

Expression, purification and characterization of the recombinant chimeric IgE Fc-fragment opossum–human–opossum (OSO), an active immunotherapeutic vaccine component

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ABSTRACT

The active vaccine component recombinant chimeric IgE Fc-fragment opossum–human–opossum (OSO) has been expressed in CHO-K1 cells. It contains two identical polypeptide chains with 338 amino acid residues in each chain connected by two disulfide bridges. The cell lines were adapted to suspension culture in a serum-free medium. An expression level of 60 mg/L was obtained after 8 days in a shaking flask at a temperature of 31.5 °C. The OSO protein has been purified to homogeneity by a combination of three chromatographic steps. Virus inactivation and reduction by solvent detergent treatment and nano-filtration were included in the process. The residual host cell protein content was less than 50 ng/mg OSO as analyzed by ELISA. Purity was analyzed by SDS–PAGE under reducing and non-reducing conditions and was estimated by densitometry to be above 99.0%. The dimer content was less than 0.1% as estimated by analytical size exclusion chromatography. The molecular mass, as estimated by SDS–PAGE, is 90 kDa. A value of around 74 kDa was calculated from its amino acid composition. This indicates that the protein is heavily glycosylated containing around 18% carbohydrate. Isoelectric focusing in polyacrylamide gel disclosed a ladder type band pattern with pI values in the pH-range 7.0–8.3, indicating a variation in the sialic acid content. The OSO protein is not stable at temperatures above 40 °C and at pH values below 4 indicating that virus inactivation by incubating the protein solution at higher temperature or at lower pH is not possible.

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Introduction

The frequency of IgE mediated allergies have increased to more than 20% of the western world population. Current treatment regimens are based mainly on the use of antihistamines and anti-inflammatory agents such as corticosteroids. For moderately or severely allergic patients, a humanized monoclonal anti-IgE antibody, Xolair, has been prescribed [1,2] and significant reduction of asthma exacerbations has been reported [3].

An alternative approach is to actively trigger the immune system to produce antibodies against self-IgE through vaccination [4]. The concept is to abrogate the allergic cascade by eliminating circulating IgE. Administration of self-IgE in a context which allows breakage of tolerance against the molecule, leads to the production of anti-IgE antibodies, which in turn decreases the level of circulating IgE [5–7].

The anti-IgE antibodies produced *in vivo* should only bind free plasma IgE and must not be able to cross-link IgE bound to Fc receptors on mast cells and basophiles, as this would induce the allergic cascade. The vaccine is therefore constructed to induce production of therapeutic antibodies against the Cε3 domain, the part of the IgE molecule that directly interacts with the Fc receptor. The amino acid residues that are involved in receptor binding are spread over most of the Cε3 domain [8,9]. Consequently, antibodies directed against the Cε3 domain of IgE are likely to clear IgE from the circulation without causing receptor cross-linking of mast cell bound IgE.

In order to break the B cell tolerance against self-IgE it is necessary to partly transform the self protein into a non-self protein by introduction of non-self T cell epitopes. At the same time, it is important to keep the conformation of self-IgE to ensure production of antibodies that also interact with circulating IgE. This has been achieved by the construction of a recombinant protein consisting of the human Cε3 domain flanked by the Cε2 and Cε4 domains from a distantly related mammal, the opossum (*Monodelphis domestica*). The IgE of the marsupial opossum and the human displays a high sequence difference [10]. The opossum domains provide the recombinant protein with the necessary foreign T cell

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epitopes, while at the same time ensuring proper conformation of the Cε3 domain.

The species specific protein, a chimeric IgE Fc-fragment consisting of the rat Cε3 domain flanked by the Cε2 and Cε4 domains of the opossum, has been tested on rat [4–7]. To be able to prove the concept in man, the objective of the work described in this paper was first to establish a stable cell line free of microbial contamination and possessing high expression level and secondly to develop a scalable and cost-effective downstream process for the production of an active vaccine component intended for clinical use.

The coding sequence for the OSO² protein was cloned in the eukaryotic expression vector pCI-neo and subsequently transfected into CHO-K1 cells. Clones with high expression levels were selected and isolated by repeated limiting dilutions. The clones obtained were further selected based on productivity and growth characteristics and the cell lines chosen were adapted to suspension culture in a serum-free medium in shaker flasks.

Cell culture conditions often have a strong effect on the extent and quality of post-translational modification, e.g. glycosylation, but the main purification problem is the separation of the target protein from variants such as nicked or truncated forms and the large number of host cell proteins. Other possible contaminants such as viruses, pyrogens and DNA fragments, may affect the safety of the purified OSO. Given the importance of a vaccine for preventive therapy and the role of the purification process in assuring the quality, consistency and safety of the product, it is clear that a combination of different chromatographic steps is required. With an emphasis on the highest possible purity and yield, cost effectiveness and ease of scaling up, a chromatographic three-step purification procedure involving immobilized metal ion affinity chromatography, hydrophobic interaction chromatography and size exclusion chromatography was developed, followed by chemical and physical characterization of the purified protein.

Materials and methods

Chimeric OSO construct and OSO production

All molecular biology work was performed according to standard methods described previously [11]. The insert is composed of 1182 bp and contains the human IgG leader sequence and encodes the C3 domain of human ε-heavy chain [12,13] flanked by the C2 and C4 domains of opossum ε-heavy chains [10]. All enzymes, otherwise specified, were purchased from GE Healthcare Bio-Sciences, Uppsala, Sweden.

Isolation of the opossum and the human sequences encoding the C2–C4 domains

A phage lambda cDNA library was constructed with mRNA isolated from a spleen sample of the grey short-tailed opossum (*M. domestica*). A clone containing the entire coding for the constant region of the opossum IgE ε-heavy-chain was isolated [10]. A IgE-producing multiple myeloma cell line U266 (American Type Culture Collection) is used for preparing human IgE. mRNA was isolated and reverse transcribed into cDNA. The human IgE constant region C2C3C4 was amplified with PCR using the forward primer containing a BamHI cleavage site and the reverse primer

containing an EcoRI site. The PCR product was cleaved with BamHI and EcoRI and cloned into the vector pGEX2 (GE Healthcare).

Construction of the recombinant OSO sequence

The opossum sequences OpC2 and OpC4 were independently amplified from the full length cDNA clone. The human C3 sequence was amplified from the C2C3C4 fragment originating from the U266 cell line. Restriction enzyme cleavage sites and other modifications were introduced into the primers by design. The resulting OpC2 fragment was flanked by an EcoRI site in the 5' end and a XbaI site at the 3' end. The human C3 fragment amplified contained an XbaI site at the 5' end and a HindIII site at the 3' end. The OpC4 fragment was flanked by a HindIII site at the 5' end and two translational stop codons followed by an XhoI restriction site at the 3' end. The fragments were then sequentially ligated into the vector pBluescript SK+/– (Stratagene). Finally, the chimeric OSO construct flanked by XhoI sites was amplified and cloned into a vector containing the human IgG leader sequence.

