SURE 静岡大学学術リポジトリ
Shizuoka University REpository

Mannose-specific lectin from the mushroom Hygrophorus russula

メタデータ	言語: eng 出版者: 公開日: 2012-12-20 キーワード (Ja): キーワード (En): 作成者: Suzuki, Tomohiro, Sugiyama, Kozue, Hirai, Hirofumi, Ito, Hiroyuki, Morita, Tatsuya, Dohra, Hideo, Murata, Takeomi, Usui, Taichi, Tateno, Hiroaki, Hirabayashi, Jun, Kobayashi, Yuka, Kawagishi, Hirokazu メールアドレス: 所属:
URL	http://hdl.handle.net/10297/6934

Mannose-specific lectin from the mushroom *Hygrophorus russula*

Tomohiro Suzuki[2], Kozue Sugiyama[3], Hirofumi Hirai[3], Hiroyuki Ito[3], Tatsuya Morita[3], Hideo Dohra[4], Takeomi Murata[3], Taichi Usui[3], Hiroaki Tateno[5], Jun Hirabayashi[5], Yuka Kobayashi[1,6], and Hirokazu Kawagishi[1,3,7]

[2]Inovation and Joint Reseach Center, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan; [3]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan; [4]Genetic Research and Biotechnology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan; [5]Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 2, Tsukuba, Ibaraki 305-8566, Japan; [6]*J*-Oil mills, INC, 11, Kagetoricho, Totsuka-ku, Yokohama, Kanagawa 245-0064, Japan; [7]Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

Running title: Man-specific lectin from *Hygrophorus russula*

[1]To whom correspondence should be addressed: Tel and Fax: +81-54-238-4885; E-mail achkawa@ipc.shizuoka.ac.jp (H. Kawagishi)/Tel +81-45-852-4001; Fax +81-45-852-6357; E-mail yuka.kobayashi@j-oil.com (Y. Kobayashi)

Supplementary;

1

Abstract

A lectin was purified from the mushroom *Hygrophorus russula* by affinity chromatography on a Sephadex G-50 column and BioAssist S cation exchange chromatography, and designated HRL. The results of SDS-PAGE, gel filtration and MALDI-TOF-mass of HRL indicated that it was composed of four identical 18.5 kDa subunits with no S-S linkage. Isoelectric focusing of the lectin showed bands near pI 6.40. The complete sequence of 175 amino acid residues was determined by amino acid sequencing of intact or enzyme-digested HRL. The sequence showed homology with *Grifola frondosa* lectin. The cDNA of HRL was cloned from RNA extracted from the mushroom. The open reading frame of the cDNA consisted of 528 bp encoding 176 amino acids. In hemagglutination inhibition assay, α1-6 mannobiose was the strongest inhibitor and isomaltose, Glcα1-6Glc, was the second strongest one, among mono- and oligo-saccharides tested. Frontal affinity chromatography indicated that HRL had the highest affinity for Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, and nonreducing terminal Manα1-6 was essential for the binding of HRL to carbohydrate chains. The sugar binding specificity of HRL was also analyzed by using BIAcore. The result from the analysis exhibited positive correlations to the result of the hemagglutination inhibition assay. All the results suggested that HRL recognized α1-6 linkage of mannose and glucose, especially Manα1-6 bond. HRL showed a mitogenic activity against spleen lymph cells of F344 rat. Furthermore, an enzyme-linked immunosorbent assay showed strong binding of HRL to HIV-1 gp120.

Key words: mushroom/fungal lectin/mannose-binding lectin/*Hygrophorus russula*

Introduction

Lectins are powerful tools for the sugar-chains structure determination. Historically their sugar-binding specificities have been used for qualitative analysis of sugar chains. Recently, new technology such as lectin array is becoming popular, therefore lectins with specific sugar binding properties are required (Hirabayashi 2004; Kuno et al. 2005; Tateno et al. 2009).

Many species produce Man-recognizing lectins. Concanavalin A (Con A) is specific to Man and has been used as a biochemical or biological agent for a long time (Loris et al. 1998). This lectin recognizes not only Man but also Glc, therefore, it is categorized as a Man/Glc-specific lectin. On the other hand, there are Man-specific lectins that bind to Man but not Glc. GNA (snowdrop; *Galanthus nivalis* agglutinin) and HHA (amaryllis; *Hippeastrum* hybrid agglutinin) are produced by plants (Howard et al. 1969; Kaku et al. 1990; Van Damme et al. 1987). FimH proteins from *Escherichia coli* and MBL (mannose-binding lectin) from mammalians are also specific to Man among monosaccharides (Krogfelt et al. 1990; Worthley et al. 2005). In the course of our continuing screening for new mushroom lectins, we found lectin activity in the extract of the mushroom *Hygrophorus russula*, and the binding affinity to sugar was specifically potent to Man. This specificity was notably new and unusual compared with characteristics of the known mushroom lectins. In particular, further study showed that this lectin activity was α1,6-Man specific and its sugar-binding specificity has not been reported yet. Here, we describe the isolation and characterization of the lectin from this mushroom.

