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S-nitrosylation of mouse galectin-2 prevents oxidative inactivation by hydrogen peroxide

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ABSTRACT

Galectins are a group of animal lectins characterized by their specificity for β-galactosides. Galectin-2 (Gal-2) is predominantly expressed in the gastrointestinal tract. A proteomic analysis identified Gal-2 as a protein that was S-nitrosylated when mouse gastric mucosal lysates were reacted with S-nitrosoglutathione, a physiologically relevant S-nitrosylating agent. In the present study, recombinant mouse (m)Gal-2 was S-nitrosylated using nitrosocysteine (CysNO), which had no effect on the sugar-binding specificity and dimerization capacity of the protein. On the other hand, mGal-2 oxidation by H_2O_2 resulted in the loss of sugar-binding ability, while S-nitrosylation prevented H_2O_2-inducted inactivation, presumably by protecting the Cys residue(s) in the protein. These results suggest that S-nitrosylation by nitric oxides protect Gal-2 from oxidative stress in the gastrointestinal tract.

1. Introduction

Galectins comprise a group of animal lectins that specifically bind to β-galactosides and are characterized by an evolutionarily conserved sequence motif in the carbohydrate-binding site [1–3]. Galectins are involved in a wide variety of biological processes, including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and immune regulation [4,5].

Interactions between lectins and their carbohydrate ligands play important roles in various biological systems. Although the basic sugar structure recognized by mammalian galectins such as human galectin-1 (hGal-1) is the *N*-acetyllactosamine disaccharide unit, the sugar-binding specificity of each galectin can be enhanced by branched, repeated, or substituted glycans [6,7], suggesting that galectins can interact with a variety of endogenous ligands possessing different carbohydrate structures.

Gal-2 is a member of the galectin family that was first found to be highly expressed in gastric cells, predominantly in epithelial cells of the rat stomach [8]. Gastric mucous cells also exhibit strong Gal-2 immunoreactivity, with expression observed in the small intestine [9] and *Gal-2* mRNA detected in human stomach [10]. The amelioration of acute and chronic colitis in mice by Gal-2 overexpression [11], and reduced Gal-2 expression in humans associated with lymph node metastasis in gastric cancer [12] and in *Helicobacter*-induced gastric cancer progression [13] suggest that Gal-2 has a protective function in the gastrointestinal tract.

Nitric oxide (NO) is an intracellular chemical messenger that is synthesized from L-arginine by NO synthase (NOS) and participates in signal transduction in variety of mammalian cell types, including neurons and vascular endothelial cells [14]. In contrast to enzymatic formation by NOS, NO is generated in the stomach by non-enzymatic acid reduction of salivary nitrite to exert various physiological functions in the gastrointestinal tract [15–18], including functioning as a barrier in the stomach [19–21]. Furthermore, orally administered nitrate ameliorated dextran sulfate sodium-induced acute experimental colitis in mice [22]. S-nitrosylation – which involves the coupling of an NO group to the reactive thiol of a Cys residue in a polypeptide – is an important post-translational

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modification detected in a variety of proteins [23]; thus, continuous exposure of gastric mucosa to high concentrations of NO is predicted to result in the S-nitrosylation of many proteins that could contribute to the protection of the stomach lining.

Gal-2 was identified through a screen of mouse gastric mucosal proteins that are uniquely sensitive to S-nitrosylation [24], suggesting Gal-2 exerts a protective effect because of S-nitrosylation by NO in the stomach. In this study, recombinant mouse (m)Gal-2 was used to test whether the protein is the target of S-nitrosylation and examine the biochemical effects thereof.