Establishment of a stable OSO expressing cell line

To obtain a stable cell line with higher expression level, the pCI-neo vector (Promega) was selected. After digestion with the restriction enzyme SmaI over night at 25 °C the vector was treated with phosphatase (20 U) for 2 h at 37 °C. The phosphatase was then inactivated for 10 min at 80 °C. The vector fragment was purified by agarose gel electrophoresis and with Qiaex kit (Qiagen). The IgG leader sequence and OSO insert fragment were purified by sequential digestion with KpnI and NsiI (Biolabs). The OSO fragment was blunted with T4 polymerase and purified as described above. 20 ng pCI-neo vector was ligated with 30 ng OSO fragment in 5 U T4 DNA ligase at 12 °C over night. The ligation mix was transformed into DH5α *Escherichia coli* competent cells according to the manufacturer's instructions (Life Technologies). DNA was prepared from overnight cultures of individual colonies using QIAprep Spin Miniprep Kit (Qiagen). Correct orientation of the OSO insert was verified by digestion with NheI, XhoI and BglII. The nucleotide sequence of the leader-OSO insert was confirmed using the dideoxy chain-termination method (Cybergene, Huddinge, Sweden).

Transfection

The CHO-K1 (Chinese Hamster Ovary) cells (ATCC) were grown in F-12 (Ham) medium (Gibco) supplemented with 50 mg/mL gentamicin (Gibco BRL), 5–10% FBS (Gibco BRL). Adherent cells were treated with trypsin/EDTA (Gibco BRL) and cultured at 37 °C, 5% CO₂ in a humidified incubator (Galaxy S, RSBiotech). The cell number and viability was measured by Trypan Blue (Invitrogen) exclusion and counted in a Bürker chamber. After growing to ~90% confluence in 75 cm² flasks (~15–20 × 10⁶ cells), the cells were washed with serum-free F-12 medium and transfected as follows: 50 µg OSO/pCI-neo plasmid was linearized with BglII. The DNA was precipitated and dissolved in 100 µL sterile water (Gibco BRL). The DNA solution and 100 µL Lipofectamine 2000 Reagent (Life Technologies) were each diluted in serum-free F-12 medium to 2.5 mL. Subsequently, the DNA and Lipofectamine preparations were mixed and incubated for 20 min at room temperature. The DNA/Lipofectamine mixture was added to the washed cells. After 12–16 h, the medium was supplemented with fetal bovine serum (FBS) to a final concentration of 10%. The first transfection was selected in flasks with culture medium supplemented with 200 µg/mL Geneticin (Gibco), 10% FBS, 10 mM Hepes (Gibco) and 1% Na-pyruvate (Gibco). Clone selection was performed after the mock transfected cells died under the selection pressure.

² Abbreviations used: CDM, chemical defined medium; CHO, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; HCP, host cell protein; MLV, Xenotropic murine leukaemia virus; MMV, Minute virus of mice; MWCO, molecular weight cut-off; OSO, chimeric IgE Fc-fragment opossum-human-opossum; S/D, solvent/detergent; TNBP, tri-*n*-butyl-phosphate.

Selection and cloning by limiting dilution

Ninety-six-well plates (VWR, Nunc) were seeded at 1, 5, 10 or 20 cells/well in selection medium as described above and supplemented with 20% conditioned medium from confluent CHO-K1 cells. Plates were incubated for 2 weeks and inspected microscopically. Wells with single clones were analyzed by ELISA. The best clones in terms of productivity related to growth were selected and expanded or subjected to next limiting dilution cloning.

Adaptation to suspension culture

Adherent growing cells were trypsinized and inoculated at a cell concentration of 1×10^6 viable cells/mL in a volume of 40 mL in selective SFX (Cambrex) supplemented with 5% FBS, 10 mM Hepes, 0.4 mM Glycine (Sigma), 4 mM L-glutamine (Gibco), 0.3 mg/mL Geneticin. The cell suspension, in a 125 mL Erlenmeyer flask, was incubated with 5% CO₂ at 37 °C in an orbital shaking incubator with a radius of rotation of 16 mm set at 110–125 rpm. After growing for 3–4 days, the cells were passaged at $0.5\text{--}1 \times 10^6$ viable cells/mL. This procedure was repeated at 3–4 days intervals until the viable cell concentration at passage was consistently at least double the seeding concentration.

Adaptation to serum-free suspension culture

The suspension-adapted cells were passaged at 1×10^6 viable cells/mL using the complete medium with 2.5% FBS and were incubated with 5% CO₂ at 37 °C in an orbital shaking incubator at 110 rpm. This step was repeated every 1–2 days until the viable cell concentration at passage was consistently at least double the seeding concentration. The cells were passaged at 1×10^6 viable cells/mL using the complete medium with 1.5% FBS and incubated under the same condition. Again this step was repeated every 1–2 days until the viable cell concentration at passage was consistently at least double the seeding concentration. The same procedure was repeated several times with reduced concentration of FBS in the medium, 0.75%, 0.4%, 0.2%, 0.1% and serum free, respectively. Once cell growth becomes consistent, seeding concentrations at passage was reduced to $0.5\text{--}1 \times 10^6$ viable cells/mL maintaining passage the cells. Stocks of the adapted cell lines were cryopreserved in complete ProFreeze-medium (Cambrex) supplemented with 7.5% DMSO (Sigma) with a cell concentration of 10×10^6 cells/mL and stored in liquid nitrogen. Cell lines were tested for protein productivity at every step of the entire adaptation process by ELISA.

Culture process for production of OSO in shaker flasks

A vial was thawed by gentle agitation in a 37 °C water bath. As soon as the contents were thawed, the cells were transferred to an Erlenmeyer flask containing 10 mL complete culture medium preheated at 37 °C. The culture media tested were ProCHO3 (Cambrex) or Sigma 5 supplemented with 10 mM Hepes, 0.4 mM glycine, 2 mM L-glutamine, 0.1 mM hypoxanthine, 0.016 mM thymidine and 0.20 mg/mL Geneticin (optional). The culture was incubated at 37 °C, 5% CO₂ in a humidified incubator in an orbital shaking at 110 rpm. Cell numbers and viability were recorded daily and were passaged at 6×10^5 viable cells/mL when densities of 1.5×10^6 /mL were reached. The cells were expanded to the required volume for production with fresh complete ProCHO3 or Sigma 5 medium at a cell density of 1×10^6 cells/mL. The cells were cultured at 37 °C, 5% CO₂ in a humidified orbital shaking at 110 rpm overnight. The flasks were then transferred to 31.5 °C and cultured for 6–8 days. 1 mL culture was withdrawn every day and the OSO productivity was measured by RP-HPLC. The OSO-containing CHO cell culture supernatants were clarified by centrifugation at 2500g for 25 min at room temperature to remove cells and other particulate material.

