Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 347 (2006) 4–11
BBRC
www.elsevier.com/locate/ybbrc

Expression and purification of the soluble isoform of human receptor for advanced glycation end products (sRAGE) from *Pichia pastoris*

Thorsten Ostendorp [a], Mirjam Weibel [b], Estelle Leclerc [b], Peter Kleinert [b], Peter M.H. Kroneck [a], Claus W. Heizmann [b], Günter Fritz [a,*]

[a] *Fachbereich Biologie, Mathematisch-Naturwissenschaftliche Sektion, Universität Konstanz, 78467 Konstanz, Germany*
[b] *Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, CH 8032 Zürich, Switzerland*

Received 11 April 2006
Available online 21 June 2006

Abstract

RAGE is a multi-ligand receptor involved in various human diseases including diabetes, cancer or Alzheimer's disease. Engagement of RAGE by its ligands triggers activation of key cellular signalling pathways such as the MAP kinase and NF-κB pathways. Whereas the main isoform of RAGE is a transmembrane receptor with both extra- and intracellular domains, a secreted soluble isoform (sRAGE), corresponding to the extracellular part only, has the ability to block RAGE signalling and suppress cellular activation. Administration of sRAGE to animal models of cancer or multiple sclerosis blocked successfully tumour growth and the course of the autoimmune disease. These findings demonstrate that sRAGE may have a potential as therapeutic. We present here a fast and simple purification protocol of sRAGE from the yeast *Pichia pastoris*. The identity of the protein was confirmed by mass spectrometry and Western blot. The protein was *N*-glycosylated and 95–98% pure as judged by SDS–PAGE.

Keywords: sRAGE; Receptor for advanced glycation endproducts; Expression; Purification

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors [1,2]. RAGE is capable to bind various ligands such as the advanced glycation end products [2], S100 proteins [3–5], amphoterin [6] and the β amyloid peptide [7]. Engagement of RAGE by these ligands triggers activation of key cell signalling pathways such as the MAP kinase, NF-κB or cdc42/rac pathways resulting in cellular reprogramming [1,8,9]. Long-term activation of RAGE is implicated in the pathogenesis of diabetic vascular disease, chronic inflammations and neurological disorders such as Alzheimer's disease [7]. Full-length RAGE is composed of an extracellular domain, a single membrane spanning helix, and a short C-terminal cytosolic domain [10]. The extracellular domain is implicated in ligand binding and recognition and contains one ''V''-type followed by two ''C''-type immunoglobulin-like domains. The intracellular domain is important for RAGE-mediated signalling. Besides full-length RAGE two other major isoforms of RAGE have been identified, which are derived from alternative splicing of the RAGE gene. One isoform, (dominant negative RAGE) is devoid of the intracellular signalling domain and prevents activation of full-length RAGE by its ligands. The second major isoform (sRAGE) is a soluble form of the receptor containing the extracellular region only. The inhibition of RAGE signalling by the secreted sRAGE might occur by sequestering circulating RAGE ligands or competing with full-length RAGE for binding to adhesion molecules such as β2-integrin Mac-1 [11]. Such an interaction would inhibit the leukocyte recruitment by blocking the adhesion to the endothelium, which is an essential step in the institution of an inflammatory response. Several studies using animal models have shown

* Corresponding author. Fax: +49 7531 88 2966.
E-mail address: guenter.fritz@uni-konstanz.de (G. Fritz).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.077

that sRAGE is a pharmaceutically relevant therapeutic molecule with applications in cancer [6], chronic autoimmune inflammatory diseases [12] or diabetic disorders [13,14]. So far the administered sRAGE was expressed and purified from a baculovirus expression system [5]. We report here a novel and rapid purification protocol of recombinant human sRAGE from *Pichia pastoris*. The purified sRAGE will enable us to investigate the binding mode of further RAGE ligands based on in vitro experiments as well as on X-ray crystallographic data.

Materials and methods

Materials. *Pichia pastoris* strains X-33 and KM71H, *Escherichia coli* strain DH5α, plasmid pPICZαA, antibiotic Zeocin and YNB (yeast nitrogen base) were purchased from Invitrogen (Karlsruhe, Germany). Yeast extract and tryptone for bacterial media preparations were obtained from Applichem (Darmstadt, Germany). Carboxymethyl-Sepharose was purchased from GE-Healthcare (Munich, Germany). All other chemicals were purchased from Sigma; Division of Fluka Chemie (Buchs, Switzerland). cDNA clones of human RAGE were a generous gift of P. Nawroth (University of Heidelberg, Germany). Purified sRAGE from HEK293 cells was a generous gift by B. Weigle (University of Dresden, Germany). For the cloning of sRAGE into *P. pastoris*, the *Taq* polymerase and the ligase were from Roche Diagnostics, the restriction enzymes were from Promega. The QIAquick gel extraction and nucleotide removal kits were from Qiagen (Hombrechtikon, Switzerland).

Generation of antibodies against human RAGE. Polyclonal antibodies against RAGE were raised in rabbits. The amino acid sequence of human RAGE (Swiss-Prot Accession No. Q15109) was used for the epitope search. One peptide from each IgG-like domain, (V-, C1- and C2-domain) was chosen for the generation of the antibodies. The selection criteria were based on a 15 amino acid length with a high content of hydrophilic residues in order to have a high probability for solvent exposure. Blast searches were carried out for the sequence of the chosen epitopes to ensure the specificity of the generated sRAGE antibodies. The three epitopes were chemically synthesized, coupled with the carrier protein keyhole limpet hemocyanin (KLH) and injected into rabbits (Eurogentec, Seraing, Belgium). The immunoreactivity of the serum of the rabbits was tested by dot blot assays using human sRAGE expressed in HEK293 cells. When antibody levels reached a plateau, the serum was collected and stored at −80 °C.

