ELSEVIER

Journal of Chromatography B, 768 (2002) 199–210

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of the affinity constants of recombinant human galectin-1 and -3 for simple saccharides by capillary affinophoresis

Kiyohito Shimura*, Yoichiro Arata, Noboru Uchiyama, Jun Hirabayashi, Ken-ichi Kasai

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan

Abstract

The affinity constants of recombinant human galectin-1 and galectin-3 for sugars were determined by capillary affinophoresis. The monoliganded affinophore contains *p*-aminophenyl-β-lactoside as an affinity ligand in the matrix of succinylglutathione and has three negative charges. An analysis of the mobility change of the lectins caused by the affinophore and its inhibition by neutral sugars allowed, for the first time, a determination of the affinity constants between the binding sites of the lectins and sugars. The relative magnitude of the affinity constants for each of the sugars in terms of dissociation constants found to be consistent with previously reported data on the concentrations of sugars that caused a 50% inhibition (I_{50}) in the binding assay of the lectin to oligosaccharide-immobilized agarose beads but the absolute values of the dissociation constants were considerably smaller than the I_{50} values. Capillary affinophoresis indicated microheterogeneity of the lectin preparations and enabled the separate analysis of the affinity of each component simultaneously showing the advantage in using a separation method for analysis of bioaffinity.

Keywords: Dissociation constant; Affinity capillary electrophoresis; Recombinant human galectins; Saccharides; Lectins

1. Introduction

The analysis of bioaffinity represents a powerful approach for elucidating the role of proteins as functional elements in cells and organisms. In this approach, the interactive nature of a protein can be quantitatively and universally described with an affinity constant for specific molecules. As a result, the determination of affinity constants represents a major issue in the biochemical characterization of proteins. Among the current techniques for the determination of affinity constants, capillary electrophoresis or chip electrophoresis increases its importance, especially from the point of view of miniaturization, high-through put and high precision [1–4]. In addition to the above benefits, the superior separation efficiency of capillary electrophoresis is powerful, especially in the analysis of bioaffinity of proteins that might be heterogeneous due to physiological and nonphysiological modifications. Such changes potentially alter the biochemical activity of proteins and can be reflected in the affinity constant for a specific molecule. The high separation efficiency of capillary electrophoresis can provide affinity data for each component of such isoforms, as well as producing information about the relative abundance of each isoform [5,6].

We previously reported the determination of affinity constants of pea lectin for simple saccharides using a capillary affinophoresis system [7]. Affinophoresis is an affinity electrophoresis system in which an affinity ligand is covalently attached to a multiply charged soluble matrix molecule, thus forming an affinophore

*Corresponding author. Fax: +81-426-853-742.

E-mail address: shimurak@pharm.teikyo-u.ac.jp (K. Shimura).

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00500-X

[8,9]. When a protein binds to the ligand on an affinophore, its electrophoretic mobility changes, as a result of the charges on the affinophore in a manner that is dependent on the concentration of the affinophore. Inhibition experiments of the affinophoresis using neutral ligands provide affinity constants for the neutral ligands. For pea lectin, the affinophore was prepared by coupling *p*-iodoacetamidophenyl α-mannoside to the thiol group of N-succinylated glutathione and the affinity constants for mono- and disaccharides were then determined [7]. In view of the successful use of capillary affinophoresis, we applied it to the determination of affinity constants of galectins for neutral sugars.

Galectins are a family of β-galactoside binding proteins that share some common amino acid sequences found in many animal species [11,12]. This family of proteins is involved in a variety of biological processes, including the modulation of cell–cell and cell–matrix interactions, T-cell apoptosis, tumor metastasis, processing of pre-mRNA in nucleus, the transformation of cells and stimulation of nerve regeneration. The binding affinity of galectins to simple saccharides have typically been estimated by the concentration of a sugar that inhibits 50% of the binding of ^{125}I-labeled galectins to agarose beads to which an oligosaccharide was immobilized [13,14]. This inhibition assay is compromised by the heterogeneity of oligosaccharide ligands on the beads, as well as by possible polyvalent interactions between the lectins and the immobilized oligosaccharide ligands. Although equilibrium dialysis can also provide an affinity constant, the fact that the method is tedious and time-consuming, and the scattering of the data often discourage its use [15]. Recently, frontal affinity chromatography has been reinforced by using a small column of immobilized galectin and a high-performance liquid chromatography (HPLC) system which enabled one to provide affinity constants for pyridylaminated oligosaccharides in a considerably small scale and a short analysis time [16]. In comparison to the preceding methods for the determination of the affinity constants of lectins for sugars, capillary affinophoresis with a monoliganded affinophore has several obvious benefits, i.e., circumvention of the polyvalency problem, the observation of interactions in free solution, no need for labeling a lectin or sugars, simultaneous and independent analysis of the affinity of isoforms of lectins, a short analysis time and small consumption of samples. Here, we report the affinity constants of human galectin-1 and -3 for simple sugars for the first time via the use of capillary affinophoresis.

2. Experimental

2.1. Chemicals

The following chemicals were obtained from commercial sources: *p*-aminophenyl β-lactoside (Sigma, St. Louis, MO, USA), *N*-acetyllactosamine (BioCarb, Lund, Sweden), mixed-bed ion-exchange resin (AG-X8 Resin, Bio-Rad Labs., Hercules, CA, USA) and fused-silica capillaries (GL Sciences, Tokyo, Japan). *N*-Iodoacetoxysuccinimide was prepared as described previously [17]. Other chemicals were the highest grade and were obtained from Wako (Osaka, Japan). The molecular extinction coefficient of the affinophore in water at 248 nm was determined to be 10 430 M^{-1} cm^{-1}.

2.2. Lectins

Recombinant human galectin-1 (rhGal-1) and its site-directed mutant protein (C2S) were produced using a pET vector system after recloning previously established plasmid clones [18]. The rhGal-1 is a dimeric protein that is composed of two identical M_r 14 000 polypeptide chain with 134 amino acid residues. The apparent difference of rhGal-1 from the native galectin-1 found in human tissues is the absence of an acetyl group at the N-terminus of the rhGal-1. The C2S mutant of rhGal-1 has one amino acid substitution at the second residue from the N-terminus, from cysteine to serine. This mutation on one of the six cysteine residues found in galectin-1 has been reported to substantially increase the stability of sugar binding activity of the lectin in the absence of reducing agents. Recombinant human galectin-3 (rhGal-3) was produced by using a pET vector system after recloning the previously established plasmid clone [19,20]. Freeze–dried galectin samples, about 100 μg, from five-times diluted buffer was redissolved in 100 μl of water and the

buffer was changed to 0.1 *M* Tris–acetic acid buffer (pH 7.9) containing 0.02% of NaN_3 (the electrophoresis buffer) with an ultrafiltration filter device (Microcon YM-10, Millipore, Bedford, MA, USA).

