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# Overall carbohydrate-binding properties of *Castanea crenata* agglutinin (CCA)

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**Abstract**—The carbohydrate-binding properties of *Castanea crenata* agglutinin (CCA) were investigated by an enzyme-linked lectin absorbent assay. The binding ability of each carbohydrate was compared using $IC_{50}$ values. CCA exhibited mannose/glucose specificity, as observed with many mannose-binding jacalin-related lectins. For oligosaccharides containing glucose, it has been shown that the degree of polymerization and the linkage mode of glucose residues have no effect on CCA–carbohydrate interaction; thus, only the non-reducing end glucose unit in glucooligosaccharides may be involved in the interaction with CCA. Among mannooligosaccharides, CCA strongly recognized α-D-Man-(1→3)-[α-D-Man-(1→6)]-D-Man, which is a core in N-linked carbohydrate chains. By considering the results with glycoproteins, it is likely that CCA binds preferentially to mono- or non-sialylated biantennary carbohydrate chains. We also obtained $K_d$ values by analysis of the dependency of the $IC_{50}$ on CCA concentration, based on the hypothesis that CCA has a single binding site or two equivalent binding sites. The estimated $K_d$ values for mannose, glucose and α-D-Man-(1→3)-[α-D-Man-(1→6)]-D-Man were 2.39, 7.19 and 0.483 mM, respectively. The relative binding abilities showed good agreement with the relative inhibition intensities. Isothermal calorimetric titration was carried out to directly estimate the dissociation constants of CCA for mannose and for α-D-Man-(1→3)-D-Man. The values were 2.34 mM for mannose and 0.507 mM α-D-Man-(1→3)-D-Man. These results suggest that the relative inhibition intensity represents the ratio of $K_d$ values and that CCA has a single or two equivalent binding sites.


*Keywords:* *Castanea crenata*; Enzyme-linked lectin assay; Carbohydrate specificity; Jacalin-related lectins

Carbohydrates are important substances in various biological systems. Thus, lectins, which can bind to carbohydrates specifically and reversibly, have been used in many biochemical and biomedical applications (for example, see reviews 1 and 2). To this purpose, it is insufficient that an investigation on the specificity of each lectin is performed only at a monosaccharide level, because in most cases lectins exhibit different binding properties for oligosaccharides even though they have same specificity for a monosaccharide. The typical example is for three mannose-binding lectins, Con A, Snowdrop lectin and jackfruit lectin (artocarpin). Among these, Con A[3] and artocarpin[4] are mannose/glucose binding lectins, while snowdrop lectin has no ability

*Abbreviations*: CCA, *Castanea crenata* agglutinin; CRLL, *Cycas revolute* leaf lectin; ELLA, enzyme-linked lectin absorbent assay; ITC, isothermal titration calorimetry; JRL, jacalin-related lectin; mJRL, mannose-binding JRL; PAL, *Phlebodium aureum* lectin; PBS, phosphate-buffered saline.

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to bind to glucose.[5] For mannobioses, Con A prefers α-D-Man-(1→2)-D-Man over α-D-Man-(1→3)-D-Man or α-D-Man-(1→6)-D-Man,[3] but α-D-Man-(1→3)-D-Man is the strongest ligand for both snowdrop lectin[6] and artocarpin.[4] Moreover, the effects of sialylation, fucosylation and GlcNAc residues at the reducing end were also different among these lectins.[3,4,6] These results indicate the necessity to elucidate the binding properties at the oligosaccharide level.

The Japanese chestnut (*Castanea crenata* Sieb. et Zucc.) agglutinin, CCA, belongs to the jacalin-related lectins (JRLs). CCA consists of 309 amino acid residues that could be divided into two domains, N- and C-domain, and both domains showed about 30% identity to other JRLs.[7] In addition, three key residues that are essential for the carbohydrate binding, for example, Gly18, Gly135 and Asp139 in heltuba,[8] are conserved in both domains. Thus, it is assumed that the structure of the CCA protomer is as if one dimeric mJRL is conjugating with the linker peptide. This indicates the possibility that CCA has two binding site per subunit, but the number of binding sites has not been clarified. For protein chemical studies, evaluation of the number of binding sites and of their separate binding properties is essential. From another point of view, however, the estimation of overall binding property of CCA is also necessary when considering direct applications. Moreover, although the expression profile of CCA suggests that CCA acts as a dual-functional protein,[9] the more precise binding property is also required to elucidate the physiological functions. To date, a conventional and semi-quantitative hapten inhibition haemagglutination assay has revealed that CCA is a mannose/glucose binding lectin.[10] Thus CCA belongs to the mannose-binding JRL (mJRL) subfamily, but the detailed binding properties for oligosaccharides have not been determined.