SDS–PAGE and Western blotting. 12.5% acrylamide/bisacrylamide gels were used for the separation of the proteins [15]. The gels were stained with Coomassie blue according to Zehr et al. [16]. For the Western blotting experiments, the proteins were transferred to PVDF membranes on a semi-dry blot device (Biometra, Goettingen, Germany). Antisera against peptides derived from human sRAGE (vide supra) were diluted 1:1000 in TBS containing 0.05% Tween 20 (TBST). After blotting, the membrane was blocked for 1 h with 10% (v/v) Rotiblock (Roth, Karlsruhe, Germany) diluted in TBS. The blot was then incubated for 2 h in the presence of the antisera raised against sRAGE. The membrane was washed three times for 10 min with TBST and incubated for 30 min with anti-rabbit alkaline-phosphatase conjugate (Sigma, Division of Fluka Chemie GmbH, Buchs, Germany) diluted 1:10,000 in TBST. Afterwards the membrane was washed three times for 5 min with TBST and the blot was stained using BCIP and NBT in 100 mM Tris–Cl, 100 mM NaCl and 5 mM $MgCl_2$, pH 9.5.

Media for P. pastoris and E.coli. LB low salt medium was prepared for growth of *E. coli* carrying plasmids with Zeocin resistance. For Zeocin to be active, the salt concentration of the medium should remain low (<90 mM) and the pH adjusted to 7.5. The medium contained 5% yeast extract, 10% tryptone and 5% NaCl; the pH was adjusted with NaOH to 7.5 prior to sterilization in an autoclave.

Pichia pastoris strains were grown on complex or on minimal media. *Pichia pastoris* growth media and expression media were prepared as described by the manufacturer (Invitrogen, Karlsruhe, Germany). For selection of transformants Zeocin was added at a final concentration of 100 μg/ml.

Cloning of sRAGE into pPICZαA. The primers used for the cloning of sRAGE into *pPICZαA* were designed using the nucleotide sequence published in the GenBank database (GenBank Accession No. AB036432) and contained the two restriction sites *Eco*RI and *Xba*I. Furthermore, a stop codon was introduced before the *Xba*I site in order to express the proteins without a C-terminal His-tag, which is encoded on pPICZαA plasmid. The following forward (5′-TAA GAA TTC GCT CAA AAC ATC ACA GCC CG-3′) and reverse primers (5′-TAA TCT AGA TCA TTC GAT GCT GAT GCT GAC-3′) were used to amplify sRAGE using the following PCR conditions: 2 min at 94 °C followed by 30 cycles of 15 s at 94 °C, 30 s at 57 °C, and 2 min at 72 °C; the final extension was for 7 min at 72 °C. The PCR products were analysed on 1% agarose gel and extracted from the agarose gel using the DNA extraction kit. The DNA was digested with *Eco*RI and *Xba*I (Catalys AG, Wallisellen, Switzerland) and purified using the Nucleotide Removal Kit (Qiagen, Hombrechtikon, Switzerland). The pPICZα vector was digested with the same two enzymes and purified from 1% agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Hombrechtikon, Switzerland).The digested human sRAGE (909 bp) was ligated into the linearised vector pPICZαA at 25 °C for 5 min using the Rapid Ligation Kit (Roche Applied Science, Rotkreuz, Switzerland). The vector contains the sequence of the *Saccharomyces cerevisiae* α-factor secretion signal which leads to secretion of the expressed gene.

Electrocompetent *E. coli* strain DH5α cells were prepared according to Sharma and Schimke [17]. The cells were transformed with 50 ng DNA of the ligation reaction mixture and selected on low salt LB agar containing 25 μg/ml Zeocin. Single colonies were selected and the sequence of the isolated plasmids was analysed to verify the presence of the correct insert. The correct plasmids were then amplified in *E. coli* DH5 cells for later transformation in *P. pastoris*.

Transformation of P. pastoris. Ten micrograms of plasmid was linearised with 3 U of *Sst*I for 2 h at 37 °C. The linearised plasmid was purified by phenol/chloroform extraction and precipitated by addition of 1:10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. After centrifugation, the resulting DNA pellet was washed with 70% ethanol, air-dried and dissolved in 10 μl water. Electrocompetent cells of *P. pastoris* were prepared according to the instruction of the manufacturer (Invitrogen, Karlsruhe, Germany) and directly used for the transformation with the linearised plasmid. Eighty microlitres of cells were transformed with 10 μg linearised plasmid in 0.2 mm cuvettes using the following conditions: a pulse of 2.5 kV, 25 microF and 200 Ohm (GenePulser, Bio-Rad, Reinach BL, Switzerland). One millilitre of 1 M ice-cold sorbitol was added immediately after the electroporation. The cells were then incubated at 30 °C without shaking for 1 h. Transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% glucose and 1 M sorbitol) plates containing 100 μg/ml Zeocin for 3–10 d at 30 °C.

Selection of P. pastoris strains for human sRAGE expression. Three different strains of *P. pastoris* were transformed with pPICZAα-sRAGE: *P. pastoris* wild-type strain X-33, strain GS115 carrying a mutation in the histidinol dehydrogenase gene, and strain KM71H carrying a defect in the alcohol oxidase AOX1 gene. The mutation in the AOX1 gene results in a strain that grows very slowly on methanol as carbon source. Growth on methanol is still possible since a second alcohol oxidase AOX2 is expressed, however, at a very slow rate compared to wild-type strain. The gene coding for sRAGE is under the control of the strong AOX1 promoter and can be induced by addition of methanol to the medium.

