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Preparation of a stable subunit of Japanese elderberry (*Sambucus sieboldiana*) bark lectin and its application for the study of cell surface carbohydrates by flow cytometry

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A stable subunit of *Sambucus sieboldiana* bark lectin (MSSA) was prepared by selective reduction of disulfide bridges between the subunits and alkylation with 4-vinylpyridine. Amino acid analysis of MSSA revealed that 1.4 cysteine residues per subunit were selectively modified. MSSA failed to agglutinate rabbit erythrocytes and precipitate fetuin. However, MSSA retained the ability to bind to fetuin, as detected by ELISA. Neu5Acα2-6lactose inhibited the binding to fetuin of both SSA and MSSA. Flow cytometric analysis showed that human histocytic lymphoma U937 cells were clearly stained with FITC-labeled MSSA (FITC-MSSA) without any detectable agglutination and that this staining was almost completely inhibited by the addition of Neu5Acα2-6lactose (2 mM). Treatment of U937 cells with native FITC-SSA at the sub-agglutinating concentration (0.3 μg/ml) showed much poorer fluorescence intensity than that of MSSA, suggesting that MSSA is an invaluable tool for the detection of cell surface glycoconjugates containing NeuAcα2-6Gal/GalNAc sequences by flow cytometry.

Elderberry bark; *Sambucus sieboldiana* lectin; Stable subunit; Monovalent lectin; Pyridylethylation; Flow cytometry

1. INTRODUCTION

Lectins are multivalent protein/glycoprotein with the ability to bind carbohydrates. Therefore, they are able to agglutinate cells and precipitate polysaccharides and glycoproteins. Because of their unique carbohydrate-binding characteristics, lectins serve as invaluable tools in biological and medical research for the separation and characterization of glycoproteins and glycopeptides, histochemistry of cells and tissues, and the study of cell differentiation [1]. However, the multivalent nature of lectins sometimes causes problems for their application. For example, direct measurement of biochemical and biophysical cellular events occurring on the surface of individual cells during differentiation, tumorogenesis etc., using flow cytometry and cell sorters has been becoming important [2]. Single cell suspensions are required for these applications to avoid stacking problems and also for accurate measurement. To attain this, lectins have to be used in the concentration range where the agglutination does not occur [3]. This has been one of the major problems for the application of lectins in this field.

Monovalent derivatives of lectins, if prepared, may serve as useful tools without the problems of agglutination while retaining carbohydrate-binding ability. They may also be valuable in the study of the biological functions of cell surface carbohydrates, similar to the Fab fragment of IgG. However, only a few monomeric, monovalent derivatives of animal and plant lectins have been prepared and characterized, e.g. *Anthocidaris crassispina* egg lectin [4], *Wistaria floribunda* lectin [5], and concanavalin A (Con A) [6], although none of them were used for any of the applications described above. Biological activities of these derivatives were reported only for Con A, where the optimal concentration of monomeric Con A for lymphocyte activation was found to be significantly different compared to that of the native Con A [7].

To avoid the agglutination problem in flow cytometry, McCoy et al. also used FITC-*Griffonia simplicifolia* I-A3B (GS I-A3B) in the presence of GalNAc as the functionally monovalent probe for the detection of cell surface α-D-Gal residue [8], although this approach can not be generally applied.

We describe herein the preparation and characterization of a stable monomeric subunit of Japanese elderberry (*Sambucus sieboldiana*) bark lectin (SSA) [9], which is specific to Neu5Acα2-6Gal/GalNAc sequences, after selective reduction and alkylation with 4-vinylpyridine. We also report the application of this

Abbreviations: SSA, *Sambucus sieboldiana* lectin; MSSA, monomeric *S*-β-(4-pyridylethyl)cysteine-SSA; Pe-cys, pyridylethylated-cysteine; FITC, fluorescence isothiocyanate; U937 cell, human histiocytic lymphoma U937 cell; PBS, 0.01 M phosphate buffered saline (pH 7.2); ELISA, enzyme-linked immunosorbent assay; HRP, horse radish peroxidase; BSA, bovine serum albumin; OVA, ovalbumin.