Purification

Starting with 5 L culture supernatant, an ÄKTAexplorer system from GE Healthcare was used for the chromatographic runs performed at room temperature.

Solvent/detergent treatment

Sodium phosphate (0.5 M) stock buffer, pH 7.2 was added to the supernatant to a final concentration of 20 mM. Tween-80 (Merck) and TNBP (Merck) was added to a final concentration of 1.0% and 0.3%, respectively. The supernatant was mixed by gentle magnet stirring for 1 h followed by incubation at room temperature for 4 h.

Immobilized metal ion affinity chromatography (IMAC)

Chelating Sepharose (250 mL) FF gel packed in a XK50 column (GE Healthcare) was saturated with zinc ions. Charging of zinc ions was performed according to the manufacturer's instruction. The column was equilibrated in 20 mM sodium phosphate, pH 7.2 containing 0.5 M NaCl at a flow rate of 120 cm/h. After loading 5 L S/D treated cell culture supernatant to the column at the same flow rate, unbound material was washed away with equilibration buffer for 5 CVs. The elution was carried out using stepwise elution with 3 CVs elution buffer (100 mM NaAc, pH 5.0 containing 0.5 M NaCl) at a flow rate of 60 cm/h. Fractions were collected when the UV absorbance at the peak front reached 100 mAU and was interrupted when the peak on the rear had reached 100 mAU. Around 350 mL eluate was collected and the pH was adjusted to 7.0 using 1 M Na₂HPO₄. After each run, the metal ions were stripped off the IMAC gel using equilibration buffer containing 50 mM EDTA at pH 7.2. The column was then sanitized using a solution containing 0.5 M NaOH and 0.5 M NaCl in reversed flow mode.

Hydrophobic interaction chromatography (HIC)

Sodium phosphate (20 mM) buffer containing 3.6 M ammonium sulfate (Merck), pH 7.0 was used as stock solution. One volume of the stock was added to 2 volumes of IMAC peak fraction with gentle stirring. The final concentration of ammonium sulfate was 1.2 M. After standing at room temperature (around 20 °C) for at least 2 h, the formed precipitate was removed by centrifugation at 2500g for 25 min. Around 500 mL supernatant was applied to the 50 mL HiLoad Phenyl Sepharose High Performance 26/10 column (GE Healthcare) previously equilibrated in 20 mM Na-phosphate buffer, pH 7.2 containing 1.2 M ammonium sulfate. After washing the column with 3 CVs equilibration buffer, elution was carried out using a 15 CVs linear descending ammonium sulfate concentration gradient from 1.2 to 0 M. The elution buffer was 20 mM Na-phosphate buffer, pH 7.2. A flow rate of 60 cm/h was used throughout the chromatographic run. The target protein was eluted early in the gradient. The more strongly bound proteins were then removed by continued washing with buffer without ammonium sulfate. About 20 mL fractions of the target protein were collected and subjected to further purification. The more strongly bound proteins were also collected and analyzed.

Size exclusion chromatography (SEC)

The HIC eluate (20 mL) was concentrated to 6 mL using an Amicon membrane centrifuge device with MWCO of 30 kDa (Millipore) at 3800g. The concentrated sample was loaded to a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare) equilibrated in phosphate buffered saline at a flow rate of 30 cm/h. The target protein peak (40 mL) was collected.

Nano-filtration

For virus removal the nano-filtration membrane ULTIPOR VF FTKDV20 with a diameter of 47 mm (Pall) was used. The filter was pre-wetted with PBS before mounting to the stainless steel

holder (Millipore). The OSO solution was filtered through a 0.22 μm filter before the nano-filtration. The protein solution was then added to the holder. A constant nitrogen pressure of 3.5 bar was applied and the filtrate flow was monitored by weighing. Samples were withdrawn before and after filtration.

Ultrafiltration

Before usage in pre-clinical studies, the purified OSO was concentrated to around 3.0 mg/mL using the Amicon membrane centrifuge device with MWCO of 30 kDa.

Virus clearance feasibility study

The virus clearance study was performed in collaboration with BioReliance, Stirling, UK. Both enveloped (MLV) and non-enveloped (MMV) viruses were independently spiked into the IMAC, HIC and nano-filtration starting materials. The experiments were performed as described above but in smaller scale. Only enveloped viruses were spiked into the SD treated starting material. Samples for virus count were withdrawn before and after the purification procedures.

Analytical techniques

Protein concentration determination

The protein concentration in the culture supernatant and during the purification process was determined using reversed-phase chromatography as described below. The final protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.41 at 1 mg/mL. This value was calculated from amino acid composition analysis data obtained from the Protein Analytical Center at the Karoliska Institute, Stockholm, Sweden.

SDS-PAGE and Western blot analysis

For purity check, estimation of molecular mass and for comparison with other OSO preparations, SDS-PAGE was performed using a pre-cast gel, Novex[®] 12% Tris-glycine gel (Invitrogen). LMW calibration proteins (GE Healthcare) were used as reference standards. The purified OSOs were analyzed under both reducing and non-reducing conditions. The samples at appropriate concentrations were mixed with Novex[®] Tris-glycine SDS sample buffer (2 $\times$) (Invitrogen) and with/without DTT. Around 2 μg protein was loaded to each lane. The running and staining procedures were carried out according to the manufacturer's instruction. SimplyBlue[™] SafeStain (Invitrogen) was used for staining.

After SDS-PAGE electrophoresis, the separated proteins were transferred to pre-wetted nitrocellulose membranes using the semi-dry method using the Multiphor II apparatus from GE Healthcare according to the manufacturer's instruction. After transfer, the membrane was blocked by 5% milk powder for at least 1 h. The membrane was then washed with 20 mM Tris-HCl, pH 7.2 containing 0.1% Tween-80. The primary antibody used was rabbit anti-OSO (1:2000 dilutions) polyclonal antibody (in house). The secondary antibody used was mouse anti-rabbit antibody alkaline phosphatase conjugate. The protein band was visualized by incubation with the substrate BCIP/NBT from Sigma.

Isoelectric focusing (IEF)

The isoelectric points of the OSO isoforms were determined by IEF in polyacrylamide gel. A broad pI range IEF gel, Novex[®] pH 3–10 (Invitrogen) and commercially available IEF Markers 3–10 (Serva) were used. An equal volume of purified protein (3 mg/mL) was mixed with Novex[®] IEF Sample Buffer pH 3–10 (2 $\times$) (Invitrogen) and around 20 μg of protein was loaded to each lane. The running and staining procedures were carried out according to the manufacturer's instruction with modification of the focusing time. The

power supply was programmed to increase the voltage in three steps: 100 V constant for 60 min, 200 V constant for 100 min and 500 V for 40 min. After electrophoresis, the gel was fixed with fixation solution (Sigma) and stained with SimplyBlue[™] SafeStain.