Four colonies of the transformed *Pichia* X-33, GS115 and KM71H strains were patched on BMGY plate containing additionally 0.5% methanol for induction of sRAGE expression. The plate was incubated at 28 °C for 24 h. A PVDF membrane was cut to the size of the plate and carefully placed on top of the yeast patches for 2 h. Afterwards the PVDF membrane was removed and carefully washed with PBS. Expression and secretion of sRAGE by the different *Pichia* strains was monitored on the PVDF membrane by immuno-staining with the antiserum directed against C2 domain of sRAGE.

Expression of sRAGE in X-33. Liquid cultures of *P. pastoris* for sRAGE expression were grown in baffled flask at 28 °C in a top shaker at 250 rpm. Twenty-five millilitres of BMMY medium containing 100 μg/ml Zeocin was inoculated with a single colony of X-33/sRAGE and grown for about 16–18 h until an OD_{600} of 2–6 was reached. This preculture was used to inoculate 400 ml of BMMY medium ($OD_{600} = 1.0$). 0.5% methanol was added every 24 h to maintain induction. After 72 h, the culture was chilled on ice and centrifuged at 4000*g*. The supernatant containing sRAGE was collected.

Expression of sRAGE in KM71H. Twenty-five millilitres of BMGY medium containing 100 μg/ml Zeocin was inoculated with a single colony of KM71H/sRAGE. Expression of sRAGE by KM71 H was performed either in BMMY or in BMGY medium (100 μg/ml Zeocin), supplemented with 0.5% methanol.

For expression in BMMY the 25 ml preculture was used to inoculate another 200 ml BMGY (100 μg/ml Zeocin) culture which was grown for 24 h until an $OD_{600} = 25$ was reached. The cells were harvested by centrifugation and resuspended in 400 ml BMMY medium (100 μg/ml Zeocin). Alternatively, the 25 ml preculture was transferred to 400 ml BMGY (100 μg/ml Zeocin) supplemented with 0.5% methanol. The culture was grown for 72 h and 0.5% methanol was added every 24 h to maintain induction. Afterwards the culture was chilled on ice and the supernatant containing sRAGE was collected. In both cases, the protein expression was maintained for 72 h by adding 0.5% methanol every 24 h. After 72 h, the culture was chilled on ice and the culture supernatant was collected after centrifugation at 4000*g*.

Purification of sRAGE. The purification of sRAGE from KM71H/sRAGE and X-33/sRAGE was performed as described below. The supernatant was first filtered through 0.2 μm, diluted 3-fold with water and the pH was adjusted to 6.0 using 1 M MES. This solution was applied either to a cation-exchange RESOURCE S30 column (1.0 × 2 cm or 1.6 × 15 cm; GE-Healthcare) connected to an FPLC system (GE-Healthcare, Munich, Germany). After loading, the column was washed with 20 mM MES, pH 6.0, until the absorption at 280 nm reached baseline. The bound sRAGE was eluted with a linear gradient of 0–50 mM MES, 1 M NaCl, pH 6.0. Alternatively the solution was applied to carboxymethyl column (2.6 × 15 ml GE-Healthcare, Munich, Germany), washed with 20 mM MES, pH 6.0, and eluted in a single step with 50 mM MES, 1 M NaCl, pH 6.0. The eluted protein was concentrated by ultrafiltration using a 30 kDa cut-off membrane (Millipore, Volketswil, Switzerland).

Mass spectrometric analysis and identification of glycosylation site. Ten microlitres of human recombinant sRAGE was desalted using C4 ZipTips® (Millipore, Bedford, MA, USA). The eluate was dried in a vacuum centrifuge and digested with 10 μl of a buffer containing 12.5 ng/μl of trypsin (Promega, Madison, WI, USA) and 25 mM NH_4HCO_3 at 37 °C overnight.

For the identification of *N*-glycosylation sites the N-linked glycans were cleaved enzymatically from the protein before digestion with trypsin. The desalted and dried sample was incubated in 10 μl of a 0.1% SDS-solution at 95 °C for 5 min. Oligosaccharides were released from the protein by incubating overnight at 37 °C in 100 μl of a solution containing 0.1 U/μl *N*-Glycosidase F (Roche Diagnostics, Mannheim, Germany) in 2 mM Tris (pH 7). The deglycosylated protein was diluted to 500 μl with water and concentrated using Centricon YM-3 Filter Devices (Millipore, Bedford, MA, USA). The concentrate was dried and digested with trypsin as described above.

0.5 μl of the protein digests was applied onto a 600 μm AnchorChip™ MALDI-target. Subsequently, 1.1 μl of matrix solution was added on top of the analyte solution and dried at room temperature. A 1:10 dilution of saturated HCCA (α-cyano-4-hydroxycinnamic acid in 33% CH_3CN, 0.1% TFA) in Ethanol/Aceton 2:1 was used as matrix solution. On-target washing was performed using 2 μl of 0.1% TFA.

Peptide mass mapping was performed with an autoflex® MALDI-TOF MS (Bruker Daltonics®, Faellanden, Switzerland).

Determination of secondary structure content using CD spectroscopy. Circular Dichroism (CD) spectra of sRAGE were recorded on a Jasco J-715 instrument (Gross-Umstadt, Germany). CD spectra were recorded in 10 mM Acetate, pH 5.0, in 0.10 cm and 0.01 cm quartz cells between 180 and 260 nm. The secondary structure content was calculated using the program CDNN 2.1 [18,19].