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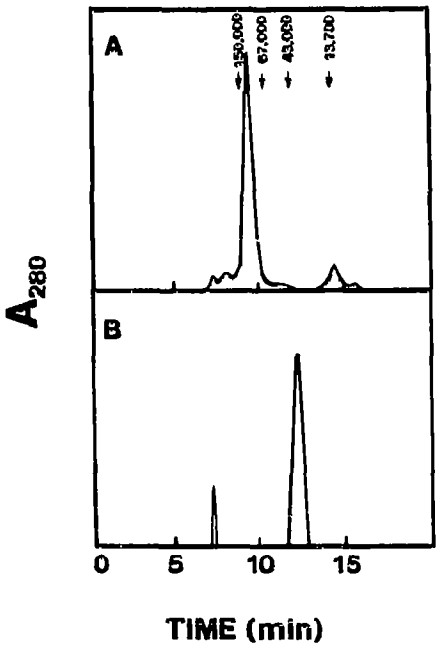

Fig. 1. HPLC analyses of SSA (A) and MSSA (B) with TSK-GEL G3000sw (0.75 × 30 cm) column eluted with PBS. Immunoglobulin (M_r 150,000), BSA (M_r 67,000), OVA (M_r 43,000) and ribonuclease (M_r 14,400) were used as protein standards.

monomeric derivative for the study of cell surface carbohydrates using flow cytometry.

2. MATERIALS AND METHODS

2.1. *Purification of SSA*

A bark lectin (SSA) was purified from the extract of the twigs of Japanese elderberry by affinity chromatography on immobilized fetuin, as previously reported [9].

2.2. *Preparation of stable SSA subunit*

Stable SSA subunit was prepared by selective reduction and alkylation of SSA according to the modified method of Tarr [10]. Briefly, SSA (5 mg/ml) in 0.4 M *N*-ethylmorpholine/acetate buffer (pH 8.3 containing 1% DTT and 0.25 M lactose was incubated at 20°C for 3 h under N_2. 4-Vinylpyridine (8 μl/ml) was added to the reaction mixture and reacted at 20°C for 15 min under N_2. The monomeric *S*-β-(4-pyridylethyl)cysteine-SSA subunit (MSSA) was purified by gel filtration on Sephadex G-25 (1.6 × 5 cm), equilibrated with PBS to remove excess reagents. Formation of MSSA was confirmed by HPLC on a TSK-GEL G3000sw column (0.75 × 30 cm) and also by SDS-PAGE in the absence of β-mercaptoethanol. MSSA, for the study of the sugar binding activity, was further purified by affinity chromatography on an immobilized fetuin–Sepharose 4B column (0.8 × 8 cm). [9].

2.3. *General analyses*

Protein content was determined by the method of Lowry et al. [11]. SDS-PAGE was carried out with Phast System (Pharmacia LKB Biotechnology Co. using an 8–25% gradient gel. Quantitative precipitation and hemagglutination assays were conducted as in [12]. Preparation of Pe-Cys-SSA under denaturing conditions [13] and amino acid analysis of SSA and MSSA [10] were performed as described.

2.4. *ELISA and hapten inhibition*

Enzyme-linked immunosorbent assay (ELISA) for the analysis of the (M)SSA–fetuin interaction was carried out as follows. A 96-well flat-bottom microtiter plate was coated with SSA or MSSA (0.1 μg/100 μl/well) in 0.1 M carbonate buffer (pH 9.6) containing 0.2 M lactose, and kept for 4 h at 37°C or overnight at 4°C. The lectin solutions were discarded and the plate was incubated with 1% OVA in the same buffer (100 μl) at 37°C for 1 h to prevent non-specific binding. The wells were washed with PBS/0.1% Tween 20 (3 times; this rinsing was repeated after every following step), then, 50 μl of serially diluted fetuin solution (PBS/0.1% OVA/0.1% Tween 20) was added to each well and incubated at 37°C for 2 h. Fetuin reacting with SSA/MSSA was detected with the use of commercial rabbit anti-bovine fetuin serum (1/2,500) and HRP-labeled goat anti-rabbit IgG (1/2,500). The color intensity at 415 nm was determined by a microplate reader, MTP-32 (Corona Electric. Co., Japan).