Purity analysis by SE-HPLC

Samples from the various purification stages of OSO were analyzed by SE-HPLC on a Superdex 200 HR 10/30 column (GE Healthcare). The column was equilibrated in PBS and the flow rate was 0.4 mL/min. A sample volume of 50 μL at a concentration of 1 mg/mL was loaded to the column. The protein was monitored using A280 nm and a HPLC system from Waters was used. The amount of dimer was estimated by integrating the UV signal area using the Empower Software.

Quantitative analysis of OSO by reversed-phase HPLC

The yield of the target protein in each purification step was determined by measuring the OSO concentration in the culture supernatant and in samples from the intermediate purification steps by reversed-phase chromatography using a RESOURCE RPC 3 mL column (GE Healthcare) attached to a Waters' HPLC system. Mobile phase A contained 0.1% trifluoroacetic acid (TFA) and 2% acetonitrile (ACN) in water and mobile phase B contained 0.1% TFA and 80% ACN in water. The flow rate was 2 mL/min and a gradient from 40% to 60% solvent B was applied in 15 CVs. Purified OSO at different loadings was used as calibration standard. The detection limit was 5 mg protein per liter supernatant.

Monosaccharide composition and sialic acid content analyses

The analyses were performed by Covance Laboratories Ltd., Otley Road, Harrogate, UK.

N-terminal sequence and MALDI-TOF MS analyses

The analyses were done by the Protein Analytical Center (PAC) in Karolinska Institutet, Stockholm, Sweden.

Residual CHO-host cell protein (HCP) analysis by ELISA

Residual CHO-host cell proteins were analyzed using an Immunoassay Kit from Cygnus Technologies according to the manufacturer's instruction.

High affinity human IgE receptor binding assay by ELISA

An ELISA method was developed for analyzing possible changes of the conformation of the C3 domain of the OSO protein. To this end, a human high affinity receptor α -chain Fc ϵ R1 was used. The receptor was produced in house using a cell line purchased from Molecular Immunology, International Center for Genetic Engineering and Biotechnology, Area Science Park, Trieste, Italy. It is a chimeric receptor consisting of the extracellular portion of the human α -chain of Fc ϵ R1 and a truncated human IgG₁ Fc. ELISA plates were coated with goat anti-human IgG (Pierce) in coating buffer to a final concentration of 1 $\mu\text{g/mL}$. The soluble dimer receptor chimera in culture medium containing 10% FBS was diluted 1:16 and added to the plates. OSO protein from stability study samples in appropriate dilutions were added to the plate. The OSO protein was detected using rabbit anti-OSO polyclonal IgG (in house production) at 1:2000 dilution. Mouse anti-rabbit IgG-AP conjugate was used as secondary antibody at 1:2000 dilution. The color was developed using the PNPP substrate (Sigma) and then measured at 405 nm against blank.

Clone selections by ELISA

The ELISA method used for the clone selection was to coat the plates with the monoclonal antibody AE/4F4 specific against the human IgE Fc ϵ 3 domain (RDI) at a concentration of 2 $\mu\text{g/mL}$. The OSO produced during the clone selection was added to the

plate after blocking and washing. The OSO protein was detected using rabbit anti-OSO polyclonal IgG (in house production) at 1:5000 dilution. Mouse anti-rabbit IgG-AP conjugate was used as secondary antibody at 1:1000 dilution. The color was developed using the PNPP substrate (Sigma) and then measured at 405 nm against blank.

Temperature and pH stability studies of the OSO protein

Temperature stability

The temperature stability was performed by incubating 50 μ L purified OSO solution (3.2 mg/mL in PBS) at 4 °C, room temperature (around 20 °C), 30, 40, 50 and 60 °C, respectively, for 1 h. The changes of the OSO conformation were analyzed by ELISA.

pH stability

Aliquots of the OSO (10 μ L) were added to 150 μ L of 100 mM glycine-HCl buffer at pH 2.6, 3.0 and 4.0, respectively. After incubation at room temperature (around 20 °C) for a period of 4 h, the changes of conformation were determined by ELISA.

Results and discussion

Expression

Vector and expression system

The cDNA encoding the human IgE C3 domain was inserted in four different eukaryotic expression vectors (data not shown). The constructs were transfected and evaluated in CHO-K1 and NS0 cells. Protein expression levels were determined in the clone mixes by ELISA. Based on the results obtained, the pCI-neo vector was selected for expression in the CHO cells. An immunoglobulin leader sequence was inserted together with the OSO sequence in the pCI-neo vector to ensure efficient secretion of the vaccine candidate into the cell culture medium [14]. The pCI-neo vector is shown in Fig. 1. The DNA sequence of the OSO insert contains 1182 bp and the deduced amino acid sequence including the IgG leader sequence is shown in Fig. 2 [15].

Cell line development

The OSO-pCI construct was linearized and transfected twice in CHO-K1 cells. Stable transfected cells were selected by neomycin

resistance. Approximately 5000 wells were screened with ELISA. The initial selection step identified 23 mixed clones with high ELISA values. Each clone mix was seeded in 96-well plates at densities of 1, 5, 10 and 20 cells/well as described above. The plates inoculated with 5 cells/well were analyzed by ELISA after an incubation time of 2 weeks, (Limiting #1). 40 wells containing one or two clones were identified with high ELISA values. Those 40 clones were seeded in 96-well plates at a density of 1 cell/well in supplemented selection medium and analyzed by ELISA (Limiting #2) after 2 weeks of incubation. And so on, two clones were selected after four separate cloning steps by limiting dilution. These clones (clones 7 and 35) were selected for more extensive characterization as well as adaptation from adherent to serum-free suspension culture.

Adaptation to serum-free suspension culture

The OSO producing clones 7 and 35 were selected for adaptation to suspension culture. Both clones were adapted successfully after a lengthy culture period in Erlenmeyer flasks on an orbital shaking platform. Subsequently, the cells were weaned into a serum-free medium (SFX) by gradual reduction of serum concentration. The SFX medium contains a low concentration of protein. Clone 35 required a slow adaptation process to serum-free culture, whereas clone 7 adapted more easily. Samples from cultures were analyzed by ELISA throughout the suspension and serum-free adaptation process in order to monitor the OSO expression levels. Clone 35 was easily adapted by a few passages from SFX to a new serum-free medium, ProCHO3-CDM. This is a chemically defined medium containing 10 mg/L of recombinant insulin as the sole protein component. Furthermore, this medium is formulated exclusively with non-animal origin components. The cell cycle time in this medium for an established culture was approximately 24 h. Cells from clone 35 were further cloned by more limiting dilution in order to ensure monoclonality after serum-free adaptation. Three single clones (A10, D3 and G12) were selected based on ELISA reactivity and growth characteristics. These clones were expanded and adapted to serum-free suspension growth in ProCHO3-CDM. Cell banks with the clones A10, D3 and G12 were thus established in serum-free medium. Viabilities were more than 95% at the time of production. The banks were submitted to tests and free from microorganisms such as bacteria or mycoplasma.

