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# Minimum stable structure of the receptor for advanced glycation end product possesses multi ligand binding ability

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ABSTRACT

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor involved in the development of diabetic complications. Although the soluble form of the extracellular domain maintains the ability to bind multi-ligands, it is unstable and degrades into several peptide species during storage. Proteolysis with thrombin or factor Xa revealed several protease sensitive sites. Most sensitive site is located between Arg228 and Val229, and peptide bond next to Arg216, Arg116, Arg114 and Trp271 are also cleaved. Seven truncated extracellular domains of RAGE were engineered in order to obtain a stable soluble fragment. RAGE 143 (Ala23-Thr143) is not only protease resistant but also shows the same ligand-binding ability as that of the full-length extracellular domain. The resultant minimum RAGE 143 works as a stable recognition devise to detect advanced glycation end products (AGEs).



Advanced glycation end products (AGEs) are protein adducts generated by non-enzymatic reactions that accumulate in various tissues with age or under diabetic conditions defined by chronic hyperglycemia [1]. Due to their diverse nature and heterogeneity, AGEs have not been well characterized [2]. However, AGEs are known to bind to the cell surface receptor for AGE (RAGE), which can lead to various diabetic complications such as nephropathy, retinopathy and neuropathy [3,4]. AGE–RAGE interaction initiates a sustained period of cellular activation mediated by receptor-dependent signaling, leading to inflammation [5].

RAGE belongs to the immunoglobulin superfamily that interacts with a broad range of ligands, including AGEs, amyloid-β peptide, amphoterin and S100 protein [6]. RAGE is composed of an N-terminal extracellular domain containing three immunoglobulin-like domains, one variable type domain (V-type domain) and two constant-type domains (C1 domain and C2 domain): a single transmembrane domain, and a C-terminal short cytoplasmic domain [7]. Recently, the extracellular domain of RAGE has been engineered and used in the detailed analysis of the kinetics of RAGE-ligand interactions *in vitro* [8–11]. In addition, the solution structure of V-domain has been elucidated [12–14]. The interaction of RAGE and AGEs is associated with the development of complications in diabetes, making this interaction a prime target for therapeutic blocking [15]. Taken together, these studies have provided new insights into RAGE–AGEs interactions.

We reasoned that soluble RAGE might be a powerful tool to detect multi ligands, which are known to cause diabetic complications. Using soluble biotinylated RAGE, we designed a novel method to detect AGEs [11]. We found that the soluble extracellular domain of RAGE without the recognition site for thrombin and factor Xa was still sensitive to both proteases. Moreover soluble RAGE was unstable and degraded into several peptide fragments during storage at 4 °C. The aim of this study was to further analyze the degradation pattern of RAGE and generate a stable form of the protein. Here, we report the minimization of soluble RAGE whilst retaining ligand-binding activity. The minimum RAGE was found to be RAGE 143 (encoding amino acids A23-T143), which is resistant to degradation and showed ligand-binding ability of the full-length extracellular domain.

## Materials and methods

*Materials*. *Escherichia coli* strain DH5α, strain Origami (DE3) and the plasmid pET-16b were purchased from Novagen (Madison, WI). Plasmid pAC-4 was obtained from Avidity (Denver, CO). Streptavidin mutein matrix was from Roche (Mannheim, Germany).

*Plasmid construction*. cDNA coding human RAGE variant 1 (GenBank accession no. AB036432) was amplified from human lung polyA$^+$ RNA (Clontech, Palo Alto, CA) by reverse transcriptase-PCR as described previously [11]. DNA fragments were PCR amplified with primers containing 5′ *Nde*I and 3′ *Xho*I restriction sites and contained the following RAGE protein sequences (excluding the 22 amino acid signal peptide): sRAGE (A23-S332:

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full-length extracellular domain), RAGE 112 (A23-N112), RAGE 120 (A23-I120), RAGE 137 (A23-V137), RAGE 143 (A23-T143), RAGE 214 (A23-L214), RAGE 223 (A23-A223) and RAGE 226 (A23-Q226). The region encoding AviTag (GLNDIFEAQKIEWHE) for *in vivo* biotinylation [16] was amplified from pAC-4 with primers containing 5′ *Xho*I and 3′ *Bgl*II restriction sites and inserted into pET16-EK (enterokinase recognition site was inserted instead of factor Xa site in pET-16b) at the *Xho*I and *BamH*I, respectively. The resultant plasmid, termed pETaviEK, confers an N-terminal His-Tag and a C-terminal AviTag onto the cloned gene products. The fragment encoding each RAGE was inserted into pETaviEK digested with *Nde*I and *Xho*I, and its identity was verified by DNA sequencing.

