J. Biochem. **143,** 229–236 (2008)
doi:10.1093/jb/mvm213

Expression and Characterization of Recombinant C-Terminal Biotinylated Extracellular Domain of Human Receptor for Advanced Glycation End Products (hsRAGE) in *Escherichia coli*

Miyuki Kumano-Kuramochi, Qiuhong Xie, Yoshikiyo Sakakibara, Setsuko Niimi, Kyoko Sekizawa, Shiro Komba and Sachiko Machida*

National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Received October 23, 2007; accepted October 29, 2007; published online November 21, 2007

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor involved in the development of diabetic complications. Using an *Escherichia coli* expression system, we have successfully expressed and purified the C-terminal biotinylated extracellular domain of human RAGE (hsRAGE), which consists of three immunoglobulin-like domains carrying three putative disulfide bonds. Over 90% of hsRAGE was expressed in soluble form in *trxB* and *gor* mutant *E. coli* strain Origami (DE3). Most hsRAGE was biotinylated with a C-terminal AviTag, and stably immobilized onto matrix *via* streptavidin without any treatment. Immobilized hsRAGE without glycosylation recognized its ligands, such as AGEs. Biotinylated hsRAGE was also able to apply in the detection of AGEs on microtitre wells like antibodies used in enzyme-linked immunoassay. SPR analysis demonstrated that the dissociation constant (K_d) of RAGE for AGE-BSA was 23.1 nM with the two-state reaction model, and 13.5 nM with the 1:1 binding model, comparable to those of RAGEs on cell surface. These results indicate that biotinylated hsRAGE must be useful not only in analysing RAGE–ligand interactions but also detect AGEs.

Key words: advanced glycation end products (AGE), AviTag, *in vivo* biotinylation, receptor for advanced glycation end products (RAGE), receptor–ligand interaction.

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; SPR, surface plasmon resonance.

Receptor for advanced glycation end products (RAGE) is a receptor of the immunoglobulin superfamily that interacts with a broad range of ligands, including advanced glycation end products (AGEs) (*1*, *2*), amyloid-β peptide (*3*), amphoterin (*4*) and S100/calgranulins (*5*). The AGEs are protein adducts generated by non-enzymatic reaction that accumulate in various tissues with age or under conditions of hyperglycaemia (*6*, *7*). The interaction of RAGE and AGE is associated with the development of complications of diabetes (*8*), making this interaction a prime target for blocking diabetic complications (*9*).

RAGE is composed of an N-terminal extracellular domain containing three immunoglobulin-like domains, one V-type domain and two C-type domains; a single transmembrane domain; and a C-terminal short cytoplasmic domain (*2*). The V-type domain may bind ligand (*10*), and the C-terminal cytoplasmic tail is important for cell signalling pathways (*11*, *12*). Recently, a secreted, soluble isoform of RAGE (sRAGE) has been identified (*13*, *14*), and administration of sRAGE to cells and animal models was shown to prevent the RAGE signalling effects (*15*, *16*), indicating that sRAGE acts as a decoy receptor. In addition, the use of sRAGE produced in insect cells has contributed to the detailed analysis of the kinetics of the RAGE–ligand interaction *in vitro* (*17*). Therefore, an effective production system to obtain functional sRAGE had been required for the detailed analysis of RAGE–ligand interactions.

Proteomics has burst onto the scientific and industrial scene in the last decade. Accordingly, many technologies and strategies have been developed for protein expression and purification. This has included the use of plasmids containing effective fusion tags (*18*), which have been used for biochemical analysis and to generate functional protein arrays (*19*, *20*). A critical step in generating functional arrays was the development of methods for spotting proteins on a solid surface without affecting activity, and at high-enough density. The biotin–streptavidin interaction has utilized as a target, leading to the development of a unique sequence, AviTag (GLNDIFEAQKIEWHE), which is recognized by biotin ligase (*21*, *22*). However, both the level of expression and the efficiency of biotinylation of the target proteins were not as high as expected.

Here, we report the use of an *Escherichia coli* expression system to produce functional human sRAGE (hsRAGE), which could be fully biotinylated *in vivo* at its C-terminus using introduced AviTag. Biotinylated hsRAGE was applicable to detect AGEs on microtitre wells as the same as the antibodies in ELISA. Moreover, biotinylated hsRAGE was effectively immobilized on a solid surface *via* biotin–streptavidin interaction without affecting its specific ability to bind to AGEs, enabling the detailed kinetic analysis of the interaction between hRAGE and AGE.

*To whom correspondence should be addressed. Tel: +81 29 838 8061, Fax: +81 29 838 7996, E-mail: lili@affrc.go.jp

MATERIAL AND METHODS

Bacterial Strains and Plasmids—*Escherichia coli* strain DH5α was used as a host for plasmid constructs, and *E. coli* strain Origami (DE3) [Δ(*ara-leu*) *7697*Δ*lacX74* Δ*phoA Pvu*II *phoR araD139 ahpC galE galK rpsL* F'[lac^+ $lacI^q$ *pro*] (DE3) *gor522*::Tn*10 trxB* (Kan^R, Str^R, Tet^R)], purchased from Novagen (Madison, WI, USA), was used as a host for the expression and *in vivo* biotinylation of hsRAGE. The plasmids pET-16b (Novagen, Madison, WI, USA) and pAC-4 (Avidity, Denver, CO, USA) were used to construct a plasmid for expression of biotinylated hsRAGE.

