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Original scientific paper

Recombinant expression of monovalent and bivalent anti-TNT--antibodies – evaluation of different expression systems

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Abstract: Monoclonal 11B3 anti-TNT (trinitrotoluene) antibody was expressed as a monovalent and bivalent form using different prokaryotic and eukaryotic expression systems. Recombinant expression in *Escherichia coli*, mammalian cells and the methylotrophic yeast *Pichia pastoris* was performed to obtain disulfide-linked and glycosylated antibody forms. The generation of antibody and subsequent evaluation of the expression rates were performed using intracellular, excretory and periplasmatic expression techniques. All methods involved striving for native expressed antibody with maintenance of its functionality only.

Keywords: TNT; antibody; recombinant expression; 11B3; scFv.

INTRODUCTION

For protein production in the laboratory, the most suitable prokaryotic system is the gram-negative bacterium *Escherichia coli* because of its rapid growth in high cell densities, easy generic and availability of a large number of vector systems. The first demonstrated expression of functional fragments of antibodies was for *E. coli* in prokaryotic periplasma. The secretion in periplasma was forced by genetic fusion of antibody fragments with a signal sequence of some periplasmatic protein.[1,2] The building of disulfide bridges is ensured through the oxidative nature of the periplasmatic compartment.[3]

Protein expression without a signal sequence results in it remaining in the cytoplasma. Native isolation with ultrasound is possible if the protein is soluble in the cytoplasma. It has to be considered that cytoplasma is not an oxidative compartment, and hence the actual protein must go through redox active systems to achieve its functionality.

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In addition to *E. coli*, other organisms can be used in which antibody fragments were successfully expressed and partially secreted into the medium, which is an enormous advantage for preparative and commercial expression. For easier laboratory expressions, each organism should be matched with *E. coli* regarding its ease of handling and diversity of possibilities.

In yeasts, the eukaryotic folding and post-translational modifications are merged through simple cultivation. The methylotrophic species *Pichia pastoris* can metabolize methanol as the only carbon source and also secrete and process recombinant proteins.[4,5] The high yields and functionality of the secreted recombinant immunoglobulins underline the importance of yeasts for this application. For scFv (single chain fragment variable) molecules, yields of over 100 mg l^{-1} were obtained, which exceed the expression in *E. coli* by more than hundred times.[6]

Without doubt, the usage of mammalian cells (COS, CHO, HEK, *etc.*) is the most suitable approach for expression of recombinant proteins. The problems with post-translational modifications are thereby almost excluded. The conversion of antibody fragments into complete immunoglobulins of different isotypes and their expression in mammalian cells have been demonstrated many times with no loss of binding activity.[7–10]

The use of recombinant antibody has significant advantages compared with conventional antibody and its use has become more popular nowadays due to the fact that no animals are required in the manufacturing procedure of the recombinant antibodies. In addition, the manufacturing time is relatively short compared with the conventional method. Moreover, the quality of the final products is higher that those manufactured by the non-recombinant method.

In this work, recombinant antibodies specific for TNT were expressed in mono- and bi-valent format in different expression systems. The aim of the work was, on the one hand, to examine the expression efficiency in prokaryotic and eukaryotic systems and, on the other, to use these antibodies for the detection of TNT and its derivatives. The best expressing system should then be used for the commercial production of the most sensitive antibody format for TNT detection.

EXPERIMENTAL

Vectors

The vectors pcDNA3.1+ and pPICZα-ABC for eukaryotic expression in mammalian cells and yeast *Pichia pastoris* were commercially purchased from Invitrogen Life Technologies (Karlsruhe, Germany). The *E. coli* expression vector pET26b(+) was purchased from Novagen (Schwalbach, Germany) and the phagemid-vector pHEN2 from G. Winter, Center of Protein Engineering, MRC Cambridge, UK.

Antibody fragments

The gene for the scFv-fragment 11B3 of mouse origin, as well as the genes for the C_H2 and C_H3 regions of human IgG was available.