Determination of CML binding affinity. Binding of carboxymethyl lysine (CML) to sRAGE was followed by the change in tryptophan fluorescence emission of sRAGE. Excitation wavelength was 290 nm and the emission spectra were recorded from 320 to 450 at 24 °C using a Perkin-Elmer (Wellesley, MA, USA) LS50-B spectrofluorimeter equipped with thermostatic cell holders. The measurements were performed in 1.2 ml of 20 mM Hepes, 100 mM NaCl, pH 7.5, with 200 nM sRAGE and sequential addition of 2 μl aliquots (5 nM) of CML. The change of fluorescence was recorded after 2 min equilibration. The binding data were modelled by a hyperbolic function assuming a single binding site of CML per sRAGE.

Results and discussion

Generation of antibodies directed against sRAGE

We decided to produce polyclonal antibodies against sRAGE in order to facilitate the selection of the expression strains and to follow each step of the purification. These antibodies could also be used in cell-based assays. The selection of the peptides of sRAGE for the generation of the antibodies was guided by an extensive analysis of the potential epitopes. Applying a fast and basic molecular modelling [20] we were able to predict long solvent exposed loops of the three Ig-like domains of RAGE. The sequences of the predicted loops were further analysed for the content of charged or bulky amino acid residues, since such epitopes contribute to the antigenicity and specificity of the generated antibodies [21]. The following epitopes were selected for the generation of antibodies: epitope 1 originates from the V domain and comprises residues 54–70 (NTGRTEAWKVLSPQGGG); epitopes 2 and 3 originate from C1 and C2 domains and comprise residues 158–179 (HLDGKPLVPNEKGVSVKEQTRR) and residues 272–293 (KDGVPLPLPPSPVLILPEIGP), respectively.

Dot blots using sRAGE expressed by HEK293 cells [22] showed that the antisera generated against the three peptides all recognise sRAGE (data not shown). The antiserum raised against the peptide originating from the C2 domain showed the highest titre and specificity. No immuno-reactivity was detected in the crude extract of either non-transformed *E. coli* or from non-transformed *P. pastoris*.

Cloning of human sRAGE

Previous studies have shown that sRAGE expressed with baculovirus in insect cells had a significant therapeutic effect when administerted to mice models of various human diseases or disorders [5,6,13,23]. The aim of this study was to establish another eukaryotic expression system which allows the production of large amount of sRAGE. *Pichia pastoris* was chosen as an expression system for several reasons: contrarily to bacteria, yeast allows the expression of glycosylated protein. It is as easy to handle as bacterial expression hosts. Furthermore, *P. pastoris* is superior to *Saccharomyces* with regard to cell density during growth, which allows higher yields of the expressed protein.

The mature protein secreted from *P. pastoris* encompassed amino acids 23–322 and contained two additional amino acid residues (E–F) at the N-terminus which originate from the cleavage of the secretion signal peptide. An additional stop codon at the 3′ end was introduced in order to obtain a protein which does not contain the c-myc tag or hexa-histidine tag coded on pPICZαA. The loss of the His-tag represented on one hand the loss of a purification tool but on the other hand, avoided that sRAGE contained other amino acids that could interfere in future animal studies.

Selection of P. pastoris strains for secreted sRAGE expression

Three different *Pichia* strains (X-33, GS115, and KM71H) were transformed with pPICZαA-sRAGE. Single colonies of the transformants were patched on BMGY plates containing 0.5% methanol in order to check the expression yields of the different strains. These plates allowed growth on methanol as well as on glycerol as carbon source. Methanol induced as well the expression of sRAGE which was secreted to the medium. The successful expression of sRAGE could be easily monitored by absorption of the protein to a PVDF membrane and detection by immunochemical techniques (Fig. 1). *Pichia pastoris* strains X-33 and KM71H showed high levels of sRAGE, whereas GS115 exhibited almost no expression.

We further compared the expression levels of sRAGE from the two strains X-33 and KM71H in BMMY medium using clones X-33 No. 2 and KM71H No. 4. Since sRAGE is a basic protein, (calculated p$I = 8.8$), we choose a cation exchange matrix to purify the protein. The level of protein expression from the two clones was compared after 72 h induction. After treatment described above, the supernatant was loaded on the resource S30 column and the protein eluted with a linear gradient from 0 to 1 M NaCl at pH 6. The elution profiles obtained with the two clones (Fig. 2) show differences in the number and intensity of eluted peaks. Two peaks were observed with X-33/sRAGE (Fig. 2A, lower trace) versus three with KM71H/sRAGE (Fig. 2A, upper trace). Analysis of the different peaks on SDS–PAGE showed that the flow-through and several fractions contained a major band around 45 kDa (Fig. 2B), that was immuno-reactive with the antibodies raised against sRAGE (data not shown). However, since the molecular mass of the expressed protein is in the range of 32 kDa, the presence of a band at 45 kDa indicated that sRAGE was glycosylated by *P. pastoris*. Noticeably, the flow-through had a yellow-brownish colour whereas the fractions collected during the gradient were colourless.

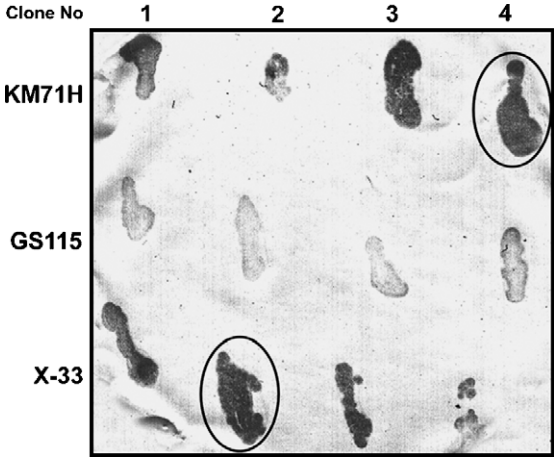

Fig. 1. Expression of sRAGE by different *Pichia pastoris* strains. Single colonies of the strains X-33, GS115 and KM71H transformed with pPICZα-sRAGE were patched on a BMGY plate containing 0.5% methanol to induce expression. After 24 h, a PVDF membrane was placed on top of the patches and secreted sRAGE was detected by immuno-staining with anti-RAGE antibodies. The clones KM71H/sRAGE No. 4 and X-33/sRAGE No. 2, which were selected for further expression tests, are indicated.