Plasmid Construction—cDNA coding human RAGE (hRAGE) variant 1 (GenBank accession no. AB036432) was amplified from human lung polyA$^+$ RNA (Clontech, Palo Alto, CA, USA) by reverse transcriptase–PCR using the primers 5′-TTGAATTCAGGATGGCAGCCGGAACAG CAG-3′ and 5′-TTCTCGAGTCAAGGCCCTCCAGTACT AC-3′ (complementary sequences of the start and stop codon are underlined). The amplified fragment coding full-length (404 amino acids) hRAGE was cloned into the plasmid pCR2.1 (Invitrogen, Carlsbad, CA, USA). This plasmid was used for the amplification of the fragment encoding the extracellular domain of RAGE (Ala_{23}–Ser_{332}, termed hsRAGE), along with the primers 5′-CTACATATGGCTCAAAACATCACAGC-3′ [containing an *Nde*I site (underlined)] and 5′-TTACTCGAGAGCCTG CAGTTGGCCC-3′ [containing an *Xho*I site (underlined)]. The hsRAGE fragment was subcloned into pCR2.1. The region coding AviTag (GLNDIFEAQKIEWHE) for *in vivo* biotinylation (*21*, *22*) was amplified from pAC-4 using primers 5′-GGAGCTCGAGGATCCCGGGCAAGC-3′, and 5′-TCGAGATCTATCAGACCGCTTCTGCG-3′, the latter containing a *Bgl*II site (underlined) and inserted into pET-16b digested with *Xho*I and BamHI. The resultant plasmid, termed pETavi, confers an N-terminal HisTag and a C-terminal AviTag onto cloned proteins. The pCR2.1 containing hsRAGE was digested with *Nde*I and *Xho*I, and fragment containing hsRAGE was inserted into pETavi digested with *Nde*I and *Xho*I. This plasmid, encoding HisTag–hsRAGE–AviTag and termed pETavi_hsRAGE, was introduced, along with plasmid pBirA (Avidity, Denver, CO, USA), into *E. coli* Origami (DE3) strain, and its identity was verified by DNA sequencing.

Expression Conditions and Purification of Biotinylated hsRAGE—Origami (DE3) co-transformed by pETavi_hsRAGE and pBirA was cultured in LB medium (0.5% yeast extract, 1% trypton, 0.5% NaCl) containing antibiotics (50 μg/ml of ampicillin, 34 μg/ml of chloramphenicol, 15 μg/ml of kanamycin, 12.5 μg/ml of tetracycline) at 25°C. When the OD_{600} of the culture reached 0.5–0.7, the expression and biotinylation of the target protein was induced by adding 1 mM IPTG and 50 μM d-biotin. After 18 h, the bacterial cells were harvested, re-suspended in buffer A (150 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole) and disrupted by sonication. The crude lysate was cleared by centrifugation at 20,000*g* for 20 min at 4°C, and biotinylated hsRAGE was purified by sequential chromatography steps. Twenty millilitres of 50% nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen, GmgH, Germany) slurry equilibrated with buffer A was added to the 80 ml of the cleared lysate and mixed gently for 1 h at 4°C. The resin, to which histidine-tagged proteins were bound, was packed into a column and washed with 150 mM NaCl, 50 mM NaH_2PO_4, 60 mM imidazole (pH 8.0). Protein bound to Ni–NTA resin was eluted with a linear gradient of imidazole (60–500 mM) using an FPLC system (GE Healthcare, Buckinghamshire, UK). Fractions showing absorbance at 280 nm were analysed by SDS–PAGE and the peak fractions containing protein at the expected molecular mass were collected (Fraction I). Fraction I was dialysed against buffer A and further purified through a streptavidin mutein matrix (Roche, Mannheim, Germany) column chromatography. Twenty millilitres of dialysed Fraction I and 2 ml of 50% streptavidin mutein matrix slurry equilibrated with buffer A were mixed for 15 min by rotary shaker, and the slurry was packed into column. The column was washed with buffer A, and hsRAGE was eluted with a linear gradient of d-biotin (0–10 mM) by FPLC. Fractions containing protein at the expected molecular mass were collected (Fraction II), and their purity was evaluated by SDS–PAGE.

Evaluation of In Vivo Biotinylation of hsRAGE by BirA—Biotinylation efficiency of hsRAGE was evaluated by a pull-down assay using streptavidin-coated matrix. Three different cultures of Origami (DE3) carrying pETavi_hsRAGE and pBirA were prepared: a normal culture induced with d-biotin, a culture induced without d-biotin and an uninduced culture. Six milligrams of cells from each culture were re-suspended in 500 μl of buffer B (150 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and disrupted by sonication. The lysates were cleared by centrifugation at 20,000*g* for 20 min at 4°C, and 100 μl of each cleared lysate were added to 20 μl of 50% streptavidin mutein matrix slurry equilibrated with buffer B and incubated for 30 min at 4°C. The mixtures were centrifuged at 2,000*g*; the resultant supernatant was used as unbound supernatant, and the resultant matrix was washed twice with wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and used as protein bound to matrix. Unbound supernatant, protein bound to matrix and untreated cleared lysate were analysed by SDS–PAGE.

Preparation of Sugar-derived AGEs-BSAs—Sugar-derived AGE-BSAs were prepared as described (*17*). Briefly, 50 mg/ml of BSA (SIGMA, Cat. No. A9418) was incubated under sterile conditions with 500 mM D(−)-fructose, D(+)-glucose or D(−)-ribose in 0.5 M sodium phosphate buffer (pH 7.4), or in buffer alone, for 12 weeks at 37°C. Unincorporated sugars were removed by dialysis against PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). The final concentration of protein was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The conditions of AGE-BSAs were confirmed by measuring the fluorescence and absorbance of each sample. Fluorescence intensities of 1 mg/ml AGE-BSAs and control BSA in PBS were measured with excitation at 335 nm and emission at 420 nm on SpectraMax GEMINI (Molecular Devices, Sunnyvale, CA, USA) using a FluoroNunc microplate (Nunc, Roskilde, Denmark). The absorbances

of AGE-BSAs at 340 nm were measured using U-3300 (Hitachi, Tokyo, Japan) with a 10 mm path length. We prepared the control BSA by incubation without sugar, and used it as a standard control for the following ligand-blotting assay, AGE-binding activity assay and surface plasmon resonance (SPR) analysis.

