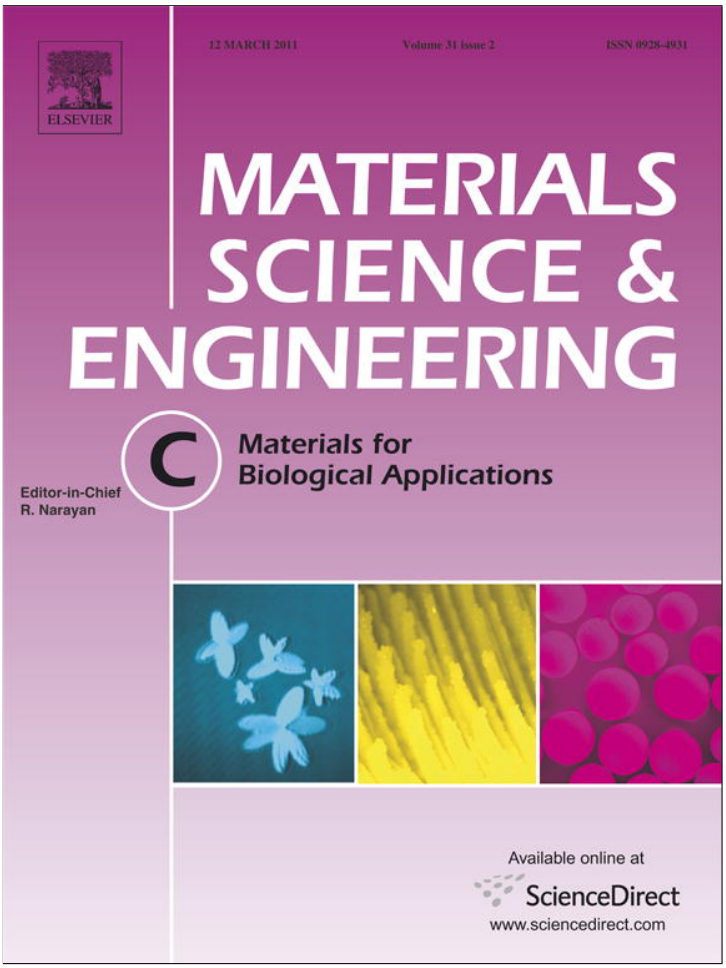

Materials Science and Engineering C 31 (2011) 158–165

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Denatured-jacalin derivatives with selective recognition for *O*-linked glycosides (ST, T, Tn, and STn Type) on IgA1 hinge region

Keiichi Miyamoto [a,*], Ayaka Kawasaki [a], Yuko Nagata [a], Masanori Uraya [a], Hisayoshi Kojima [a], Takanori Ito [a], Takashi Horiuchi [a], Nagisa Asakawa [b], Shinsuke Nomura [b]

[a] *Department of Chemistry for Materials, Graduate School of Engineering, Mie University, 1577 Kurima-machiya-cho, Tsu, Mie 514-8507, Japan*
[b] *Department of Cardiology and Nephrology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan*

ARTICLE INFO

Article history:
Received 23 February 2010
Received in revised form 18 June 2010
Accepted 8 August 2010
Available online 25 August 2010

Keywords:
Jacalin
IgA1
IgA nephropaty
O-linked glycoside

ABSTRACT

Immunoglobulin A1 (IgA1) concentration in the plasma of patients with IgA nephropathy (IgAN) as the cause of renal failure is higher than that in the plasma of normal controls. IgA1 with abnormal sugars is considered to deposit in the glomerular mesangium, aggravating nephritis in IgAN. Jacalin is a lectin that recognizes sugars on IgA1. However, its selective-recognition for normal-type (ST type, NeuAc-α(2,3)-Gal-β(1,3)-GalNAc) and abnormal-type (T type, Gal-β(1,3)-GalNAc; Tn type, GalNAc; STn type, NeuAc-α(2,6)-GalNAc) sugars α-*O*-linked to serine/threonine in IgA1 is weak. Therefore, jacalin cannot be used for recognizing specific sugar types on IgA1. We attempted to develop a new recognition method for specific sugar types on IgA1 by utilizing the multirecognition capability of jacalin. Its binding abilities were regulated by heat denaturation with suitable template sugar (galactose or *N*-acetylgalactosamine). Further, we successfully prepared denatured-jacalin derivatives, which recognized ST-/T-type sugars on IgA1, by sugar-immobilized affinity chromatography. Enzyme-linked immunosorbent assay of denatured-jacalin derivatives, showed the ratios of abnormal sugars on IgA1 in the plasma of IgAN patients and normal controls to be approximately 60% and 20%, respectively. The results proved that profiling of sugar types in IgAN can successfully be performed by solely using jacalin derivatives.

1. Introduction

Immunoglobulin A (IgA) is the main antibody involved in mucosal immunity. IgA has 2 isotypes–IgA1 with sugars on the hinge region and IgA2 with usually no sugars on the hinge region [1]. The concentration of IgA1 in the plasma of patients with IgA nephropathy (IgAN), the most common form of glomerulonephritis, is higher than that in the plasma of normal controls [2]. It is considered that IgA1 deposits in the glomerular mesangium, aggravating nephritis in IgAN [3,4]. Further, it has recently been reported that most of the IgA1 deposited in the mesangium has abnormal sugar type [5–8]. The normal sugar type, known as ST type, is *N*-acetylneuraminic acid (NeuAc)-α(2,3)-galactose (Gal)-β(1,3)-*N*-acetylgalactosamine (GalNAc), which binds to serine or threonine in the hinge region of IgA1 by an α-*O*-glycosidic linkage. T-type sugar is similar to ST-type sugar except that it lacks NeuAc, and Tn-type sugar is similar to T-type sugar except that it lacks Gal. T and Tn types are considered as abnormal sugar types on IgA1 (Fig. 1) [9]. STn-type sugar, in which NeuAc binds to GalNAc of Tn-type sugar by α(2,6)-glycosidic linkage, is another abnormal-type sugar.

IgAN patients do not show subjective symptoms until the condition worsens, and it is considered that IgAN slowly progresses nearly over 10 years. Therefore, analysis of abnormal sugars on IgA1 could be considered for the diagnosis of IgAN. Structural analysis by mass spectroscopy and detection of sugars by specific antibodies has been attempted [10]. However, mass spectroscopy [6,7,9] has a less widespread use because of the operational difficulties encountered and the high costs involved. In addition, preparation of antibodies specific for each sugar is very difficult. Therefore, most studies on recognition of sugars on IgA1 are attempted using lectins, which recognize sugar structures [10,11]. For example, *Artocarpus integrifolia* aggulutinin (jacalin) [13,14] and *Arachis hypogaea* agglutinin (peanut agglutinin (PNA)) recognize Gal-β(1,3)-GalNAc [14], and *Vicia villosa* agglutinin (VVL) and *Helix aspersa* aggulutinin (HAA) recognize GalNAc [15,16]. However, selective recognition of these sugars by the lectins has been recognized to be weak, since these lectins can also bind to several other sugars. In addition, many lectins, except jacalin, can bind to both *O*-linked glycosides on IgA1 and *N*-linked glycosides on other molecules in the plasma. Therefore, a two-step method was needed for the recognition of sugar type on IgA1, with the first step being the isolation of IgA1 from the plasma and the second step being the selective recognition of sugar by suitable lectins. However, this two-step method was disadvantageous in that its results were not very accurate.