In this paper, we elucidated the overall binding property of CCA at the mono- and oligosaccharide levels by comparing CCA with other mJRLs using an enzyme-linked lectin absorbent assay. Then, we discuss the relationships between $IC_{50}$ and the dissociation constant by kinetic analysis (Fig. 1).

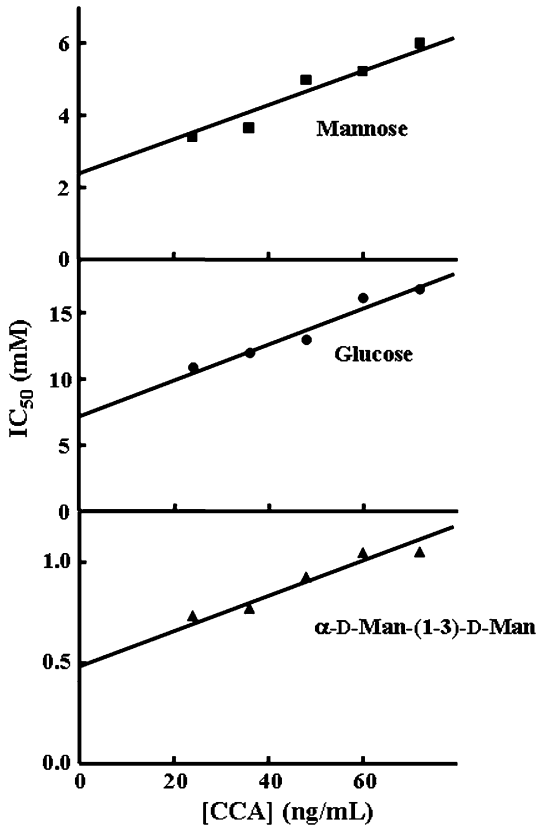


**Figure 1.** Relationships between $IC_{50}$ and initial concentration of CCA. Circles, squares and triangles represent the result of mannose, glucose α-D-Man-(1→3)-D-Man, respectively. The results were analyzed by linear regression using Prism software. The intercept at the *y*-axis corresponds to the dissociation constant (see Eq. 1 in the text).

## 1. Results and discussion

### 1.1. Binding of mono- and oligosaccharides

The $IC_{50}$ values of mono- and oligosaccharides and the relative inhibition intensity to mannose are summarized in Table 1 compared with other mJRLs. CCA can bind to both mannose and glucose like other mJRLs, but the relative intensities of Glc to Man varied from 0.05 (Calsepa) to 0.47 (BanLec) among the mJRLs. In addition, by comparison with the binding properties of each mJRL, some unique features were found. Although GlcNAc exhibited weak binding, ManNAc has no binding ability. Thus, it could be concluded that bulkiness at the C-2 axial position considerably interferes in interactions with mJRLs. Whereas addition of a methyl group to the C-1 OH group did not exhibit negative effects on the binding to mJRLs, because MeαMan was a similar or stronger inhibitor than mannose. Fructose is also a poor inhibitor for most mJRLs, but only BanLec exhibited the unique property of preferring fructose over mannose. Lastly, no mJRL could specifically recognize fucose.

For oligosaccharides containing a glucose unit, their binding abilities to CCA were similar to those of glucose, except for sucrose. That is, neither chain length nor binding mode has an affect on the interaction with CCA. This indicates that CCA recognizes only one glucose unit at the non-reducing side. A remarkable weak interaction with sucrose suggests that a furanose ring at the second site interferes with binding. In contrast to CCA, other mJRLs showed a dependency on the binding mode. BanLec is similar to CCA but prefers nigerose and trehalose over glucose. In CRLL, the ability of maltose is half that of glucose but trehalose binds