*Expression and purification of biotinylated RAGE fragments*. Origami B(DE3) co-transformed by pETaviEK carrying each RAGE fragment and pBirA (Avidity, Denver, CO) was cultured, and the biotinylated RAGE fragments were induced by adding 1 mM IPTG and 50 μM D-biotin. After 18 h incubation at 25 °C, the bacterial cells were harvested, resuspended in TBS (20 mM Tris–HCl, 150 mM NaCl, pH 7.6) containing 10 mM imidazole and disrupted by sonication. The biotinylated sRAGEs were purified by sequential chromatography steps as described previously [11]. Briefly, cleared lysate was gently mixed with 1/4 volume of 50% nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen GmbH, Hilden, Germany) slurry for 30 min at 4 °C. The resin was packed into a column and washed with TBS containing 60 mM imidazole. Bound proteins were then eluted with a linear gradient of imidazole (60–500 mM) using an FPLC system (GE Healthcare, Amersham, UK). After dialysis against TBS the protein was further purified using a streptavidin mutein matrix (Roche) as follows. A 10% volume of 50% streptavidin mutein matrix slurry equilibrated with TBS was added to the dialyzed solution and gently mixed for 15 min at 4 °C before packing into a column. The column was washed with TBS, and RAGE fragments were then eluted with a linear gradient of D-biotin (0–2 mM) using an FPLC. Fractions containing protein of the expected molecular mass were collected and their purity was assessed by SDS–PAGE.

*Protease treatment and determination of the protease sensitive sites*. sRAGE was incubated with thrombin or factor Xa (both from Novagen) according to the conditions recommended by the manufacturer except for the ratio of target protein to protease. The reaction was stopped by adding 2 mM (final concentration) PMSF, and the mixture was analyzed by SDS–PAGE or SDS–PAGE followed by blotting onto a PVDF membrane. However, no PMSF was added to samples that were subsequently analyzed by mass spectrometry. The reaction mixture with or without protease was analyzed with MALDI-TOF mass spectrometry (Reflex II, Brucker Daltonik GmbH, Bremen, Germany) and ESI-QIT mass spectrometer (LCQ, Thermo Fischer Scientific, Waltham, MA) equipped with a nano LC system coupled to a Chorus220 nano-LC pump and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) *via* the XYZ NanoESI interface (AMR Inc., Tokyo, Japan).

The blotted PVDF membrane was stained with Coomassie (CBBR) and the amino terminal sequence of each fragment was determined using a protein sequencer G1000A (Hewlett–Packard, Palo Alto, CA). SDS–PAGE gels were stained with CBBR and tryptic peptides derived from gel excised CBBR stained bands were analyzed by MALDI-TOF mass spectrometry.

*Preparation of sugar-derived advanced glycation end products*. Sugar-derived AGE–BSAs were prepared as described previously [17]. We prepared the control BSA by incubation without sugar.

*Detection of AGEs on microtiter wells*. Detection of AGEs on microtiter wells was performed described previously [11]. The specific binding of AGEs was determined by subtraction of the absorbance obtained with TBS instead of AGE–BSAs.