* Corresponding author. Fax: +81 59 231 9480.
E-mail address: miyamoto@chem.mie-u.ac.jp (K. Miyamoto).

0928-4931/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2010.08.011

ST: NeuNA -α(2,3)-Gal-β(1,3)-GalNAc-α-*O*-linked (Ser/Thr)

T : Gal-β(1,3)-GalNAc-α-*O*-linked (Ser/Thr)

Tn: GalNAc-α-*O*-linked (Ser/Thr)

STn: NeuNA- α(2,6)-GalNAc-α-*O*-linked (Ser/Thr)

Fig. 1. Chemical composition of *O*-linked glycosides (ST, T, Tn, and STn type). NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine Ser/Thr, serine/threonine α-*O*-linked in the IgA1 hinge region.

Jacalin is a lectin present in the seeds of jackfruit [13], and it recognizes *O*-linked glycosides on IgA1. Although the main sugar recognized by jacalin is T-type sugar, the other possible *O*-linked glycosides recognized by jacalin are ST and Tn type [16]. Jacalin is used for the purification and detection of IgA1 by recognition of the *O*-linked glycoside on it. However, selective recognition of sugar type for IgAN diagnosis by using jacalin is difficult because of the multirecognition capability of jacalin for *O*-linked glycosides. We focused on this point that jacalin has the ability to recognize most of the sugar types on IgA1. If its efficiency of binding to several sugars (multirecognition) is transformed into that of binding to a specific sugar (selective recognition), the newly prepared jacalin derivatives that require a one-step recognition method for sugars can be used as new diagnostic agents for IgAN. Jacalin contains 4 α-subunits of 133 amino acid residues (molecular weight, 10–15 kDa) and 4 β-subunits of 20 amino acid residues (molecular weight, 2.1 kDa) [18]. The recognition site for a specific sugar structure is considered to be present in the α-subunit. The sugar-recognition site in jacalin does not contain a linear sequence but is considered to consist of 3 loops in which the loop strands are connected by 2 β-sheets in an α-subunit. Sugar and jacalin interact through the hydrogen bonds present in the 3 loops ($_{46}$Ser-$_{52}$Thr, $_{76}$Ser-$_{82}$Arg, and $_{122}$Tyr-$_{125}$Asp) at different positions in the primary structure. The 3 binding sites have been reported as the primary site, secondary site A, and secondary site B with specific arrangement of amino acid residues ($_{78}$Tyr, $_{79}$Val, $_{122}$Tyr, $_{123}$Trp, and $_{125}$Asp). Therefore, we considered that the binding properties of jacalin would largely change by subjecting the lectin to protein denaturation by heating, because its recognition site for specific sugars will partly be denatured.

In this study, we attempted to perform the challenging task of preparation of jacalin derivatives with recognition for selective sugars on IgA1. As our trial strategy, the recognition site for a specific sugar was protected by heating with a suitable template sugar, whereas those for other sugars were denatured on heating. Jacalin derivatives with selective recognition for ST-, T-, and Tn-type sugars on IgA1s were prepared by this partial denaturation of jacalin, and their interaction with IgA1 having abnormal sugars was examined. Finally, we analyzed the plasma of IgAN patients by using the novel jacalin derivatives.

2. Materials and methods

2.1. Extraction and purification of jacalin

Jacalin was extracted from the seeds of jackfruits (Okinawa, Japan). The seeds (100 g) were crushed in a food processor, and the crushed sample was obtained as a suspension in deionized water. The suspension was filtered through a membrane filter (pore size, 0.22 μm; Millipore, Billerica, MA, USA) for sterilization, and the filtrate was dialyzed against deionized water in a cellulose acetate tube (cutoff molecular weight, 10 kDa; Sanko Junyaku, Tokyo, Japan) at 4 °C for 48 h for removed the low molecules such as vitamin, minerals and oligosaccharides. The dialyzed filtrate was freeze-dried to yield 3-g powder of crude extract of jacalin.

2.2. Denaturation point of jacalin

Denaturation point of jacalin in aqueous solution was decided on the basis of change in the visible light transmittance of the solution at 400 nm, measured using UV-160A (SHIMADZU, Osaka, Japan). In addition, absorbance of ultraviolet (UV) rays at 250 nm (A_{250}) and 280 nm (A_{280}) was measured to determine structural transformation by calculating the absorbance ratio (A_{250}/A_{280}). Absorbance of the jacalin solution (1 wt.%) taken in a crystal glass cell (path length = 1 cm) was measured while heating the solution from 10 °C to 60 °C at the rate of 1 °C/min and while cooling it from 60 °C to 10 °C at the same rate.

2.3. Preparation of denatured-jacalin derivatives

Crude extract of jacalin (100 mg) was dissolved in 10 ml of 0.05 M phosphate-buffered saline (PBS; pH 7.5), and 1-g sodium dodecyl sulfate (SDS; Wako, Osaka, Japan) was added to the solution. Denatured-jacalin derivatives – termed Gal-J and GalNAc-J – were obtained by heating the solution with Gal (90 mg) and GalNAc (110 mg), respectively. Each solution was filtered through a membrane filter (pore size, 0.22 μm; Millipore) for sterilization, and the filtrate was dialyzed against deionized water at 4 °C for 48 h. Gal-J and GalNAc-J powders were obtained after freeze-drying the dialyzed filtrate.

2.4. Mean molecular weights of denatured-jacalin derivatives

Mean molecular weights of the denatured-jacalin derivatives were measured by high-performance liquid chromatography (HPLC), using 3000 SW columns (Tosoh, Tokyo, Japan). Absorbance of the elution solution was monitored at 280 nm by using a UV detector (Hitachi, Japan). The elution buffer was 0.1 M PBS (pH 7.0) containing 0.1 M sodium chloride (NaCl), and sample concentration was 5 mg/ml (loop volume, 20 μl). The flow rate was 0.1 ml/min. A working curve of elution time and molecular weight was prepared using standards (Bio-Rad, CA, USA), and mean molecular weights of the denatured-jacalin derivatives were calculated from the working curves.