**Table 1.** $IC_{50}$ values of CCA of mono- and oligosaccharides and comparison of the relative inhibition intensity with other mannose-binding JRLs[a]

| Carbohydrates | $IC_{50}$ for CCA (mM) | Relative inhibition intensity | | | | | |
|---|---|---|---|---|---|---|---|
| | | CCA | Artocarpin[b] | BanLec[c] | CRLL[d] | PAL[e] | Calsepa[f] |
| Mannose | 4.89 ± 0.16 | 1 | 1 | 1 | 1 | 1 | 1 |
| MeαMannose | 3.73 ± 0.11 | 1.3 | 1.8 | 2.1 | 1.0 | 1 | 6.3 |
| ManNAc | NI[300] | | NI[100] | — | — | — | <0.02 |
| Glucose | 17.8 ± 0.2 | 0.28 | 0.11 | 0.47 | 0.125 | 0.25 | 0.05 |
| GlcNAc | 28.1 ± 0.2 | 0.17 | 0.12 | 0.50 | 0.25 | 0.13 | 0.1 |
| Fructose | 32.3 ± 1.3 | 0.15 | — | 1.4 | 0.5 | 0.13 | 0.17 |
| Fucose | NI[400] | | NI[100] | — | 0.015 | NI[50] | — |
| Maltose (Glcα1→4Glc) | 18.9 ± 0.2 | 0.26 | — | 0.41 | 0.062 | 0.03 | 2.0 |
| Maltotriose | 18.9 ± 0.5 | 0.26 | — | 0.49 | — | 0.06 | — |
| Maltotetraose | 16.3 ± 0.5 | 0.30 | — | — | — | — | — |
| Nigerose (Glcα1→3Glc) | 15.5 ± 0.4 | 0.32 | — | 0.67 | NI[50] | 0.13 | — |
| Trehalose (Glcα→αGlc) | 15.0 ± 0.5 | 0.33 | — | 0.9 | 0.25 | — | <0.02 |
| Sucrose | 80.1 ± 1.9 | 0.06 | — | NI[100] | NI[200] | — | <0.02 |
| α-D-Man-(1→2)-D-Man | 4.46 ± 0.12 | 1.1 | 0.14 | 1.9 | 0.70 | 1.3 | — |
| α-D-Man-(1→3)-D-Man | 1.14 ± 0.04 | 4.3 | 3.00 | 2.1 | 0.70 | 8.0 | — |
| α-D-Man-(1→4)-D-Man | 5.31 ± 0.12 | 0.9 | 0.12 | — | 0.70 | (0.26) | — |
| α-D-Man-(1→6)-D-Man | 3.24 ± 0.17 | 1.5 | 0.53 | 2.0 | (0.35) | 1.0 | — |
| α-(1→3)-D-Man-[α-D-Man-(1→6)]-D-Man | 0.86 ± 0.04 | 5.7 | 13.0 | — | (2.78) | 1.0 | — |

[a] Galactose, GalNAc, lactose, fucose, rhamnose, arabinose, xylose, ribose and deoxyribose showed no inhibition at 400 mM. Values in parentheses are for the *O*-methyl derivatives of carbohydrates.
[b] Ref. 11.
[c] Ref. 12.
[d] Ref. 13.
[e] Ref. 14.
[f] Ref. 15.

two times stronger than glucose. Only calsepa showed a stronger binding ability to maltose than glucose. These results reveal that only CCA among the mJRLs does not interact with a second glucose unit. Since lactose has no binding ability to any mJRLs, it could be concluded that the configuration of the C-4 hydroxyl group in glucose at the non-reducing end is essential for the interaction of the lectin with carbohydrates.

No mannooligosaccharide showed weaker binding ability to CCA than mannose. α-(1→3)-D-Man-[α-D-Man-(1→6)]-D-Man was the preferred inhibitor for CCA, and the potency was 5.7 times that of mannose. α-D-Man-(1→3)-D-Man and α-D-Man-(1→6)-D-Man were stronger than mannose, but α-D-Man-(1→2)-D-Man and α-D-Man-(1→4)-D-Man exhibited a similar intensity as that of mannose. These results indicate that the binding mode of mannooligosaccharides is an important factor, but an unfavourable mode does not interfere with the carbohydrate–CCA interaction at the second site. In contrast to this, artocarpin prefers only α-D-Man-(1→3)-D-Man and shows the strongest binding to α-(1→3)-D-Man-[α-D-Man-(1→6)]-D-Man though α-D-Man-(1→6)-D-Man is a weaker inhibitor than mannose. In addition, CRLL exhibited unique features that all mannobioses are less effective than mannose. Thus, an unfavourable binding mode may be rejected at the second binding site in artocarpin and CRLL.

In summary, although the six lectins listed in Table 1 belong to the same category, these carbohydrate-binding properties are rather different. Among these, CCA and BanLec are assumed to have relatively loose specificity, because no carbohydrates showed extremely strong or weak inhibition to them.