Oligonucleotides

The employed oligonucleotides were synthesized by Metabion (Martinsried) (Table I).

TABLE I. Utilized oligonucleotides

Name	Sequence 5`-3`	Target region	Restriction sites
11B3 Bsi for	Gatccgtacgtgtgggatggcccaggtgaag	11B3-scFv	*BsiW*I
11B3 Asc back	Gatcggcgcgccacctaggacggtcagcttg	11B3-scFv	*Asc*I
11B3 Sfi for (pPICZαB)	Gatcggcccagccggccttatggcccaggtgaag	11B3-scFv	*Sfi*I
11B3 Nde for	Ggaattccatatggcccaggtgaagctg	11B3-scFv	*Nde*I
11B3 Not back (pET)	Attcttatgcggccgcccgttttatttccagctt	11B3-scFv	*Not*I

Standard molecular biology techniques

PCR, ligation, restriction, DNA dephosphorylation, agarose gel electrophoresis, DNA extraction from agarose gels, classic plasmid preparation, alcohol precipitation, DNA quantification, *etc.* were performed according to standard protocols.[11]

Production of competent E. coli *cells, transformation and expression*

Electrocompetent *E. coli* cells, transformation, periplasmatic and intracellular expression were performed according to standard protocols.[11-13]

Standard techniques in protein biochemistry

Immobilized metal ion affinity chromatography for protein purification using a Ni-NTA (nickel nitrilotriacetate) matrix, PAGE (polyacrylamide gel electrophoresis), protein determination, Western blot, dialysis, antibody purification *via* the Fc region using protein A/G-PLUS-agarose, *etc.* were generally performed according to standard protocols.[11]

Eukaryotic expression in yeast and mammalia

Antibody expression in yeast *P. pastoris* and mammalia was performed according to standard protocols.[11,14] For the expression in *P. pastoris,* the standard vector pPICZIgGscFv-Fc, which already contained an α-factor as a signal sequence and an expression cassette (C_H2 and C_H3 genes), was available. Using restriction sites *Sfi*I and *Asc*I, any scFv can be cloned into the cassette and a dimer IgGΔC1, with constant C_H2 and C_H3 regions, is generated as the result of the expression.

For expression of the IgGΔC1-dimer in mammalian cells, HEK (humane embryo kidney) cells were mostly used. The available vector was pcDNA3.1/Zeo, which contained a CMV promoter, a rat signal sequence, a gene for zeocine resistance and an expression cassette with the genes for the IgG domains C_H2 and C_H3.

RESULTS

Starting from the TNT-specific scFv-fragment of mouse origin, named 11B3, monovalent and bivalent antibodies were generated. The fragment was produced in different formats, *i.e.*, it was expressed in a monovalent form as scFv and the scFv were further used for the generation of bivalent antibodies. In order to use the scFv in the bivalent form, the IgGΔC1 constructs were made. To analyze the binding properties of antibodies, the free TNP–Tris (trinitrophenol–tris-(hydroxymethyl)aminomethane), as well as the TNP–protein conjugates were used.

Monovalent antibody 11B3-scFv

For the cloning and expression of the monovalent antibody, a vector pet26b(+) with a stronger T7-promoter was chosen, in which the amplified 11B3-scFv gene was inserted between the restriction sites *Nde*I and *Not*I. During the insertion, the signal sequence was removed, which meant a predisposition for intracellular expression in *E. coli* strain BL21 DE3. The expression was performed for 3 hours at 25 °C using the lactose analog IPTG (isopropyl-β-D-thiogalactopyranoside) as inducer. The protein was purified from the cytosol supernatant with a NiNTA-matrix and subsequently analyzed by SDS-PAGE and immunoblot. The band of 28 kDa on the membrane for the Western-blot confirmed the successful expression of the antibody (Fig. 1).