2.5. Preparation of columns immobilized with IgA1 having T-/Tn-type sugar (T-/Tn-IgA1)

Cyanogen bromide (CNBr)-activated Sepharose 4B beads (3.34 g; GE Healthcare, Amersham, UK) and normal IgA (1000 μg; Calbiochem, San Diego, CA, USA) were suspended in 20-ml carbonate buffer (pH 8.3) containing 0.1 M NaCl and allowed to react for 2 h. IgA1-immobilized beads were packed into 3 columns (C columns; diameter, 10 mm; volume, 5 ml; GE Healthcare). One column was used as the control column. The second column, T-IgA1-immobilized column, was prepared by injecting neuraminidase (300 mU; Seikagaku Kogyo, Tokyo, Japan) into the packed column and allowing reaction for 12 h at 4 °C. The third column, Tn-IgA1-immobilized column, was prepared by injecting both neuraminidase (300 mU) and β-galactosidase (300 mU; Calbiochem) into the packed column and allowing reaction for 12 h at 4 °C. All the columns were washed and equilibrated with 0.05 M PBS (pH 7.5).

2.6. Sugar recognition by denatured-jacalin derivatives

Sugar recognition by denatured-jacalin derivatives (Gal-J and GalNAc-J) was examined by affinity chromatography with a column immobilized with IgA1 having selective sugar type. The denatured-jacalin derivatives (sample concentration, 5 mg/ml; loop volume, 500 μl) in 0.1 M Tris–HCl (pH 7.5) were passed through the sugar-IgA1-immobilized columns. After elution of the non-adsorbed fraction, the adsorbed fraction was eluted with 0.1 M Tris–HCl

containing 0.5 M Gal. The content ratio of the adsorbed fraction to the non-adsorbed fraction for each column was calculated from the obtained chromatogram. The content ratio of the adsorbed fraction to the non-adsorbed fraction for each column of untreated jacalin was used as the standard, and the recognition coefficient was obtained as follows.

$$\text{Recognition coefficient} = \text{Adsorption ratio of [(Gal-J or GalNAc-J)} - \text{Heat-J]} \div \text{Adsorption ratio of [untreated jacalin} - \text{Heat-J]} \quad (1)$$

Here, Heat-J represents the denatured jacalin obtained by heating without any template sugar. Recognition ratio of Gal-J or GalNAc-J was defined as the value obtained relative to the recognition ability of untreated jacalin for each sugar.

2.7. Preparation of columns immobilized with different O-linked glycosides

Columns were immobilized with different types of *O*-linked glycosides, i.e., ST, T, and Tn, for preparing highly pure jacalin derivatives. T-type sugar-*p*-nitrophenyl (T-*p*NP; 3.5 mg) and α(2,3)-(*O*)-sialyltransferase (100 mU; Calbiochem) were added into a solution (100 μl; Calbiochem) of 100 mM cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP-NeuAc) containing 50 mM morpholinoethansulfonic acid (MES; Dojindo, Kumamoto, Japan), 2 mM $CaCl_2$, 2 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.6% Triton X-100, and T-*p*NP was converted to ST-*p*NP. T-*p*NP (3.5 mg) and β-galactosidase (600 U) were added into a solution of the same composition, and T-*p*NP was converted to Tn-*p*NP. After the solution was incubated at 37 °C for 48 h, the reaction was terminated by incubation at 100 °C for 5 min. The enzymes were removed by precipitation by using a centrifugal separator (Hitachi) at 15,000 rpm for 30 min.

The sugar-*p*NPs (ST-*p*NP, T-*p*NP, and Tn-*p*NP) were reacted with 0.1 M sodium thiosulfate (Wako) at room temperature for 12 h. Sodium cyanoborohydride ($NaBH_4CN$; 72 mg) was added into each sugar-*p*NP solution and allowed to react at room temperature for 5 h. The sugar-*p*NPs were transformed into sugar-*p*-aminophenyl derivatives (sugar-*p*APs). Each sugar-*p*AP (0.5–10 mg/ml) in a solution of 0.2 M sodium hydrogencarbonate ($NaHCO_3$) and 0.5 M NaCl (pH 8.3) was injected into the HiTrap *N*-hydroxysuccinimide (NHS)-activated column (GE Healthcare) for immobilization. After blocking with 0.5 M ethanolamine containing 0.5 M NaCl (pH 8.3), the column was equilibrated with 0.1 M PBS (pH 7.0) containing 0.1 M NaCl.

2.8. High-grade purification of sugar type-recognizing jacalin derivatives

T-type-recognizing jacalin ((T)-Jacalin) was purified as follows. The ST-*p*AP-immobilized column (primary) and T-*p*AP-immobilized column (secondary) were coupled (ST-T coupled column), and the coupled column was equilibrated with 175 mM Tris–HCl (pH 7.4). Gal-J was passed through the ST-T coupled column, and the non-adsorbed fraction was eluted. The coupled column was then separated into individual columns, and the adsorbed fraction was eluted from the T-*p*AP-immobilized column with 175 mM Tris–HCl containing 0.1 M Gal. For the purification of ST-type-recognizing jacalin ((ST)-Jacalin), the columns were coupled in the reverse order (T-ST coupled column). Untreated jacalin was passed through the coupled column, and the adsorbed fraction was eluted from the ST-*p*AP-immobilized column with 175 mM Tris–HCl containing 0.1 M Gal. For the purification of Tn-type-recognizing jacalin ((Tn)-Jacalin), untreated jacalin was passed through the Tn-*p*AP-immobilized column, and the adsorbed fraction was eluted from the column with 175 mM Tris–HCl containing 0.1 M Gal.

2.9. Enzyme-linked immunosorbent assay (ELISA) of denatured-jacalin derivatives

Concentrations of abnormal sugar types on IgA1 were measured by jacalin-binding ELISA. (ST)-Jacalin and (T)-Jacalin were labeled with horseradish peroxidase (HRP) by using an HRP labeling kit (Dojindo). The standard IgA1s having specific sugar types on them were obtained from human plasma by using Hi-Trap NHS-activated columns immobilized with sugar type-recognizing jacalins. The other standard IgAs used were total IgA (Human IgA ELISA Quantitation kit; BETHYL, Montgomery, TX, USA), IgA1 (Sigma-Aldrich, St.Louis, MO, USA), and IgA2 (Sigma-Aldrich). Concentrations of the IgAs were normalized against that in the international standard plasma (IRMM ERM-DA470; ReCCS, Tokyo, Japan). Each of the anti-human IgA1 and anti-human IgA2 was added into a 96-well plate (100 μl/well) and coated by incubation for 1 h. After the plate was washed with PBS containing 0.05% Tween 20 (PBST), it was incubated for 30 min with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) solution for blocking. The samples were added into the wells (100 μl/well) and incubated for 1 h. After washing with PBST, HRP-labeled jacalin recognizing a specific sugar type was added into each well and incubated for 1 h. After washing again with PBST, a color reagent, *o*-phenylenediamine (OPD), was added to the plates, and they were left for 10 min. Next, 2 N sulfuric acid was added and absorbance was measured at 490 nm by using a microplate reader (Bio-Rad). Concentrations of the proteins were calculated from the curves prepared with reference to the standard molecules.