2. Material and methods

2.1. Construction of wild-type and mutant recombinant mGal-2 expression plasmids

A DNA fragment encoding mGal-2 was amplified by PCR using mouse stomach first strand cDNA (MD-05, GenoStaff Co., Ltd., Tokyo, Japan) as a template. Forward and reverse primer sequences containing *Nde*I and *Bam*HI sites (underlined), respectively, were 5′-CATATGTCGGAGAAATTTGAGGTC-3′ and 5′-GGATCCGGCTAAGGTCTTCTGAGG-3′, respectively. The PCR product was ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The pCR2.1-TOPO-mGal-2 plasmid was digested with *Nde*I and *Bam*HI, and the DNA fragment was inserted into the corresponding restriction sites of the pET21a vector for protein expression.

cDNAs for mGal-2 mutants C57M, C75S, and C57M/C75S were generated by PCR using the pCR2.1-TOPO-mGal-2 plasmid and the following primers (substitution sites are underlined), as previously described [25]: C57M, 5′-ATGAACACCAGTGAAGGTG-3′ and 5′-GACAATGGTGGATTCATC-3'; C75S, 5′-AGCTTCAGTCCAGGGTCA-3′ and 5′-CATGTGATTTTCTCGTTG-3'; C57M/C75S, 5′-CATTGTCATGAACACCAGTGAAGG-3′ and 5′-GTGGATTCATCGAAGCGAGG-3'. The PCR products were inserted into the pET21a vector for protein expression as described above.

2.2. Purification of recombinant mGal-2

The expression and purification of recombinant wild-type and mutated forms (C57M, C75S, and C57M/C75S) of mGal-2 were performed as previously described [26].

2.3. Measurement of protein concentration

Protein concentration was determined by Bradford's method [27] using bovine serum albumin as standard.

2.4. Estimation of sulfhydryl groups

The number of free sulhydryl groups on Cys residues of recombinant mGal-2 proteins was estimated using Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]) and by measuring the absorbance at 412 nm [28] using Cys as standard.

2.5. S-nitrosylation

The wild-type and mutated forms of mGal-2 protein (1 mg/ml) were incubated in ethylenediamine tetraacetic acid-phosphate-buffered saline (EDTA-PBS) (20 mM NaH_2PO_4, 150 mM NaCl, 1 mM EDTA, pH 7.2) with or without 1 mM S-nitrosocysteine (CysNO) for 60 min at room temperature in the dark with constant rotation. CysNO was prepared at the time of use by mixing equal amounts of 200 mM Cys in 100 mM HCl and 210 mM sodium nitrite in 100 mM HCl. To remove unreacted CysNO, samples were ultrafiltered with Amicon Ultra (Millipore, Billerica, MA, USA).

2.6. Saville-Griess assay

The number of S-nitrosylated molecules was measured using the Saville-Griess assay as previously described [29]. Briefly, S-nitrosylated mGal-2 (wild-type or mutated forms) was incubated with 1% sulfanilamide and 0.1% *N*-(naphthyl)ethylenediaminde dihydrochloride in the presence 4 mM $CuCl_2$ for 20 min and S-nitrosylated content was measured photometrically at 540 nm. The amount was calculated using defined concentrations of S-nitrosoglutathione as a standard.

2.7. Oxidation of mGal-2 by hydrogen peroxide (H_2O_2)

H_2O_2 concentration was measured at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$). S-nitrosylated mGal-2 (0.1 mg/ml, 1 ml) was incubated with 10 mM H_2O_2 for 2 h in the dark followed by addition of catalase from bovine liver at a final concentration of 100 U/ml (Wako Pure Chemical Industries, Osaka, Japan) to quench excess H_2O_2.

2.8. Binding of Gal-2 to lactose

S-nitrosylated mGal-2 treated with H_2O_2 was applied to a lactose-immobilized agarose (J-Oil Mills, Inc., Tokyo, Japan) column (bed volume 1 ml). After washing with 5 ml EDTA-PBS, the bound mGal-2 was eluted with 2 ml EDTA-PBS containing 0.1 M lactose. The eluted fraction was collected (0.5 ml/fraction), and each fraction was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Bio-Safe Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA).