Hapten inhibition experiments were carried out by the addition of increasing amounts of Neu5Ac α2-6lactose or lactose to the microtiter plate wells. Briefly, varying amounts of oligosaccharide (25 μl) was added to the SSA- or MSSA-coated plate. After incubation at 37°C

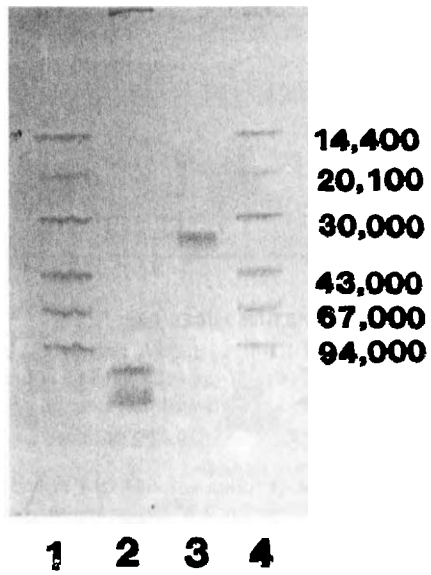

Fig. 2. SDS-PAGE of SSA and MSSA on an 8–25% gradient gel in the absence of β-mercaptoethanol. Lane 2, SSA; lane 3, MSSA. Lanes 1 and 4 contain M_r phosphorylase *b* (94,000), BSA (67,000), OVA (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400).

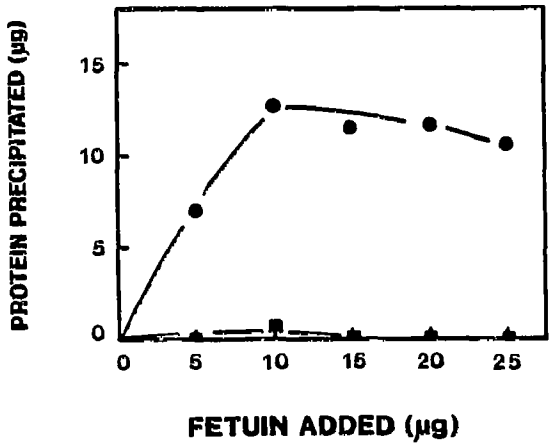

Fig. 3. Quantitative precipitation of fetuin with SSA and MSSA. SSA (●) and MSSA (■) (15 μg) was added to varying amounts of fetuin in a total volume of 150 μl.

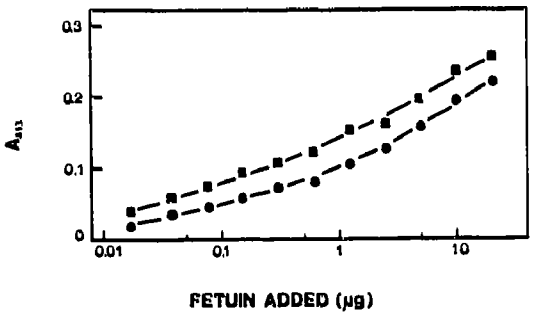

Fig. 4. The interaction of fetuin with SSA and MSSA analyzed by ELISA. SSA (■) or MSSA (●) (0.1 μg/well) solution containing 0.2 M lactose was coated on a 96-well, flat-bottom plate.

for 30 min, a 25 μl aliquot of fetuin solution (0.1 mg/ml) was added to each well and kept at 37°C for 2 h. Determination of the bound fetuin with the commercial antiserum/IgG was carried out as described above.

2.5. *Flow cytometry*

FITC-labelling of SSA and MSSA, and staining of U937 cells with FITC-lectins were carried out by the method of McCoy et al. [8]. The molar ratio of fluorescein/protein of SSA and MSSA was 0.2 for both of them. U937 cells stained with FITC-lectins were analyzed using flow cytometry (Epics Profile-II, Coulter Co., Hialeah, FL). The cell culture of the U937 cell line was maintained by the method of Sundström and Nilsson [14].