Plasma samples of IgAN patients were obtained from Mie University Hospital on the consent of the patients and in agreement with the ethical guidelines of the medical department. A total of 26

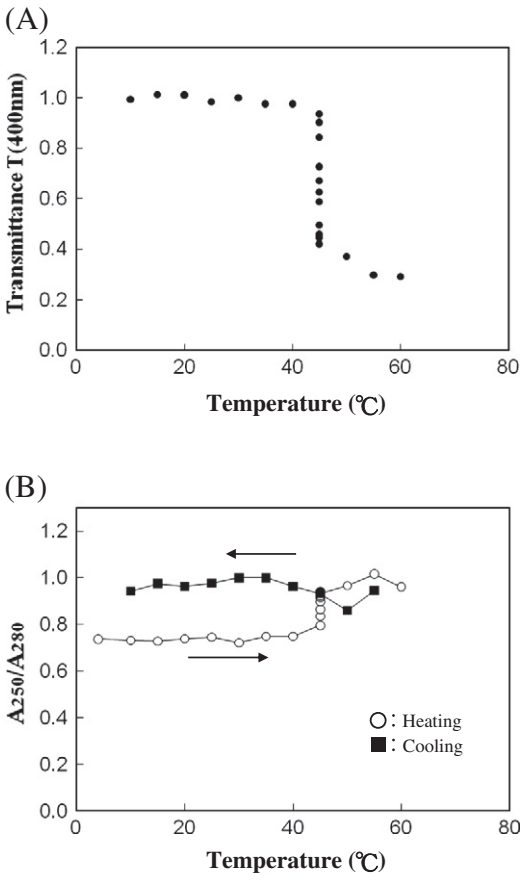

Fig. 2. Denaturation point of jacalin solution. (A) Temperature dependence of transmittance at 499 nm. (B) Temperature dependence of absorbance ratio (A_{250}/A_{280}).

plasma samples of IgAN patients and 6 plasma samples of healthy volunteers (normal controls) were analyzed.

2.10. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical significance was set at a P value of <0.05. Calculations were performed using the statistical package StatView 5.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Physiochemical properties of denatured-jacalin derivatives

Fig. 2 shows the denaturation point of jacalin, determined by measuring the turbidity of its aqueous solution at 400 nm and calculating the absorbance ratio (A_{250}/A_{280}). The denaturation point was decided to be 45 °C, as the point of change in the transmittance of aggregated jacalin molecules at 400 nm (Fig. 2A). The A_{250}/A_{280} value indicated change in the structure of jacalin in the solution, and 45 °C was decided as the denaturation point (Fig. 2B).

Fig. 3 shows the chromatogram of untreated jacalin (Fig. 3A) and Gal-J (Fig. 3B). The average molecular weight of untreated jacalin was 10–15 kDa, and this size was considered to be appropriate for one α-subunit of jacalin. The result clearly indicated that jacalin aggregated with several molecules on heat treatment. When the reaction solution was heated with <0.01 g/ml SDS, aggregation was accelerated and the solution precipitated, indicating complete denaturation. In the case when >0.01 g/ml of SDS was used, the jacalin solution did not precipitate on heating. As shown in Fig. 3, the single fraction of untreated jacalin was changed into 3–4 fractions by heat treatment of the jacalin solution. Native-type jacalin has the range of molecular weight of 10–60 kDa and this size was considered to be appropriate as one-four complex of α-subunit of jacalin.

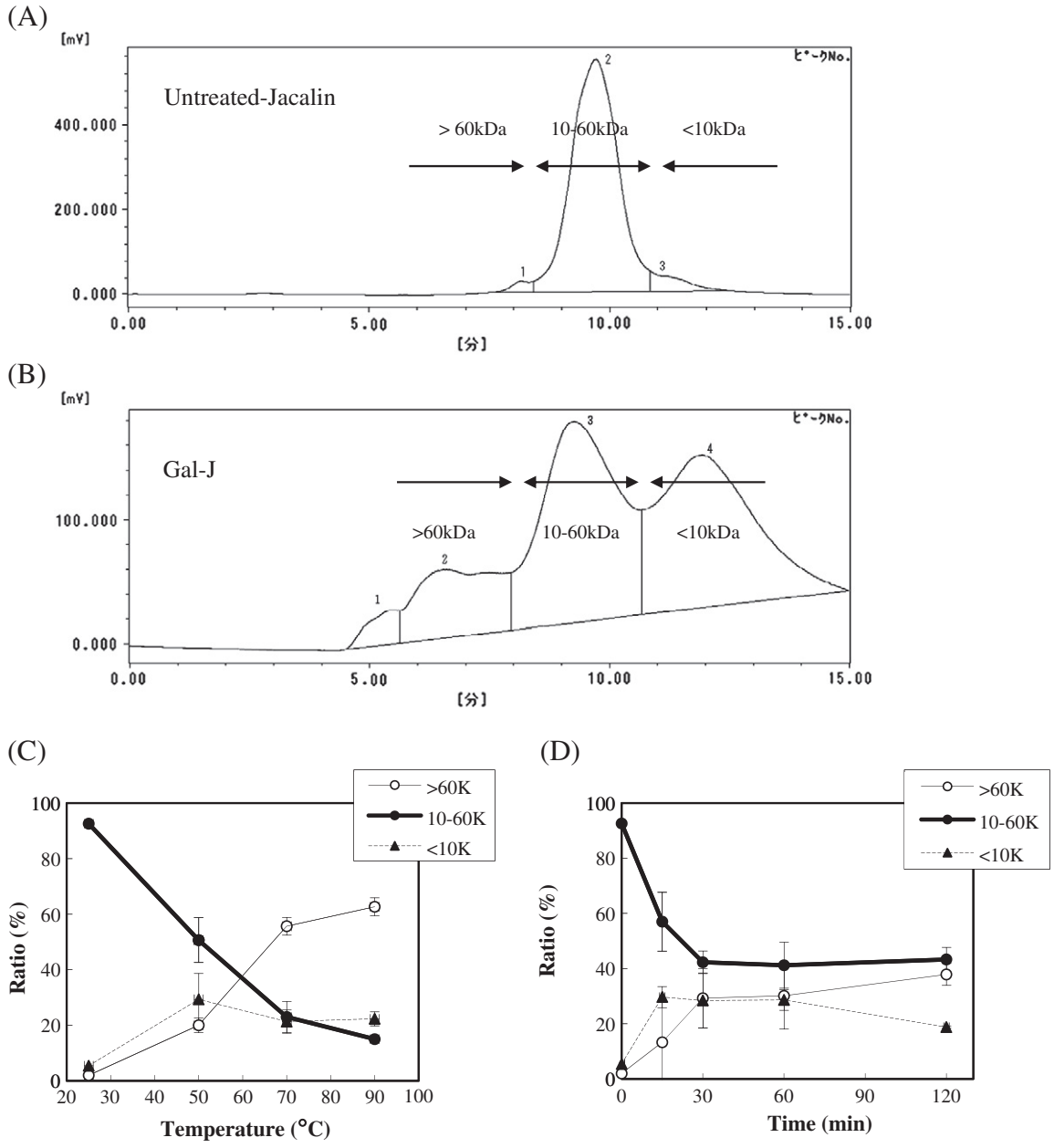

Fig. 3. Chromatogram of (A) untreated jacalin and (B) galactose-treated jacalin (Gal-J). (C) Temperature dependence (°C) of the content ratio of Gal-J, with 30-min reaction time and (D) time (min) dependence of the content ratio of Gal-J, with 50 °C temperature.

