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One-Step Purification of Lectins from Banana Pulp Using Sugar-Immobilized Gold Nano-Particles

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To obtain lectins without tedious purification steps, we developed a convenient method for a one-step purification of lectins using sugar-immobilized gold nano-particles (SGNPs). Proteins in crude extracts from plant materials were precipitated with 60% ammonium sulphate, and the precipitate was re-dissolved in a small volume of phosphate buffer. The resultant solution was then mixed with appropriate SGNPs under an optimized condition. After incubating overnight at 4°C, lectins in the mixture formed aggregate with SGNPs, which was visually detected and easily sedimented by centrifugation. The aggregate was dissolved by adding inhibitory sugars, which were identical to the non-reducing sugar moieties on the SGNPs. According to SDS–PAGE and MS of thus obtained proteins, it was found that SGNPs isolated lectins with a high purity. For example, a protein isolated from banana using Glcα-GNP (α-glucose-immobilized gold nano-particle) was identified as banana lectin by trypsin-digested peptide-MS finger printing method.

Key words: gold nano particle, lectin, peptide MS fingerprinting, purification, sugar chain.

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; Glcα-GNP, alpha-glucose-immobilized gold nano-particle; GlcNAcα-GNP, alpha-*N*-acetyl-glucosamine-immobilized gold nano-particle; Manα-GNP, alpha-mannose-immobilized gold nano-particle; SA, 3,5-dimethyl-4-hydroxycinnamic acid; SGNPs, sugar-immobilized gold nano-particles.

Lectins are carbohydrate-binding proteins, which can specifically recognize sugar structures (*1*). Their physiological functions have been argued for a long time, and were recently determined for several lectins. Selectins mediate the adhesion of leucocytes and the endothelial cells of blood vessels. Some plant lectins serve as defence factors against phytopathogenic fungi, insect and animals by interacting with their glycans (*2–4*). According to these examples, lectin–glycan interactions are recognized as important in biological processes in both plant and animal bodies. To understand the functions of lectins at the molecular level in detail, purification and subsequent characterization are the most crucial.

To purify lectins from crude extract, several chromatography techniques, such as affinity chromatography, ion-exchange chromatography and gel permeation chromatography, are generally used. However, such chromatographic purification needs lengthy and tedious steps, preventing the studies of lectins especially in case of small amount of target lectins in the starting materials. To overcome this problem, a simple and effective method is desired. Use of gold nano-particles having glycans is one of the most promising for the purpose.

Gold nano-particles having glycans were rapidly developed in this decade, and utilized to analyse lectins, to estimate their affinity strength or to visualize them with electron microscopy (*5–7*). Recently, we established an efficient technique for the immobilization of glycans on gold nano-particles (*8*, *9*). The produced gold nano-particles, designated sugar-immobilized gold nano-particles (SGNPs), were homogeneous in size and amount of glycans. Importantly, they are easily sedimented by forming aggregate with lectins, suggesting that they are promising for capturing lectins. In this study, we established an effective method for purification of lectins using the SGNPs. As a result, a lectin with high purity was successfully obtained from plant extract.

MATERIALS AND METHODS

Materials—All reagents were used without further purification. Banana was obtained from a grocery store and stored at −20°C until use. Sugars were purchased as follows: maltose, cellobiose and lactose were obtained from Nacalai tesque (Kyoto, Japan); GalNAcβ1-3Gal and α1-2 mannobiose from Dextra Lab. (Reading, UK); melibiose from TCI (Tokyo, Japan). GlcNAcα1-6Glc, GlcNAcβ1-6Glc, GalNAcα1-6Glc, Fucα1-6Glc, Fucβ1-6Glc were generous gifts from Dr Wakao (Kagoshima University).