2.9. Frontal affinity chromatography analysis

Frontal affinity chromatography was carried out as previously described [30–32]. Briefly, pyridylaminated (PA)-oligosaccharides were dissolved in EDTA-PBS at a concentration of 5 nM and applied to the column (10 × 4.0 mm; 0.126 ml/bed volume) through a 2-ml sample loop connected to a Rheodyne 7725i injector. The flow rate was controlled by a LC-20AT pump (Shimadzu, Kyoto, Japan) at 0.25 ml/min. The sample loop and column were immersed in a 20 °C water bath. Elution of PA-oligosaccharide from the column was monitored with a Shimadzu RF-10A_{XL} fluorescence detector at 380 nm (with excitation at 310 nm). The elution volume of the PA-oligosaccharide of interest was determined as previously described. PA-rhamnose was used to determine the elution volume of a PA-oligosaccharide with no affinity for galectins. The PA-oligosaccharides used to verify the sugar-binding profile of mGal-2 were PA-001, 002, 004, 022, 023, 041, 042, 043, 044, 045, 046, 047, and 049, whose structures were described in our previous reports [31,33].

2.10. Hemagglutination assay

Hemagglutination activity was measured as previously reported [34]. Briefly, a 25-μl sample of S-nitrosylated mGal-2 was serially diluted 2-fold in a 96-well V-shaped microtiter plate, followed by the addition of either 50 μl of EDTA-PBS or 20 mM H_2O_2 in EDTA-PBS. After incubation at room temperature for 2 h, 25 μl of 4% (v/v) glutaraldehyde-fixed rabbit erythrocytes in PBS were added. After standing for 1 h at room temperature, the end-point showing the minimum concentration resulting in hemagglutination was determined.

2.11. Crosslink reaction using BS^3

After the S-nitrosylation reaction, recombinant mGal-2 (0.01 mg in 1 ml) was incubated with 0.3 mM bis (sulfosuccinimidyl) suberate (BS^3) crosslinking reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 30 min in EDTA-PBS at room temperature. The reaction was terminated by the addition of Tris to a concentration of 50 mM. Samples were resolved by SDS-PAGE, and the gel was stained with Oriole stain (Bio-Rad).

3. Results

3.1. S-nitrosylation of recombinant mGal-2

Recombinant mGal-2 (wild-type or mutated forms) was expressed in *Escherichia coli* and purified by affinity chromatography. The purified protein was S-nitrosylated using CysNO as an NO donor and the stoichiometry of S-nitrosylation per sulfhydryl group (−SH) was measured with the Saville-Griess assay and Ellman's method. The ratio of mGal-2 S-nitrosylation for wild-type mGal-2 was calculated as 1.07 moles/mole of −SH. To determine the position(s) of S-nitrosylation, mutant forms of mGal-2 (C57M, C75S, and C57M/C75S) were generated as recombinant proteins. The Cys^{57} residue was changed to Met instead of Ser because the C57S mutant had low expression (data not shown) and solubility, as previously reported [35]. For the two mutant forms of mGal-2 with only one Cys remaining in the polypeptide (C57M and C75S), the S-nitrosylation ratios were approximately 0.86 and 0.74 moles/mole of −SH, respectively. Furthermore, no S-nitrosylation was observed for the C57M/C75S mutant lacking Cys residues.