Ligand Blotting Assay—Sugar-derived AGE-BSAs and control BSA prepared without sugar were serially diluted in PBS and spotted onto nitrocellulose membranes (Pall Life Sciences, Ann Arbor, MI, USA). The dried membranes were blocked for 1 h at room temperature with 5% skimmed milk in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T), washed with TBS-T and incubated with 10 μg/ml of hsRAGE in buffer B for 1 h at room temperature. The membranes were washed three times with TBS-T and incubated with 1000-fold diluted anti-RAGE rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at room temperature. The membranes were again washed with TBS-T and incubated with 1000-fold diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. The hsRAGE bound to AGE-BSAs was detected using an ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK).

AGE-Binding Activity Assay—AGE-BSA-conjugated sepharose beads were prepared using NHS-activated Sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK), according to manufacturer's instructions. Briefly, NHS-activated sepharose was washed by 1 mM HCl. AGE-BSA coupling was performed by mixing the washed sepharose medium and fructose-derived AGE-BSA in coupling buffer (200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) for 4 h at room temperature. We also prepared control BSA (prepared by incubation without sugar)-conjugated sepharose and non-protein-conjugated sepharose. To block non-reacted groups after protein coupling, the medium was kept in blocking buffer (500 mM ethanolamine, 500 mM NaCl, pH 8.3) for 4 h at room temperature. Then, medium was washed with three cycles of buffer W1 (100 mM Tris–HCl, pH 8.5) and buffer W2 (100 mM sodium acetate, 500 mM NaCl, pH 4.5), and equilibrated with buffer C (100 mM NaCl, 50 mM NaH_2PO_4, pH8.0). Purified hsRAGE in buffer C was added to AGE-BSA-conjugated sepharose medium with its final concentration at 18 μg/ml. Then mixtures were incubated at 4°C overnight. After incubation, medium was separated into supernatant and sepharose by centrifugation. Sepharose was then washed twice with buffer C. Supernatant of mixture before and after incubation, and sepharose pellet after incubation were collected and analysed by SDS–PAGE. We also performed the same reaction using control BSA-conjugated sepharose and non-protein-conjugated sepharose by mixing them with hsRAGE at the concentration of 70 μg/ml.

Detection of AGEs on Microtitre Wells—One hundred microlitres of AGE-BSAs solution was incubated in each microtitre wells (Nunc, Roskilde, Denmark) at 4°C for 12 h. After the solution was discarded, the wells were filled with 300 μl of non-protein blocking buffer (Pierce, Rockford, IL, USA) at room temperature for 1 h. After the blocking solution was discarded, 100 μl of biotinylated hsRAGE solution (5 μg/ml) were applied to the each well, and incubated at room temperature for 2 h. Then the wells were washed five times with TBS. The wells were incubated with 100 μl of streptavidin-HRP conjugate solution (Roche, Mannheim, Germany, diluted 1:100,000 in TBS) for 1 h at room temperature. After washing the well five times with TBS, 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB); reactive substrate solution (Sigma, St Louis, MO, USA) was added, and the plate was incubated for 10 min at room. The enzyme reaction was stopped using 50 μl of 2N HCl, and the absorbance at 450 nm was measured with micro plate reader (Bio-Rad, Hercules, CA, USA).

CD Spectroscopy—Purified biotinylated hsRAGE was dialysed against buffer B and CD spectra in the wavelength range 200–250 nm was recorded on a J-820 spectropolarimeter (JASCO, Tokyo, Japan) using a quartz cell with a path length of 1 mm. The sample concentration was 10 μM, and spectra were scanned five times at 10 nm/min at 25°C.

SPR Analysis—The interaction between hsRAGE and fructose-derived AGE-BSA was analysed using BIAcore 2000 (Biacore AB, Uppsala, Sweden). Buffer B was used as running buffer. Biotinylated hsRAGE was immobilized onto a SA sensor chip. To block the reference flow cell surface, BSA was chemically biotinylated by Sulfo-NHS-SS-Biotin (Pirece, Rockford, IL, USA) according to the manufacturer's instructions and immobilized to the reference cell. The SPR analysis was performed after we determined regeneration conditions of RAGE-AGE binding. AGE-BSA (4–128 μg/ml) diluted with running buffer was injected over flow cells at a flow rate of 10 μl/min for 3 min. The flow cells were allowed 3 min for dissociation and were subsequently regenerated by two repetitive injections of 10 mM glycine (pH 2.0) for 30 s. Specific binding curves were obtained by subtracting the reference curve from each binding curve and analysed using BIAevaluation software, version 3.0. Here we assumed that the molecular mass of fructose-derived AGE-BSA was 71.2 kDa, according to the average molecular weight of fructose-derived AGE-BSA (*17*).

RESULTS AND DISCUSSION

Expression and Purification of Biotinylated hsRAGE—The extracellular domain of hRAGE, corresponding to residues Ala_{23}–Ser_{332} of hRAGE and termed hsRAGE (Fig. 1, upper black solid bar) was introduced into pETavi and expressed in *E. coli* strain Origami (DE3), resulting in high levels of expression of hsRAGE with functional N- and C- terminal tags (Fig. 2). At 25°C, hsRAGE was one of the main soluble proteins (Fig. 2, lane 2), with over 90% expressed as a soluble form (Fig. 2, lanes 3 and 4). In contrast, almost all the hsRAGE expressed in *E. coli* strain BL21 (DE3) was insoluble (data not shown). *Escherichia coli* strain Origami (DE3) is a *trxB* and *gor* mutant, which allows more efficient formation of disulphide bonds (*23*). RAGE is a member of the immunoglobulin superfamily, with three sets of conserved cysteine residues (*2*). A disulfide linkage in an immunoglobulin fold is important for

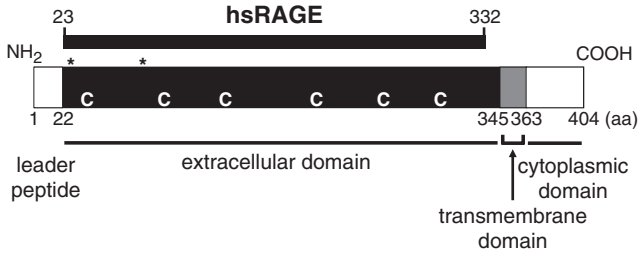

Fig. 1. **Schematic outline of full-length hRAGE domains.** Numbers correspond to the amino acid (aa) sequence of full-length hRAGE, a 404 amino acid protein composed of an extracellular domain (aa 23–344) containing three immunoglobulin-like domains, a single transmembrane domain (aa 345–363) and a cytoplasmic domain (aa 364–404). Asterisks represent potential *N*-glycosylation sites; C represents cysteine residues involved in S–S linkages. The upper solid black bar represents the hsRAGE region (aa 23–332) used in this study.