In some experiments, the recombinant lectin preparation was treated with 10 m*M* dithiothreitol at 37°C for 1 h in the electrophoresis buffer and used as a sample for the capillary affinophoresis as it was or after removal of the reducing agent by using an ultrafiltration filter device.

2.3. Affinophore

The affinophore, which contained *p*-aminophenyl β-lactoside as a ligand on an affinophore matrix of succinylglutathione was prepared as previously described [7] with minor modifications. In the previous preparation, *p*-iodoacetamidophenylglycoside was coupled to succinylated glutathione. Since the chromatographic separation of succinylated reduced glutathione from the dithiothreitol used for reductive cleavage of succinylated oxidized form of glutathione was not easily accomplished even by HPLC, *p*-iodoacetamidophenyl β-lactoside was first coupled to reduced glutathione and the product was subsequently succinylated.

p-Aminophenyl β-lactoside (25 mg, 57.7 μmol) was dissolved in 1 ml of 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH buffer (pH 6.0), and *N*-iodoacetoxysuccinimide (25 mg, 88 μmol) in 80 μl of *N*,*N*-dimethylformamide was then added to the solution. After a 1-h reaction at room temperature in the dark, a mixed-bed ion-exchange resin (45 mg, Bio-Rad AG-X8 resin) was added to the mixture and it was vortexed mainly to remove free iodoacetic acid. The supernatant was removed and 300 μl of 0.5 *M* sodium phosphate buffer (pH 7.5) containing 25 m*M* EDTA was mixed with the supernatant. Glutathione (reduced form, 23 mg, 75 μmol) was added to the mixture and allowed to react for 2 h at room temperature in the dark. The mixture was acidified to pH 2–3 by the addition of 1 *M* hydrochloric acid. The coupling product (GS-AP-Lac) was purified by HPLC on a reversed-phase chromatographic column (TSK-Gel ODS-80TS, 25 cm×4.6 mm I.D.) with a cartridge guard column (TSK guard gel ODS-80TS, 1.5 cm×3.2 mm I.D.) (Tosoh, Tokyo, Japan). About one fifth each of the reaction mixtures was applied to the column, which had been equilibrated with 0.1% trifluoroacetic acid, and eluted with a gradient of 0 to 25% acetonitrile in 0.1% trifluoroacetic acid over a period of 25 min at a flow-rate of 1 ml/min at room temperature, with detection by measurement of the absorption at 280 nm. GS-AP-Lac, which eluted at 14.5 min was collected, combined and dried by evaporation. The GS-AP-Lac preparation was dissolved in 700 μl of 0.1 *M* sodium phosphate buffer (pH 7.5) containing 25 m*M* EDTA and succinic anhydride, 20 mg, was added to the solution. The mixture was allowed to react for 10 min, while maintaining the pH at 7–8 by the addition of 5 *M* NaOH. Aliquots, 100 μl, of the reaction mixture were acidified to pH 2–3 by the addition of hydrochloric acid, and applied to the chromatographic column, followed by elution under the same conditions as described above. The affinophore, sGS-AP-Lac, eluted at 15.3 min and was collected, combined and dried by evaporation. It was then dissolved in 1 ml of water and stored in a freezer. The concentration of the affinophore was determined by means of the phenol–sulfuric acid method with a lactose solution as a standard. The overall yield of the affinophore from the *p*-aminophenyl β-lactoside was 40–50%.

2.4. Capillary affinophoresis

Affinophoresis was carried out with an automated capillary electrophoresis instrument (P/ACE 5010 with a UV detector, Beckman Coulter, Fullerton, CA, USA). The inner wall of a fused-silica capillary (50 μm I.D., 375 μm O.D.) was coated with succinylpolylysine as described previously [21], and then placed in a cartridge with a separation distance of 20 cm. The capillary was filled with the affinophore (sGS-AP-Lac) in the electrophoresis buffer [0.1 *M* Tris–acetic acid buffer (pH 7.9) containing 0.02% of NaN_3 as a preservative] in the high-pressure-rinse mode (20 p.s.i.) for 30 s (1 p.s.i.= 6894.76 Pa). The electrophoresis marker solution, 1 m*M* acrylamide and 0.3 m*M* acetyl-L-tryptophan in the electrophoresis buffer, and lectin sample solution, 1 μg/μL in the buffer, was consecutively injected at the positive end for 1 s each under a pressure of 0.5 p.s.i. (injection volume of 2.2 nl of each solution). Solutions, 20 μl each, of the affinophore, lectin and

marker was placed in a polypropylene mini vial (part number 727013, Beckman Coulter) and overlaid with 20 μl of mineral oil (Light White Oil, density=0.84 g/ml, M-3516, Sigma) to prevent the evaporation of water [22,23]. The vials were set in a temperature-controlled sample tray using vial holders (part number 727012, Beckman Coulter). Electrophoresis was carried out at a field strength of 300 V/cm with an electric current of about 25 μA. The cartridge temperature was set at 20°C and galectins were detected by measuring the absorption at 214 nm. The electrophoresis buffer was used as an electrode solution (about 4 ml) in each electrode vessel. In the case of competition experiments with neutral sugars, a solution of a sugar in the electrophoresis buffer was used as a positive-electrode solution (200 μl) with a 20-μl overlay of the mineral oil. After electrophoresis, the capillary was rinsed with water in the high-pressure-rinse mode for 3 min. Acetyltryptophan or acrylamide was used as a reference ion for the analysis of affinophoresis of rhGal-1 and rhGal-3, respectively.

2.5. Theory

Galectin-1 is a dimeric protein that consists of two identical subunits, with one sugar-binding site on each subunit. When the affinophore binds to the protein, its electrophoretic mobility of the protein is changed in two steps depending on the number of affinophores that are associated with it. Previous work on the affinophoresis of pea lectin with this type of monoliganded affinophore showed that the same equation as that used to describe the mobility change of a monovalent protein can be applicable for divalent proteins when the following assumption is valid, i.e., the equivalency and the independence of the two binding site, and approximately the same mobility change occurs for the successive binding of the affinophores [7]. A linear form of the equation, which is identical to a Woolf–Hofstee plot of enzyme kinetics, is suitable for the mobility change analysis of affinophoresis. The equation is:

$$\Delta\mu = -K_{\mathrm{d}}^{\mathrm{int}}\Delta\mu/[\mathrm{A}] + \Delta\mu_{\max} \quad (1)$$

where $K_{\mathrm{d}}^{\mathrm{int}}$ is the intrinsic or microscopic dissociation constant of the complex between each binding site and the affinophore. $\Delta\mu$ is the mobility change of lectins caused by the affinophore at the concentration of [A]. $\Delta\mu_{\max}$ is the maximum mobility change of the lectin at infinite concentration of A and is identical to the mobility difference between free lectin and the lectin–affinophore complex in which the two binding sites are filled with the affinophore.