Production of OSO

A process for production of OSO in shaker flasks was used. Cell numbers and viability were monitored and supernatant was harvested when high cell densities were reached. However, relatively low amounts of OSO (less than 10 mg/L) were detected in the supernatants at production temperature of 37 °C. Therefore, a number of parameters for the culture process were studied in order to find optimal conditions for OSO expression. Preliminary observations suggested that OSO was mainly produced when cells reached confluence or were arrested from proliferation. Thus, the effect of cell density on the OSO expression level was studied by varying the inoculation density and the temperature during the production phase. The OSO expression level increased dramatically after lowering the temperature (data not shown). The productivity was almost 10-fold higher when the production took place at 30 °C in comparison with 37 °C. The cell viability was >80% after 8 production days at 30 °C. The optimal seeding density was $1.25\text{--}1.5 \times 10^6$ viable cells/mL. Culture media from different vendors were studied. An important observation was that different OSO variants were produced in different media and this will be discussed later in this paper. The productivity of OSO in Sigma 5 medium reached a level of 60 mg/L after 8 days of culture at 31.5 °C. No difference in OSO productivity could be seen between a 30 mL and 1 L scale.

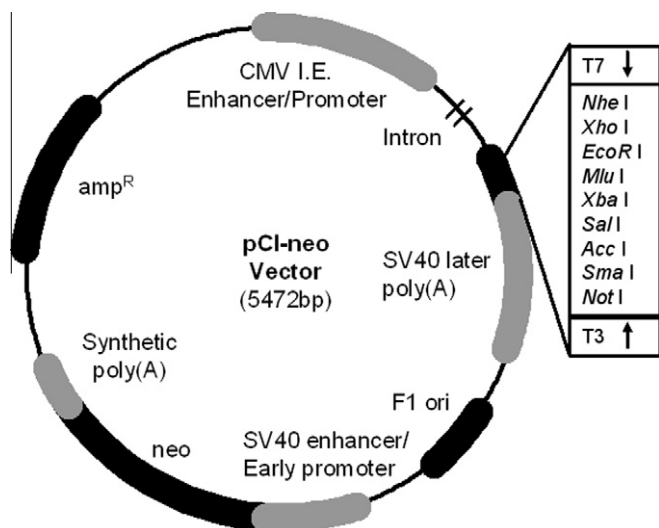


Fig. 1. Diagram of the pCI-neo vector. Multiple cloning sites are between 1085 and 1137. The DNA was inserted to the SmaI site.

CGGGCCGACCTCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGT	91
G P T S P W D G A V S S S S W St Q Q L Q V R G S Q St Q A St G	
CTGGACATATATATGGGTGACAAATGACATCCACTTTGGCCTTTCTCTCCACAGGTGTGCATTCTCTCGAGTACTTTATCTCTCCAGAAAGT	181
L D I Y M G D N D I H F A F L S T G V H S S S T L S L P E S	
GGCCCTGTGACAAATCATCCACCTACAGTGAAGCTCTTCCACTCATCTGTGACCCCCGAGGGGATGCTCATTCCACCATCCAGCTGCTC	271
G P V T I I P P T V K L F H S S C D P R G D A H S T I Q L L	
TGCTTTGTCTCTGGCTTCTCCCCAGCCAAGGTCCATGTGACCTGGCTGGTAGATGGACAGGAGGCTGAAAATCTCTTTCCCTATACAACC	361
C L V S G F S P A K V H V T W L V D G Q E A E N L F P Y T T	
AGACCTAAGAGGGAAGGGGGACAGACTTTTTCTCTACAAAGTGAAGTCAACATCACACAGGGCCAGTGGATGTCATCAAACACCTACACC	451
R P K R E G G Q T F S L Q S E V N I T Q G Q W M S S N T Y T	
TGCCATGTCAAGCACAAATGGCAGCATCTTTGAAGACAGTCTCTAGAAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCTACCTAAGC	541
C H V K H N G S I F E D S S R K C A D S N P R G V S A Y L S	
CGGCCCAGCCCGTTTCGACCTGTTTCATCCGAAGTCGCCACGATCACCTGTCTGGTGGTGGACCTGGCACCCAGCAAGGGGACCGTGAAC	631
R P S P F D L F I R K S P T I T C L V V D L A P S K G T V N	
CTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAACCACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCC	721
L T W S R A S G K P V N H S T R K E E K Q R N G T L T V T S	
ACCCTGCCGTTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCAGTGCAGGGTGACCCACCCCACTGCCAGGGCCCTCATGCGG	811
T L P V G T R D W I E G E T Y Q C R V T H P H L P R A L M R	
TCCACGACCAGCTTGTCTAGC	901
S T T K L A S P G K R L A P E V Y M L P P S P E E T G T T R	
ACTGTAACTGCTTAATTCGGGGTTTCTACCCTTCTGAAATATCTGTCCAATGGCTGTTTAATAACGAAGAGGACCACACTGGACACCAT	991
T V T C L I R G F Y P S E I S V Q W L F N N E E D H T G H H	
ACTACCACCCGTCCCCAAAAGGACCACGGAACGGATCCTTCTTCTTCTCTACAGCCGAATGCTTGTCACAAAGTCTATTTGGGAAAAA	1081
T T T R P Q K D H G T D P S F F L Y S R M L V N K S I W E K	
GGCAATCTCGTCACCTGCCGTGTGGTGCATGAAGCCCTACCTGGCTCCCGCACCCTGGAAAAAAGCCTGCATTACTCAGCTGGTAAC	1171
G N L V T C R V V H E A L P G S R T L E K S L H Y S A G N St	
TCTCGAGTGCA	1182
S R V	

Fig. 2. The DNA sequence of the OSO insert contains 1182 bp. The deduced amino acid sequence is below the DNA sequence. Underlined bases indicate the signal sequence. Double underlined bases indicate the introduced restriction enzyme cleavage sites flanking the opossum C2-sapien C3-opossum C4 domains and not part of the original nucleotide sequence. Dashed underlined bases indicate translational start and stop positions. Underlined amino acids correspond to the start position of mature OSO as determined by N-terminal sequence analysis. St indicates the stop codons. The mature protein contains 338 amino acid residues.