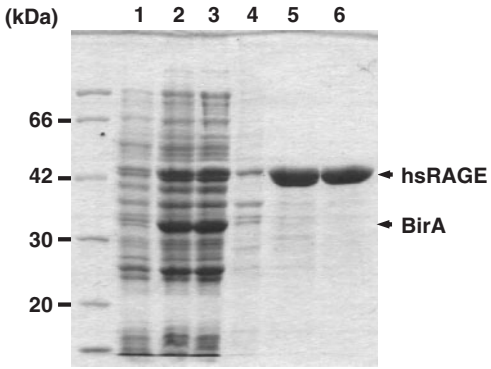

Fig. 2. **SDS–PAGE (12%) showing the expression, solubility and purity for each purification step of hsRAGE.** Lane 1, total protein of Origami (DE3) containing pETavi_hsRAGE and pBirA before induction; lane 2, total protein of Origami (DE3) after induction of hsRAGE and BirA; lane 3, soluble protein of Origami (DE3) after induction; lane 4, insoluble protein of Origami (DE3) after induction; lane 5, Ni–NTA-purified protein; lane 6, streptavidin mutein-purified protein after Ni–NTA purification.

maintaining the stability of most proteins in the immunoglobulin superfamily (*24*, *25*). The ability to express soluble hsRAGE in *E. coli* Origami (DE3) indicates the importance of disulfide linkages for the stability of this protein.

The hsRAGE expressed in this system carried an N-terminal HisTag and a C-terminal AviTag. Those double tags worked effectively in purification step. Soluble lysate containing double-tagged hsRAGE was purified through a Ni–NTA column, following which it was about 90% pure (Fig. 2, lane 5). The protein was further purified using a mutein matrix column. Although biotin–streptavidin binding has been often used for molecular interaction or protein purification, the recovery of biotinylated protein bound to streptavidin-coated matrix is limited because of its strong affinity ($K_d \sim 10^{-15}$ M) (*26*). The streptavidin mutein is mutant protein with reduced binding to biotin ($K_d \sim 10^{-7}$ M) (*27*). Using an eluant containing d-biotin, the immobilized

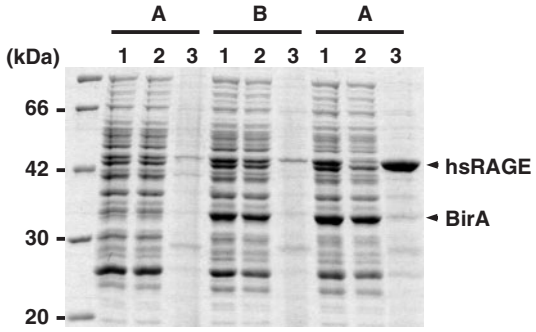

Fig. 3. **SDS–PAGE (12%) showing the binding of biotinylated hsRAGE to streptavidin-coated matrix.** Lysate of Origami (DE3) was obtained from cultures A, without protein induction; B, induced without d-biotin; C, induced with d-biotin. Each lysate was mixed with streptavidin mutein matrix and separated into supernatant and matrix by centrifugation. Untreated lysate (1), supernatant containing unbound proteins to matrix (2) and matrix bound to biotinylated protein (3) were analysed.

biotinylated protein was easily released from the mutein matrix. Following this step, the biotinylated hsRAGE was over 95% pure (Fig. 2, lane 6). The final yield of biotinylated hsRAGE was 2.2 mg/1 l culture.

Biotinylation Efficiency of hsRAGE Using AviTag—The plasmid encoding hsRAGE was co-transferred with pBirA, carrying biotin ligase, into *E. coli* strain Origami (DE3). We expected that hsRAGE would be biotinylated at C-terminal AviTag by BirA when induced in culture medium containing d-biotin. To evaluate the efficiency of *in vivo* biotinylation of hsRAGE, we examined the binding of the expected biotinylated hsRAGE to streptavidin-coated matrix. We found that hsRAGE expression was detectable only under induced conditions and that the expression level was high (Fig. 3, lanes B1 and C1). The same results were observed for BirA (Fig. 3, lanes B1 and C1), indicating that pETavi and pBirA were stable, and that their expression was completely regulated in *E. coli* Origami (DE3). When hsRAGE and BirA were induced in cultures containing d-biotin, hsRAGE was fully bound to the matrix, whereas no other protein was bound (Fig. 3, lanes C2 and C3). This binding did not occur in uninduced cultures (Fig. 3, lane A2 and A3), or in cultures induced in the absence of d-biotin (Fig. 3, lane B2 and B3). These results indicate that C-terminal AviTag fused to hsRAGE was successfully biotinylated in culture medium containing d-biotin, and biotinylation by BirA was highly specific to AviTag. Other expression systems producing biotinylated proteins have been described, *e.g.* pAC (Avidity, Denver, CO, USA) and PinPoint (Promega, Madison, WI, USA), but both these systems sometimes result in low expression and biotinylation of the target protein (data not shown). These problems have been the bottleneck of biotinylated protein productions, however, overcome by the system introduced in this study.