The addition of a neutral sugar that competes for the binding to the lectin with the affinophore reduces the mobility change caused by the affinophore form $\Delta\mu$ to $\Delta\mu_{\mathrm{i}}$. This can be written as:

$$\Delta\mu_{\mathrm{i}} = \Delta\mu_{\max}[\mathrm{A}]/\left(K_{\mathrm{d\ app}}^{\mathrm{int}} + [\mathrm{A}]\right) \quad (2)$$

where:

$$K_{\mathrm{d\ app}}^{\mathrm{int}} = K_{\mathrm{d}}^{\mathrm{int}} \cdot \left((1 + [\mathrm{I}]/K_{\mathrm{i}}^{\mathrm{int}}\right) \quad (3)$$

Since $\Delta\mu_{\max}$ is already known, $K_{\mathrm{d\ app}}^{\mathrm{int}}$ can be determined by measurement of $\Delta\mu_{\mathrm{i}}$ at known concentrations of A and I. The dissociation constant of the complex, LI, between a lectin binding site, L, and a neutral sugar, I, is written as: $K_{\mathrm{i}}^{\mathrm{int}} = [\mathrm{L}][\mathrm{I}]/[\mathrm{LI}]$. Rearrangement of Eq. (3) gives:

$$K_{\mathrm{i}}^{\mathrm{int}} = [\mathrm{I}]K_{\mathrm{d}}^{\mathrm{int}}/\left(K_{\mathrm{d\ app}}^{\mathrm{int}} - K_{\mathrm{d}}^{\mathrm{int}}\right) \quad (4)$$

where $K_{\mathrm{d}}^{\mathrm{int}}$ also represents a known quantity. The analysis based on the above equations is valid for the divalent binding protein under the conditions described above, but it should be noted that the analysis is also appropriate for a monovalent protein, as in the case of galectin-3.

The mobility change ($\Delta\mu$) caused by the affinophore was calculated from the detection time data for the lectin peak (t) and the reference marker peak (t_{r}) using the following equation:

$$\Delta\mu = \frac{L}{E} \cdot \left\{ \left(\frac{1}{t} - \frac{1}{t_{\mathrm{r}}} \right) - \left(\frac{1}{t_0} - \frac{1}{t_{\mathrm{r0}}} \right) \right\} \quad (5)$$

where L is the migration distance (from the injection end to the detection point), and E the field strength. The subscript 0 indicates that the values are for the experiment in the absence of the affinophore [21].

3. Results and discussion

3.1. Lactose affinophore

The lactose affinophore was prepared by coupling *p*-iodoacetamidophenyl β-lactoside to the thiol group of glutathione and, the amino group of the gluta-

thione was then succinylated. The lactose moiety functions as an affinity ligand and a portion of the succinylated glutathione as an affinophore matrix (Fig. 1). The procedure for the preparation was slightly modified from the previous preparation of the mannose affinophore [7], in which *p*-iodoacetamidophenyl α-mannoside was coupled to the reduced form of N-succinylated glutathione. The new scheme for the preparation of the affinophore circumvents the delicate separation of the reduced form of *N*-succinylglutathione from dithiothreitol used for the reductive cleavage of the oxidized form of *N*-succinylglutathione. The affinophore bears three negative charges that are responsible for the change in the mobility of the lectin when it binds the affinophore.

3.2. Affinophoresis of C2S mutant of rhGal-1

Galectin-1 rapidly loses its sugar binding activity in the absence of reducing agents such as 2-mercaptoethanol or dithiothreitol. The formation of intramolecular disulfide(s) between the six-cysteine residue is believed to be involved in the inactivation process of sugar binding activity [24]. Galectin-1 is thought to modulate cell–cell and cell–matrix interactions by its sugar binding activity [11], while the oxidized form, having lost the sugar binding activity, is reported to present cell-growth-stimulating activity that is not shared by the reduced form [25,26]. The substitution of the cysteine residue at the second position from the N-terminus to serine increases the stability relative to sugar binding activity in the absence of reducing agents without the apparent loss of sugar binding ability [18]. The substitution seems to decelerate a critical step of the process that may eventually lead to the loss of the sugar binding activity. This background promoted us to initially use C2S as a stable galectin-1 mutant for the analysis of the sugar binding activity by capillary affinophoresis.

The inner surface of the fused-silica capillary was covalently coated with succinylpolylysine [21]. This coating preserves the high electroosmosis as a bare fused-silica capillary, but provides more stability, probably due to the reduced adsorption of proteins. The capillary was filled with the affinophore solution and the lectin solution was injected at the anodic end with a neutral and a mono-charged marker, acrylamide and acetyl-L-tryptophan, respectively. The relative magnitude of the mobility of the migrating species and electroosmosis (EO) is EO> affinophore>acetyltryptophan>lectin, although the direction of EO is opposite to the others. Consequently, the lectin migrates in the solution of the affinophore throughout the electrophoresis process.

The electropherograms obtained in the presence of the affinophore at different concentrations are shown in Fig. 2. In the absence of the affinophore, the C2S preparation appeared as a relatively broad single peak at around 6.5 min, whereas acrylamide and acetyltryptophan were observed at around 4 and 10 min, respectively. With increasing concentration of the affinophore, the apparent mobility of the lectin became larger due to complexation and the peak was split into three, designated as peaks 1 to 3 in the order of higher to lower mobility, i.e., from the right to left on the electropherogram. A Woolf–Hofstee plot according to Eq. (1) was made for each peak (Fig. 3) and K_d^{int} and $\Delta\mu_{max}$ were determined from five independent experiments for peaks 1 to 3 as follows, $K_d^{int}=0.37$ m*M* (SD 0.015), 0.47 m*M* (SD 0.005), 0.55 m*M* (SD 0.008); $\Delta\mu_{max}=4.73\cdot10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ (SD 0.06), $4.71\cdot10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ (SD 0.03), $4.55\cdot10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ (SD 0.02), respectively. The three values for $\Delta\mu_{max}$ were very close and within a difference of 4%, while the K_d^{int} for peak 2 is larger than that of peak 1 by about 30%

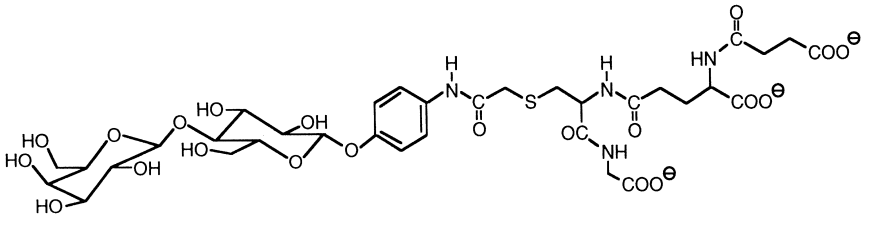

Fig. 1. Structure of lactose affinophore.