OSO purification

The design of a manufacturing process for a vaccine component based on animal cell culture requires certification that no viruses are present in the purified product. Literature data have shown that immobilized metal ion affinity chromatography may reduce the level of both enveloped viruses (6.5 log) and non-enveloped viruses (4.0 log) [17]. However, under the IMAC conditions used in this work, the reduction log for both types of viruses was poor. This is why it is required that the other chromatographic steps used in the purification process must contribute significantly to the virus clearance. Thus the hydrophobic interaction chromatography step reduced the levels of both MMV (1.92 log) and MLV (2.24 log). However, in order to further reduce the virus levels other removal/inactivation steps were required. Results from short-term stability studies indicated that inactivation using low pH or high temperature was not possible due to the instability of the recombinant OSO protein (Fig. 3). The OSO protein is not stable at temperatures above 40 °C. 98% of the OSO proteins were denatured at 60 °C which is a common temperature used for virus inactivation. Neither is OSO stable at low pH; the protein entirely lost its activity at pH 4.0. This is why inactivation of enveloped viruses by solvent/detergent treatment was incorporated into the process. The current condition (0.3% TNBP and 1% Tween-80 for

an incubation period of 4 h) is commonly used during plasma protein fractionation [18]. The reduction log was above 6 after incubation for 5 h. No effect on the OSO protein was observed after incubation with solvent/detergent for less than 8 h. Both enveloped and non-enveloped viruses were further reduced after introduction of a nano-filtration step where a reduction log of ≥ 7.53 and 5.88 was obtained for MLV and MMV, respectively.

Due to the large volume of starting material, a combination of both concentration and purification is desirable as a first step in the purification procedure. OSO contains as many as 28 histidine residues in its two polypeptide chains why the use of IMAC as a first purification step is a suitable choice. Zinc ions were used to saturate the Chelating Sepharose FF gel to give a suitable selectivity. A 5-fold purification was obtained in this step with a 15-fold volume reduction and a recovery of 80–90%. Imidazole, the reagent used as displacer in common IMAC step, was not used due to possible toxicity since the process will later produce material for clinical trials. Instead, elution by lowering the pH using acetate buffer was used and turned out to be very successful. Safety reasons are also why zinc ions were chosen instead of nickel ions. Sodium acetate is an inexpensive buffer salt commonly used in biopharmaceutical processes. Using a sodium acetate buffer at pH 5.5 only 50% of the target protein was eluted as estimated by RP-HPLC. However, reducing the pH to 5.0 allowed 100% elution of OSO. A buffer

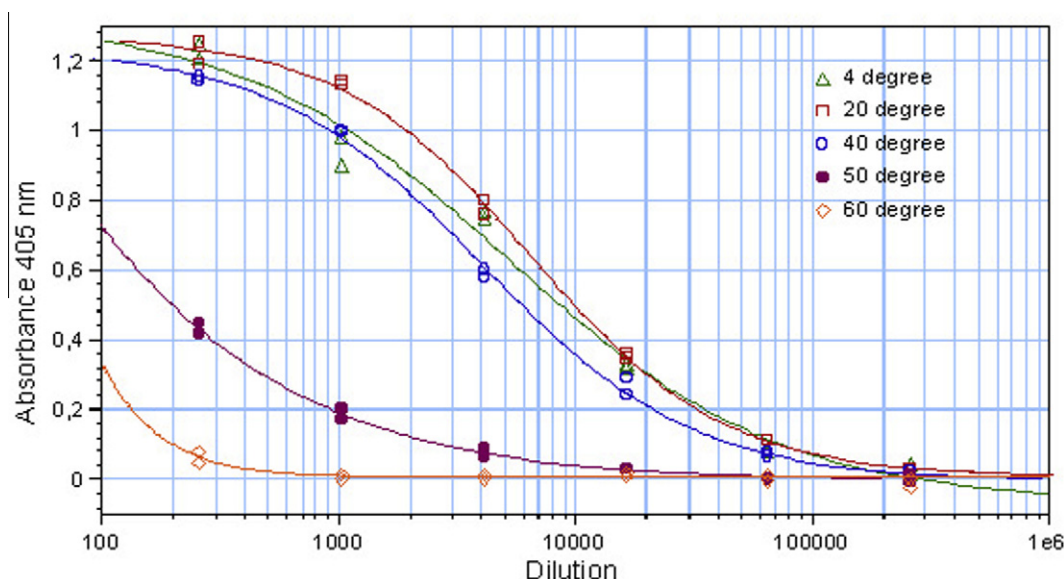


Fig. 3. OSO temperature stability study. Changes of the OSO conformation were analyzed by ELISA. The study was performed by incubating 50 μ L purified OSO solution (3.2 mg/mL in PBS) for 1 h at 4 $^{\circ}$ C, room temperature (around 20 $^{\circ}$ C), 30, 40, 50 and 60 $^{\circ}$ C, respectively.

strength of 100 mM gave an adequately sharp peak. The recovery of the target protein was comparable to that obtained with imidazole as elution agent.

In order to achieve a sufficient binding strength of OSO to the Phenyl Sepharose HP gel, an ammonium sulfate concentration of 1.2 M was required. After an incubation period of 2 h the precipitate formed was removed by centrifugation. This precipitation step turned out to be highly advantageous since around 50% of the host cell proteins remaining after the IMAC step were removed in this way. The OSO primary structure reveals regions rich in hydrophobic amino acid residues, some of which are likely to be exposed at or near the protein surface. So, HIC is a logical choice for a second step, particularly so as the OSO solution eluted from the IMAC column is high in salt. Immunoglobulins are known for their affinity towards thioether-substituted organic sulfone compounds, the effect sometimes called “thiophilic” interaction [19]. Since adsorption depends on the presence of a cosmotropic salt (e.g. ammonium sulfate), this method combines the advantage of an easily controllable adsorption behavior with chemical stability of the matrix and high protein binding capacity. Two gel media worked very well including HiTrap IgY (2-mercaptopyridyl groups coupled to Sepharose High Performance) and Phenyl Sepharose HP. Phenyl Sepharose FF could not be used because the target protein binds to the column irreversibly. A relatively sharp peak was obtained on both the HiTrap IgY column and the Phenyl Sepharose HP gel due to small particle size ($\sim 34 \mu$ m). Finally, mainly for economic reasons, Phenyl Sepharose High Performance was chosen for the second purification step. The major advantage of HIC is that different variants of OSO can be separated which may not be possible using other methods. Thus the desired intact OSO eluted early in the descending ammonium sulfate gradient, whereas OSO aggregates and degradation products eluted later (Fig. 4A). These OSO variants will be further discussed in the characterization section.

Size exclusion chromatography on Superdex 200 was used as a final polishing step. Trace amounts of contaminant proteins, possible OSO degradation products or aggregates were removed and the product was transferred to the desired formulation buffer (PBS).