Binding of hsRAGE to AGE-BSAs—The ability of biotinylated hsRAGE to bind to ligand was examined using sugar-derived AGE-BSAs. We found that biotinylated hsRAGE bound specifically to various

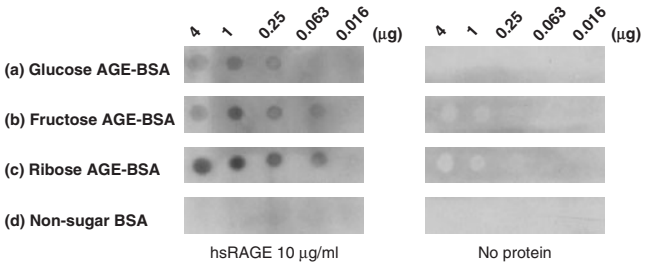

Fig. 4. **Ligand blotting assay showing interaction between purified hsRAGE and AGE-BSAs.** The indicated protein amounts (0.016–4 μg) of sugar-derived AGE-BSAs (glucose, fructose, ribose) and control BSA were spotted onto membranes, and membranes were incubated with (left panel) or without (right panel) hsRAGE (10 μg/ml). The membranes were exposed to anti-RAGE rabbit antibody and HRP-conjugated anti-rabbit IgG, and the interactions between hsRAGE and AGE-BSAs were visualized by ECL system.

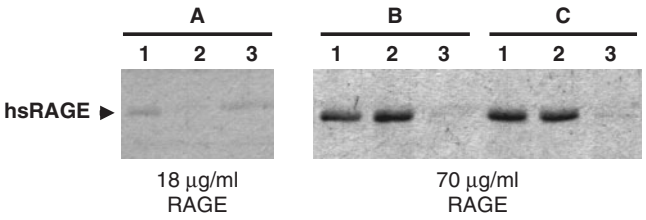

Fig. 5. **SDS–PAGE showing the binding activity of purified hsRAGE to AGE-BSA.** Purified hsRAGE at indicated concentration (18 μg/ml, 70 μg/ml) was incubated with AGE-BSA-conjugated sepharose (A), non-protein-conjugated sepharose (B) and BSA-conjugated sepharose (C), respectively. Supernatant of mixture before incubation (1), supernatant of mixture after incubation (2) and sepharose after incubation (3) were analysed by SDS–PAGE.

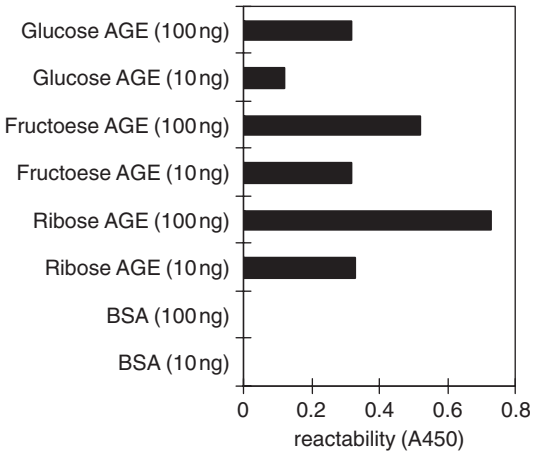

Fig. 6. **Detection of various sugar derived AGEs on microtitre wells.** Microtitre wells were coated with 10 or 100 ng of control BSA or various sugar derived AGE-BSAs. Biotinylated hsRAGE was applied to each well, followed by incubation with streptavidin-HRP conjugate. Finally, the absorbance at 450 nm was measured as described under MATERIAL AND METHODS section. Values are means of five wells of three independent experiments.

sugar-derived AGE-BSAs spotted onto nitrocellulose membrane (Fig. 4, left panel a–c), whereas it did not bind to BSA alone (Fig. 4, left panel d). No non-specific signal was detected from incubation in the absence of protein (Fig. 4, right panel). RAGE is a glycosylated protein (*2*, *28*, *29*), and glycan has been reported to mediate multiple biological functions (*30*, *31*). Human RAGE has two potential *N*-glycosylation sites in its N-terminal region (Fig. 1), a domain for the ligand binding, suggesting that its *N*-glycans were involved in binding. Ostendorp *et al*. (*32*) reported a method to produce highly *N*-glycosylated hsRAGE in *Pichia pastoris*, and that the hsRAGE from *P. pastoris* bound to carboxymethyl lysine (CML), one of the major AGEs. However, biotinylated hsRAGE expressed in *E. coli* bind AGEs, indicating that *N*-glycosylation is not necessary for hsRAGE to recognize AGEs. In addition, binding affinity of hsRAGE to each sugar-derived AGE-BSA was different, with a relative binding affinity of ribose > fructose > glucose, consistent with that reported for glycosylated RAGE expressed in insect cells (*17*).

We also determined what percentage of the recombinant hsRAGE retains AGE-binding activity by pull-down assay using fructose-derived AGE-BSA-conjugated sepharose. As shown in Fig. 5, most of the purified hsRAGE specifically bound to AGE-BSA-conjugated sepharose (Fig. 5, lane A3). The hsRAGE did not bind to either of non-protein-conjugated sepharose or control BSA-conjugated sepharose (Fig. 5, lanes B3 and C3), even though four times of concentration of hsRAGE was used.

These results suggest that almost 100% of the purified hsRAGE retained its ability to bind to AGE, and biotinylated hsRAGE maintains its respective binding specificities to various sugar-derived AGE-BSAs. It is possible to prepare biotinylated hsRAGE by *in vitro* biotinylation of hsRAGE produced from *P. pastoris* using purified biotin ligase. However, hsRAGE produced in *E. coli* is simple protein without *N*-glycans possesses its specific binding ability, and almost all of the hsRAGE is biotinylated without any treatment. These are the advantages of *in vivo* biotinylation using *E. coli* system in this study, although the yield of hsRAGE from *E. coli* was less than that from *P. pastoris*.

Detection of AGEs on Microtitre Wells—The ability of biotinylated hsRAGE to detect AGEs on microtitre wells was examined. Figure 6 showed the specificity of biotinylated hsRAGE to various AGEs on microtitre well. C-terminal biotin ligated to hsRAGE was effectively recognized by streptavidin–HPR conjugate, and AGEs on microtitre wells were measurable by absorbance at 450 nm. There was the strong signal from AGE-BSAs (10 ng/well was enough for detection), but almost no signal was detected from control BSA. Ribose-AGE-BSA showed the strongest signal, consistent with that reported for glycosylated RAGE (*17*). AGEs are general terms and consist of many kinds of molecular species. It is necessary to produce antibodies against each AGE species in order to detect each AGEs by ELISA. But these results indicated that biotinylated hsRAGE could work as the recognition device instead of antibodies in ELISA.