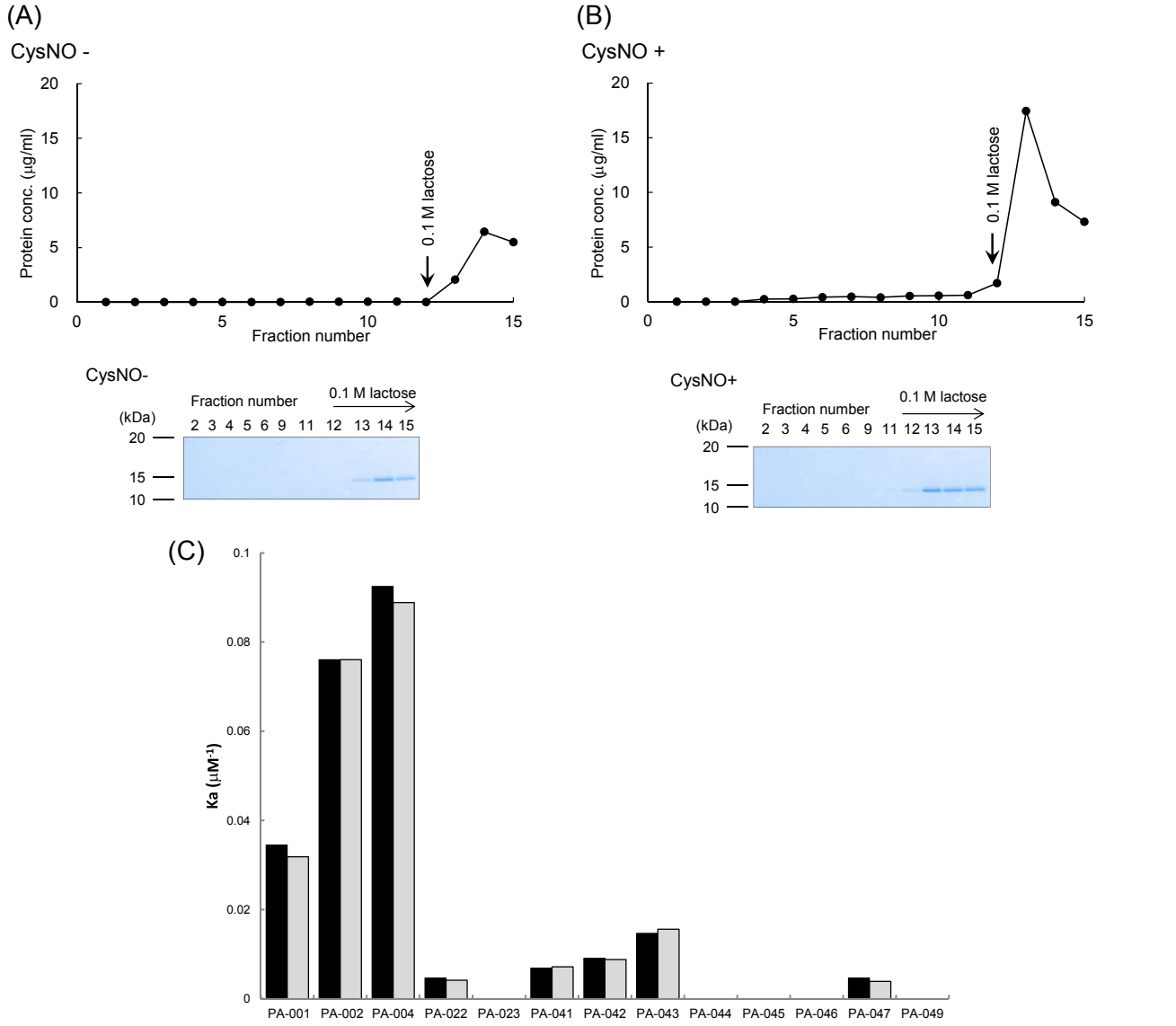

Fig. 1. Effect of S-nitrosylation on sugar-binding profiles of recombinant mGal-2. (A, B) Adsorbance to a lactose-immobilized agarose column. Purified recombinant mGal-2 was added to the column following the S-nitrosylation reaction. The column was washed with EDTA-PBS and adsorbed proteins were eluted with EDTA-PBS containing 0.1 M lactose. The protein concentration of each fraction was measured and analyzed by SDS-PAGE followed by gel staining with Coomassie Brilliant Blue. Since only recombinant mGal-2 protein was applied to the column, the bands at 15 kDa corresponded to recombinant mGal-2; bands for each fraction are shown at corresponding areas in each gel. Profiles of recombinant non-nitrosylated mGal-2 (A) and nitrosylated mGal-2 (B) eluted from lactose-immobilized agarose. (C) Frontal affinity chromatography analysis of recombinant mGal-2 with or without S-nitrosylation. After the S-nitrosylation reaction, recombinant mGal-2 protein was immobilized on *N*-hydroxysuccinimide-activated agarose. *Ka* values of immobilized mGal-2 after and without S-nitrosylation are shown as gray and black bars, respectively.