Results

Purification of HRL

The purification procedure is summarized in Table I. In the present study, Sephadex G-50 was chosen as the affinity support since the hemagglutination activity of the crude extract from the mushroom was inhibited by Man and Glc. Initially, the extract of *H. russula* was applied to Sephadex G-50. Almost all the lectin activity from the extract was adsorbed to the affinity column and eluted with 0.3 M Man. The eluate was further purified by BioAssist S cation exchange chromatography, and a pure lectin, which was named HRL, was obtained.

Molecular properties of HRL

HRL gave a single band with an apparent mass of 18.5 kDa on SDS-PAGE (Figure 1A) regardless of

the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol. The isoelectric focusing of HRL gave a band with a pI of 6.4 (Figure 1B). HPLC gel filtration of the protein gave a single symmetrical peak at an elution volume corresponding to a molecular mass of 74 kDa (Supplementary Figure 1). The MALDI-TOF mass spectrum gave a main peak at *m/z* 18511 and a small peak at *m/z* 9267 (Figure 1C). The results of SDS-PAGE, gel filtration, and MALDI-TOF mass analysis indicated that the molecular mass of HRL was 74 kDa and it was composed of four identical 18.5 kDa-subunits with no disulfide bonds.

The protein content of HRL was about 94.4% (Supplementary Figure 2A). The amino acid composition revealed a high content of Gly, Asx, and Thr (Supplementary Table 1). *N*-Terminal amino acid sequence analysis of intact HRL gave a sequence of 30 amino acids from the terminal (Figure 2, lane 1). The protein was digested with *Lysobacter enzymogenes* protease (Lys-C), *Clostridium histolyticum* proteinase (Arg-C), or *Pseudomonas fragi* mutant protease (Asp-N), and the resulting peptides were isolated by reverse-phase HPLC. Each purified peptide sequence was determined by *N*-terminal amino acid sequence analysis (Figure 2, lanes 2 to 7). As a result, the complete amino acid sequence of HRL was determined as shown in Figure 2 (lane 8). This lectin had heterogeneity in the sequence at the position of 52 (Gln or Trp) from the *N*-terminus. The neutral carbohydrate content amounted to 3.5% (Supplementary Figure 2B), and the components of the sugars in the glycoprotein were identified as L-Fuc : Glc : Man : Gal : GlcN : GalN : Xyl in a 3.4 : 3.3 : 2.9 : 2.2 : 1.3 : 1.0 : 0.3 molar ratio. HRL did not contain any sialic acid.

cDNA cloning and structural analysis of HRL

The cDNA of HRL was cloned from total RNA extracted from fruiting bodies of the fungus using RT-PCR coupled with RACE-PCR. (GenBank™ accession no. AB586742). The open reading frame of the cDNA consisted of 528 bp encoding 176 amino acids (Figure 3). No signal sequence was present in the cDNA. Two amino acids, Asp^{111} and Cys^{111} were detected in the cDNA.

The amino acid sequence of HRL was analyzed by the BLAST program, and the sequence showed homology to the lectin from the mushroom *Grifola frondosa* (GFL) (38%) and the lectin from Japanese sago palm *Cycas revoluta* (CRLL) (27%) (Figure 4).

.

Properties of HRL as a lectin

HRL was stable below 60°C, but rapidly inactivated at temperatures above 60ºC (Supplementary Figure 3A). HRL was relatively stable over a wide range of pH values, especially between pH 2.0 and

9.5 (Supplementary Figure 3B). EDTA-treatment and metal cations addition to the pre-treated lectin did not affect the lectin activity. The lectin agglutinated Pronase- and neuraminidase-treated human erythrocytes (Table II). Table III shows the inhibition of hemagglutination activity of HRL by various monosaccharides, oligosaccharides, and glycopeptides. Among the mono- and oligo-saccharides tested, the most potent inhibitor was α1-6 mannobiose (MIC, 39.1 μM). Isomaltose, isomaltotriose, isomaltotetraose isomaltopentaose, and isomaltohexaose were also inhibitory at higher concentrations, 156, 316, 316, 316, and 316 μM, respectively, than α1-6 mannobiose. Thyroglobulin, ribonuclease B, asialo-PSM, and PSM inhibited agglutination. Comparison of the sugar-binding specificity of HRL with those of other Man/Glc-specific lectins, ConA, PSA, BanLec, GNA, HHA and TxLc-I, is shown in Table IV. ConA, PSA, BanLec, GNA, HHA and TxLc-I bound to α-mannosides regardless of their linkage positions and some of them bound to Glcα1-6 linkage, too. However, the binding specificity to mannosides and glucosides of HRL was strictly limited to α1-6 linkage, especially Manα1-6 one.