### 1.2. Binding of glycoproteins

The $IC_{50}$ of glycoproteins could not be compared simply because of the heterogeneity of the carbohydrate chains and the difference in molar concentrations. However, some interesting features were found: Ovomucoid, of which most chains contain bisected GlcNAc,[17] were weakly inhibitory. CCA may dislike a sialylated triantennary structure, because transferrin[18] and fetuin[19] were also poor inhibitors. Although desialylation of fetuin enhanced the binding ability about 10-fold, the inhibitory intensity was still lower than tyroglobulin. Therefore, the most preferred scaffold for CCA is assumed to be a biantennary structure, even one that is monosialylated.[20] The enhancement of desialylation also has been reported for artocarpin, but there was no or little effect on BanLec, CRLL and calsepa. Whereas, the effect of fucose on CCA should be considered, because most carbohydrate chains in thyroglobulin contain fucose at the reducing terminal GlcNAc residue.[20] Indeed, fucose alone could not inhibit the CCA–carbohydrate interaction, but artocarpin, which cannot bind to xylose, showed strong affinity for the xylose-containing heptasaccharide.[4] Therefore, the effect of monosaccha-

**Table 2.** $IC_{50}$ values of CCA of glycoproteins and comparison of the relative inhibition intensity with other mJRLs

| Glycoproteins | $IC_{50}$ (μg/mL) | | | | |
|---|---|---|---|---|---|
| | CCA | Artocarpin[a] | BanLec[b] | CRLL[c] | Calsepa[d] |
| Transferrin (human) | 788 ± 83 | NI | — | >350 | — |
| Thyroglobulin | 7.50 ± 0.40 | — | 8 | 10 | 167 |
| Ovomucoid | 776 ± 15 | — | >2000 | 125 | 100 |
| Fetuin | 583 ± 38 | — | 250 | 125 | 333 |
| Asialofetuin | 54.3 ± 4.2 | — | 250 | — | 177 |
| Asialothyroglobulin | — | — | — | 5 | 82.5 |
| Asialotransferrin | — | 8.6 μM | — | >350 | — |

[a] Ref. 3.
[b] Ref. 16.
[c] Ref. 13.
[d] Ref. 15.

ride should be considered in both states, free and as part of the sugar chain. From the results in Table 2, it can be inferred that there is no common feature among the binding specificity for glycoproteins among mJRLs.

### 1.3. $IC_{50}$ values depend on the CCA concentration

As described in the Experimental section, ELLA is a modified method of competitive ligand binding. Thus, using the assumption that CCA has one binding site, the $IC_{50}$ can be presented as follows:

$$IC_{50} = (K_I/K_d)[CCA]_0 + K_I \quad (1)$$

where $K_d$, $K_I$ and $[CCA]_0$ denote the dissociation constant of the base–CCA complex, the dissociation constant of the inhibitor–CCA complex and the initial CCA concentration, respectively. This equation shows that $IC_{50}$ depends on both base used and initial CCA concentration; namely, the intercept on the *y*-axis and slope are $K_I$ and the ratio of $K_I$ to $K_d$, respectively, in the $IC_{50}$ versus [CCA] plot (Fig. 1). From these intercepts on the *y*-axis, the dissociation constants for mannose, glucose and α-D-Man-(1→3)-D-Man were estimated to be 2.39 ± 0.15, 7.19 ± 0.30, 0.483 ± 0.024 mM, respectively (Table 3). In addition, the relative values, $K_{Man}/K_{Glc}$ and $K_{Man}/K_{Biose}$ (0.33 and 4.9, Table 3) agreed with the relative inhibitory intensities (0.28 and 4.3, in Table 1) within experimental error. These results indicate that the relative value of $IC_{50}$ means the ratio of dissociation constants of each inhibitor. This means that direct comparison of $IC_{50}$ values among different experiments is not significant, but the relative values of $IC_{50}$ reflect the ratio of dissociation constants under identical conditions. If the conditions are appropriate, $[CCA]_0 \ll K_d$ the $IC_{50}$ value approximately represents the dissociation constant. This result is also obtained if CCA has two equivalent binding sites, because, in this case, $[CCA]_0$ changes to $2[CCA]_0$ in Eq. 1 without affecting the intercept on the *y*-axis. Therefore, the above results suggest that CCA has one or two equivalent binding sites.