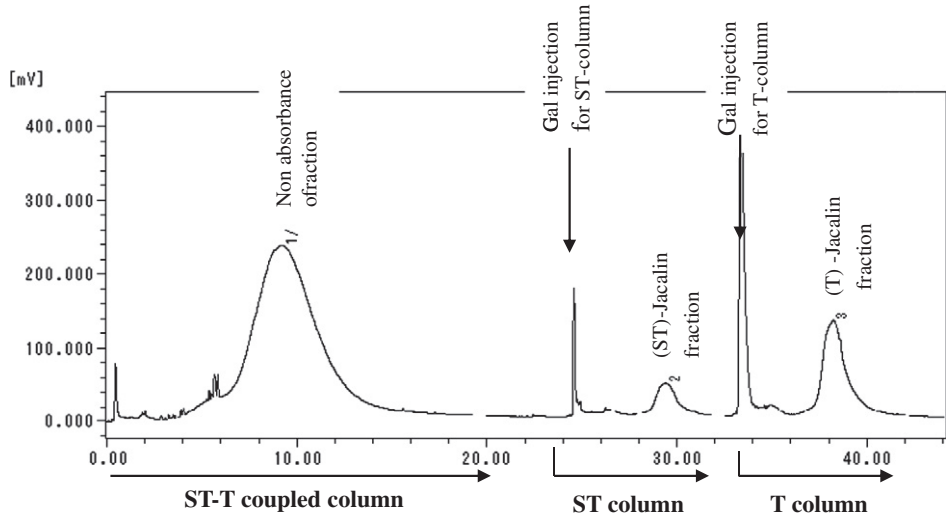

Fig. 4. Affinity chromatogram of Gal-J eluted with Gal, for ST-*p*AP- and T-*p*AP-immobilized coupled column (ST-T coupled column). The purified jacalin derivatives eluted with Gal from ST-*p*AP- and T-*p*AP-immobilized columns are named (ST)-Jacalin and (T)-Jacalin, respectively.

Therefore, the chromatogram area was divided into 3 fields–<10 kDa, 10–60 kDa, and >60 kDa–for determining the ratio of chromatogram areas as the content ratios of the denatured-jacalin derivatives.

Fig. 3C shows the temperature dependence of the content ratio of Gal-J in the 3 fields on heating for 30 min. The content ratio in the 10–60-kDa field decreased with increase in temperature. When the reaction temperature was 25 °C, the denaturation on jacalin by SDS was not observed. When the reaction temperature was >50 °C, the content ratio in the >60-kDa field showed a 20% increase. On heating at 90 °C, the content ratio in the >60-kDa field showed a 60% increase. Fig. 3D shows the time dependence of the content ratio of Gal-J at the heating temperature of 50 °C. No noticeable change was observed after 30 min of the reaction. From these results, the suitable condition for denaturation was determined as heat treatment at 50 °C for 30 min.

3.2. Measurement of the sugar-recognition ability of denatured-jacalin derivatives

Fig. 4 shows the affinity chromatogram of Gal-J passed through the ST-T coupled column. The coupled column was separated into individual columns (ST-*p*AP- and T-*p*AP-immobilized columns) after elution of the non-adsorbed fraction, and the adsorbed fractions in each column were eluted with Gal. The ratio of the maximum adsorption area to the maximum non-adsorption area of the column was calculated as the recognition ability.

Table 1 shows the recognition abilities of Gal-J and GalNAc-J samples for ST-IgA1, T-IgA1, Tn-IgA1, ST-*p*AP, T-*p*AP, and Tn-*p*AP. The recognition coefficients of Gal-J for ST-IgA1, T-IgA1, and Tn-IgA1 were calculated from the areas of the chromatogram and normalized to be 0.3, 1.9, and 4.5, respectively, whereas those for ST-*p*AP, T-*p*AP, and Tn-*p*AP were calculated and normalized to be 0.4, 1.2, and 1.0, respectively. The recognition coefficients of GalNAc for ST-IgA1, T-IgA1, and Tn-IgA1 were normalized to be 0.2, 2.1, and 1.7, respectively. It is evident that the recognition ability of jacalin for ST-type sugar was reduced on heating with Gal or GalNAc. These results show that the denatured-jacalin derivatives Gal-J and GalNAc-J had relatively higher recognition abilities for abnormal sugars than untreated jacalin.

Table 1
Recognition abilities of heat-denatured jacalin derivatives (Gal-J and GalNAc-J) for ST, T-, and Tn-type sugars.

	ST-IgA1	ST-*p*AP[a]	T-IgA1	T-*p*AP	Tn-IgA1	Tn-*p*AP
Jacalin	1.0	1.0	1.0	1.0	1.0	1.0
Gal-J	0.3	0.4	1.9	1.2	4.5	1.0
GalNAc-J	0.2	–	2.1	–	1.7	–

[a] *p*AP: *p*-aminophenol.

3.3. High-grade purification of sugar type-recognizing jacalin derivatives

Gal-J solution was passed through the ST-T coupled column. By this method, (T)-Jacalin fraction with no recognition for ST-type sugar was collected. Untreated jacalin solution was passed through the T-ST coupled column. By this method, (ST)-Jacalin fraction with no recognition for T-type sugar was collected.

3.4. ELISA for abnormal sugar types on IgA1

Fig. 5A, B, and C shows the standard calibration curves of total IgA1, ST-IgA1 and T-IgA1 for ELISA with anti-IgA1 antibody, (ST)-Jacalin-HRP, and (T)-Jacalin-HRP, respectively. Thus, it was proved that the denatured-jacalin derivatives could be used as molecules for the recognition of abnormal sugar types on IgA1.

Fig. 6A shows the concentration of total IgA in the plasma of IgAN patients and healthy volunteers (normal controls). Table 2 shows the concentration of IgAs and ratios of abnormal sugars in the plasma of IgAN patients and normal controls. The average concentrations of total IgA in the plasma of IgAN patients and normal controls were 282.6 ± 60.4 mg/dl and 198.3 ± 36.9 mg/dl, respectively, and those of IgA1 were 234.5 ± 53.6 mg/dl and 178.0 ± 31.7 mg/dl, respectively. The IgA and IgA1 concentrations in the plasma of IgAN patients were higher than those in the plasma of normal controls. However, the concentration ratio of IgA1 to total IgA in the plasma of IgAN patients was 83%, which was quite close to that in the plasma of normal controls (89%).