Secondary Structure of hsRAGE—The secondary structural properties of hsRAGE were examined by CD spectroscopy, which showed minima at 216–218 nm, a feature of β-sheets (Fig. 7). Although the tertiary structure of RAGE is not yet known, this result indicated that biotinylated hsRAGE contains a substantial amount of β-sheet structure, in agreement with the typical secondary structure of immunoglobulin superfamily proteins (*33*, *34*).

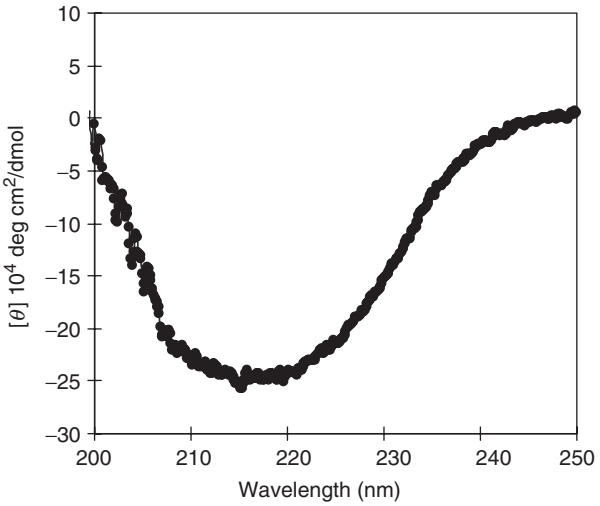

Fig. 7. **CD spectra of biotinylated hsRAGE purified from *E. coli*.** The CD spectra of purified hsRAGE (10 μM) in buffer B (150 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) was recorded five times at 10 nm/min at 25°C over a wavelength range from 200 to 250 nm. Contribution from the buffer was removed by subtracting the spectra, and ellipticity (θ) was reported as the mean molecular residue ellipticity expressed in deg cm^2/dmol.

Kinetic Analysis of Binding of hsRAGE and AGE-BSA—SPR experiments were performed to analyse the binding kinetics of biotinylated hsRAGE to AGE-BSA using BIAcore 2000. As hsRAGE had already been biotinylated *in vivo* at its C-terminal using AviTag, it could be immobilized onto a SA sensor chip *via* biotin on AviTag by injection of hsRAGE solution in running buffer without any treatment. This simple procedure results in a minimal denaturation of hsRAGE while immobilization. Moreover, all the molecules immobilized on the SA chip should have the same orientation. Sensor grams showed a rapid increase in RU reflective of AGE-BSA binding (association), and a decrease of RU consistent with a loss of mass from on washout (dissociation). Binding to AGE-BSA was concentration dependent (Fig. 8) and was not observed upon injection of control BSA, which was prepared by incubation without sugar (data not shown). These results suggest that immobilized C-terminal biotinylated hsRAGE maintains its ability to specifically bind to AGE-BSA. In SPR analysis, designing of regeneration condition of immobilized molecule is important for stable sensor grams of ligand binding. After we determined regeneration condition of hsRAGE, we obtained a good binding response and stable regeneration. While routinely used for kinetic measurement, BIAcore sometimes generates non-specific binding of analytes to the flow cell, increasing the RU of the reference flow cell. We therefore immobilized biotinylated BSA onto the reference flow cell to prevent this non-specific binding. The masking by biotinylated BSA worked well and almost no non-specific binding to the flow cell was detected (data not shown).

Data analysis was carried out using BIAevaluation 3.0 software. We analysed the sensor grams by fitting them with possible binding models. The sensor grams fitted best with a two-state reaction model. The χ^2-value of the