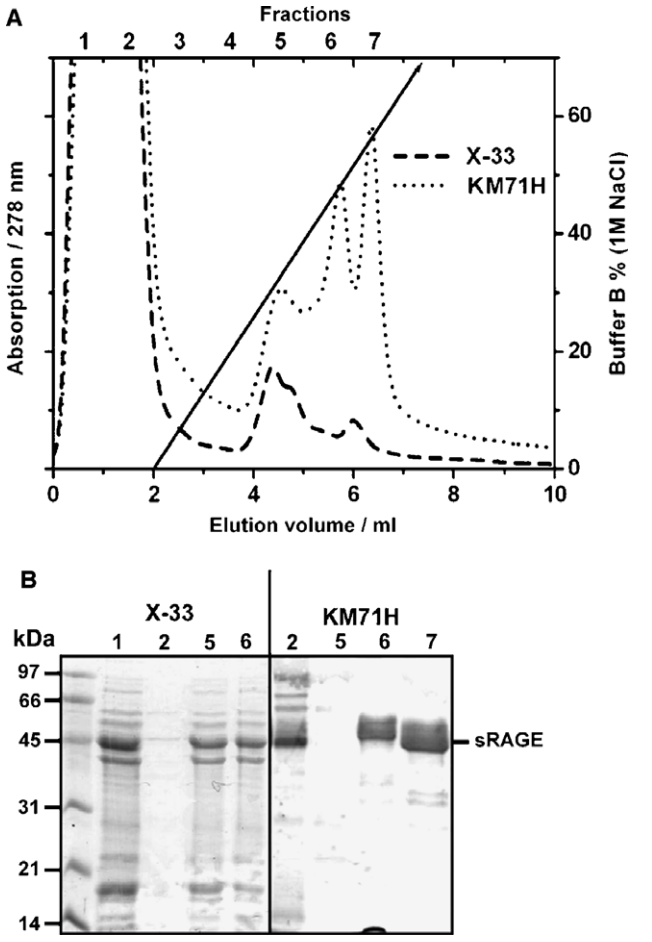

Fig. 2. Purification of sRAGE from *P. pastoris* strains X-33/sRAGE and KM71H-sRAGE. (A) Elution profiles of treated supernatant from X-33/sRAGE (dashed line) and KM71H/sRAGE (dotted line) via cation exchange (Resource S30) chromatography. The solid line indicates the salt gradient. (B) SDS–PAGE analysis of sRAGE expression. Collected fraction of the eluted sRAGE from strains X-33-sRAGE and KM71H-sRAGE was analysed on SDS–PAGE. Whereas the fractions from X-33-sRAGE supernatant revealed several bands in SDS–PAGE, those from strain KM71H-sRAGE showed only one major band.

Analysis of the flow-through revealed that a yellow-brownish substance stuck to the protein which could not be removed by chromatographic methods. Therefore, the flow-through was discarded and only the eluted fractions were used. The fractions 5 and 6 obtained from the culture medium of X-33-sRAGE contained both a major band at 45 kDa as well as two other bands at 40 kDa and 18 kDa (Fig. 2B). Both 45 and 40 kDa bands were sRAGE immuno-reactive with the generated antibodies derived from the three peptides. The different molecular masses most likely reflect different grades of glycosylation or proteolytic degradation levels of the protein. The fraction 5 obtained from the elution profile of KM71H-sRAGE (Fig. 2A) showed no protein by SDS–PAGE analysis whereas fractions 6 and 7 corresponding to 450 and 600 mM NaCl contained bands at 48 and 45 kDa corresponding to sRAGE (Fig. 2B). The comparison of the yield of sRAGE obtained from strains X-33/sRAGE and KM71H/sRAGE shows that KM71H/sRAGE produced about 4-fold higher yields than X-33-sRAGE.

Optimization of sRAGE purification

Further optimization of the expression and purification of sRAGE was made with regard to the growth conditions of strain KM71H-sRAGE. Since KM71H has a mutation in the AOX1 gene, it grows only very slowly on methanol-containing medium and therefore had first to be grown on glycerol-containing medium (BMGY) until an adequate cell density was reached before being transferred to a methanol-containing medium for protein expression. In order to simplify the protein purification procedure, we tested whether we could grow KM71H/sRAGE and induce sRAGE expression in a single medium. For this reason we tested whether BMGY + 0.5% methanol could serve as growth and expression medium for KM71H-sRAGE. For comparison the clone was grown in BMMY or BMGY + 0.5% methanol. After 96 h, the supernatants from both cultures revealed a major band at 45 kDa on SDS–PAGE demonstrating that expression of sRAGE is feasible in BMGY + methanol (Fig. 3). Western blotting of the protein proved that the 45 kDa band was sRAGE. When KM71H-sRAGE was grown on BMMY, two minor bands at 35 and 37 kDa were detected, which originate most likely from a partially glycosylated protein.