## Results

### *Sensitivity of hsRAGE to proteases of the coagulation cascade*

Thrombin and factor Xa, proteases belonging to the coagulation cascade, are widely used for cleavage of affinity tags during purification of recombinant proteins. However, despite the absence of a recognition site for either thrombin or factor Xa, unexpected cleavage of sRAGE occurred upon treatment with a slight excess of these proteases. Thus, we introduced an enterokinase recognition site between the N-terminal His-Tag and sRAGE in this study. Purified sRAGE was then analyzed by MALDI-TOF mass spectrometry and nano ESI-QIT mass spectrometry. The molecular mass of the protein was identical to the anticipated value based on the primary amino acid sequence with no modifications other than the expected biotinylation at the C-terminus. There was, however, no change in sensitivity of sRAGE to thrombin or factor Xa proteases. Furthermore, our results show that sRAGE is unstable and undergoes degradation during storage at 4 °C (Fig. 1B, left panel). Proteolysis by these two proteases was performed in order to characterize the sensitive sites of sRAGE.

Two major species appeared after 18 h treatment with thrombin; one migrated around 30 kDa (Fig. 1B, arrow a) another around 16 kDa (Fig. 1B, arrow b), with an additional very weak signal detected below 14 kDa (Fig. 1B, arrow c). N-terminal peptide sequencing revealed that peptide "a" started from the amino terminus of the full-length protein and "b" from V229. Peptide "c" was a mixture of two species starting from the N-terminus and from V117. In-gel digestion of each band with trypsin followed by MALDI-TOF mass spectrometry analysis showed the following results. Peptide "a" comprises the N-terminus of the full-length protein to R228 and R216. Peptide "b" includes V229 to the C-terminus. Peptide "c" contains two peptide species from the N-terminus to R116 and from V117 to R228.

Treatment with factor Xa generated three major peptide fragments (Fig. 1B, arrows d–f). N-terminal peptide sequencing showed that "d" started from the N-terminus, and "f" was a mixture of three peptides starting from the N-terminus, V115 and M272. Analysis of peptide "e" was unclear. Each band on the gel was further analyzed by MALDI-TOF mass spectrometry, as described for the thrombin-treated peptides. Our results show that peptide "d" comprises the N-terminal amino acid of the full-length protein to R216. Peptide "e" consists of residue V117 to R228. Peptide "f" is a mixture of three peptides encoding the N-terminus to R116, V115 to R216, and M272 to the C-terminus. The identified fragments are illustrated in the lower panel to Fig. 1A along with the corresponding amino acid sequences.

A time course of digestion by thrombin showed that the major fragment, migrating with an apparent molecular mass of ~30 kDa, appeared early on in the digest and was subsequently cleaved into two fragments (Fig. 1D). Detailed analysis of the peptides by MALDI-TOF mass spectrometry indicated that the R228 site was cleaved first (Fig. 1D, arrow 1) followed by cleavage at R216. These results demonstrated that the peptide bond between R228 and V229 was the most sensitive site to proteolysis. Once cleavage had started, digestion between R216 and H217 occurred resulting in the appearance of a peptide fragment from the N-terminus to R216 (Fig. 1D, arrow 2).

Taken together, our results identified the protease sensitive sites of sRAGE, which are summarized in Fig. 1A, upper panel and in Fig. 1C, sRAGE is composed of three immunoglobulin-like domains; one V-type and two C-type domains. Protease sensitive sites are located near the end of these domains, with the most sensitive site located between R228 and V229 near the end of the C1 domain.