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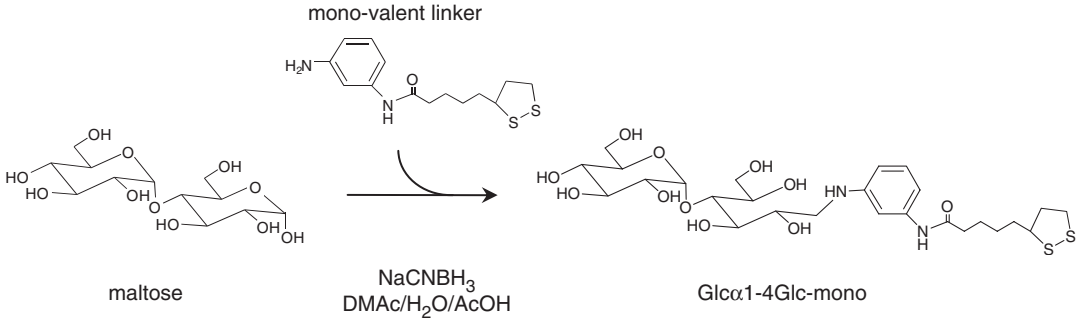


Fig. 1. Synthesis of ligand-conjugate containing α-D-glucoside (Glcα1-4Glc-mono).

Synthesis of Ligand-Conjugate Containing Sugar Moieties—Ligand-conjugates containing sugar-moieties were prepared according to the previous report (*8*, *9*). For preparation of ligand-conjugate containing α-D-glucoside (abbreviated as Glcα1-4Glc-mono), mono-valent linker compound (10.0 mg, 34 μmol) (*9*) dissolved in 1.0 ml of dimethylacetoamide (DMAc) was mixed with maltose (12.2 mg, 34 μmol, dissolved in 0.8 ml of distilled water) and 0.2 ml of acetic acid (Fig. 1). After incubation at 37°C for 4 h, $NaCNBH_3$ (21.3 mg, 340 μmol) dissolved in 0.2 ml of distilled water was added to the solution. After further incubation at 37°C for 72 h, the reaction was lyophilized. The obtained ligand-conjugate was purified by reverse-phase chromatography using Chromatorex ODS (Fuji Silysia Chemical, Aichi, Japan) equilibrated with 45% methanol at the flow rate of 0.8 ml/min. The obtained ligand conjugate was elucidated by reverse-phase chromatography using Inertsil ODS-3 (GL Science, Tokyo, Japan), MS (Voyager DE-Pro, Applied Biosystems, CA, USA) and ^{1}H NMR (ECA-600, JOEL, Tokyo, Japan).

By a similar protocol described above, the objected compound was prepared from appropriate materials, i.e. Glcβ1-4Glc-mono, Galα1-6Glc-mono, Galβ1-4Glc-mono, GlcNAcα1-6Glc-mono, GlcNAcβ1-6Glc-mono, GalNAcα1-6Glc-mono, GalNAcβ1-3Gal-mono, Fucα1-6Glc-mono, Fucβ1-6Glc-mono and Manα1-2Man-mono were prepared from cellobiose, melibiose, lactose, GlcNAcα1-6Glc, GlcNAcβ1-6Glc, GalNAcα1-6Glc, GalNAcβ1-3Gal, Fucα1-6Glc, Fucβ1-6Glc and α1-2 mannobiose, respectively.

Synthesis of SGNPs—Sugar-immobilized gold nanoparticles (SGNPs) were prepared according to the previous report (*8*). To synthesize α-D-glucoside immobilized SGNP (Glcα-GNP), 5 mM (final concentration) of $NaBH_4$ was added to 1 mM of aqueous solution of $NaAuCl_4$ with stirring. Above prepared 100 μM of ligand-conjugate (Glcα1-4Glc-mono) was then added to the solution with stirring. The resulting solution was subsequently dialysed against distilled water and PBST [100 mM phosphate buffer, pH 7.2, containing 0.9% (w/v) NaCl and 0.05% (v/v) Tween-20]. By TEM analysis, diameter of obtained particles was estimated to be 2–10 nm, and most of them showed around 5 nm.

Glcβ-GNP, Galα-GNP, Galβ-GNP, GlcNAcα-GNP, Glc NAcβ-GNP, GalNAcα-GNP, GalNAcβ-GNP, Fucα-GNP, Fucβ-GNP and Manα-GNP were prepared from appropriate ligand-conjugate, i.e. Glcβ1-4Glc-mono, Galα1-6Glc-mono, Galβ1-4Glc-mono, GlcNAcα1-6Glc-mono, GlcNAcβ1-6Glc-mono, GalNAcα1-6Glc-mono, GalNAcβ1-3Gal-mono, Fucα1-6Glc-mono, Fucβ1-6Glc-mono and Manα1-2Man-mono, with similar protocol. The SGNPs prepared were elucidated by binding experiment using lectins, e.g. Concanavalin A (Con A) purchased from EY Laboratories (CA, USA) and RCA120 from Vector Laboratories (CA, USA). The amount of sugar ligand attached to the gold nanoparticles was estimated by elemental analysis. As a result, 50–70 ligand-conjugates were immobilized on the surface of one gold nano-particle of 5 nm diameter.