Simplification of cation exchange step: single-step elution from cation exchange column vs. gradient elution

The purification of sRAGE on a Resource S30 column requires that the column is connected to an FPLC system or similar pump system that can generate a pressure high enough to run the column. We have shown that sRAGE is the only protein from culture supernatants that binds to a cation exchange column and elutes at high ionic strength. Therefore, we tested the carboxymethyl Sepharose cation exchange matrix which can be run by a simple

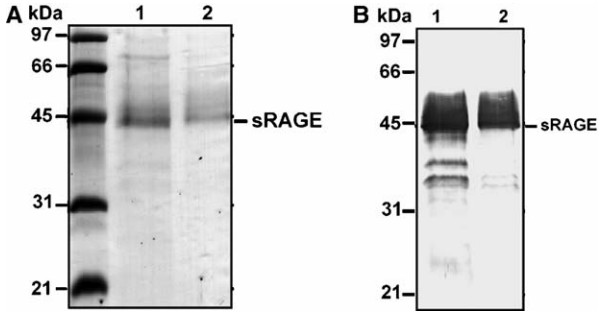

Fig. 3. Expression of sRAGE by *P. pastoris* strain KM71H-sRAGE/human. KM71H-sRAGE/human was grown on BMGY + 0.5% methanol or BBMY. After induction for 96 h, supernatant of each culture medium was applied to SDS–PAGE. (A) Coomassie staining of SDS–PAGE; lane 1, 2.9 μg protein from BMMY; lane 2, 2.3 μg protein from BMGY + 0.5% methanol. (B) Western blot of (A); immuno-staining with antisera against peptide deriving from C2 domain. Lane 1, BMMY culture medium; lane 2 BMGY medium + 0.5% methanol.

peristaltic pump or by gravity flow due to the lower back-pressure of the material. Culture supernatant from KM71H-sRAGE was applied to the column. Again, some sRAGE was found in the flow-through. An one-step elution was performed with 1 M NaCl in buffer and yielded a colourless fraction containing sRAGE (Fig. 4A). The protein was 95% pure as judged by SDS–PAGE. Only a minor band around 35 kDa was visible on the Coomassie-stained gel (Fig. 4B).

Characterization of human recombinant sRAGE

Secondary structure analysis

The proper folding of the recombinant protein was checked by circular dichroism (CD) spectroscopy (Fig. 5). Analysis by deconvolution of the spectrum using CDNN 2.1 gave a secondary structure content of 35% antiparallel β-sheet, 4% parallel β-sheet, 22% β-turns, 31% random coil, and 10% α-helix. Except for the small content in α-helix, these values are in very good agreement with the secondary structure content of homologous Ig domains with known structure like, e.g., NCAM [24] or axonin [25] analysed with DSSP [26]. The CD data show that the recombinant produced sRAGE is properly folded and adopts a fold similar to homologous proteins.

Mass spectrometric analysis and identification of N-glycosylation sites

Purified human recombinant sRAGE was subjected to tryptic digest and the peptides were analysed by mass spectrometry. Two *N*-glycosylation sites in human sRAGE are predicted; one site at position 25 in the V-domain and the other at position 81 in the C1-domain. After tryptic digestion of sRAGE, the N-terminal peptide (E^{17} A E A E F A Q N^{25} I T A R^{29}) of the mature protein was not detected at 1449.7 Da by MS analysis, possibly indicating a posttranslational modification of this peptide. The other predicted

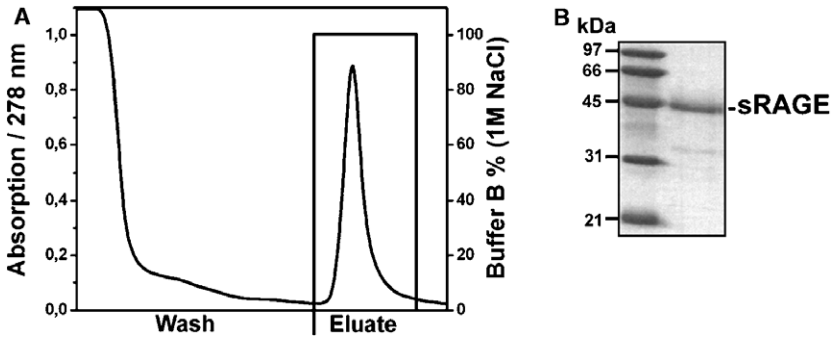

Fig. 4. One-step purification of sRAGE from KM71H-sRAGE/human culture supernatant on carboxymethyl column. (A) Chromatogram of sRAGE purified on carboxymethyl column. Bound sRAGE is eluted in a single step with 50 MES, 1.0 M NaCl, pH 6.0. (B) SDS–PAGE of eluted fraction showing a single band at 45 kDa, which was identified as sRAGE.

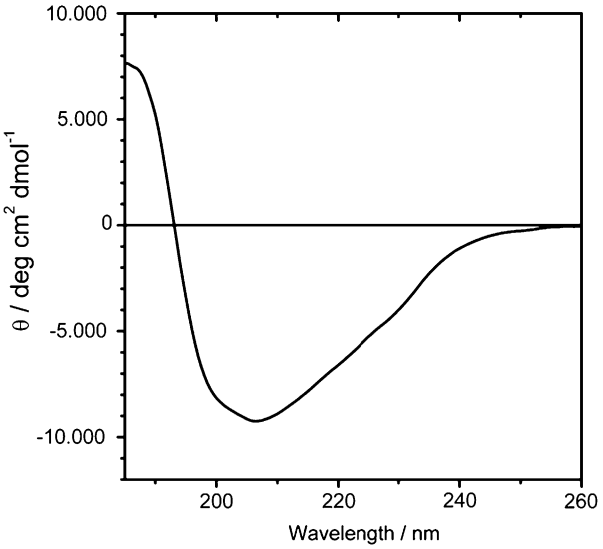

Fig. 5. Circular dichroism spectrum of human recombinant sRAGE. Analysis by deconvolution of the spectrum using CDNN 2.1 gave a secondary structure content of 35% antiparallel β-sheet, 4% parallel β-sheet, 22% β-turns, 31% random coil, and 10% α-helix.