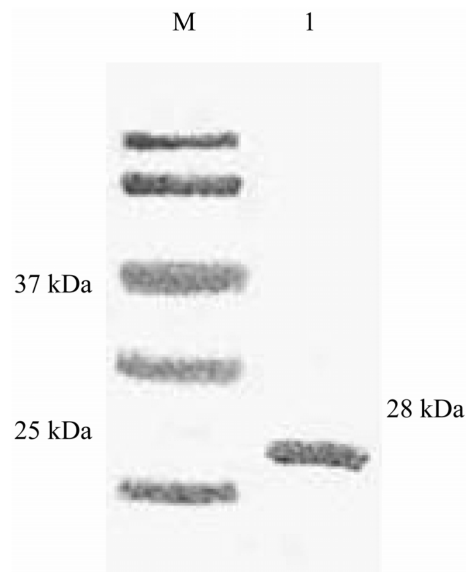

Fig. 1. Analysis of purified 11B3-scFv using immunoblot. 15 µl of protein probe (1) was separated under reducing conditions on a 12 % SDS-gel together with 10 µl of protein marker (M). The presence of protein was confirmed after transfer onto a PVDF-membrane and detection with murine anti-His-IgG-antibody (1: 2500) and anti-mouse-IgG–AP conjugate (1: 2500).

The determination of the protein concentration gave a value of 65 µg/ml. The antibody was further biotinylated for usage in the TNP-assay.

Bivalent construct 11B3-IgGΔC1

The bivalent construct 11B3-IgGΔC1 is a synthetic molecule obtained after cloning the murine 11B3-gene in the expression cassette with constant C_H2 and C_H3 regions of the human gamma heavy chain. Expression of 11B3-IgGΔC1 was performed parallel in two expression systems, *i.e.*, yeast *Pichia pastoris* and the human cell line HEK293.

Expression in human HEK293 cells

As the expression cassette, the vector pcDNA3.1 was available, which had a strong viral promoter and genes for the constant regions of the human gamma

heavy chain. The restriction sites *Bsi*I and *Asc*I were chosen for the cloning of the 11B3-gene. After ligation and transformation, characterization of the clones was performed with PCR. The obtained amplificates confirmed the cloning of the11B3-scFv insert in the vector.

The recombinant DNA was further employed for the production of stable secreting cell lines. From *ca.* 300 ml expression supernatant, the antibody was purified using protein A-agarose and analyzed by Western blot (Fig. 2). The eluted amount of 4.5 ml gave a total protein quantity of about 410 μg (90 μg/ml); hence the expression rate for the 300 ml culture was about 1.37 μg/ml. The purified protein was directly used in the assay for confirming its functionality. This immunocomponent proved to be very sensitive in the assay for detection of TNT-derivatives. Using 60 μl of antibody solution at a concentration of 225 ng/ml, 25 fmol TNP–Tris was identified.

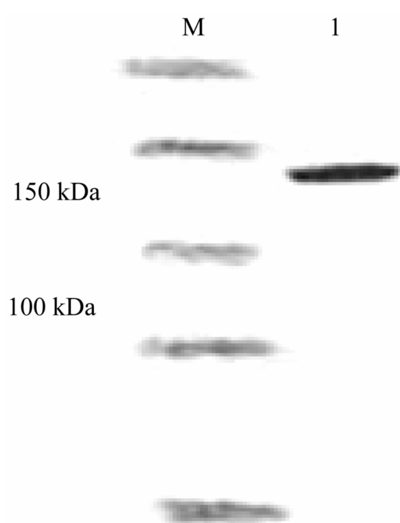

Fig. 2. Immunoblot of purified 11B3-IgGΔC1. 10 μl of protein marker (M) and 10 μl of protein sample (1) were separated on a 7.5 % SDS-gel under non-reducing conditions. The presence of protein was verified after transfer onto a PVDF-membrane and detection with anti-human-IgG–AP conjugate (1:2500).