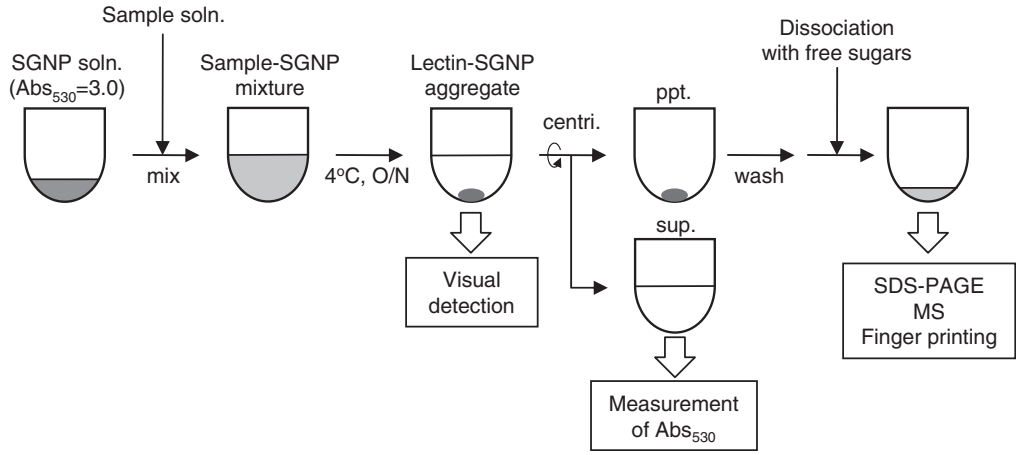
Preparation of Plant Extract—Matured banana pulp (1 g) was homogenized in 5 ml of PBS [100 mM phosphate buffer, pH 7.2, containing 0.9% (w/v) NaCl] containing 10 mM 2-mercaptoethanol. The extract was stirred at 4°C for 2 h, and the homogenate was centrifuged at 8,000 r.p.m. for 30 min. Obtained supernatant was then filtered with DISMIC® ($\phi = 0.45$ μm, ADVANTEC, CA, USA). After adding 60% (w/v) ammonium sulphate to the filtrate, the precipitate was obtained by centrifugation at 10,000 r.p.m. for 10 min at 4°C. Thus, obtained precipitate was dissolved in 2.5 ml of PBS containing 10 mM 2-mercaptoethanol. This solution was used for the following purification steps of lectins.

Screening with SGNPs—To evaluate the sugar chain-binding properties of the extract from banana, a series of SGNPs, i.e. Glcα-GNP, Glcβ-GNP, Galα-GNP, Galβ-GNP, GlcNAcα-GNP, GlcNAcβ-GNP, GalNAcα-GNP, GalNAcβ-GNP, Fucα-GNP, Fucβ-GNP and Manα-GNP, was used for screening. In brief, the above 11 kinds of SGNP solution (30 μl, adjusted to $Abs_{530} = 3.0$) were mixed with 10 μl of sample solutions in round-bottomed microtitre plate wells, respectively, and incubated overnight at 4°C. Also, the extent of aggregation was determined by measuring the absorbance of supernatant at 530 nm. Activity was calculated according to the following equation:

$$\text{Precipitation (\%)} = 100 - \frac{\text{Abs}^{\text{a}}_{530}}{\text{Abs}^{\text{b}}_{530}} \times 100$$

where Abs^{b}_{530} and Abs^{a}_{530} indicate absorbance at 530 nm before and after overnight incubation, respectively.

Dissociation of SGNP–Lectin Complex with Free Sugars—Dissociation effects of free sugars against SGNP aggregates were examined. Similar to the screening described above, 30 μl of Glcα-GNP was mixed with



Scheme 1. **The procedure for capturing lectins using SGNPs**. Several SGNPs, e.g. Manα-GNP, Glcα-GNP and Galβ-GNP, can be used. After subsequent washing with appropriate buffer and distilled water, aggregate was dissolved in inhibitory sugar solutions and applied to subsequent analyses.