glycosylation site at position 81 was checked by mass spectrometry as well. However, this tryptic peptide (V^{78} L P N^{81} G S L F L P A V G I Q D E G I F R^{98}) was detected at 2242.2 Da. Comparing to the digests of mature sRAGE, the tryptic digests of sRAGE pretreated with *N*-glycosidase F exhibited additional signals at 1450.7 and 2243.2 Da. Cleavage of glycosylamino linkage by *N*-glycosidase F will result in conversion of the asparagine to an aspartic acid residue. Consequently, the molecular mass of a deglycosylated peptide will increase by 1 Da (per glycosylation site), thus indicating the posttranslational modification. From these results we conclude that the peaks at 1450.7 Da and 2243.2 Da correspond to the deglycosylated peptides aa (17–29) and aa (78–98), respectively. These results were further confirmed by postsource decay (PSD) analysis of the peptide aa (17–29). In summary, we could show that human recombinant sRAGE was glycosylated at the two predicted *N*-glycosylation sites. Asn 25 seems to be completely glycosylated, whereas Asn 81 turned out to be partially glycosylated.

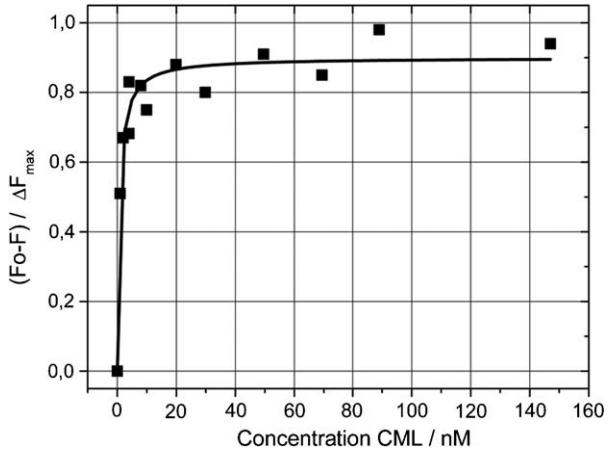

Fig. 6. Carboxymethyl lysine (CML) binding to human recombinant sRAGE. Binding of CML was followed by changes in tryptophan fluorescence of sRAGE. The change in fluorescence is plotted against the CML concentration in solution. The solid line represents a hyperbolic fit ($\frac{F_o - F}{\Delta F_{max}} = \frac{[CML]}{K_d + [CML]}$) of the data with a K_d of 4 ± 0.5 nM.

Binding of AGE carboxymethyl lysine

The functionality of human recombinant sRAGE was tested by binding to the advanced glycation endproduct carboxymethyl lysine (CML), which binds with high affinity to RAGE [27]. We found that sRAGE bound CML tightly displaying a K_d of 4 ± 0.5 nM (Fig. 6). The secondary structure analysis and the high affinity binding of CML to sRAGE illustrate that the recombinant protein is fully folded and functional, a prerequisite for further studies using recombinant sRAGE.

Conclusion and outlook

We successfully cloned and purified human sRAGE from *P. pastoris*. So far sRAGE for in vivo experiments was expressed in baculovirus transfected insect cells. The new expression system can be easily scaled up for expression and purification of sRAGE. The expression in yeast has several advantages over the baculovirus system. The media for *Pichia* are by far not as cost-intensive as the cell

culture media needed for the insect cells. Yeast is a single cell microorganism and can be plated for selection. The transformation is stably inserted into the genome and the transformation does not need to be repeated as with insect cells that have to be transfected prior to every expression. *Pichia* grows to very high cell densities and is most likely able to produce larger amounts of sRAGE per culture medium than baculovirus transfected insect cells. After 96 h of expression, KM71H-sRAGE reached a protein concentration of 150 mg/l culture medium. Assuming that about 30–40% percent are lost during the chromatography the yields for sRAGE could reach 100 mg/l culture medium. The secondary structure analysis and the high affinity binding of CML to sRAGE illustrate that the recombinant protein is fully folded and functional, a prerequisite for further studies using recombinant sRAGE.

We are currently further optimizing the growth and expression conditions of sRAGE in *P. pastoris* to establish large scale purification of the protein for further biochemical, cellular and structural studies.

Acknowledgments

We thank Prof. Dr. P. Nawroth (University of Heidelberg) for providing us the cDNA for human RAGE and Dr. B. Weigle for providing us human sRAGE. This work was supported by Transregio Sonderforschungsbereich TR SFB11 Konstanz/Zürich (to C.H., G.F., P.K.), and by the Wilhelm Sander-Stiftung.

References

[1] R. Ramasamy, S.J. Vannucci, S.S. Yan, K. Herold, S.F. Yan, A.M. Schmidt, Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation, Glycobiology 15 (2005) 16R–28R.

[2] M. Neeper, A.M. Schmidt, J. Brett, S.D. Yan, F. Wang, Y.C. Pan, K. Elliston, D. Stern, A. Shaw, Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins, J. Biol. Chem. 267 (1992) 14998–15004.

[3] S.S. Shaw, A.M. Schmidt, A.K. Banes, X. Wang, D.M. Stern, M.B. Marrero, S100B-RAGE-mediated augmentation of angiotensin II-induced activation of JAK2 in vascular smooth muscle cells is dependent on PLD2, Diabetes 52 (2003) 2381–2388.

[4] T. Arumugam, D.M. Simeone, A.M. Schmidt, C.D. Logsdon, S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE), J. Biol. Chem. 279 (2004) 5059–5065.