3. RESULTS

A stable subunit of SSA (MSSA) was prepared by selective reduction and alkylation of disulfide bridges between the subunits. MSSA showed a single peak corresponding to a M_r of 34,000 by gel filtration on a TSK-GEL G3000sw column (Fig. 1B). The M_r of MSSA was also obtained from SDS-PAGE in the absence of β-mercaptoethanol (Fig. 2). On the other hand, native SSA gave one major peak with a M_r 1.4×10^5 by HPLC analysis, while SDS-PAGE of native SSA in the absence of β-mercaptoethanol showed the presence of two bands corresponding to a tetrameric and octomeric molecule (Fig. 2).

The number of total pyridylethylated-cysteine (Pe-Cys) residues obtained from completely denatured and reduced SSA, which corresponds to the number of total Cys residues in the subunit, was ca. 6 per subunit (data not shown). On the other hand, MSSA gave 1.4 Pe-Cys residues per subunit, suggesting that most of these residues were derived from the selective reduction and alkylation of disulfide bridges between the subunits.

MSSA failed to agglutinate rabbit erythrocytes (data not shown) and also to form a precipitate with fetuin (Fig. 3). However, MSSA was shown to react strongly with fetuin, using an ELISA-type assay in which a MSSA-coated microtiter plate was reacted with fetuin, followed by the detection of bound fetuin with rabbit anti-fetuin serum and HRP-labeled goat anti-rabbit IgG (Fig. 4). Moreover, MSSA displayed a similar

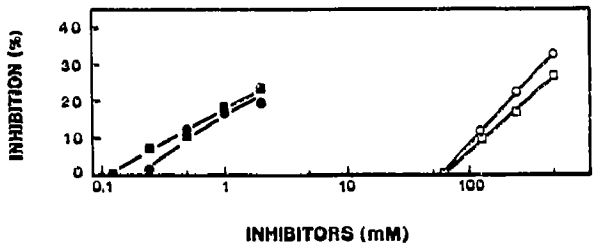

Fig. 5. Inhibition of SSA/MSSA–fetuin interaction by haptenic sugars. ■, □ inhibition for SSA-fetuin interaction; ●, ○ inhibition for MSSA-fetuin interaction. Open symbols, lactose; closed symbols, Neu5Acα2-6lactose.

binding curve to that of native SSA (Fig. 4). Both SSA– and MSSA–fetuin interactions were inhibited by Neu5Acα2-6lactose or lactose, where Neu5Acα2-6lactose was a 500 times more potent inhibitor than lactose for both MSSA and native SSA (Fig. 5). These results demonstrated that MSSA retained original carbohydrate binding ability without any significant changes to its specificity for the Neu5Acα2-6Gal/GalNAc sequence.

The results of titration of U937 cells with FITC-MSSA by flow cytometry are shown in Fig. 6. U937 cells were slightly fluorescent by themselves (Fig. 6A). The fluoroscence intensity of U937 cells treated with FITC/MSSA increased with the increase in lectin concentration (from 1 to 5 μg/ml, Fig. 6B–F). The interaction of FITC-MSSA with U937 cells was not affected by the addition of the non-competitive sugar, D-glucose (final conc. 100 mM, Fig. 7B). However, the fluorescence intensity of U937 cells were reduced to the level of control cells by the addition of Neu5Acα2-6lactose (2 mM, Fig. 7C), indicating the staining was due to the specific binding of FITC-MSSA to cell surface glycoconjugates. Sub-agglutinating concentrations of FITC-SSA (0.3 μg/ml) stained U937 cells only slightly, resulting in a comparable level of fluorescence intensity to that of U937 cells stained with FITC-MSSA in the presence of Neu5Acα2-6lactose (Fig. 7D).