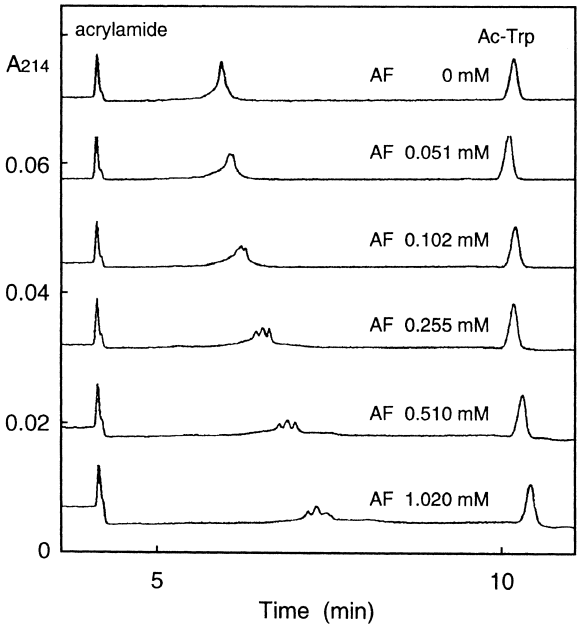

Fig. 2. Affinophoresis of C2S mutant of rhGal-1 with the lactose affinophore prior to thiol treatment. C2S was subjected to capillary affinophoresis using the lactose affinophore (AF) at concentrations indicated above each electropherogram. Acrylamide and acetyltryptophan (Ac-Trp) were injected with the lectin sample as electrophoresis markers. Three peaks separated at higher concentration of the affinophore were designated as peak 1 to 3 in decreasing order of migration time.

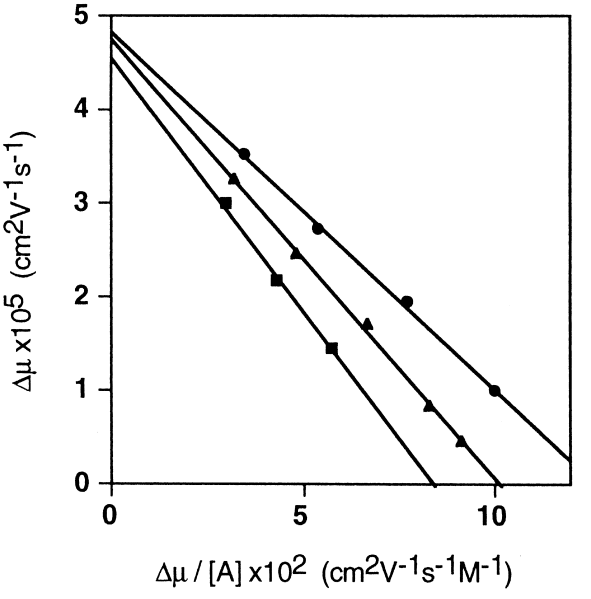

Fig. 3. Woolf–Hofstee plots for the affinophoresis of the C2S mutant of rhGal-1 according to Eq. (1). Plots were carried out for the three peaks of C2S separated by affinophoresis as shown in Fig. 2. The symbols are; ●, peak 1; ▲, peak 2; ■, peak 3.

and that for peak 3 is larger by 50%. The results show that the C2S mutant preparation of rhGal-1 contains at least three molecular species that are different in terms of affinity for the affinophore but are identical in terms of hydrodynamic drag when complexed with the affinophore, i.e., identical molecular shapes.

As noted above, galectin-1 has six cysteine residues in each subunit and its sugar-binding activity is sensitive to oxidation, although the stability is greatly increased in the C2S mutant. To test the possibility of the involvement of an oxidation reaction in the heterogeneity of the C2S preparation, it was treated with dithiothreitol and subjected to affinophoresis (Fig. 4). The treatment with the thiol sharpened the C2S peak and revealed the presence of an additional peak, even in the absence of the affinophore. The affinophore increased the mobility of the both peaks but peak splitting was not observed for the thiol-treated C2S mutant. The Woolf–Hofstee plot for the two peaks gave K_d^{int} values and $\Delta\mu_{max}$ values for the main and the first migrating species as follows, $K_d^{int}=0.40$ mM (SD 0.005), 0.42 mM (SD 0.000) and $\Delta\mu_{max}=4.69\cdot10^{-5}$ cm^2 V^{-1} s^{-1} (SD 0.03), $4.65\cdot10^{-5}$ cm^2 V^{-1} s^{-1} (SD 0.02), respectively. The two sets of values are close, and the agreement of the values to those of peaks 1 or 2 of the non-treated C2S sample is apparent. We concluded that the original C2S mutant preparation contained at least three isoforms, peaks 1–3, that are different in the state of thiol groups, e.g., the formation of an intra- or intermolecular disulfide bond with a small thiol compound and also in their affinity for the affinophore. The treatment by the thiol converted them back to the most reduced state, which probably has the highest affinity to the affinophore, i.e., peak 1. The minor peak observed in the thiol-treated C2S increased consistently and gradually when the protein was stored in a refrigerator and more rapidly at an incubation temperature of 37°C with the formation of an additional peak with an even higher mobility. The K_d^{int} value and the $\Delta\mu_{max}$ value of the minor peak are indistinguishable from that of the