A 56-fold purification based on the protein content of the original cell culture supernatant was achieved in the OSO process. After the purification, a total recovery of around 33% was obtained as

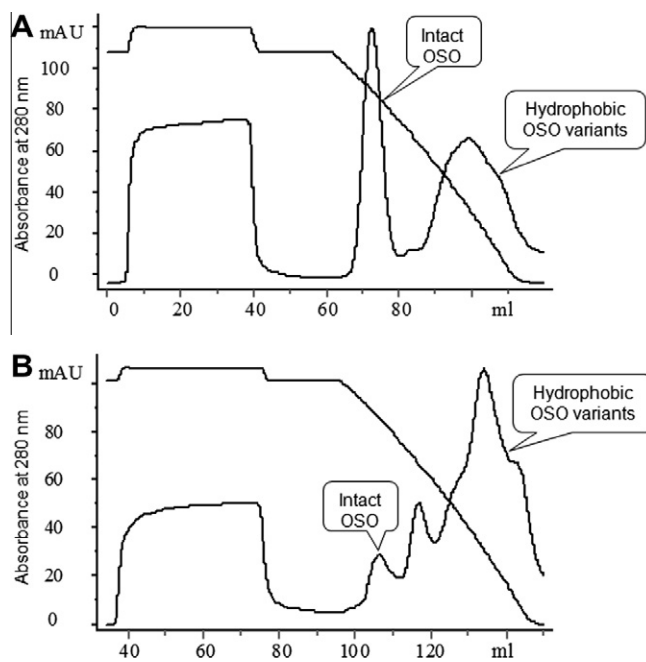


Fig. 4. Separation of OSO variants by hydrophobic interaction chromatography on Phenyl Sepharose HP. A descending ammonium sulfate concentration gradient in 15 CVs was applied. The starting material was OSO produced (A) in Sigma 5 medium and (B) in Sigma 2 medium. The intact OSO and hydrophobic OSO variants are indicated. The solid line represents absorbance at 280 nm and the dotted line represents conductivity.

determined by RP-HPLC (Table 1). Approximately 89 mg pure protein was obtained from 5 liters cell culture supernatant.

Binding assay and stability of OSO

In order to study the conformation of the CHO cell produced OSO, a soluble receptor-Ig chimera, in which the two extracellular domains of human Fc ϵ RI α -chain (D1 and D2) were fused to the dimerized C-terminal domain of human IgG1 heavy chain (gamma1-CH3), was used [16]. This protein binds secreted and membrane IgE of native conformation via the C3 domain. The OSO protein should also bind the high affinity receptor if the C3

Table 1

Recovery of OSO purified from the CHO cell culture supernatant.

Step	Amount of OSO (mg) ^a	Recovery (%)
Harvest	310	100
Solvent/detergent treatment	308	99
Chelating Sepharose FF	262	85
Phenyl Sepharose HP (desired OSO)	136	52
Phenyl Sepharose HP (non-desired OSO)	105	40
UF and Superdex 200 pg	110	81
Manofiltration	89	94
Total recovery of desired OSO		33

^a Results are expressed in terms of mg OSO determined by RP-HPLC.

domain of OSO is folded like native IgE. An ELISA method was established using the FcεRI alpha-chain chimeric protein as a capture reagent for OSO. A polyclonal antiserum (purified IgG) against opossum IgE was used for detection of bound protein. The ELISA re-

sults show that the OSO produced in the CHO cell lines binds to the high affinity receptor of IgE. Purified OSO protein which has been subject to heat and low pH could not be recognized by the alpha-chain indicating that proper folding is important. Temperature stability data are shown in Fig. 3.

Characterization of OSO

N-terminal sequence analysis of 18 amino acid residues was used to confirm the OSO identity (Fig. 1). In Fig. 5 is shown the result of SDS-PAGE analysis under reducing conditions of samples from the different steps of the purification process. The corresponding analysis under non-reducing conditions of purified OSO is shown in lane 7 of Fig. 5. The position of the single band representing pure OSO after the SEC step indicates the presence of two identical polypeptide chains with a molecular weight around 50 kDa. The purity is estimated to be above 99%.

The purified OSO was also analyzed by SEC and no aggregates or degradation products could be observed in the final purified protein preparation. The dimer content was estimated to around 0.1% (data not shown).

The molecular weight of OSO was determined as 89,417 Da by MALDI-TOF MS (Fig. 6). The theoretical molecular weight of the two peptides is 75,092 Da indicating that the protein is glycosylated. There are five putative glycosylation sites in each peptide chain as predicted using the NetNGlyc 1.0 Server [20] in the ExPASy Proteomics Server of the Swiss Institute of Bioinformatics. All immunoglobulins are glycoproteins and the glycosylation sites are found mainly in the constant regions of the heavy chain [21]. The monosaccharide composition of the OSO protein includes glucosamine (51.0%), mannose (25.7%), galactose (15.5%) and small amounts of glucose, fucose and galactosamine.

IEF analyses gave rise to typical ladder patterns with isoelectric points in the pH-range 7.0–8.3 indicating the presence of sialic acid (Fig. 7). The sialic acid content has been determined and is composed of 1.1% *N*-glycosylneuraminic acid and 98.9% *N*-acetylneuraminic acid.

The presence of around 30 ng residual CHO-host cell proteins per mg purified OSO could be detected in the final purified protein solution which should be compared with a starting concentration of 1.2×10^5 ng/mg OSO in the harvested cell culture supernatant. Thus a 4000-fold reduction was achieved. The endotoxin levels were below 2 EU/mg OSO protein.

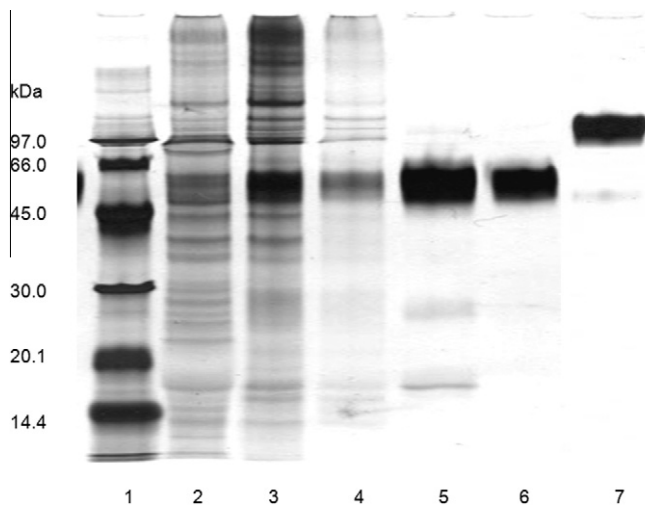


Fig. 5. SDS-PAGE analysis of OSO fractions withdrawn during different chromatographic steps on a 12% gel under both reducing (from lane 1 to 6) and non-reducing (lane 7) conditions. 2 mg was loaded to each lane. Lane 1, LMW marker; lane 2, cell culture supernatant; lane 3, IMAC eluate; lane 4, After ammonium sulfate precipitation; lane 5, HIC eluate, lane 6, purified OSO and lane 7, purified OSO.