10 μl of sample solution. After standing at room temperature for 10 min, the formed SGNP aggregate was sedimented by centrifugation at 1,800 r.p.m. for 1 min at room temperature. After removal of supernatant, 100 μl of sugar solutions (0.2 M glucose, GlcNAc, mannose and galactose dissolved in distilled water) were added to each well.

Capturing Lectins—Sugar-binding proteins (lectins) were captured by SGNPs and characterized. The overall approach was shown in Scheme 1. From data of screening, 30 μl of Glcα-GNP, GlcNAcα-GNP or Manα-GNP was added to 10 μl of banana extract, and thoroughly mixed by pipetting. After incubation overnight at 4°C, the formed aggregate was sedimented by centrifugation at 10,500 r.p.m. for 10 min, and the supernatant was removed. After subsequent washing with 50 μl each of PBST and distilled water, the aggregate was dissolved by adding 10 μl of inhibitory monosaccharides, *i.e.* 0.2 M glucose, 0.2 M GlcNAc and 0.2 M mannose were used for Glcα-GNP, GlcNAcα-GNP and Manα-GNP, respectively. Thus, captured proteins were analysed by SDS–PAGE under non-reducing condition using 15% gel without further purification. Also, they were applied to the subsequent analyses described below.

Proteolytic Digestion by Trypsin—After re-dissolving in inhibitory sugar solutions, 10 μl aliquot of sample solution (corresponding to 2.3 μg protein) was added to the same volume of 75 mM NH_4HCO_3. Proteins in the solution were denatured by boiling for 5 min, and then 10 μl of trypsin (Sigma–Aldrich, MO, USA) dissolved in distilled water (5 μg/ml) was added. The protein digestion was performed by incubating the reaction solution at 37°C for 2 h. The resulting digest was analysed by MALDI-TOF/MS without further purification.

MALDI-TOF Mass Spectrometry—The MALDI-TOF mass spectrometer used was a Voyager DE-Pro (Applied Biosystems). α-cyano-4-hydroxycinnamic acid (CHCA) or 3,5-dimethyl-4-hydroxycinnamic acid (SA) as MALDI matrix was dissolved in the aqueous solution containing 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) to make 10 mg/ml. SGNP-captured proteins and the trypsin-digested peptides were co-crystallized with CHCA or SA matrix. The MS analyses were performed with a reflector and positive-ion mode. The spectra were acquired with 300 shots of a 337 nm nitrogen laser operating at 3 Hz. Angiotensin (SIGMA) and Calibration mixture 2 (PE Biosystems, CA, USA) were used as MS calibration standards. Protein identification was performed by searching the National Center for Biotechnology Information (NCBI) non-redundant database using Mascot search engine (http://www.matrixscience.com/search_form_select.html). The following parameters were used for database searches with MALDI-TOF peptide mass fingerprinting: monoisotopic mass, ±1.2 Da peptide mass tolerance, trypsin as digestion enzyme with one missed cleavage allowed, no modification of a cysteine residue.

RESULTS AND DISCUSSION

To purify lectins from biological materials, the most efficient way may be to utilize their affinity for sugar chains. Since lectins specifically recognize sugar structures, it is essential to select appropriate sugar chains. Thus, we first screened the sugar-binding property of the extract using a series of SGNPs, i.e. Glcα-GNP, Glcβ-GNP, Galα-GNP, Galβ-GNP, GlcNAcα-GNP, GlcNAcβ-GNP, GalNAcα-GNP, GalNAcβ-GNP, Fucα-GNP, Fucβ-GNP and Manα-GNP. The banana extract was thoroughly mixed with 11 kinds of SGNPs and allowed to stand at 4°C. When banana extract includes agglutinin having affinity for particular SGNPs, lectin-SGNP aggregate may be formed, and it is visually detected as precipitate. As a result, three of the SGNPs tested, i.e. Glcα-GNP, GlcNAcα-GNP and Manα-GNP, obviously formed precipitate (Fig. 2). In contrast, no precipitate was detected for the other SGNPs including Glcβ-GNP and GlcNAcβ-GNP. To know the extent of aggregation, the absorbance of supernatant at 530 nm was measured. As shown in Fig. 2, 91.2%, 92.4% and 92.8% of Glcα-GNP, GlcNAcα-GNP and Manα-GNP, respectively, in the wells precipitated, while the β-anomers showed no or, if any, weak affinity for banana extract. These results clearly