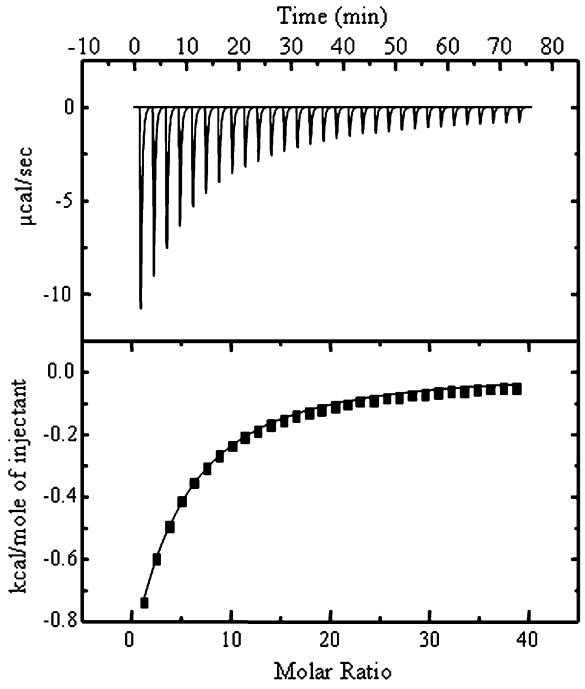


**Figure 2.** Calorimetric titration of CCA with mannose at 300 K. Top, thermogram obtained for 30 automatic injections, each 2 μL, of 200 mM mannose; bottom, the integrated curve shows experimental point and the best fit.

### 1.4. Isothermal calorimetric titration (ITC)

To confirm the reliability of the estimated dissociation constants and the number of binding sites, we analyzed the interactions of CCA with mannose or α-D-Man-(1→3)-D-Man by ITC. A typical titration calorimetry measurement with mannose is shown in Figure 2. When analyzed by a one-set site model, the dissociation constants, calculated as a reciprocal of the obtained binding constant, for the mannose–CCA complex and α-D-Man-(1→3)-D-Man-CCA complex were 2.34 ± 0.03 mM and 0.507 ± 0.071 mM, respectively (Table 3). These values are in good agreement with the above values obtained from concentration dependency of $IC_{50}$. Consequently,

**Table 3.** Comparison of $IC_{50}$, $K_d$ and their relative values obtained by ELLA and ITC

| Carbohydrates | ELLA | | | | ITC | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (mM) | Relative value | $K_d$ (mM) | Relative value | $K_d$ (mM) | Relative value |
| Mannose | 4.89 ± 0.16 | 1 | 2.39 ± 0.15 | 1 | 2.34 ± 0.03 | 1 |
| Glucose | 17.8 ± 0.0 | 0.28 | 7.19 ± 0.30 | 0.33 | — | — |
| α-D-Man-(1→3)-D-Man | 1.14 ± 0.04 | 4.3 | 0.483 ± 0.024 | 4.9 | 0.507 ± 0.071 | 4.6 |

the ratio of $K_{Man}/K_{Biose} = 4.6$ also agreed with the relative intensity (Table 3). Since neither two-set site nor multiple interacting site models fit in both cases, it could be concluded that CCA has a single or two equivalent binding sites. In addition, these results support the dissociation constants estimated by ELLA. In artocarpin, the coordination of relative affinity estimated by ITC and relative inhibitory intensity measured by ELLA has also been reported,[11] but the dissociation constants have not been compared. This is the first report on dissociation constants of lectin–carbohydrate complexes evaluated by ELLA directly.

In conclusion, it was revealed that CCA strongly recognizes α-(1→3)-D-Man-[α-D-Man-(1→6)]-D-Man unit, which is a core structure in N-linked glycans. In addition, CCA cannot distinguish glucooligosaccharides by chain length or by binding mode. Among mJRLs, no common properties were found at either the oligosaccharide level or sugar chain level. Thus, finer analyses at the sugar chain level are required for these applications. The number of carbohydrate-binding sites in CCA is supposed to be one or equivalent two. At present, there is a stronger possibility that CCA has two equivalent sites, because amino acid residues contributing to sugar binding in other mJRL are conserved in both the N- and C-domains.[7] If CCA has two equivalent binding sites, the values obtained in this study are the average value of the two sites. The word 'equivalent' used here means undistinguishable, but not equal. As repeated above, since the carbohydrate specificities were rather different among mJRLs, the fine specificity also differs between the supposed two sites in CCA. Because the sequence identity between the N- and C-domains in CCA is similar to that among mJRLs.[7,13,14] Therefore, in this case, the $IC_{50}$, $K_d$ and relative intensities obtained here are only average; in other words, the overall properties of these two site. However, these overall properties should be important for direct applications. In many applications, lectins have often been used in an immobilized form, such as lectin columns or lectin beads. In such cases, it is necessary to confirm whether the carbohydrate specificity changes through immobilization to a matrix. For this purpose, surface plasmon resonance (SPR) or frontal-affinity chromatography, which has been developed by Hirabayashi et al.,[21] are powerful tools. Both determination of binding site number and elucidation of sugar-chain specificity for the immobilized form are required for future studies on CCA–carbohydrate interactions.