4. DISCUSSION

The Japanese elderberry bark lectin is a tetrameric glycoprotein connected with disulfide bridges as previously reported [9], and no free cysteine residues have been found in the molecule (unpublished results). Approximately 1.4 cysteine residues per subunit were modified in parallel with the dissociation into a stable monomer (MSSA) by selective reduction and subsequent pyridylethylation, suggesting that a large part of these modified residues were derived from the disulfide bridges originally present between subunits. The MSSA completely retained sugar-binding ability, including the specificity to the Neu5Acα2-6Gal sequence. In contrast, SSA derivatized in the presence of the denaturing agent,

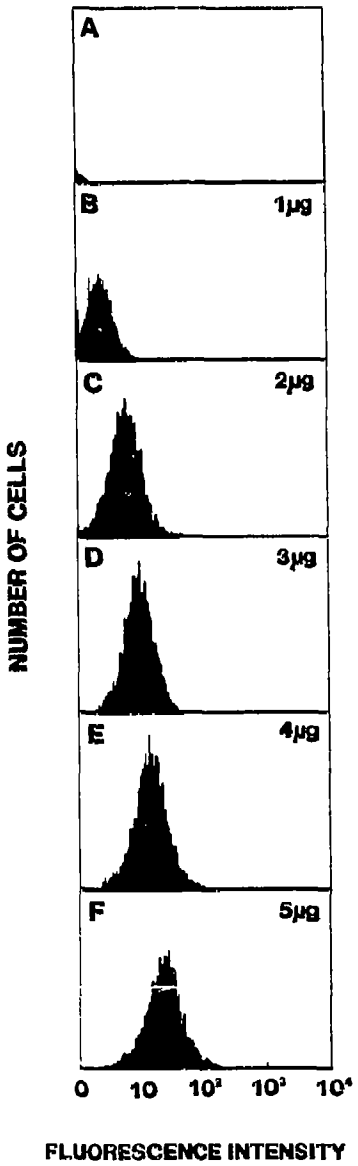

Fig. 6. Flow cytometric analyses of U937 cells stained with various concentrations of FITC-MSSA. Five thousand cells were used in each case. A, 0 μg/ml; B, 1 μg/ml; C, 2 μg/ml; D, 3 μg/ml; E, 4 μg/ml; F, 5 μg/ml.

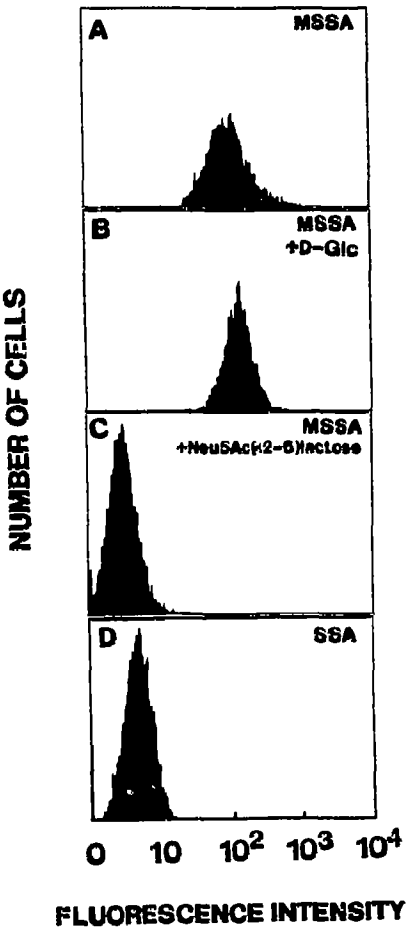

Fig. 7. Flow cytometric analyses of U937 cells stained with FITC-MSSA or SSA. Five thousand cells were used in each case. A, MSSA (4 μg/ml); B, MSSA (4 μg/ml) containing 100 mM D-glucose; C, MSSA (4 μg/ml) containing 2 mM Neu5Acα2-6lactose; D, SSA (0.3 μg/ml).

guanidine HCl, completely abolished the sugar-binding activity (data not shown).

FITC-labeled MSSA was able to stain the cells in a Neu5Acα2-6Gal/GalNAc-specific manner without detectable agglutination which were successfully applied to flow cytometric analyses. This is an advantage of MSSA compared to the original tetrameric SSA molecule, which suffered from extensive agglutination when used for a similar application in the form of a FITC derivative. Thus, the monomeric derivative of SSA can be an invaluable tool for the study of cell surface glycoconjugates containing the Neu5Acα2-6Gal/GalNAc sequence, especially with the use of a flow cytometer/cell sorter.

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