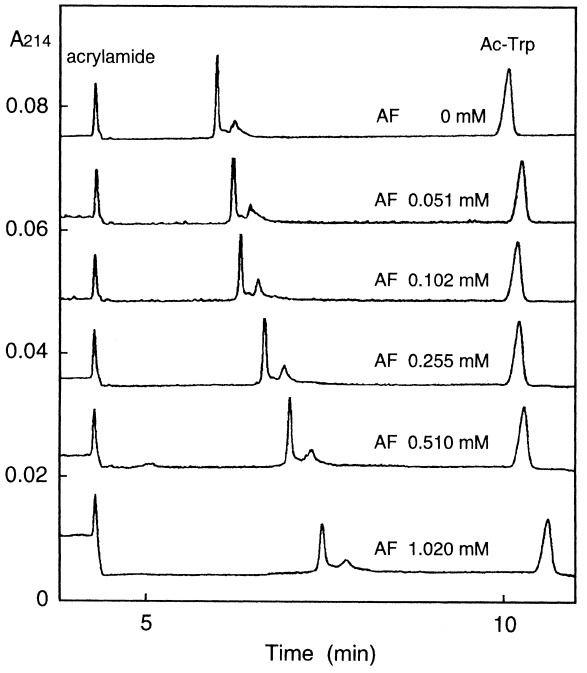

Fig. 4. Affinophoresis of thiol treated C2S with the lactose affinophore. C2S treated with dithiothreitol was subjected to capillary affinophoresis using the lactose affinophore (AF) at concentrations indicated above each electropherogram. Acrylamide and acetyltryptophan (Ac-Trp) were added as electrophoresis markers.

major peak. Heterogeneity in terms of isoelectric point of human galectins has been reported [13]. The origin of the minor peak found in the thiol treated C2S sample is most probably the deamidation of the original protein, the major peak in the thiol-treated sample.

Some increment of the detection time of acetyltryptophan was observed in the presence of the affinophore at high concentration (Figs. 2 and 4). Although the observed increase in the detection time ran up to 5.6% at the largest case (Fig. 4, at the highest concentration of the affinophore), the increase of the mobility itself was calculated to be 3.1% using acrylamide as the electroosmosis marker. Such a mobility change of acetyltryptophan in the presence of the affinophore, however, was not observed consistently and does not seem to represent the weak interactions between acetyltryptophan and the affinophore. In theory, either acrylamide or acetyltryptophan can be used as the reference marker for the calculation of $\Delta\mu$ according to Eq. (5). For example, the K_d^{int} values that were calculated using the different markers for the results in Fig. 4 agreed within the difference of 9%. For repeated experiment of K_d^{int} determination, however, the use of acetyltryptophan yielded consistently smaller variations in the determined K_d^{int} values than that of acrylamide. The source of the variation of the mobility of the reference markers is not clear at present but has to be investigated to further improve the precision of the determination of affinity constants.

When we first used capillary affinophoresis for determining the affinity constants of pea lectin, a longer migration distance of 50 cm was adopted, in order to reduce the proportion of the capillary that was not temperature-controlled, i.e., about 4 cm. To shorten the time of electrophoresis and increase the throughput of the analysis, a migration distance of 20 cm was examined. The dissociation constant of C2S for the affinophore as determined using the migration distance of 20 cm was about 5% larger than that obtained by using a migration distance of 50 cm. The difference can be attributed to the increase in the segment that was not temperature controlled, since most lectins have a reduced affinity for sugars at higher temperature. Although the dissociation constant can be larger by about 5%, the migration distance of 20 cm was selected, for the sake of higher throughput of analysis.

3.3. Determination of affinity constants of C2S to neutral sugars

Although the chemical nature of the different oxidation state is unknown, further analysis was focused on the major peak of the thiol-treated C2S, since it likely corresponds to the native galectin-1 in which all the cysteine residues are in the reduced form. The affinity constants for neutral sugars were determined by their inhibition of affinophoresis with a fixed concentration of the affinophore at 0.51 m*M* that was roughly equal to the K_d^{int} value. In these experiments, the neutral sugar was added to the electrode solution at the anode. The sugar solution is transferred into the capillary by the electroosmosis behind the neutral marker and the lectin migrates in the presence of both the affinophore and the neutral

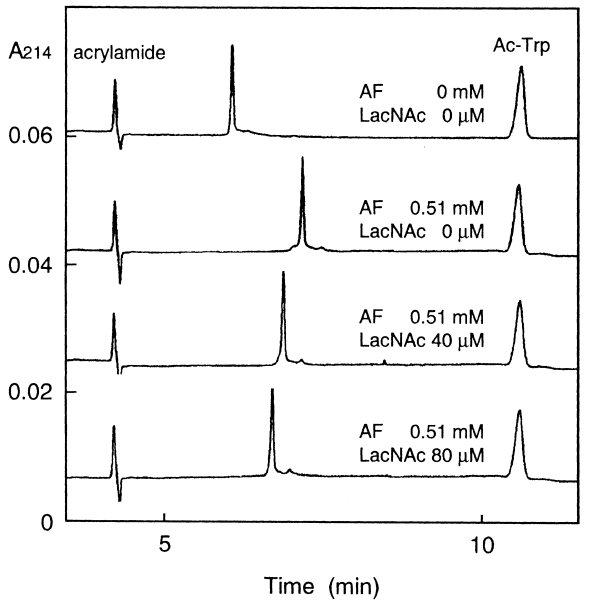

Fig. 5. Inhibition of affinophoresis of thiol treated C2S by *N*-acetyllactosamine. Affinophoresis of C2S was carried out with the lactose affinophore (AF) at a concentration of 0.51 m*M*. *N*-Acetyllactosamine (LacNAc) was added to the electrophoresis buffer at the anode at the concentrations indicated above each electropherogram. Acrylamide and acetyltryptophan (Ac-Trp) were injected with the lectin sample as electrophoresis markers.

sugar. The inhibition of affinophoresis for the case of C2S as the result of the addition of *N*-acetyllactosamine is shown in Fig. 5. Since the $\Delta\mu_{max}$ values are already known, the measurement of $\Delta\mu_i$ in the presence of the neutral sugar, I, allows the calculation of $K^{int}_{d\ app}$ according to Eq. (2). The calculation using Eq. (4) gives the values for K^{int}_i, that is the dissociation constant of the complex between the single binding site and a sugar. A plot of $K^{int}_{d\ app}/K^{int}_d$ vs. [I] gives a linear line with a slope of $1/K^{int}_i$ as shown by Eq. (3). Plots for the collection of neutral sugars used here are shown in Fig. 6. The steeper the slope, the higher is the affinity to C2S. Since the affinity for *N*-acetyllactosamine and lactose is considerably higher than the others, they were plotted separately (Fig. 6A). The determined K^{int}_i values are summarized in Table 1. It would be preferable to take the concentration of the sugar around its K^{int}_i value but the affinity for some sugars are so low to realize such conditions and this may have affected the precision in the determination of some of the K^{int}_i values.