Fig. 6B shows the relationship between IgA1 concentration and ratio of abnormal sugars on IgA1. Although the IgA1 concentration in the plasma of IgAN patients was higher than that in the plasma of normal controls, the IgA1 concentration and ratio of abnormal sugars on IgA1 did not show any specific relation. This was observed for both IgAN patients and normal controls. The average ratio of abnormal sugars on IgA1 in the plasma of IgAN patients (62.6%) was remarkably higher than that in the plasma of normal controls (20.9%). Although the ratio of abnormal sugars on IgA1 in the plasma of IgAN patients was high, the ratios of T-IgA1 to total IgA1 in the plasma of IgAN patients and normal controls were $18\% \pm 13\%$ and $23\% \pm 8\%$,

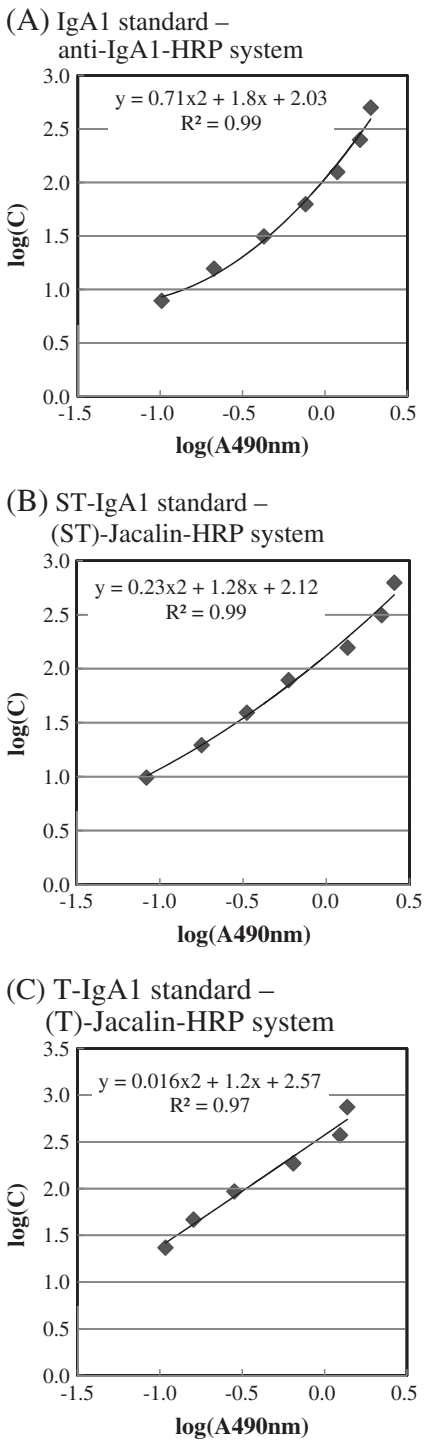

Fig. 5. IgA1 standard curves for jacalin-binding enzyme-linked immunosorbent assay (ELISA). (A) anti-IgA1 antibody, (B) horseradish peroxidase (HRP)-labeled (ST)-Jacalin (ST-Jacalin-HRP), and (C) (T)-Jacalin-HRP.

respectively, that is the concentration ratio of T-IgA1 in the plasma of IgAN patients was lower than that in the plasma of normal controls.

4. Discussion

4.1. Sugar recognition by jacalin

Jacalin is a lectin originally known to recognize T-type sugar. However, it also shows a low affinity for both ST- and Tn-type sugars

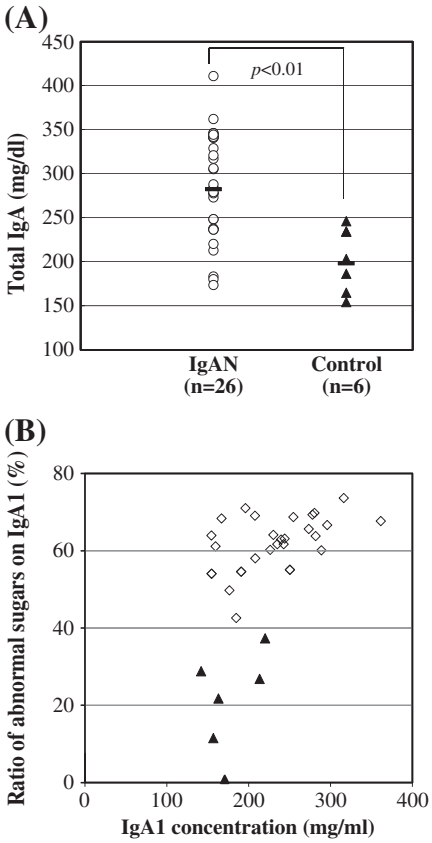

Fig. 6. (A) Total IgA concentration in the plasma of patients with IgA nephropathy (IgAN) (○) and normal controls (▲) and (B) relationship of IgA1 concentration with the ratio of abnormal sugars on IgA1 with IgAN (◇) and normal controls (▲).

$$\text{Total IgA} = \text{IgA2} + \text{IgA1} = \text{IgA2} + \text{ST-IgA1} + (\text{T-IgA1} + \text{Tn-/STn-IgA1})$$

$$\text{Tn-/STn-IgA1} = \text{Total IgA1} - (\text{ST-IgA1} + \text{T-IgA1}).$$

Concentrations of ST-IgA1 and T-IgA1 were measured by ELISA with (ST)-Jacalin and (T)-Jacalin.

$$\text{Ratio of abnormal sugars on IgA1} = [\text{IgA1} - (\text{ST-IgA1})] / \text{IgA1} = [(\text{T-IgA1}) + (\text{Tn-/STn-IgA1})] / \text{IgA1}.$$

[17]. Therefore, the general method for purification of IgA1 from blood plasma was by using a jacalin-immobilized column [13]. The investigation regarding abnormal sugars on IgA1 in the plasma of IgAN patients began on the observation of difference between the plasma of IgAN patients and normal controls. Jacalin does not have many recognition sites; however, it is considered that the same site has the structure for the recognition of different sugars such as Gal and GalNAc.

In general, lectins do not possess molecular-recognition properties with high affinity for the molecules, as in the case of antigen–antibody

Table 2
IgA-related values measured by jacalin-binding ELISA in the plasma of IgAN patients and normal controls.