[5] M.A. Hofmann, S. Drury, C. Fu, W. Qu, A. Taguchi, Y. Lu, C. Avila, N. Kambham, A. Bierhaus, P. Nawroth, M.F. Neurath, T. Slattery, D. Beach, J. McClary, M. Nagashima, J. Morser, D. Stern, A.M. Schmidt, RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides, Cell 97 (1999) 889–901.

[6] A. Taguchi, D.C. Blood, G. del Toro, A. Canet, D.C. Lee, W. Qu, N. Tanji, Y. Lu, E. Lalla, C. Fu, M.A. Hofmann, T. Kislinger, M. Ingram, A. Lu, H. Tanaka, O. Hori, S. Ogawa, D.M. Stern, A.M. Schmidt, Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases, Nature 405 (2000) 354–360.

[7] S. Du Yan, H. Zhu, J. Fu, S.F. Yan, A. Roher, W.W. Tourtellotte, T. Rajavashisth, X. Chen, G.C. Godman, D. Stern, A.M. Schmidt, Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease, Proc. Natl. Acad. Sci. USA 94 (1997) 5296–5301.

[8] T. Chavakis, A. Bierhaus, P.P. Nawroth, RAGE (receptor for advanced glycation end products): a central player in the inflammatory response, Microbes. Infect. 6 (2004) 1219–1225.

[9] A.M. Schmidt, S.D. Yan, S.F. Yan, D.M. Stern, The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses, J. Clin. Invest. 108 (2001) 949–955.

[10] H.J. Huttunen, J. Kuja-Panula, G. Sorci, A.L. Agneletti, R. Donato, H. Rauvala, Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation, J. Biol. Chem. 275 (2000) 40096–40105.

[11] T. Chavakis, A. Bierhaus, N. Al-Fakhri, D. Schneider, S. Witte, T. Linn, M. Nagashima, J. Morser, B. Arnold, K.T. Preissner, P.P. Nawroth, The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment, J. Exp. Med. 198 (2003) 1507–1515.

[12] S.S. Yan, Z.Y. Wu, H.P. Zhang, G. Furtado, X. Chen, S.F. Yan, A.M. Schmidt, C. Brown, A. Stern, J. LaFaille, L. Chess, D.M. Stern, H. Jiang, Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system, Nat. Med. 9 (2003) 287–293.

[13] T. Wendt, E. Harja, L. Bucciarelli, W. Qu, Y. Lu, L.L. Rong, D.G. Jenkins, G. Stein, A.M. Schmidt, S.F. Yan, RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes, Atherosclerosis 185 (2006) 70–77.

[14] T. Wendt, N. Tanji, J. Guo, B.I. Hudson, A. Bierhaus, R. Ramasamy, B. Arnold, P.P. Nawroth, S.F. Yan, V. D'Agati, A.M. Schmidt, Glucose, glycation, and RAGE: implications for amplification of cellular dysfunction in diabetic nephropathy, J. Am. Soc. Nephrol. 14 (2003) 1383–1395.

[15] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[16] B.D. Zehr, T.J. Savin, R.E. Hall, A one-step, low background coomassie staining procedure for polyacrylamide gels, Anal. Biochem. 182 (1989) 157–159.

[17] R.C. Sharma, R.T. Schimke, Preparation of electrocompetent *E. coli* using salt-free growth medium, Biotechniques 20 (1996) 42–44.

[18] G. Böhm, CDNN 2.1 CD Spectra Deconvolution , Halle, 1997.

[19] G. Böhm, R. Muhr, R. Jaenicke, Quantitative analysis of protein far UV circular dichroism spectra by neural networks, Protein Eng. 5 (1992) 191–195.

[20] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.

[21] M.H. Van Regenmortel, Which structural features determine protein antigenicity? Trends Biochem. Sci. 11 (1986) 36–39.

[22] H.L. Hsieh, B.W. Schafer, B. Weigle, C.W. Heizmann, S100 protein translocation in response to extracellular S100 is mediated by receptor for advanced glycation endproducts in human endothelial cells, Biochem. Biophys. Res. Commun. 316 (2004) 949–959.

[23] B. Liliensiek, M.A. Weigand, A. Bierhaus, W. Nicklas, M. Kasper, S. Hofer, J. Plachky, H.J. Grone, F.C. Kurschus, A.M. Schmidt, S.D. Yan, E. Martin, E. Schleicher, D.M. Stern, G.G. Hammerling, P.P. Nawroth, B. Arnold, Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response, J. Clin. Invest. 113 (2004) 1641–1650.

[24] V. Soroka, K. Kolkova, J.S. Kastrup, K. Diederichs, J. Breed, V.V. Kiselyov, F.M. Poulsen, I.K. Larsen, W. Welte, V. Berezin, E. Bock, C. Kasper, Structure and interactions of NCAM Ig1-2-3 suggest a novel zipper mechanism for homophilic adhesion, Structure 11 (2003) 1291–1301.

[25] J. Freigang, K. Proba, L. Leder, K. Diederichs, P. Sonderegger, W. Welte, The crystal structure of the ligand binding module of axonin-1/TAG-1 suggests a zipper mechanism for neural cell adhesion, Cell 101 (2000) 425–433.

[26] W. Kabsch, C. Sander, Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features, Biopolymers 22 (1983) 2577–2637.

[27] T. Kislinger, C. Fu, B. Huber, W. Qu, A. Taguchi, S. Du Yan, M. Hofmann, S.F. Yan, M. Pischetsrieder, D. Stern, A.M. Schmidt, N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression, J. Biol. Chem. 274 (1999) 31740–31749.