The striking preference of the galectin-1 mutant for *N*-acetyllactosamine and lactose was clear, as has been repeatedly reported [13,14]. The most relevant work that can be compared with the present results is

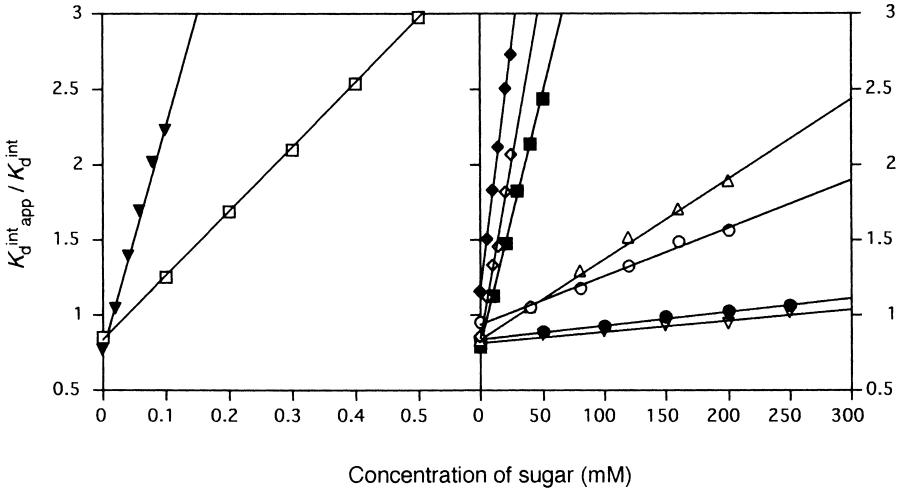

Fig. 6. Inhibition plots for the affinophoresis of C2S with neutral sugars. Note the difference in the concentration range of sugars for the two panels. The symbols are; ▼, *N*-acetyllactosamine; □, lactose; ♦, methyl-α-galactoside; ◇, melibiose; ■, galactose; △, sucrose; ○, maltose; ●, glucose; ▽, mannose.

Table 1
Summary of K_i^{int} values of galectins for sugars

No.	Sugar	K_i^{int} (SD) (m*M*)	Concentration range[a] (m*M*)	Relative affinity[b] (Lac = 100)
C2S				
1	*N*-Acetyllactosamine (Galβ1–4GlcNAc)	0.067 (0.003)	0.02–0.1 (5)	352
2	Lactose (Galβ1–4Glc)	0.23 (0.005)	0.1–0.5 (5)	100
3	Melibiose (Galα1–6Glc)	21 (1.8)	5–25 (5)	1.1
4	Maltose (Glcα1–4Glc)	310 (44)	40–200 (5)	0.07
5	Maltotriose (Glcα1–4Glcα1–4Glc)	280 (27)	40–200 (5)	0.08
6	Sucrose (Glcα1–β2Flc)	190 (5)	40–200 (5)	0.12
7	Galactose	30 (0.5)	10–50 (5)	0.78
8	Methyl-α-D-galactoside	16 (0.5)	5–25 (5)	1.5
9	Methyl-β-D-galactoside	39 (2)	10–50 (5)	0.6
10	Isopropyl-β-D-thiogalactoside	19 (3)	5–25 (3)	1.2
11	*N*-Acetylgalactosamine	170 (1)	40–120 (3)	0.14
12	Glucose	1080 (90)	50–250 (5)	0.02
13	Mannose	1300 (150)	50–250 (5)	0.017
14	Xylose	640 (70)	50–250 (3)	0.04
15	Methyl-β-D-glucoside	1010	200 (1)	0.02
16	*N*-Acetylglucosamine	2600	200 (1)	0.009
17	Methyl-α-D-mannoside	1600	200 (1)	0.015
18	L-Fucose	1200	200 (1)	0.02
19	Rhamnose	960	200 (1)	0.02
20	L-Arabinose	420	200 (1)	0.06
rhGal-1				
1	*N*-Acetyllactosamine (Galβ1–4GlcNAc)	0.062 (0.007)	0.02–0.1 (5)	367
2	Lactose (Galβ1–4Glc)	0.23 (0.014)	0.1–0.5 (5)	100
7	Galactose	22 (14)	10–50 (5)	1.0
rhGal-3				
1	*N*-Acetyllactosamine (Galβ1–4GlcNAc)	0.032 (0.004)	0.01–0.05 (5)	231
2	Lactose (Galβ1–4Glc)	0.074 (0.004)	0.04–0.2 (5)	100
3	Melibiose (Galα1–6Glc)	3.0 (0.3)	1–5 (5)	2.4
6	Sucrose (Glcα1–β2Flc)	150 (16)	40–200 (5)	0.05
7	Galactose	2.5 (0.23)	1–4 (4)	2.9
8	Methyl-α-D-galactoside	2.6 (0.15)	1–3 (3)	2.8
9	Methyl-β-D-galactoside	2.7 (0.15)	1–4 (4)	2.7
10	Isopropyl-β-D-thiogalactoside	2.5 (0.08)	1–3 (3)	2.9

[a] The range of concentration of sugars used for inhibition analyses with the number of data points in parentheses.
[b] The value of $1/K_i^{int}$ relative to that of lactose for each lectin.

that reported by Lee et al. [14]. They reported the concentrations, I_{50}, for various sugars that caused a 50% reduction in the binding of ^{125}I-labeled galectin-1 to a lactose-immobilized agarose beads. The values of I_{50} with the relative value of $1/I_{50}$ in parentheses were as follows, *N*-acetyllactosamine, 0.13 m*M* (350%); lactose, 0.46 m*M* (100%); methyl-α-galactoside, 26 m*M* (1.8%); melibiose, 30 m*M* (1.5%); galactose, 63 m*M* (0.7%) and methyl-β-galactoside, 85 m*M* (0.5%). Although these I_{50} values are about twice larger than the K_i^{int} values reported in the present paper, the consistency of the relative magnitude of the affinity is remarkable. When lactose-immobilized beads are used at a concentration that is equivalent to the K_d value between the lectin and the modified beads, as it was the case in their experiments [14], the I_{50} value for each sugar should be twice as large as the corresponding K_i^{int} value. This theory may simply explain the difference, although there are some additional

factors to be considered, i.e., the divalency of the lectin for the interaction with the lactose-immobilized beads and the difference in the temperature between their experiments, 25°C, and ours, 20°C.