	IgAN ($n=26$)	Control ($n=6$)	P value
Total IgA (mg/dl)	282.6 ± 60.4	198.3 ± 36.9	<0.01
IgA1 (mg/dl)	234 5 ± 53 6	178 ± 31 7	<0 01
IgA2 (mg/dl)	48.0 ± 25.0	20.3 ± 8.5	<0.01
Abnormal sugar-IgA1 (mg/dl)	148.5 ± 44.0	38.8 ± 29.3	<0.01
Ratio of abnormal sugar (%)	62 6 ± 7 1	20 9 ± 13 6	<0 01
ST-IgA1/IgA1 (%)	37.5 ± 7.1	79.2 ± 13.7	<0.01
T-IgA1/IgA1 (%)	18 ± 13	23 ± 8	NS
T-IgA1/abnormal sugar-IgA1 (%)	29.6 ± 22.3	103.8 ± 31.2	<0.01

reactions. Therefore, their use for selective recognition of sugars poses difficulties. However, we analyzed this problem from a different perspective. Jacalin is considered the most suitable lectin for recognition of sugar types on IgA1, and the major reason is that only jacalin can recognize most of the *O*-linked glycosides on IgA1. Therefore, we attempted to develop a new method for the preparation of jacalin derivatives with selective recognition for sugar types, in which several recognition sites of jacalin were denatured. Jacalin was completely denatured by heating at 90 °C. However, we discovered that when jacalin was partly denatured by heating with a suitable template sugar, only the recognition site for the selective sugar was protected and the other sugar-binding sites were denatured. In addition, highly pure denatured-jacalin derivatives were obtained by affinity chromatography with sugar-immobilized columns. Table 1 shows the recognition abilities of denatured-jacalin derivatives for abnormal sugar types on IgA1. It is clearly indicated that the recognition ability of Gal-J for T- and Tn-type sugars was higher than that of untreated jacalin. Thus, denaturation with a suitable template sugar was recognized as a suitable method for enhancing the recognition ability for T- or Tn-type sugar. Purification of (T)-Jacalin and (Tn)-Jacalin by affinity chromatography increased the recognition efficiency and selectivity. Recognition for ST-type and STn-type sugars were considered to decrease by partial denaturation of the binding site for NeuAc reduced without protection with Gal.

In this study, we prepared 3 types of denatured-jacalin derivatives that recognized ST-, T-, and Tn-type sugars. The basic mechanism underlying the recognition of a specific sugar structure by partly denatured jacalin is shown in Fig. 7. The Gal-recognition site of untreated (original) jacalin was protected by blocking it with Gal, whereas the other recognition sites, especially those of NeuAc recognition (denatured sites 1 and 2), were denatured by heat treatment. Thus, we successfully prepared denatured-jacalin derivatives with selective recognition for abnormal sugars. The method of denaturation with a suitable template sugar was a new method of enhancing the recognition abilities of proteins for sugars, and it indicated a potential for further investigation of the sugar-recognition mechanism of lectins.

Although IgA1 with STn-type sugar as the *O*-linked glycoside was not included in the analysis, it was considered that the total untreated jacalin would have the fraction recognizing this sugar type. However, the content ratio of all the fractions was not determined, which is considered a hindrance for future investigation.

4.2. Ratio of abnormal sugars to total IgA1 in IgAN

Concentrations of sugars on IgA1 determined by differential lectin-binding assay have not been reported by many researchers [5,9,12,15,19] because of the difficulties encountered in the preparation of standard IgA1s. Therefore, most reports present relative values.

In this study, we resolved this problem by using the international standard plasma, purified ST-IgA1, and purified T-IgA1. In addition, total IgA1 concentration was calculated by subtracting the concentration of IgA2 from that of total IgA. The concentration of IgA1 with abnormal sugar types was calculated by subtracting the concentration of ST-IgA1 from that of total IgA1. This was done to compare the concentration of abnormal sugar types on IgA1 with that of the normal sugar type. In addition, the value obtained by subtracting the concentration of T-IgA1 from that of total IgA1 with abnormal sugar types indicated the concentration of IgA1 with other abnormal sugar types, namely, Tn and STn type.

The concentration ratio of T-IgA1 to total IgA1 was 18% ± 13% in the plasma of IgAN patients and 23% ± 8% in that of normal controls, that is, the concentration ratio of T-IgA1 to total IgA1 was low in the plasma of IgAN patients. The results demonstrated that the concentration of T-IgA1 decreased and those of Tn-IgA1 and STn-IgA1 increased. The ratio of abnormal sugars in the plasma of IgAN patients was approximately 60%, which was 3 times the ratio detected in the plasma of normal controls (Fig. 6, Table 2). However, a high ratio is not always detected in the plasma of IgAN patients. The ratio is considered to depend on disease progression or recovery after treatment. In

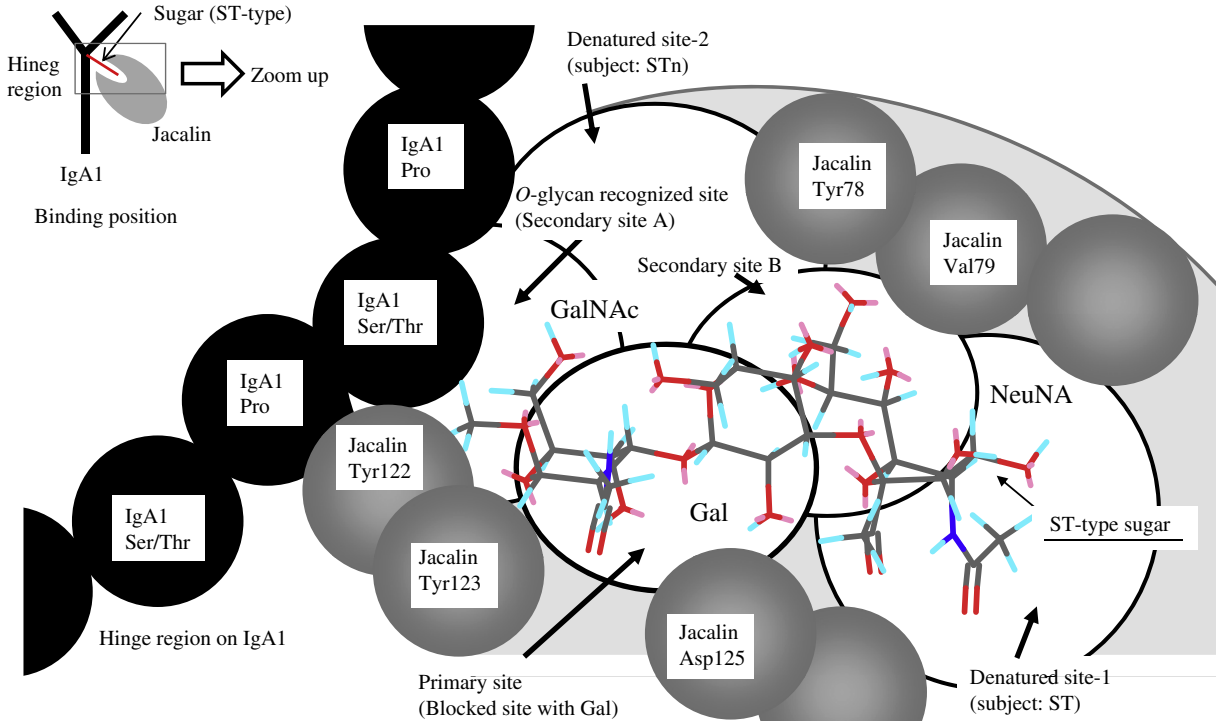

Fig. 7. Diagram showing blocked and denatured sites on heat denaturation with template sugar. The primary site was blocked with Gal, and the denatured sites were denatured by heating. Thus, the primary and secondary sites with specific structures would recognize T- and Tn-type sugar, respectively, and not ST- or STn-type sugar on IgA1.