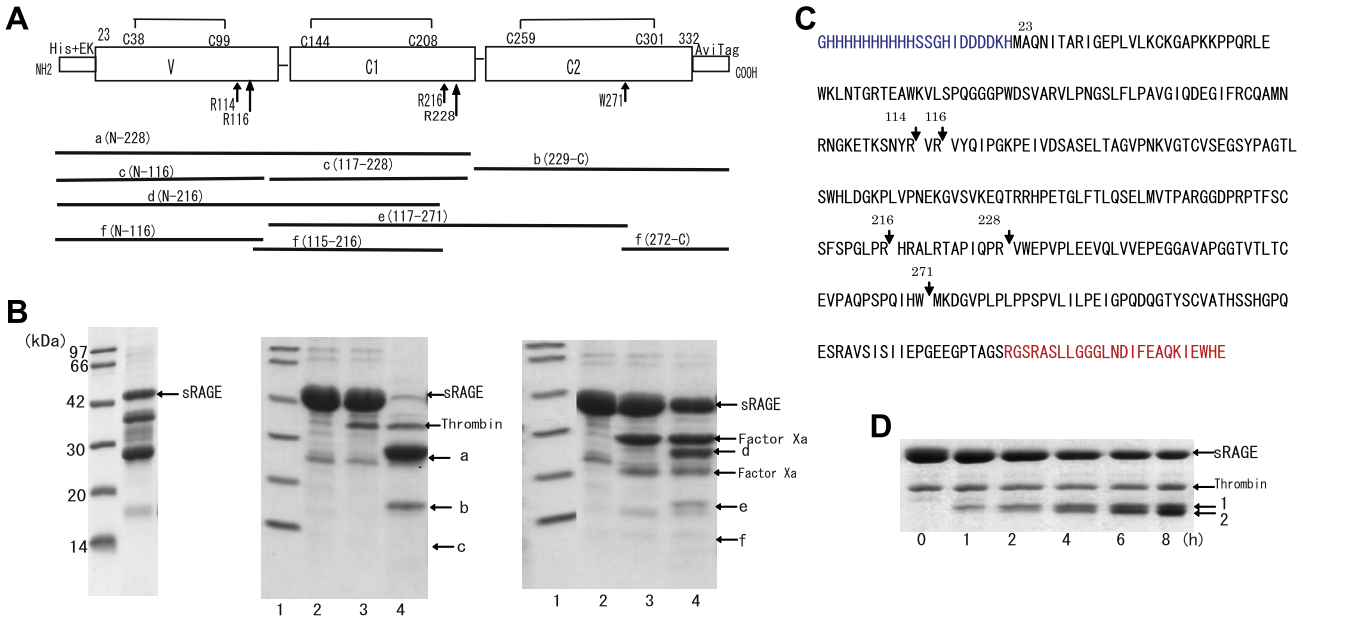


**Figure 1.** Sensitivity of sRAGE to thrombin and factor Xa. (A) The upper bar: schematic diagram of the structure of human RAGE used in this study (carrying an N-terminal His-Tag and C-terminal AviTag) and protease sensitive recognition sites. Numbers correspond to the amino acid sequence of full-length human RAGE. C: represents Cys involved in disulfide linkages. Lower part: identified peptide fragment generated by treatment with thrombin or factor Xa. Fragments of a–f correspond to arrows in Fig. 1B. N in parentheses means N-terminus and C means C-terminus. (B) Left panel: degradation pattern of sRAGE during storage at 4 °C. A few months after purification, 4 μg of protein was applied to SDS–PAGE followed by CBBR staining. Central and right panel: SDS–PAGE of sRAGE digested with proteases. Fifteen micrograms of sRAGE/30 μl solution was treated with 2 U of thrombin (central panel) or 12 U of factor Xa (right panel) for 18 h at 25 °C. The reaction was quenched by adding PMSF to a final concentration of 2 mM. A 10 μl aliquot of the reaction mixture was then analyzed by SDS–PAGE followed by CBBR staining. Lane 1: molecular weight marker, 2: sRAGE incubated without protease, 3: 0 h after adding protease, 4: 18 h after adding protease. (C) Amino acid sequence of recombinant sRAGE used in this study. The N-terminal His-Tag and enterokinase recognition site for removing the His-Tag are highlighted in blue. Ala 23 is the first amino acid of the mature protein after the signal sequence is removed. The 25 amino acids at the C-terminus (highlighted in red) comprise the AviTag (15 amino acids) for biotinylation and another 10 amino acids inserted as a result of the cloning strategy. Arrows indicate the protease sensitive sites. (D) Time course of cleavage by thrombin treatment. Sixteen micrograms of sRAGE/70 μl solution was treated with 2 U of thrombin at 25 °C. A 10 μl aliquot of reaction mixture was removed at the indicated time and the reaction stopped by addition of PMSF. The reaction mixtures were then analyzed by SDS–PAGE. Time points are given in hours.