3.4. Affinophoresis of rhGal-1

The thiol-treated rhGal-1 was used as the sample for the affinophoresis without removing the added dithiothreitol. Oxidized dithiothreitol almost overlapped with the peak of acrylamide and dithiothreitol appeared a short distance behind these peaks. The rhGal-1 preparation appeared at around 6 min with a fast migrating minor peak, which is thought to be deamidation products. Overall electropherograms was very similar to those presented in Fig. 4 for the reduced C2S mutant. A Woolf–Hofstee plot was made for the main peak and K_d^{int} and $\Delta\mu_{max}$ values were determined as $K_d^{int}=0.41$ m*M*, $\Delta\mu_{max}=4.73\cdot 10^{-5}$ cm^2 V^{-1} s^{-1} ($R^2=1.000$). These values are nearly indistinguishable from the values obtained for the major peak of the C2S mutant sample.

3.5. Determination of affinity constants of rhGal-1 to neutral sugars

Based on the results that the main peak of rhGal-1 is not distinguishable from that of C2S in terms of the affinity for the affinophore, inhibition analysis of rhGal-1 with lactose, *N*-acetyllactosamine and galactose was carried out under the identical conditions to C2S. The results were plotted according to Eq. (3) in Fig. 7 and fitted lines were represented with solid lines. The K_i^{int} values are summarized in Table 1. Although the number of sugars tested was not large, the values are very similar to those of C2S and two proteins can be considered to be identical in terms of affinity for sugars. The present results extend the previous findings that the substitution of the second cysteine to serine has no effect on the sugar-binding activity of the lectin [18].

3.6. Affinophoresis of rhGal-3

Galectin-3 is an M_r 26 200 protein and the carbohydrate-binding domain that resembles to the subunit of Gal-1 is located at its C-terminal half. A short N-terminal domain is linked to the C-terminal domain through an intervening proline, glycine, and tyrosine-rich domain. Gal-3 behaves as a monomer in a solution but was reported to multimerize on binding to surfaces that contain glycoconjugate ligands, and the N-terminal portion of the protein is responsible for this property [11]. The capillary affinophoresis of rhGal-3 shows that the mobility of the protein is very small in comparison to rhGal-1 (Fig. 8). The isoelectric point of rhGal-3 is calcu-

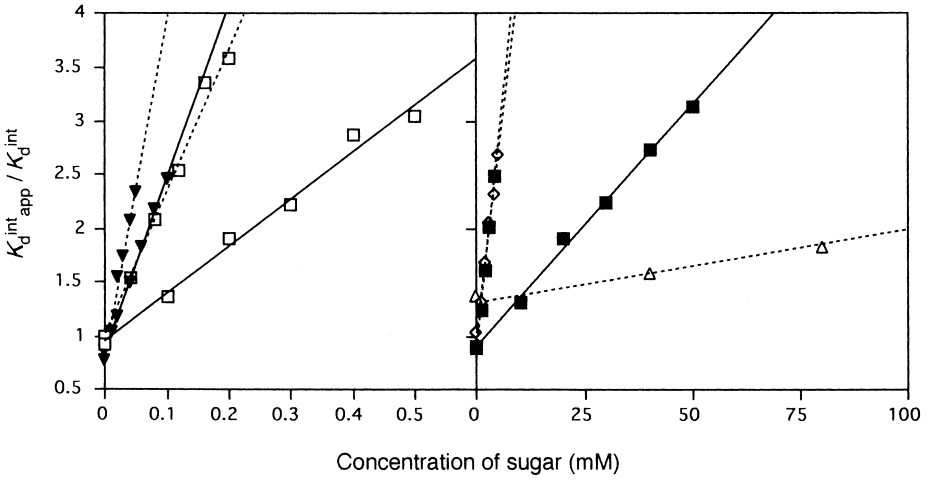

Fig. 7. Inhibition plots for the affinophoresis of rhGal-1 and rhGal-3 with neutral sugars. Note the difference in the concentration range of sugars for the two panels. The symbols are; ▼, *N*-acetyllactosamine; □, lactose; ◇, melibiose; ■, galactose; △, sucrose. Solid lines are for rhGal-1 and broken lines for rhGal-3.

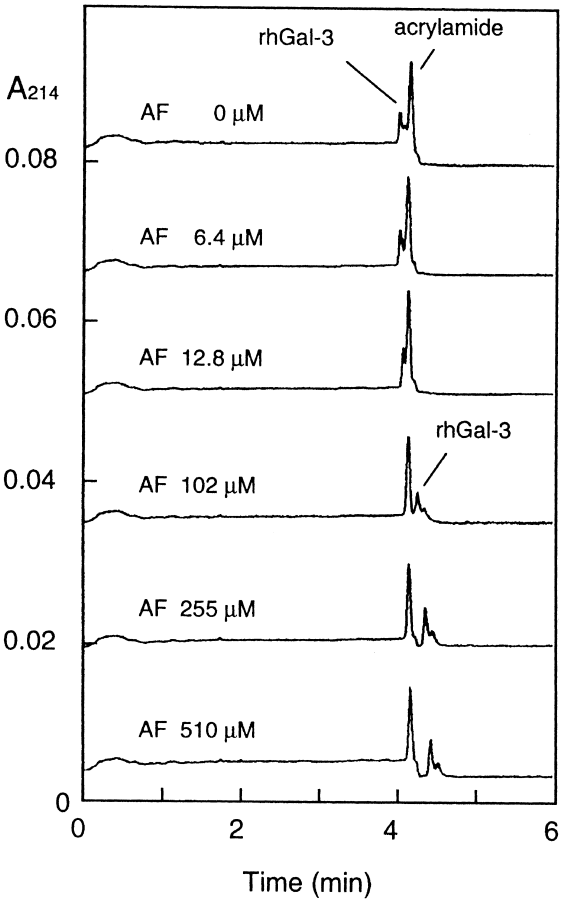

Fig. 8. Affinophoresis of rhGal-3 with the lactose affinophore. rhGal-3 was subjected to capillary affinophoresis using the lactose affinophore (AF) at concentrations indicated above each electropherogram. Acrylamide was injected with the lectin sample as an electrophoresis marker.

lated to be 8.9, which is consistent with the result that the lectin appeared before the neutral marker in the absence of the affinophore. As the concentration of the affinophore increased, the detection time of the lectin peak increased and eventually exceeded that of the neutral marker. The lectin preparation is composed of two peaks, i.e., a major peak and a minor one that is more negatively charged than the former. A Woolf–Hofstee plot according to Eq. (1) for three sets of experiments gave the K_d^{int} and $\Delta\mu_{max}$ values for the major and minor peak as follows, $K_d^{int}=0.059$ m*M* (SD 0.001) and 0.066 m*M* (SD 0.001); $\Delta\mu_{max}=2.72\cdot10^{-5}$ cm^2 V^{-1} s^{-1} (SD 0.01) and $2.86\cdot10^{-5}$ cm^2 V^{-1} s^{-1} (SD 0.02), respectively. The differences are 12% in K_d^{int} and 5% in $\Delta\mu_{max}$ values. The difference in the sugar binding activity was small and the origin of the two peaks should be again attributed to deamidation reactions. The affinity of rhGal-3 for the affinophore was about seven-times higher than that of rhGal-1.