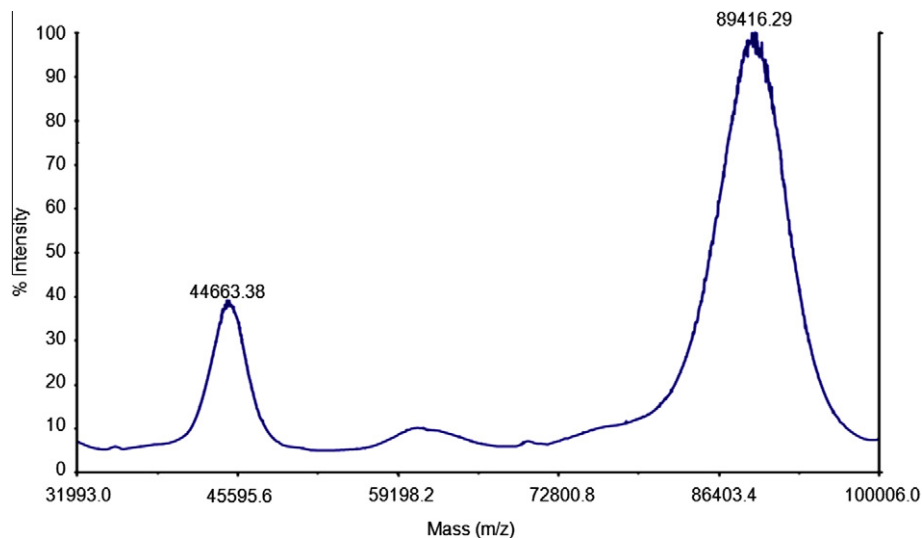


Fig. 6. MALDI-TOF analysis of OSO. The analysis was performed at the Protein Analysis Center (PAC), Karolinska Institute, Stockholm, Sweden. The mass obtained was 89,417 Da.

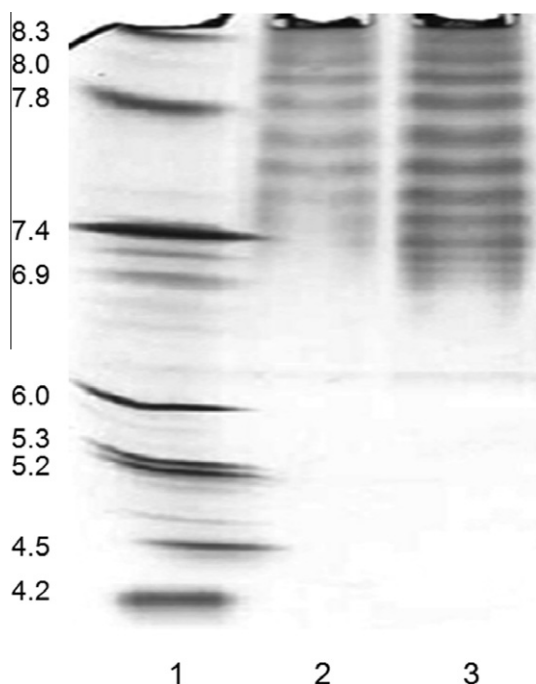


Fig. 7. IEF analysis of OSO on Novex® pH 3–10 (Invitrogen). Lane 1, broad pI marker; lane 2, 5 µg sample and lane 3, 20 µg sample.

As discussed above, HIC is a powerful step for the separation of the desired intact OSO from its variants, e.g., aggregates or degradation products that are more hydrophobic. Thus, aggregates of

OSO eluted later in the descending ammonium sulfate concentration gradient. During the expression studies, it was found that different cell culture media gave different proportions of the desired intact form of OSO. Two HIC chromatograms from separation of OSO produced in different media are shown in Fig. 4. The more favorable OSO composition is shown in Fig. 4A. The ratio between the intact and the aggregated/degraded forms of OSO varied from 60:40 to 20:80. The HIC fraction containing the non-desired more hydrophobic form of OSO has been further purified by size exclusion chromatography on Superdex 200 prep grade (Fig. 8A). Comparison with the Gel Filtration HMW Calibration Kit (GE Healthcare) running on the same column under the same conditions, showed that the more hydrophobic OSO is composed of molecules varying in size from around 90 to 500 kDa. This result is confirmed by SDS–PAGE under non-reducing conditions and Western blot analysis of corresponding fractions. However, the high molecular weight aggregates can be reduced by DTT or 2-mercaptoethanol (Fig. 8B). After SDS–PAGE under reducing conditions and transfer to a cellulose membrane, the bands obtained were stained with Coomassie blue and subjected to N-terminal sequence analysis. Two degradation sites were identified (Fig. 8B). It is thus important that the more hydrophobic forms of OSO produced during cell culturing are removed during the downstream process to ensure adequate quality of the final product.

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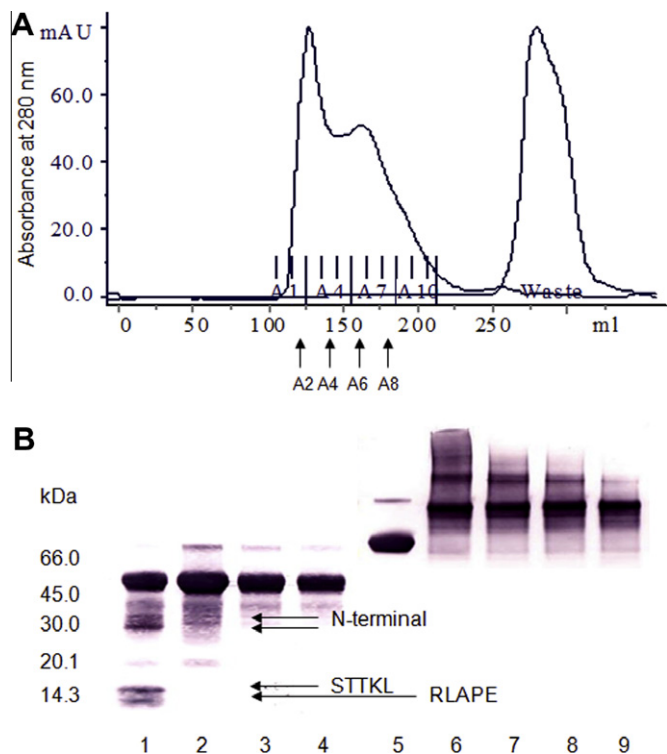


Fig. 8. (A) Preparative separation of the hydrophobic OSO variants by size exclusion chromatography on a Superdex 200 pg 26/60 column. The solid line represents absorbance at 280 nm, while the dotted line represents conductivity. (B) The SEC fractions were analyzed by SDS–PAGE under both reducing (from lane 1 to 4) and non-reducing (from lane 5 to 9) conditions followed by Western blot analysis. Lane 1 and 6, fraction A2; lane 2 and 7, fraction A4; lane 3 and 8, fraction A6; lane 4 and 9 and lane 5, purified intact OSO. The N-terminal sequences of the degradation products are indicated.

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