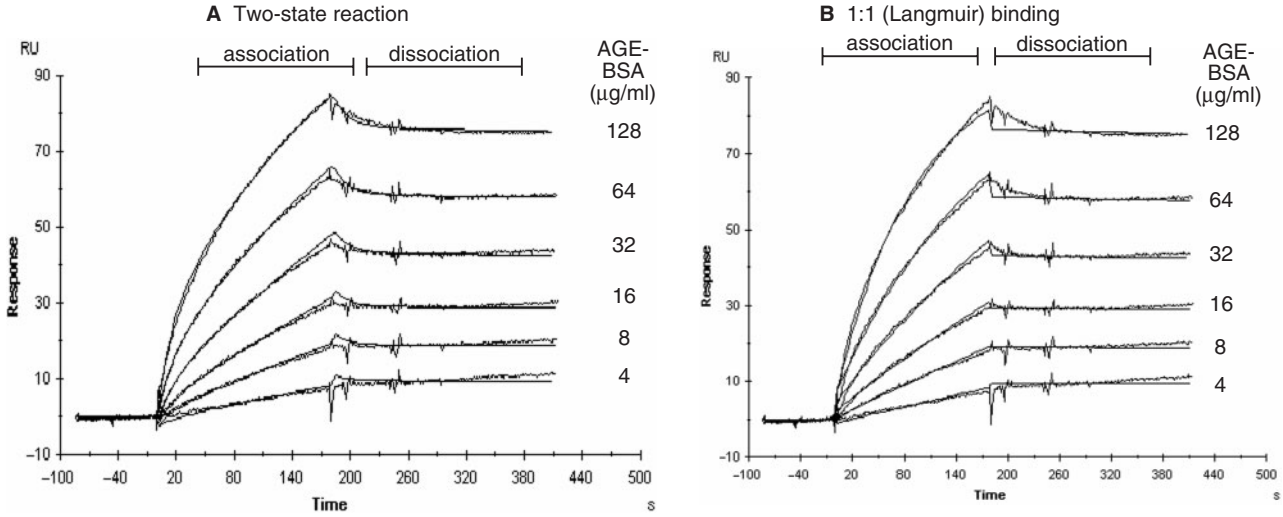

Fig. 8. **SPR sensor grams of specific binding of serially diluted fructose-derived AGE-BSA (4–128 μg/ml) to immobilized biotinylated hsRAGE on the surface of the SA sensor chip.** AGE-BSA diluted with running buffer was injected over flow cells at a flow rate of 10 μl/min for 3 min, and flow cells were allowed 3 min for dissociation. Curves from reference cell immobilized with chemical biotinylated BSA were respectively subtracted from binding curves. The dissociation constant of AGE-BSA and hsRAGE was calculated based on the non-linear curve fitting with the two-state reaction model (A, $K_d = 23.1$ nM, $\chi^2 = 0.582$) and the 1:1 (Langmuir) binding model (B, $K_d = 13.5$ nM, $\chi^2 = 0.557$).

fit was <0.6, with K_{d1} of 3.8 μM and K_{d2} of 6.1 mM. The equilibrium constant K_d was calculated as 23.1 nM, using an equation of $K_d = (K_{d1} \ K_{d2})/(K_{d2} + 1)$. This result suggests that the conformation change of RAGE might occur due to its binding with AGE-BSA. The engagement of receptors–ligands often induces the receptor clustering and/or conformation change, which modulates the subsequent signal transductions, such as kinase activation. Ishihara *et al*. (*35*) reported that the C-terminal intracellular domain of RAGE directly binds to ERK-1/2, and that kinase activity of ERK was enhanced under the presence of amphoterin, a ligand of RAGE. Whether the conformation change occurs through its engagement to ligandsor it is necessary for the RAGE-dependent signalling, is not elucidated. However, this study implied that the binding between RAGE and AGEs might be accompanied with a conformation change.

We also analysed the sensor grams using the 1:1 (Langmuir) binding model to compare with the past reports of binding between RAGE and AGE, which were analysed using the 1:1 binding model. The 1:1 binding model also gave fits with χ^2-value <0.6. The analysis showed that the dissociation constant (K_d) for binding of AGE-BSA to hsRAGE was 13.5 nM. This was in good agreement with the K_d of ^{125}I-AGE-BSA to RAGE isolated from bovine lung (~60 nM) (*1*), and with similar binding parameters for the binding of AGEs to endothelium ($K_d \sim 70$ nM), macrophages ($K_d \sim 40$ nM) and RAGE-transfected cells ($K_d \sim 100$ nM) (*1*, *2*, *36*).

Wilton *et al*. (*37*) reported a method for expressing and purifying the extracellular domain of human RAGE with a C-terminal HisTag. However, the SPR analysis using RAGE immobilized onto NTA sensor chip *via* C-terminal HisTag failed to analyse the kinetics of AGE-BSA binding to immobilized RAGE (*37*). In this present study, we expressed and purified the C-terminal biotinylated hsRAGE in *E. coli*, and succeeded in analysing the binding kinetics of AGE-BSA to hsRAGE. This achievement is due to the biotinylation of hsRAGE in *E. coli* with high efficiency; as a result, hsRAGE was stably and strongly immobilized on a solid matrix, with the molecules arranged in the same orientation. It is the first successful case for the detailed analysis of RAGE-AGE interaction using hsRAGE expressed in *E. coli*.

These results indicate that the biotinylated hsRAGE is the advanced molecule for the detailed analysis of RAGE–ligand interaction.

This work was supported in part by the 'Research Program on development of evaluation and management methods for supply of safe, reliable and functional food and farm produce' of the Ministry of Agriculture, Forestry, and Fisheries of Japan, and the 'Research Program on development of innovative technology' of the Japan Science and Technology Agency.

REFERENCES

1. Schmidt, A.M., Vianna, M., Gerlach, M., Brett, J., Ryan, J., Kao, J., Esposito, C., Hegarty, H., Hurley, W., Clauss, M., Wang, F., Pan, Y.C., Christopher, T., and Stern, D. (1992) Isolation and characterization of two binding proteins for advanced glycation end products from bovine lung which are present on the endothelial surface. *J. Biol. Chem.* **267**, 14987–14997
2. Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D., and Shaw, A. (1992) Cloning and expression of a cell surface receptor for advanced glycation end products. *J. Biol. Chem.* **267**, 14998–15004
3. Yan, S.D., Chen, X., Fu, J., Chen, M., Zhu, H., Roher, A., Slattery, T., Zhao, L., Nagashima, M., Morser, J., Migheli, A., Nawroth, P., Stern, D., and Schmidt, A.M. (1996) RAGE and amyloid–beta peptide neurotoxicity in Alzheimer's disease. *Nature* **382**, 685–691
4. Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J.X., Nagashima, M., Lundh, E.R., Vijay, S., Nitecki, D., Morser, J., Stern, D., and Schmidt, A.M. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J. Biol. Chem.* **270**, 25752–25761
5. Hofmann, M.A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M.F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., and Schmidt, A.M. (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* **97**, 889–901
6. Brownlee, M., Cerami, A., and Vlassara, H. (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* **318**, 1315–1321
7. Sell, D.R. and Monnier, V.M. (1989) Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J. Biol. Chem.* **264**, 21597–21602
8. Wautier, J.L., Wautier, M.P., Schmidt, A.M., Anderson, G.M., Hori, O., Zoukourian, C., Capron, L., Chappey, O., Yan, S.D., Brett, J., Guillausseau, P.J., and Stern, D. (1994) Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc. Natl Acad. Sci. USA* **91**, 7742–7746
9. Hudson, B.I. and Schmidt, A.M. (2004) RAGE: a novel target for drug intervention in diabetic vascular disease. *Pharm. Res.* **21**, 1079–1086
10. Kislinger, T., Fu, C., Huber, B., Qu, W., Taguchi, A., Yan, S.D., Hofmann, M., Yan, S.F., Pischetsrieder, M., Stern, D., and Schmidt, A.M. (1999) N^{ϵ}-(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**, 31740–31749
11. Huttunen, H.J., Fages, C., and Rauvala, H. (1999) Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-κB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J. Biol. Chem.* **274**, 19919–19924
12. Li, J.H., Wang, W., Huang, X.R., Oldfield, M., Schmidt, A.M., Cooper, M.E., and Lan, H.Y. (2004) Advanced glycation end products induce tubular epithelial-myofibroblast transition through the RAGE-ERK1/2 MAP kinase signaling pathway. *Am. J. Pathol.* **164**, 1389–1397
13. Malherbe, P., Richards, J.G., Gaillard, H., Thompson, A., Diener, C., Schuler, A., and Huber, G. (1999) cDNA cloning of a novel secreted isoform of the human receptor for advanced glycation end products and characterization of cells co-expressing cell-surface scavenger receptors and Swedish mutant amyloid precursor protein. *Brain Res. Mol. Brain Res.* **71**, 159–170