3.7. Determination of affinity constants of rhGal-3 to neutral sugars

Since the peak of rhGal-3 overlapped with the peak of acrylamide at concentrations around the K_d^{int} value, 0.059 m*M*, inhibition experiments were carried out with the affinophore at a considerably higher concentration of 0.255 m*M* than the K_d^{int} value. The plots for the inhibition experiments according to Eq. (3) are shown in Fig. 7 with fitted lines represented by dotted lines and the K_i^{int} values are summarized in Table 1. The exclusively high affinity of rhGal-3 for *N*-acetyllactosamine and lactose is clear. rhGal-3 is shown to have 2–10-times higher affinity than rhGal-1 for the sugars containing galactose at their non-reducing ends, while the affinity for sucrose were almost the same. This result indicates a higher preference of galectin-3 to galactosides than galectin-1. The preference for methyl-α-galactoside over methyl-β-galactoside was not observed for rhGal-3.

Sparrow et al. reported I_{50} values for variety of sugars and oligosaccharides in the binding of human galectin-3 (called HL-29 at that time) to asialofetuin-agarose beads [13]. A part of their data that are relevant to our results is as follows with a relative value of $1/I_{50}$ to lactose in parentheses, lactose (100%), *N*-acetyllactosamine (1130%), galactose (1.8%), methyl-α-galactoside (2.6%) and methyl-β-galactoside (1.6%). Overall agreement in the relative affinity data based on the I_{50} values and the K_i^{int} values are apparent, although the affinity for *N*-acetyllactosamine had been emphasized in comparison to the relative affinity based on the affinity constants.

4. Conclusion

In the analysis of the specific affinity of rhGal-1 and its mutant C2S, the advantages of the use of capillary electrophoresis are clearly demonstrated. If the separation method had not been used in the

analysis, the presence of heterogeneity in these samples would have been overlooked. Although further analysis is needed to characterize the nature of the heterogeneity observed in the sample prior to thiol treatment, the separation could be a key in revealing the oxidation process of the protein, which could eventually lead to the appearance of a different type of biological activity [25,26].

Although divalency should play a crucial role in the actual function of the lectins in cells or tissues, it poses an awkward problem in the determination of affinity constants (K_d^{int}), when a polyliganded surface or insoluble support, such as a microtiter plate well, agarose beads, a sensor chip of the surface plasmon resonance analyzer is used in the analysis. The problem arises from the fact that the degree of divalent interaction between lectins and polyliganded matrices is unknown and inhibition experiments should give a smaller dissociation constant for free ligand than the real value depending on the degree of divalency [10]. Although the I_{50} values that have been reported thus far should, conversely, be larger than the affinity constant from reasons discussed in Section 3.3, the consistency between the relative affinities based on I_{50} values and that based on affinity constants is noteworthy. The value of capillary affinophoresis with a monoliganded affinophore for the determination of the affinity constants between the binding site of lectins and sugars is unequivocal.

References

[1] Y.-H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 461.
[2] K. Shimura, K. Kasai, Anal. Biochem. 251 (1997) 1.
[3] N.H.H. Heegaard, R.T. Kennedy, Electrophoresis 20 (1999) 3122.
[4] R.M.G. Duijn, J. Frank, G.W.K. van Dedem, E. Baltussen, Electrophoresis 21 (2000) 3905.
[5] J. Gao, M. Mammen, G.M. Whitesides, Science 272 (1996) 535.
[6] K. Shimura, K. Kasai, Jpn. J. Electrophoresis 41 (1997) 179.
[7] K. Shimura, K. Kasai, J. Biochem. 120 (1996) 1146.
[8] K. Shimura, J. Chromatogr. 510 (1990) 251.
[9] K. Shimura, K. Kasai, Methods Enzymol. 271 (1996) 203.
[10] K. Shimura, K. Kasai, Electrophoresis 19 (1998) 397.
[11] S.H. Barondes, D.N.W. Cooper, M.A. Gitt, H. Leffler, J. Biol. Chem. 269 (1994) 20807.
[12] K. Kasai, J. Hirabayashi, J. Biochem. 119 (1996) 1.
[13] C.P. Sparrow, H. Leffler, S.H. Barondes, J. Biol. Chem. 262 (1987) 7383.
[14] R.T. Lee, Y. Ichikawa, H.J. Allen, Y.C. Lee, J. Biol. Chem. 265 (1990) 7864.
[15] R.N. Knibbs, N. Agrwal, J.L. Wang, I.J. Goldstein, J. Biol. Chem. 268 (1993) 14940.
[16] J. Hirabayashi, Y. Arata, K. Kasai, J. Chromatogr. A 890 (2000) 261.
[17] A. Hampton, L.A. Slotin, R.R. Chawla, J. Med. Chem. 19 (1976) 1279.
[18] J. Hirabayashi, K. Kasai, J. Biol. Chem. 266 (1991) 23648.
[19] Y. Oda, H. Leffler, Y. Sakakura, K. Kasai, S.H. Barondes, Gene 99 (1991) 279.
[20] J. Hirabayashi, Y. Sakakura, K. Kasai, in: H.-J. Gabius, S. Gabius (Eds.), Lectins and Glycobiology, Springer-Verlag, Berlin, 1993, p. 474.
[21] K. Shimura, K. Kasai, Anal. Biochem. 227 (1995) 186.
[22] N.H.H. Heegaard, J.W. Sen, M.H. Nissen, J. Chromatogr. A 894 (2000) 319.
[23] K. Shimura, N. Uchiyama, K. Kasai, Electrophoresis 22 (2001) 3471.
[24] P.L. Whitney, J.T. Powell, G.L. Sanford, Biochem. J. 238 (1986) 683.
[25] K. Yamaoka, A. Ingendoh, S. Tsubuki, Y. Nagai, Y. Sanai, J. Biochem. 119 (1996) 878.
[26] Y. Inagaki, Y. Sohma, H. Horie, R. Nozawa, T. Kadoya, Eur. J. Biochem. 267 (2000) 2955.