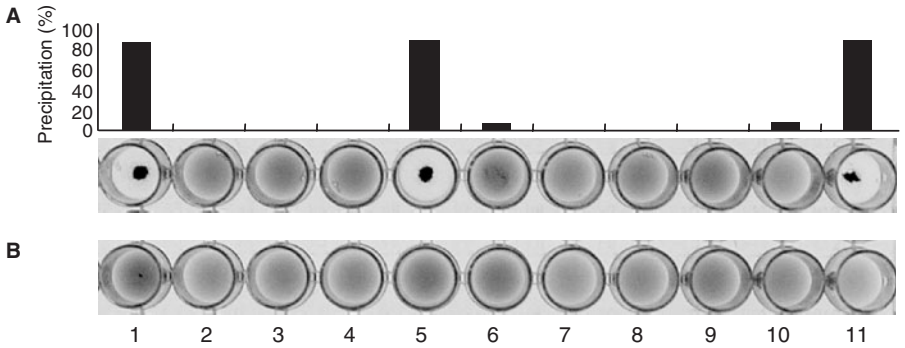


Fig. 2. **Screening of sugar-immobilized gold nano-particles (SGNPs).** Ten microlitres of sample solutions were added to 30 μl of each SGNP in round-bottomed microtitre plate wells. When the sample solution includes lectin(s) having affinity for particular SGNP(s), lectin–SGNP aggregate is formed and observed as a precipitate. Graphs indicate the extent of aggregation determined by measuring the absorbance of supernatant at 530 nm. (A) Banana extract, (B) PBST as negative control. 1: Glcα-GNP, 2: Glcβ-GNP, 3: Galα-GNP, 4: Galβ-GNP, 5: GlcNAcα-GNP, 6: GlcNAcβ-GNP, 7: GalNAcα-GNP, 8: GalNAcβ-GNP, 9: Fucα-GNP, 10: Fucβ-GNP, 11: Manα-GNP.

indicated that banana extract included agglutinin having affinity for α-glucose, α-GlcNAc and α-mannose. The sugar-binding specificity observed here agrees well with previous reports describing that lectin in banana pulp shows affinity for mannose, glucose, GlcNAc and their derivatives (*10–13*).

To clarify sugar-binding specificity in detail, dissociation effects of free sugars were examined using Glcα-GNP. Similar to the case of screening described above, 30 μl of Glcα-GNP was mixed with 10 μl of banana extract. After standing at room temperature for 10 min, aggregate was sedimented by centrifugation at 1,800 r.p.m. for 1 min, and supernatant was removed. To the wells, 100 μl of sugar solutions, i.e. 0.2 M glucose, 0.2 M GlcNAc, 0.2 M mannose or 0.2 M galactose dissolved in distilled water, was added, respectively. As expected, Glcα-GNP-aggregate was re-dissolved in glucose solution (Fig. 3). In addition, it was also re-dissolved in GlcNAc and mannose solution, but not at all in galactose solution (Fig. 3). The result indicated that the banana extract included an agglutinin having affinity for glucose, mannose and GlcNAc. In other words, the protein aggregated with Glcα-GNP, GlcNAcα-GNP or Manα-GNP may be identical.

Using Glcα-GNP, GlcNAcα-GNP or Manα-GNP, the purification of the agglutinin from banana extract was performed. As shown in Scheme 1, 10 μl of banana extract was added to 30 μl of each SGNP. The formed aggregates were sedimented by centrifugation, and supernatant was transferred to other tubes. Precipitates were subsequently washed with PBS containing 0.05% Tween-20 (PBST) and distilled water to remove non-specifically bound proteins and salt, and then re-dissolved in inhibitory sugar solutions. As estimated by quantifying the protein using a dye-binding assay (*14*), 2.3, 4.3 and 3.6 μg proteins were captured from 10 μl of extract (corresponding to 4 mg starting plant material) using Glcα-GNP, GlcNAcα-GNP and Manα-GNP, respectively. Higher yield relative to previous report (*11*) was probably achieved by one-tube reaction. Upon SDS–PAGE under non-reducing conditions, every SGNP-captured protein showed a single protein band at a molecular mass 13.6 kDa (Fig. 4, lanes 4, 6 and 8). No band was detected at the corresponding mass in