3.2. Sugar-binding capacity of mGal-2 is unaffected by S-nitrosylation

To determine the effect of S-nitrosylation on mGal-2 function, the sugar-binding capacity of mGal-2 was assessed. Following S-nitrosylation, the protein was exposed to a lactose-immobilized agarose column. In the absence of S-nitrosylation, recombinant mGal-2 was adsorbed to the column and eluted with 0.1 M lactose (Fig. 1A). Similarly, S-nitrosylated mGal-2 was adsorbed to the column and eluted with 0.1 M lactose (Fig. 1B), suggesting that S-nitrosylation did not alter the β-galactoside-binding capacity of mGal-2. To examine the effects of S-nitrosylation on sugar-binding by mGal-2 in more detail, recombinant mGal-2 protein with or without S-nitrosylation was immobilized to agarose resin and analyzed by frontal affinity chromatography. There were no significant changes in the binding affinity of mGal-2 to the PA-oligosaccharides examined (Fig. 1C). Furthermore, when recombinant and S-nitrosylated mGal-2 were labeled with Cy3 and their sugar-binding profiles were analyzed by glycoconjugate microarray [36], there were no significant differences detected (data not shown).

3.3. Dimerization of mGal-2 is unaffected by S-nitrosylation

mGal-2 dimerization was analyzed by incubating the protein with the crosslinker BS^3, followed by SDS-PAGE and Oriole staining (Fig. 2). A similar dimer-monomer ratio was observed for mGal-2 with and without S-nitrosylation.

3.4. H_2O_2-induced mGal-2 inactivation is abrogated by S-nitrosylation

Unlike untreated mGal-2 (Fig. 1A), recombinant mGal-2 was inactivated by treatment with 10 mM H_2O_2 for 2 h, as evidenced by the absence of interaction between H_2O_2-treated mGal-2 and immobilized lactose in the agarose column (Fig. 3A). In contrast, S-nitrosylated mGal-2 was able to bind to the lactose-immobilized agarose column even after H_2O_2 treatment and was eluted with lactose (Fig. 3B), suggesting that the oxidative inactivation of mGal-2 was prevented by S-nitrosylation. Similarly, S-nitrosylation preserved the hemagglutination activity of mGal-2. As shown in Fig. 4, the hemagglutination activity was lost upon exposure to H_2O_2 prior to the addition of a rabbit erythrocyte suspension (line C), even when the mGal-2 concentration was 2^5-fold higher than the minimum concentration that induced hemagglutination in the absence of H_2O_2 (line A). S-nitrosylated mGal-2 retained hemagglutination activity (line B) even after H_2O_2 treatment (line D). These results indicate that S-nitrosylation protects mGal-2 against oxidative inactivation by H_2O_2.

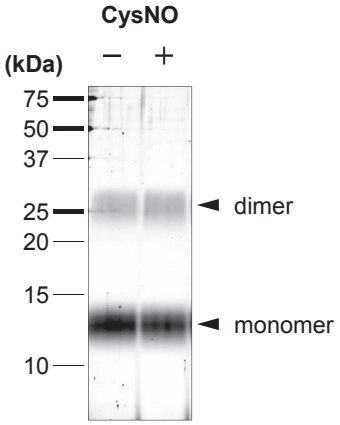

Fig. 2. Detection of mGal-2 dimer formation using the BS^3 crosslinker. BS^3 was added to recombinant mGal-2 solution with (CysNO+) or without (CysNO−) S-nitrosylation. The reaction mixture was analyzed by SDS-PAGE and the gel was stained with Oriole.