## 2. Experimental

### 2.1. *Castanea crenata* agglutinin (CCA)

Japanese chestnut (*C. crenata* Sieb. et Zucc.) agglutinin (CCA) was purified from its seeds as described previously.[10] The protein concentration was determined by amino acid analysis.

### 2.2. Chemicals

Methyl-α-D-mannose, *N*-acetyl-D-mannosamin, α-D-Man-(1→2)-D-Man, α-D-Man-(1→3)-D-Man, α-D-Man-(1→4)-D-Man, α-D-Man-(1→6)-D-Man, α-(1→3)-D-Man-[α-D-Man-(1→6)]-D-Man, nigerose, thyroglobulin, fetuin and asialofetuin were purchased from Sigma–Aldrich chemical Co. Glucose, maltotriose, maltotetraose and ovomucoid were purchased from Wako Pure Chemical Industries Ltd. Fructose was purchased from the Funakoshi Co. Other monosaccharides, oligosaccharides, yeast mannan and bovine serum albumin (BSA) were purchased from the Nakarai Tesque Co. Anti-rabbit IgG conjuging horseradish peroxidase and 3,3′,5,5′-tetramethylbezidine (TMB) were purchased from Promega.

### 2.3. Enzyme-linked lectin assay (ELLA)

ELLA was carried out to estimate the carbohydrate specificity of CCA according to the method of Eck et al.[22] Briefly, 50 μL of yeast mannan (0.2 mg/mL) dissolved in ELLA–PBS (10 mM phosphate buffer, pH 7.2, containing 130 mM NaCl) was transferred into 96-well microplates (Greiner Bio-One) and incubated at room temperature for 16 h. After washing three times with washing buffer (ELLA–PBS containing 0.05% (v/v) Tween 20), the coated wells were blocked with 200 μL of blocking buffer (ELLA–PBS containing 1% (w/v) BSA and 0.1% (v/v) Tween 20) and incubated at 37 °C for 1 h. After washing as above, 50 μL of CCA (usually 70 ng/mL) was loaded into wells and reacted with immobilized matrix. Following incubation and washing as above, 50 μL of anti-CCA antibody (1:3000 dilutions in blocking buffer) was added to the wells for detection of the CCA binding to yeast mannan, and the plate was incubated at 37 °C for 1 h. After washing, the immune complexes were detected with 50 μL of anti-rabbit IgG antibody conjugating horseradish peroxidase (1:8000

dilution in ELLA–PBS containing 1% (w/v) BSA), and then the plate was incubated at 37 °C for 1 h. Subsequent to addition of TMB into the washed wells, the reaction was terminated with 1 N HCl after 10 min. The absorbance at 415 nm was measured using a Model 550 microplate reader (BIO-RAD).

Carbohydrate-binding specificity was analyzed using an inhibition assay. CCA was pre-incubated with carbohydrate at room temperature for 1 h, and transferred into yeast mannan-coated wells. The $IC_{50}$ values, which indicate the concentration of inhibitor for 50% inhibition in the ELLA system, were calculated using Graphpad Prism (GraphPad Software) from the residual binding based on the absorbance without inhibitor.

### 2.4. Isothermal calorimetric titration

Isothermal calorimetric titration measurements were performed using an OMEGA titration calorimeter (Microcal Inc.) at 300 K equipped with a 1.385-mL cell. CCA dissolved in 50 mM phosphate buffer, pH 7.0, at a subunit concentration of 0.28 mM was titrated with ligands in the same buffer. In the case of mannose, titration was carried out with 2 μL of 200 mM mannose at a time, whereas for α-D-Man-(1→3)-D-Man titration 3 μL of 10 mM solution was used. We also tried to titrate with glucose, but reliable results could not be obtained because of low $C$ values ($C = K_b \times [\mathrm{CCA}]$, where $K_b$ is binding constant) caused by high $K_b$ and low solubility of CCA.

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