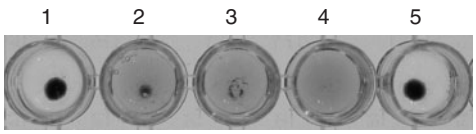


Fig. 3. **Dissociation of SGNP–lectin complex with free sugars.** Lectin–Glcα-GNP aggregate is first formed. After removal of supernatant, sugar solutions were added to each well. When lectin–SGNP interaction is inhibited by free sugars added, lectin will dissociate from the SGNP and the aggregate disappears. (1) PBS, (2) glucose, (3) GlcNAc, (4) mannose, (5) galactose.

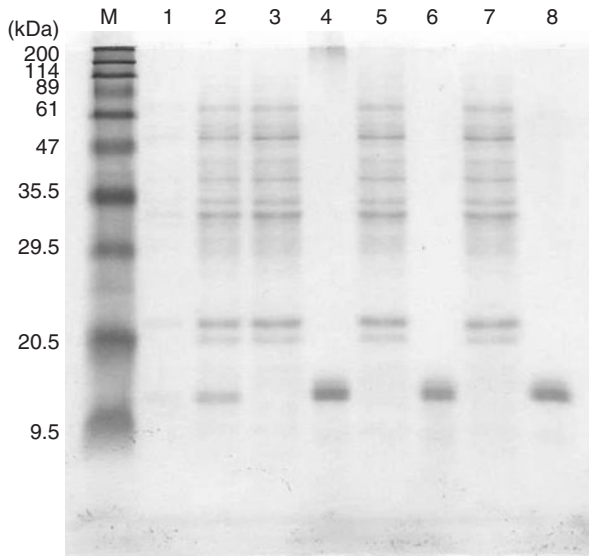


Fig. 4. **SDS–PAGE of proteins obtained from banana stained with CBB.** M: Molecular marker, lane 1: crude extract from plant materials, lane 2: extract after concentration with 60% (w/v) ammonium sulphate, lanes 3 and 4: Glcα-GNP, lanes 5 and 6: GlcNAcα-GNP, lanes 7 and 8: Manα-GNP. Lanes 3, 5 and 7: supernatant after aggregation, lanes 4, 6 and 8: precipitation after aggregation.