4. Discussion

In this study, the ratio of recombinant wild-type mGal-2 S-nitrosylated by CysNO was calculated as 1.07 moles of S-nitrosylation/mole of −SH, suggesting that the two Cys residues (Cys^{57} and Cys^{75}) were both fully S-nitrosylated, which was supported by the fact that no S-nitrosylation was observed for the C57M/C75S mutant lacking Cys. When equivalent amounts of wild-type or mutated protein were used for S-nitrosylation, the ratio was 1:0.48:0.42:0 for wild-type:C57M:C75S:C57M/C75S. Furthermore, the ratio of S-nitrosylation was approximately 0.86 and 0.74 moles/mole of −SH for the C57M and C75S mGal-2 mutants, respectively. These results suggest that the single remaining Cys in the two mutants (Cys^{75} in C57M mutant and Cys^{57} in C75S) were both S-nitrosylated by CysNO.

Since the generated mutants were adsorbed to β-galactoside-containing columns and eluted with lactose, and no differences in sugar-binding profiles were detected by frontal affinity chromatography—implying that sugar-binding profiles were similar between mutant and wild-type mGal-2—it was concluded that the mutants have the same biochemical properties as wild-type mGal-2.

Cys^{57} and Cys^{75} are highly conserved in Gal-2 across mammals. According to the X-ray crystal structure of human Gal-2 in complex with lactose [37], Cys^{57} is located in the middle of the carbohydrate-binding cassette with its side chain sulfhydryl group (a potential site for S-nitrosylation) protruding to the side opposite the β-sheet that binds lactose.

We speculated that S-nitrosylation could alter the sugar-binding capacity of mGal-2, especially at Cys^{57}; S-nitrosylated mGal-2 was therefore assessed for its capacity to bind to lactose-immobilized agarose columns and was also analyzed by frontal affinity chromatography and glycoconjugate microarray. There were no differences in the sugar-binding profile or hemagglutination activity between mGal-2 with and without S-nitrosylation, even though Cys^{57} appeared to be S-nitrosylated. These combined with the results from the BS^3 crosslinking experiment indicate that S-nitrosylation does not affect mGal-2 dimerization; this is likely because—based on the crystal structure of human Gal-2—the two Cys residues are distant from the surface that engages in dimer formation.

However, an interesting difference between mGal-2 with and without and S-nitrosylation was observed when the microtiter V-plate was left on the bench after the measurement of hemagglutination activity: erythrocytes remained hemagglutinated by S-nitrosylated mGal-2 but not by mGal-2 after a few days at room temperature. Since it was assumed that the loss of hemagglutination activity was due to mGal-2 oxidation, mGal-2 was oxidized by treatment with H_2O_2, one type of reactive oxygen species (ROS) produced in the stomach upon inflammation caused by *Helicobacter pylori* or other types of infection [38]. H_2O_2 treatment resulted in the loss of mGal-2 activity, an effect that was abrogated by S-nitrosylation. Although a small amount of mGal-2 was still adsorbed to the lactose-immobilized agarose column and was eluted with 0.1 M lactose, this appeared to be the result of insufficient oxidation by H_2O_2.

It is possible that S-nitrosylation of the two Cys residues inhibited the oxidation of these residues. Some proteins such as