particular, the ratios in the plasma of IgAN patients who underwent treatment such as steroid pulse therapy and tonsillectomy ranged from 40% to 60%, whereas those in the plasma of untreated patients ranged from 60% to >80% (data not shown). Thus, the ratio of abnormal sugar types (profiling of sugars on IgA1) was indicated as a factor for analyzing the effects of treatment.

IgA1 with abnormal sugar types is known to deposit in the mesangium of glomeruli. Aggregation of more than 2 molecules of IgA produces polymeric IgA (poly-IgA) [3]. The concentration of poly-IgA in the serum of IgAN patients is higher than that in the serum of normal controls, and the affinity of poly-IgA toward the mesangial cell surface receptor is higher than that of monomeric IgA (mono-IgA) [20]. Deposition of poly-IgA on the cell surface is considered to affect mesangial proliferation and overproduction of extracellular matrix, which are considered to indicate the initiation of the mechanism underlying inflammation. It has been reported that most of the aggregated IgA is Tn-IgA1 [21,22], compared with the non-aggregated IgA. In addition, because poly-IgA, in its aggregated state in the blood, circulates in the whole body, the aggregation properties of IgA1 in the blood were investigated by recognition by jacalin derivatives for the diagnosis of IgAN. Because our newly developed method is a relatively easy method for the recognition of sugar type on IgA1 than mass spectroscopy, it will prove to be advantageous as a support method or clinical technique for early diagnosis of IgAN in a large sample.

5. Conclusion

We proved that IgAN can successfully be diagnosed by solely using jacalin by utilizing the selective recognition ability of the lectin for sugar types on IgA1. Jacalin derivatives with selective recognition for sugar types on IgA1 were produced by regulating the binding abilities of jacalin by its heat denaturation.

References

[1] S. Kawamura, K. Omoto, S. Ueda, J. Mol. Biol. 215 (1990) 201.
[2] M.E. Conley, M.D. Cooper, A.F. Michael, J. Clin. Invest. 66 (1980) 1432.
[3] Y.H. Gao, L.X. Xu, J.J. Zhang, M.H. Zhao, H.Y. Wang, Clin. Exp. Immunol. 148 (2007) 507.
[4] Y. Yasuda, A. Horie, H. Odani, H. Iwase, Y. Hiki, Contrib. Nephrol. 141 (2004) 170.
[5] M. Tomana, K. Matousovic, B.A. Julian, J. Radl, K. Konecny, J. Mestecky, Kidney Int. 52 (1997) 509.
[6] H. Odani, Y. Hiki, M. Takahashi, A. Nishimoto, Y. Yasuda, H. Iwase, T. Shinzato, K. Maeda, Biochem. Biophys. Res. Commun. 217 (2000) 268.
[7] Y. Hiki, H. Odani, M. Takahashi, Y. Yasuda, A. Nishimoto, H. Iwasw, T. Shinzato, Y. Kobayashi, K. Maeda, Kidney Int. 59 (2001) 1077.
[8] A. Horie, Y. Hiki, H. Odani, Y. Yasuda, M. Takahashi, M. Kato, H. Iwase, Y. Kobayashi, I. Nakashima, K. Maeda, Am. J. Kidney Dis. 42 (2003) 486.
[9] K. Takahashi, Y. Hiki, H. Odani, S. Shimozato, H. Iwase, S. Sugiyama, N. Usuda, Biochem. Biophys. Res. Commun. 350 (2006) 580.
[10] T. Kokubo, Y. Hiki, H. Iwase, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, Nephrol. Dial. Transplant. 14 (1999) 81.
[11] L.X. Xu, M.H. Zhao, Kidney Int. 68 (2005) 167.
[12] J.S. Moore, R. Kulhavy, M. Tomana, Z. Moldoveanu, H. Suzuki, R. Brown, S. Hall, M. Kikian, K. Poulsen, J. Mestecky, B.A. Julian, J. Novak, Mol. Immunol. 44 (2007) 2598.
[13] S. Kabir, J. Immunol. 212 (1998) 193.
[14] M.V. Sastry, P. Banarjee, S.R. Patanjali, M.J. Swamy, G.V. Swarnalatha, A. Surolia, J. Biol. Chem. 261 (1986) 11726.
[15] J.S. Moore, R. Kulhavy, M. Tomana, Z. Moldoveanu, H. Suzuki, R. Brown, S. Hall, M. KKilian, K. Poulsen, J. Mestecky, B.A. Julian, J. Novak, Mol. Immunol. 44 (2007) 2598.
[16] S. Shimozato, Y. Hiki, H. Odani, K. Takahashi, K. Yamamoto, S. Sugiyama, Nephrol. Dial. Transplant. 23 (2008) 1931.
[17] K. Tachibana, S. Nakamura, H. Wang, H. Iwasaki, K. Tachibana, K. Maebara, L. Cheng, J. Hirabayashi, H. Narimatsu, Glycobiology 16 (2006) 46.
[18] A.A. Jeyaprakash, S. Katiyar, C.P. Swaminathan, K. Sekar, A. Surolia, M. Vijayan, J. Mol. Biol. 332 (2003) 217.
[19] H. Suzuki, Z. Moldoveanu, S. Hall, R. Brown, H.L. Vu, L. Novak, B.A. Julian, M. Tomana, R.J. Wyatt, J.C. Edberg, G.S. Alarcón, R.P. Kimberly, Y. Tomio, J. Mestecky, J. Novak, J. Clin. Invest. 118 (2008) 629.
[20] I.C. Moura, M. Arcos-Fajardo, A. Gdoura, V. Leroy, C. Sadaka, N. Mahlaoui, Y. Lepelletier, F. Vrtovsnik, E. Haddad, M. Benhamou, R.C. Monteiro, J. Am. Soc. Nephrol. 16 (2005) 2667.
[21] J. Novak, M. Tomana, K. Matousovic, R. Brown, S. Hall, L. Novak, B.A. Julian, R.J. Wyatt, J. Mestecky, Kidney Int. 67 (2005) 504.
[22] J. Novak, H.L. Vu, L. Novak, B.A. Julian, J. Mestecky, M. Tomana, Kidney Int. 62 (2002) 465.