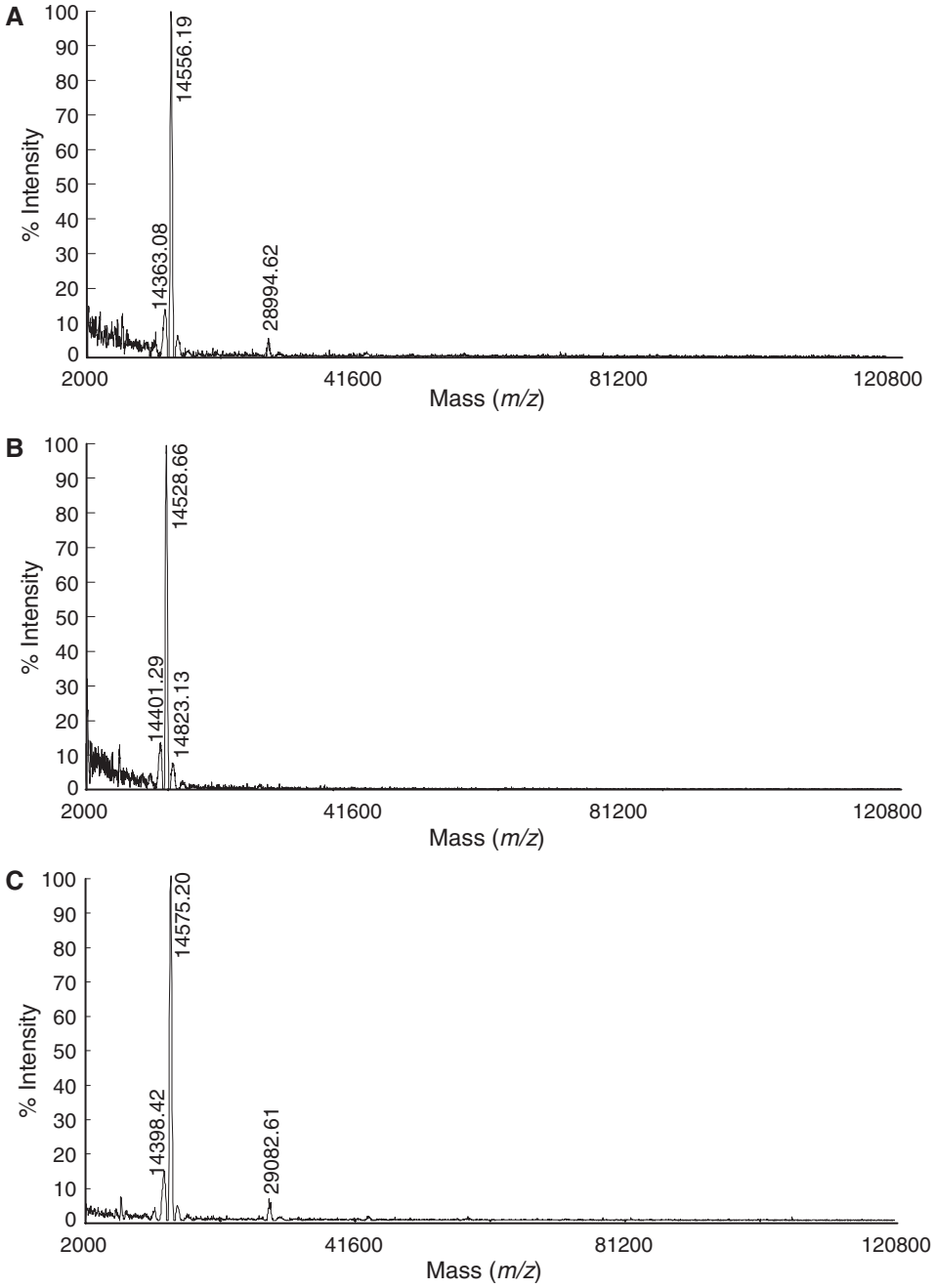


Fig. 5. **MALDI-TOF mass spectra of proteins obtained from banana extract using Glcα-GNP (A), GlcNAcα-GNP (B) and Manα-GNP (C).**

supernatant (Fig. 4, lanes 3, 5 and 7). This result indicated that the protein with molecular mass 13.6 kDa in the extract was completely captured by SGNPs. Also, the captured proteins are very pure, as far as stained with CBB.

Exact molecular mass of captured proteins was then measured By MALDI-TOF MS analysis. Since aggregates were washed with distilled water and dissolved in sugar solutions without salts, SGNP-captured proteins were used for MS analysis without further purification and desalting steps. For analysis, 1.2, 2.2 and 1.8 μg of Glcα-GNP-, GlcNAcα-GNP- and Manα-GNP-captured proteins (corresponding to 5 μl of extract, *i.e.* 2 mg of starting plant material), respectively, was co-crystallized with SA, and directly analysed by MALDI-TOF mass spectrometer. As a result, intense signals were detected for all the three samples (Fig. 5), in spite of remaining free SGNPs. The result indicates that free SGNPs did not disturb the ionization of proteins in MALDI-TOF/MS, suggesting that the SGNP was not needed to be removed from the analytical samples. In case of Glcα-GNP-captured protein, only one major peak was detected at m/z value of 14,556 (Fig. 5A). According to the previous report, mannose/glucose-binding lectin from banana pulp