*Minimization of RAGE extracellular domain*

The extracellular portion of RAGE is composed of three independent domains [10], each domain connected by a short linker region (Fig. 1A). The V-domain, thought to be essential for AGE binding [18], contains the two protease sensitive sites (Arg114 and Arg116) near the linker region. Three truncated forms of RAGE (RAGE 112 (A23-N112), RAGE 120 (A23-I120) and RAGE 137 (A23-V137)) covering the V domain were designed in order to generate a protease resistant protein. RAGE 112 and RAGE 120, lacking a protease sensitive site, were found to be completely insoluble. A proportion (~20%) of RAGE 137 encoding the V-domain and a linker region was expressed in a soluble form.

Judging from the time course of thrombin treatment, V-C1 was relatively resistant to proteolysis. We also designed a longer mini RAGE compared to the V domain that did not include the highly sensitive protease recognition sites. The most sensitive site was R228, so the longest mini RAGE was designed from the amino terminus to Q226. The C-terminus of R216 was also a protease sensitive site, so a mini RAGE shorter than the amino terminus to R216 was also designed. Care was taken not to disrupt the intramolecular S–S bond of the C1 domain (C144–C208). Finally RAGE 143 (A23-T143), RAGE 214 (A23-L214), RAGE 223(A23-A223) and RAGE 226 (A23-Q226) were designed. All four mini-RAGEs were expressed in a fully soluble form, and RAGEs 143, 223 and 226 were successfully purified to homogeneity (Fig. 2A) using the same protocol as described for sRAGE. The protein yield after purification of the mini RAGEs were similar for each other (around 0.8 mg/L culture) but lower than that of sRAGE (2.2 mg/L culture). However, all three mini-RAGEs were stable during storage. Indeed, no aggregation or degradation was detected after several months' storage (date not shown). These results indicated that the problem of protein instability had been overcome by minimization.

*Evaluation of the ligand binding ability of each mini RAGE*

The ability to bind AGEs was evaluated using ELISA. C-terminal biotin ligated to mini RAGEs was effectively recognized by streptavidin–HRP conjugate. Furthermore, AGEs on a microtitre well are measurable by absorbance at 450 nm without using an antibody. Indeed, the absorbance is directly correlated to the amount of binding. The binding ability of each mini RAGE to AGEs was compared to that of sRAGE. All three mini RAGEs recognized the various AGEs (Fig. 2B-2, 3, 4), but failed to recognize BSA used as a negative control (Fig. 2B-1). The results indicated that RAGE 143, 223, 226 retain the specific binding ability to AGEs. Moreover they showed the highest affinity for Ribose–AGE, consistent with that reported for glycosylated RAGE expressed on the cell surface [17]. Comparison between the sensitivity of each mini RAGE to that of sRAGE showed that RAGE 223 displayed the strongest binding ability for each AGE. Indeed, RAGE 223 shows the strongest binding ability to all three AGEs. Thus, RAGE 223 has the potential to work as the most sensitive devise for detecting AGEs. We also found that all of mini RAGEs can recognize 10 ng/ml of AGEs. Although RAGE 143 comprised only the V domain and lacks the C1 domain, its binding ability was slightly stronger than that of sRAGE at low concentrations of AGEs.

The sensitivity of mini RAGEs to proteolytic digestion were also investigated. It is said that 0.001 units of thrombin cleaved 1 μg control protein. Although sRAGE lacks any thrombin sensitive rec-