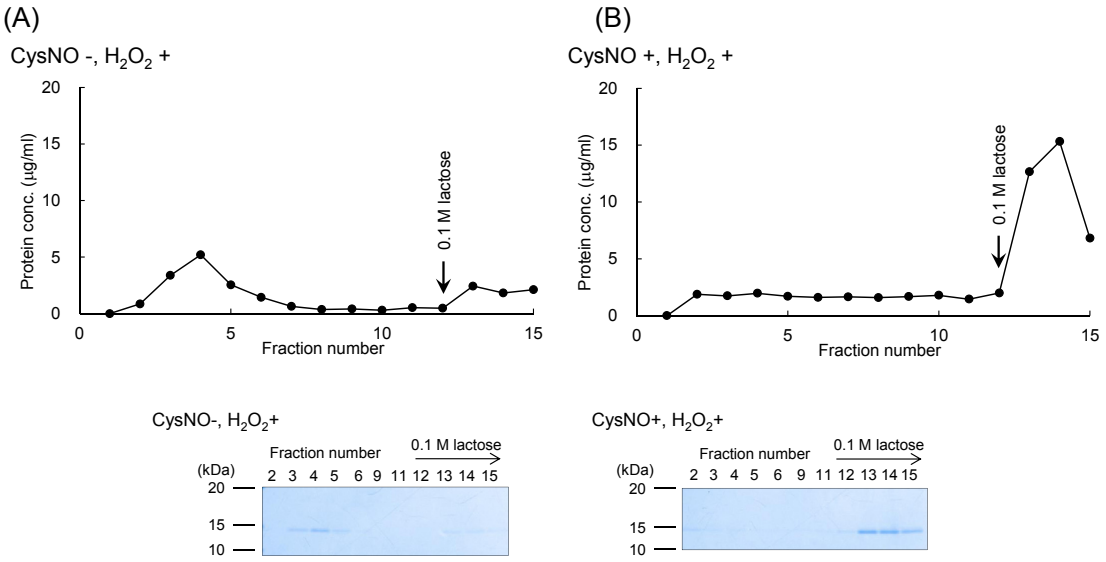

Fig. 3. Loss of sugar-binding capacity of recombinant mGal-2 after H_2O_2 treatment and protective effect of S-nitrosylation against oxidation-induced protein inactivation. Purified recombinant mGal-2 was treated with 10 mM H_2O_2 for 2 h in the dark, and was added to a lactose-immobilized agarose column, washed with EDTA-PBS, and eluted with lactose. Elution profiles of mGal-2 were verified by measuring the protein concentration of each fraction (0.5 ml each), which was analyzed by SDS-PAGE. Bands at around 15 kDa, the molecular weight of the mGal-2 monomer, were detected by Coomassie Brilliant Blue staining. (A, B) Profiles of recombinant non-nitrosylated mGal-2 (A) and mGal-2 nitrosylated before H_2O_2 treatment (B) eluted from lactose-immobilized agarose.

protein-tyrosine phosphatase 1B are protected against oxidation-induced inactivation by S-nitrosylation of Cys residues [39]. Since Gal-2 exists predominantly in the gastrointestinal tract where it has a protective role, NO produced in the stomach may induce S-nitrosylation of Gal-2, thereby protecting the protein from oxidation by ROS so that it retains its activity.

On the other hand, a very small amount of S-nitrosylated mGal-2 lost lectin activity by oxidation and were therefore not retained by the lactose-immobilized agarose column, possibly because H_2O_2 treatment may have resulted in oxidation of residues other than the two Cys residues.

Gal-1 has six Cys residues whose oxidative states dictate Gal-1 function [40–44]. On the other hand, the monomer–dimer equilibrium of Gal-1 regulates its sensitivity to oxidative inactivation [45]. Furthermore, human Gal-1 may also be a substrate for S-nitrosylation [46]. Taken together, these results suggest that S-nitrosylation is an important modification for regulating galectin activity in animals.

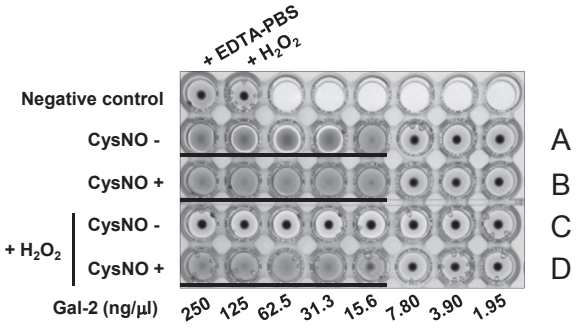

Fig. 4. Loss of hemagglutination activity of recombinant mGal-2 after H_2O_2 treatment and protective effect of S-nitrosylation against oxidation-induced protein inactivation. Purified recombinant mGal-2 (250 μg/ml in EDTA-PBS) was diluted 2-fold in a 96-well microtiter plate and treated with H_2O_2 for 2 h in the dark. A suspension of glutaraldehyde-fixed rabbit erythrocytes in EDTA-PBS was added and hemagglutination activity was determined after 1-h incubation.

Conflict of interest

None declared.

Acknowledgments

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