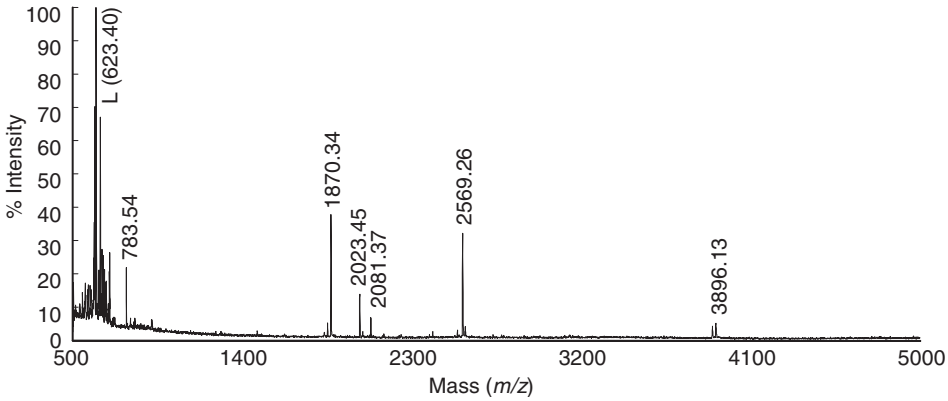


Fig. 6. **MALDI-TOF mass spectra from tryptic digestion of proteins obtained from banana extract using Glcα-GNP.** All the six peaks submitted to the Mascot search engine for database searching was matched for total sequence coverage of 76%. L denotes *m*/*z* value of ligand conjugate.

Table 1. **Peptide peaks detected from the tryptic digestion of Glcα-GNP-captured proteins.**

	m/*z*	Protein	Start–End	Missed cleavage	Sequence
1	1870.40	Banana lectin	7–25	0	VGAWGGNGGSAFDMGPAYR
2	2081.37	Banana lectin	31–49	0	IFSGDVVDGVDVTFTYYGK
3	2569.26	Banana lectin	31–53	1	IFSGDVVDGVDVTFTYYGKTETR
4	3896.13	Banana lectin	54–91	0	HYGGSGGTPHEIVLQEGEYLVGMAGEVANYHGAVVLGK
5	2023.45	Banana lectin	100–120	0	AYGPFGNTGGTPFSLPIAAGK
6	783.55	Banana lectin	121–127	0	ISGFFGR

is a dimeric protein composed of 15 kDa subunits estimated by SDS–PAGE (*10*, *11*). In addition, its molecular weight calculated from sequence (Accession No: 2BMYA) is 14,554 Da (*15*). Thus, the captured protein is supposed to be a banana lectin. Since gene of banana lectin is a member of a multi-gene family (*11*), a weak signal at *m*/*z* value of 14,363 might be derived from them. Another minor signal at *m*/*z* value of 28,994 was corresponding to a dimer of banana lectin. Very similar MS profiles were observed for GlcNAcα-GNP and Manα-GNP (Fig. 5B and C). In both cases, only one major peak was detected around m/z = 14,550. The *m*/*z* value of major peaks observed for GlcNAcα-GNP and Manα-GNP were 14,528 and 14,575, respectively, which are corresponding to the previous report, too (*11*). According to the results of MS analysis together with those of SDS–PAGE, the purity of captured proteins was high enough to apply further analysis.

To identify SGNP-captured protein, we carried out peptide-mass fingerprinting. Judging from the results of dissociation of SGNP–lectin complex with free sugars, SDS–PAGE and MS spectrometry described above (Figs 3, 4 and 5), all the proteins captured by Glcα-GNP, GlcNAcα-GNP and Manα-GNP were supposed to be identical. Thus, Glcα-GNP-captured protein was used for the purpose. As described under MATERIALS AND METHODS section, the captured protein (2.3 μg protein) was digested by trypsin, and the resulting digests were analysed by the MALDI-TOF/MS (Fig. 6). Detected peaks at *m*/*z* 783.54, 1870.34, 2023.45, 2081.37, 2569.26 and 3896.13 were searched against the Swiss-Prot protein database for the identification of source proteins. All six peaks matched the database. The peptide sequences from the digested protein are listed in Table 1. As expected, all the peaks are revealed to be derived from banana lectin. The sequence coverage was 76%. Thus, Glcα-GNP-captured protein was identified as banana lectin. This result indicated that the purity of protein captured by Glcα-GNP was sufficient to identify the source protein.

In conclusion, we established an effective method for a one-step purification of lectin from extracts using SGNPs. Compared with conventional methods, several advantages of SGNPs were found, e.g. small amount of start materials (in case of banana lectin, <1 g), simple operation (only centrifugation) and direct analysis by SDS–PAGE and MS spectrometry (without further purification or concentration steps). Although SGNPs having simple saccharides were used here, a lectin was successfully purified with such high purity as to identify the source protein. Using a similar protocol, we have also performed easy and quick purification of lectins from soybean. Since, in general, affinities of lectins for oligosaccharides are relatively high compared with those for simple saccharides, utilization of SGNPs having complex glycans is promising for easy purification of less abundant carbohydrate-binding proteins.

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