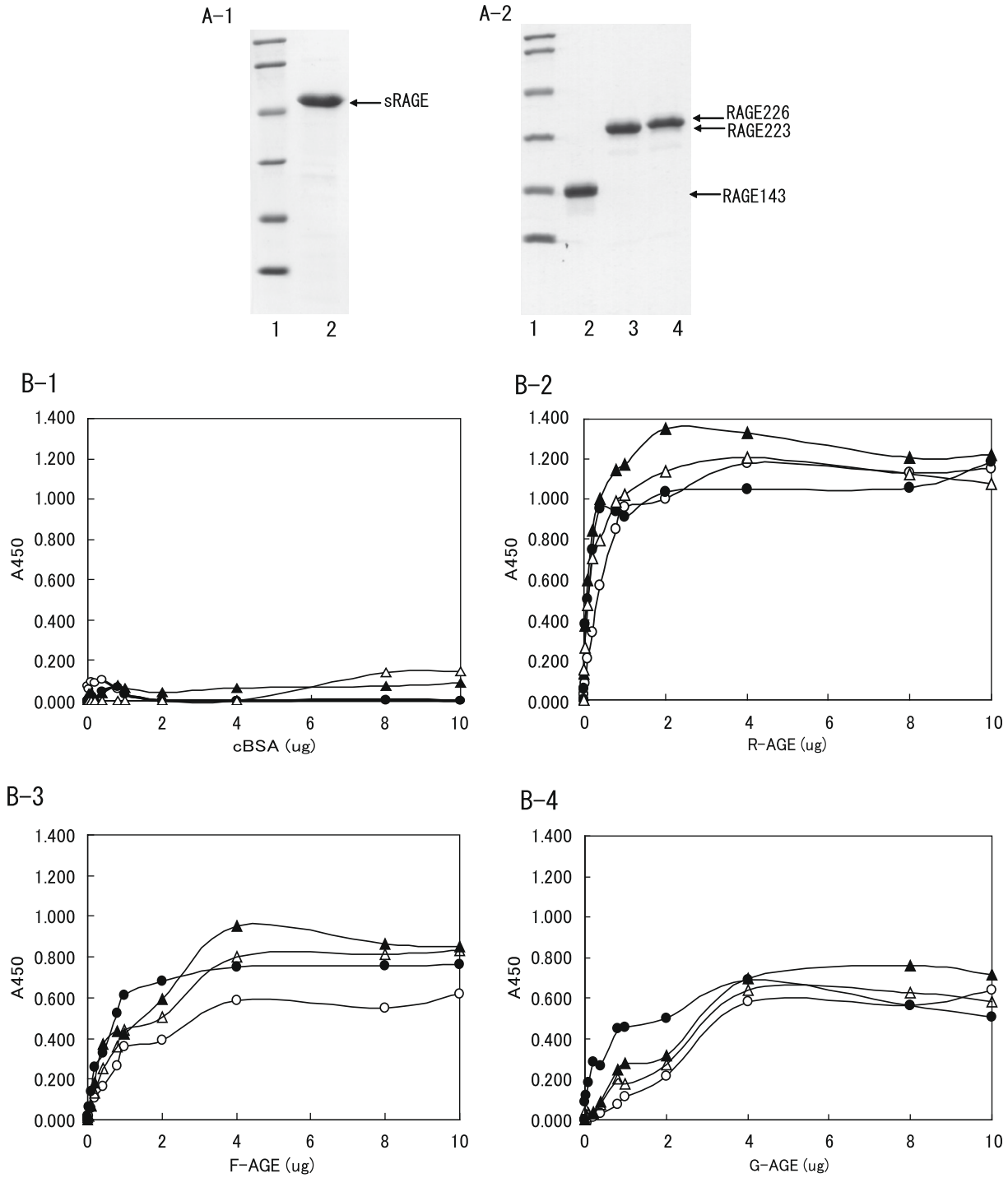


**Figure 2.** (A) SDS–PAGE of purified sRAGE (A-1) and each mini RAGE (A-2). Two micrograms of fresh RAGEs were applied to SDS–PAGE. (B) Binding ability of soluble RAGEs to different AGEs. Open circle, sRAGE; closed circle, RAGE 143; closed triangle, RAGE 223; open triangle, RAGE 226. B-1, binding ability to control BSA; B-2, binding ability to Ribose AGE; B-3, binding ability to Fructose AGE; B-4, binding ability to Glucose AGE. Values are means of three wells of two independent experiments.

ognition sites, cleavage was detected in the presence of a slight excess of protease (0.01 U to 1 μg protein: 10-fold over digest). RAGE 143 includes R114 and R116, which are sensitive to thrombin digestion as indicated in the above section. Nevertheless, RAGE 143 was not cleaved by treatment with an excess amount of thrombin compared to sRAGE (Fig. 3). RAGE 223 and RAGE 226 were stable during storage, though they were cleaved at the C-terminus of R216 by thrombin treatment (date not shown). These results indicated that RAGE 143 is the minimum stable soluble RAGE that still retains the specific ligand binding ability of the full-length protein.