14. Schlueter, C., Hauke, S., Flohr, A.M., Rogalla, P., and Bullerdiek, J. (2003) Tissue-specific expression patterns of the RAGE receptor and its soluble forms—a result of regulated alternative splicing? *Biochim. Biophys. Acta* **1630**, 1–6
15. Wautier, J.L., Zoukourian, C., Chappey, O., Wautier, M.P., Guillausseau, P.J., Cao, R., Hori, O., Stern, D., and Schmidt, A.M. (1999) Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy. Soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. *J. Clin. Invest.* **97**, 238–243
16. Park, L., Raman, K.G., Lee, K.J., Lu, Y., Ferran, L.J. Jr., Chow, W.S., Stern, D., and Schmidt, A.M. (1998) Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat. Med.* **4**, 1925–1031
17. Valencia, J.V., Weldon, S.C., Quinn, D., Kiers, G.H., DeGoot, J., TeKoppele, J.M., and Hughes, T.E. (2004) Advanced glycation end product ligands for the receptor for advanced glycation end products: biochemical characterization and formation kinetics. *Anal. Biochem.* **324**, 68–78
18. Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., and Nygren, P.A. (1997) Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expr. Purif.* **11**, 1–16
19. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R.A., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips. *Science* **293**, 2101–2105
20. Lue, R.Y., Chen, G.Y., Zhu, Q., Lesaicherre, M.L., and Yao, S.Q. (2004) Site-specific immobilization of biotinylated proteins for protein microarray analysis. *Methods Mol. Biol.* **264**, 85–100
21. Schatz, P.J. (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology* **11**, 1138–1143
22. Beckett, D., Kovaleva, E., and Schatz, P.J. (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci.* **8**, 921–929
23. Prinz, W.A., Aslund, F., Holmgren, A., and Beckwith, J. (1997) The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J. Biol. Chem.* **272**, 15661–15667
24. Goto, Y. and Hamaguchi, K. (1979) The role of the intrachain disulfide bond in the conformation and stability of the constant fragment of the immunoglobulin light chain. *J. Biochem.* **86**, 1433–1441
25. Glockshuber, R., Schmidt, T., and Plueckthun, A. (1992) The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in *Escherichia coli*. *Biochemistry* **31**, 1270–1279
26. Green, N.M. (1990) Avidin and streptavidin. *Methods Enzymol.* **184**, 51–67
27. Qureshi, M.H., Yeung, J.C., Wu, S.C., and Wong, S.L. (2001) Development and characterization of a series of soluble tetrameric and monomeric streptavidin muteins with differential biotin binding affinities. *J. Biol. Chem.* **276**, 46422–46428
28. Srikrishna, G., Huttunen, H.J., Johansson, L., Weigle, B., Yamaguchi, Y., Rauvala, H., and Freeze, H.H. (2002) *N*-Glycans on the receptor for advanced glycation end products influence amphoterin binding and neurite outgrowth. *J. Neurochem.* **80**, 998–1008
29. Hanford, L.E., Enghild, J.J., Valnickova, Z., Petersen, S.V., Schaefer, L.M., Schaefer, T.M., Reinhart, T.A., and Oury, T.D. (2004) Purification and characterization of mouse soluble receptor for advanced glycation end products (sRAGE). *J. Biol. Chem.* **279**, 50019–50024
30. Helenius, A. and Aebi, M. (2001) Intracellular functions of N-linked glycans. *Science* **291**, 2364–2369
31. Yoshida, Y. (2003) A novel role for *N*-glycans in the ERAD system. *J. Biochem.* **134**, 183–190
32. Ostendorp, T., Weibel, M., Leclerc, E., Kleinert, P., Kroneck, P.M., Heizmann, C. W., and Fritz, G. (2006) Expression and purification of the soluble isoform of human receptor for advanced glycation end products (sRAGE) from *Pichia pastoris*. *Biochem. Biophys. Res. Commun.* **347**, 4–11
33. Williams, A.F. and Barclay, A.N. (1988) The immunoglobulin superfamily—domains for cell surface recognition. *Annu. Rev. Immunol.* **6**, 381–405
34. Hunkapiller, T. and Hood, L. (1989) Diversity of the immunoglobulin gene superfamily. *Adv. Immunol.* **44**, 1–63
35. Ishihara, K., Tsutsumi, K., Kawane, S., Nakajima, M., and Kasaoka, T. (2003) The receptor for advanced glycation endproducts (RAGE) directly binds to ERK by a D-domain-like docking site. *FEBS Lett.* **550**, 107–113
36. Yang, Z., Makita, Z., Horii, Y., Brunelle, S., Cerami, A., Sehajpal, P., Suthanthiran, M., and Vlassara, H. (1991) Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose-modified proteins. *J. Exp. Med.* **174**, 515–524
37. Wilton, R., Yousef, M.A., Saxena, P., Szpunar, M., and Stevens, F.J. (2006) Expression and purification of recombinant human receptor for advanced glycation endproducts in *Escherichia coli*. *Protein Expr. Purif.* **47**, 25–35