Expression in yeast

After ligation of scFv and transformation, the clones were controlled using PCR. One of the selected clones was chosen for inoculation of the overnight culture with zeocine and subsequently for DNA isolation. The isolated DNA was then linearized and inserted into yeast cells using electroporation.

After yeast electroporation with yeast DNA, a three-day-expression was performed with one of selected clones. A very small quantity of the protein was detected on a PVDF (poly(vinylidene difluoride)) membrane with anti-human-IgG (1:2500) conjugated with AP (alkaline phosphatase) or HRP (horseradish peroxidase). No protein was visualized on an SDS-gel. Accordingly, expression in HEK-cells was preferred.

DISCUSSION

Cloning and expression of recombinant immunoreagents

The bivalent 11B3-IgGΔC1-construct was successfully expressed in the stable human embryo cell line HEK293. Deletion of the C_H1 region offered the advantage of a lower molecular weight, which contributed to the enhanced secretion. The high expression yield facilitated the purification of the 11B3-antibody on a protein A-matrix. The only problem with this antibody was the determination of its molecular weight, which was necessary for the precise determination of the protein concentration. According to the primary sequence, computer calculations gave a value of 104 kDa. An apparent appearance of these antibodies on an SDS-gel and Western blot was at about 130 kDa due to glycosylation. The functionality of this antibody and its binding properties were successfully verified in ELISA for detection of TNT-derivatives. According to these results, it can be concluded that this construct expressed in mammalian showed complete and correct folding and oxidation. Alternatively, 11B3-IgGΔC1 was expressed in yeast cells but, due to low yields, this expression was not the subject of further investigations.

Monoclonal 11B3-scFv was expressed in two different systems. First, it was supposed to have been expressed under a Lac-promoter with a signal sequence, but this failed as the antibody was expressed mostly within inclusion bodies in the cytosol. It was further attempted to perform intracellular expression without a signal sequence under the stronger T7-promoter. This expression gave better antibody yields, which were about 0.5 μg per ml of culture. For detection of monovalent antibodies in ELISA, biotinylation was performed in order to detect these antibodies with the streptavidine–HRP conjugate.

The reason for the bad expression efficiency can be a frequent presence of codons for some amino acids in the gene sequence.[15] The origin of the scFv-gene can be the reason for the difference in expression efficiency. Another reason could be potential problems in the post-translational modifications or folding at 11B3-scFv.[3] It could also be possible that more hydrophobic domains were formed in 11B3-scFv, whereby insolubility of the protein occurred. Some experiments concerning higher yields of expressed protein were performed under a lower temperature and IPTG concentration,[16] but no improved expression was registered. Solubility enhancement of 11B3-scFv can be achieved by co-expression of bacterial chaperons[16] or eukaryotic disulfide isomerases,[17] which should be a future line of research. It has already been confirmed that the fusion of 11B3-scFv with the enzyme *β*-lactamase gave a higher expression rate than for the single antibody.

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ИЗВОД

РЕКОМБИНАНТНА ЕКСПРЕСИЈА МОНОВАЛЕНТНИХ И БИВАЛЕНТНИХ АНТИ-ТNT-АНТИТЕЛА – ЕВАЛУАЦИЈА РАЗЛИЧИТИХ ЕКСПРЕСИОНИХ СИСТЕМА

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Моноклонско 11B3 антитело специфично за TNT (тринитротолуен) експримирано је у моновалентном и бивалентном облику користећи различите прокариотске и еукариотске експресионе системе. Рекомбинантна експресија у *Escherichia coli*, ћелијама сисара и метилотрофном квасцу *Pichia pastoris* изведена је да би се добиле гликозиловане и дисулфидним мостовима везане форме антитела. Стварање антитела и евалуација приноса експресије изведени су применом техника интрацелуларне, екскреторне и периплазматске експресије. Све методе укључивале су тежњу за експресијом антитела искључиво у нативном облику што је очувало његову функционалност.

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