## Discussion

Because soluble RAGE is invaluable for the detection of AGEs, several methods to produce correctly folded soluble RAGEs have been published [8–11]. We previously demonstrated that the C-

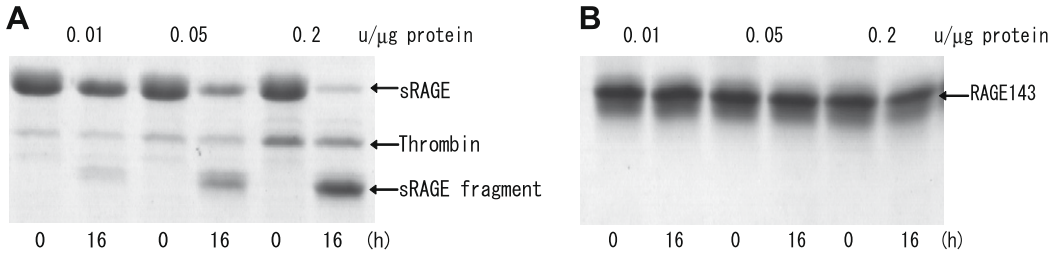


**Figure 3.** Sensitivity of sRAGE and RAGE 143 to thrombin. Two micrograms of each RAGE were treated with the indicated ratio of thrombin for 16 h at 25 °C. The reaction was stopped by adding 2 mM (final concentration) PMSF and applied to SDS–PAGE. Left panel: sRAGE, right panel: RAGE 143.

terminal biotinylated extracellular domain of RAGE is a powerful tool for the detection of multi ligands [11]. Unfortunately the soluble form of the extracellular domain is unstable and is therefore an unsuitable device for the detection of AGEs. In this study we determined the protease sensitive sites of soluble RAGE. We have also generated a minimum stable RAGE, which possesses the specific binding ability to AGEs displayed by the full-length protein.

Five protease sensitive sites were determined, all of which were located near the end of domains (especially the C1 domain). Dattilo et al. indicated that the V and C1 domains form an integrated structural unit, rather than two independent domains [12]. Consistent with this hypothesis, the mini RAGE comprising the V and C1 domain (RAGE 223 and 226 in this study) are free from degradation and show specific binding to AGEs. The initial cleavage site is thought to be between R228 and V229 near the end of the C1 domain. Both RAGE 223 and 226 lack this site, which could account for their enhanced resistance to degradation. Moreover they showed higher sensitivity to AGEs compared to the full-length extracellular domain composed of V, C1 and C2 (sRAGE in this study). The V domain in isolation (RAGE 112, 120 in this study) could not fold properly, resulting in the formation of inclusion bodies. However, correct folding occurred when the V domain was attached to a linker region and a short part of the C1 domain (RAGE 143 in this study). Furthermore, this mini RAGE was insensitive to thrombin, despite the presence of two thrombin recognition sites (R114, R116). This result agreed with the report that the isolated V-domain displays enhanced stability [12]. The resultant mini RAGE 143 was stable during long term storage and acted as a specific recognition device. RAGE 143 shows strong binding ability to AGEs, but no detectable binding to control BSA. Furthermore, the order of affinity for the different AGEs is identical to that displayed by RAGE on the cell surface [17].

Recently, a much smaller RAGE (A23–P121) was successfully expressed in a soluble form using the thioredoxin tag and the solution structure analyzed [14]. In this study, trials to make smaller RAGEs lacking the protease sensitive sites (R114 and R116) resulted in folding problems. These two protease recognition sites are present in RAGE 143. Nevertheless, RAGE 143 folded correctly and showed dramatically reduced sensitivity to proteolytic cleavage compared with full-length protein. In conclusion, we have engineered a stable soluble RAGE possessing specific binding properties, which will serve as a valuable recognition devise.

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