of c-ANCA positive patients.

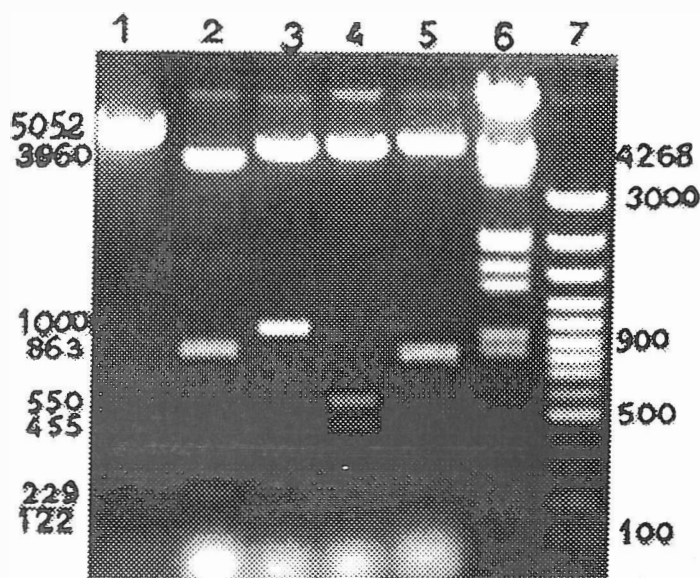
	IIF	Anti-rPR3	Anti-native PR3
c-ANCA positive	14	12	12
c-ANCA negative	0	2	2
Normal	10	10	10
Total	24	22	22

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b

Fig. 1. b) 1= Size marker 100-3000 b.p., 2= pUK21-SPR3, 3=pUK21-SPR3 digested by HindIII and XbaI (3035, 882), 4= pUK21-SPR3 digested by HinIII and BamHI (3058, 859), 5= pUK21-SPR3 digested by KpnI (3795, 122), 6= pUK21-SPR3 digested by PstI (3542, 375), 7= pUK21-SPR3 digested by SacI (3256, 554, 107).



c

Fig. 1. c) 1= pME18-SPR3 digested by BamHI (229, 863, 3960), 2= pME18-SPR3 digested by KpnI (122, 1000, 4087), 3= pME18-SPR3 digested by SacI (455, 550, 4047), 4= pME18-SPR3 digested by EcoRI and XbaI (750, 4459), 5= Size marker, 6= Size marker 100-3000 b.p.

bands around 29 kDa which is similar to native PR3 (Fig. 1d).

DISCUSSION

We have cloned and expressed PR3, which is a major

antigen implicated in WG. The signal sequence of Scd89 was selected and was added in front of PR3 cDNA. The restriction with appropriate restriction endonuclease showed the signal sequence and PR3 cDNA had correct length and was inserted correctly together and into the vectors. pME18-

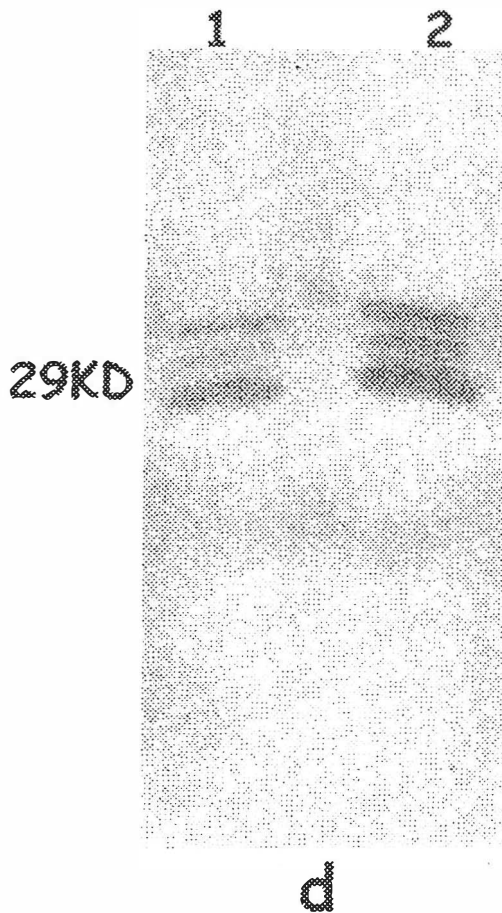


Fig. 1. d) 1= native PR3, 2= rPR3.

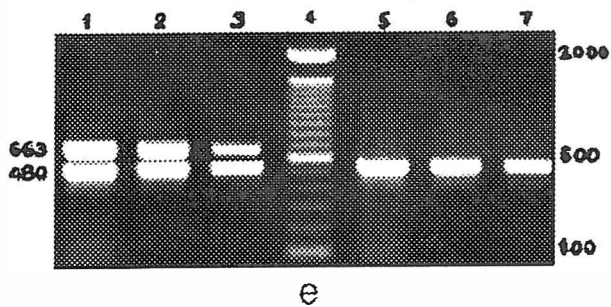


Fig. 1. e) 1-3= PR3 and -actin RT-PCR products of transfected CHO-cells (1/100, 1/1000, 1/10000), 4= Size marker 100-2000 b.p, 5-8= PR3 and -actin RT-PCR products of untransfected CHO-cells (1/100, 1/1000, 1/10000).

SPR3 was sent to Eurogenetec Company for sequencing and verification. Data showed that there is one mutation in PR3 cDNA, A instead of G base, that replaces isoleucine instead of valine in rPR3. Nevertheless both amino-acids are non-polar and branching and have common properties. Therefore, it appears that this alteration doesn't create considerable differentiation in rPR3 structure. Transfected cells were

separated using G418. The rPR3 was detectable 10 days after transfection in culture medium. Finally 4 monoclonal CHO-cells were obtained, out of which one of them with high rPR3 production was propagated. The transfected cells' total RNA was isolated and Reverse Transcription-PCR(RT-PCR) was performed using 2 and 3 and also β -actin primers (Fig. 1e). There was a strong band around 663 base pairs that indicated PR3 cDNA has cloned in CHO-cell chromosomes and has performed transcription. The secreted rPR3 was measured in culture medium using a capture-ELISA, the producer cells secreted 12 ng/mL rPR3 in culture medium. Nevertheless, there are 6 base pairs between signal sequence and PR3 cDNA (AgeI site), those encoding threonine and glycine, which were added to the N-terminal of rPR3, are not found in native PR3 and create some alteration in rPR3 structure. However, these two amino-acids (Thr and Gly) differ from the two amino acids (Val and Gla) that are found at the N-terminal of pre-pro-PR3. Anyway rPR3 can be recognized as the native PR3 by ANCA in ELISA and has enzymatic activity. In order to assay PR3-ANCA with ELISA previous reports have suggested directed coating of the purified antigen to the plastic plate. But in this manner immobilization may result in partial denaturation of the antigen with alteration of conformational epitopes. So we used capture ELISA and our results are similar to other reports. Although the rPR3 concentration in culture medium is low and sera with rheumatoid factor (RF) can interfere in ANCA measurement in capture ELISA,¹² the results showed that the recombinant molecule can replace native PR3 for clinical purposes. Of course rPR3 production reported by others and isolation of human PR3 from blood involves many limitations.^{1,3,5,7} ANCA measurement while coating PR3 should also have problems.¹² Our results presented here showed that the rPR3 produced had good enzymatic activity and exhibited proper epitopes for ANCA, which could enable us to measure this factor in sera samples. The results also indicated that CHO-cells are an appropriate host for expression of recombinant serine proteases.

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