The sugar-binding specificity of HRL was also investigated by SPR analysis. HRL was immobilized on the sensor chip CM-5 by amine coupling and ten glycoproteins were used as analytes. The sensorgrams and the kinetic data of the binding are shown in Supplementary Figure 4 and Table V, respectively. The binding of all the glycoproteins to the immobilized lectin fitted best the 1:1 binding model among various models in the evaluating software (Supplementary Figures 4 and 5). Asialo-PSM, PSM and thyrogloblin, which inhibited the HRL-mediated hemagglutination, showed similar kinetic parameters to each other. Asialo-BSM, asialo-fetuin, BSM and fetuin that showed weaker inhibitory activity in the hemagglutination assay also bound to the immobilized lectin. On the other hand, α_1-acid glycoprotein and transferrin, which were not inhibitory in the hemagglutination assay, did not bind to the immobilized lectin at all. The binding specificity of the immobilized HRL toward α1-6 mannobiose or other α-mannobioses was analyzed by the co-injection method (Kobayashi et al. 2005). After binding of PSM, dissociation was initiated by α1-6 mannobiose at various concentrations. The dissociation of the glycoprotein by the disaccharide was promoted dose-dependently (Figure 6A). Furthermore, disassociation of the glycoprotein caused by α1-6 mannobiose and other α-mannobioses was compared with one another. As shown in Figure 6B, the binding of PSM was abruptly reversed by injection of α1-6 mannobiose, whose MIC in the hemagglutination inhibition assay was 39.1 μM (Table III). On the other hand, the effects of the other

mannosides, α1-3 mannobiose (MIC, 2.50 mM), α1-2 mannobiose (10.0 mM), and α1-4 mannobiose (10.0 mM) were very small.

The sugar-binding specificity of HRL was further elucidated by FAC analysis using 125 kinds of PA-glycans (Supplementary Figure 6). The amounts of immobilized HRL was determined to be 10 μg/mL gel. For evaluation of the prepared columns, it was necessary to determine the effective ligand content (Bt) based on the concentration dependence analysis. Concentration dependence analysis was performed with methotrexate (MTX) derivatized Manα1–3(Manα1–6)Manβ1-4GlcNAcβ1-4GlcNAc (Nakamura et al. 2005) at various concentrations ranging from 4.2 to 33.3 μL. As a result, Bt, *K*d and *K*a values were determined to be 1.84 nmol, 1.63×10^{-5} M and 6.13×10^{-4} M^{-1}, respectively (Supplementary Figure 7). The strength of affinity of each PA-glycans for the immobilized lectin was shown as V- V_0 value (μL). HRL bound to high mannose type sugar chains (Figure 6). The lectin showed the highest affinity for Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (PA-003). Manα1-6(Manα1-3) Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (PA-004) and Manα1-6(Manα1-3) Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (PA-005) were also very strong haptenic sugars.

Mitogen activity of HRL

HRL stimulated the production of IL-6, IFN-γ and IL-10 dose-dependently when rat spleen cells were treated with the lectin (Figure 7, Supplementary Figure 8). The stimulatory effect of HRL on both IL-6 and IFN-γ production was suppressed by addition of α1-6 mannnobiose. The IL-6 production by HRL is much stronger than that by ConA.

Binding of HRL to gp 120

In order to examine anti-HIV of HRL, interaction of gp120 and HRL was analyzed by plate assay using immobilized gp120 on the plate and was compared with other lectins (Figure 8). HRL and BanLec showed strong affinity to gp120.

Discussion

A novel Man-specific lectin, HRL, was purified from the edible mushroom *Hygrophorus russula* by

repeated chromatography. The complete amino acid sequence of HRL was determined and this lectin had heterogeneity in the sequence at the position of 52 (Gln or Trp) from the *N*-terminus. (Figure 2). HRL was composed of 175 amino acid residues and its calculated molecular mass was 18484 Da (Gln^{52}) or 18542 Da (Trp^{52}). The theoretical molecular mass was in good agreement with the value (18,511 Da) obtained by MALDI-TOF mass spectrometry (Figure 1). The elimination of sugar chains from glycoproteins might be accounted due to the condition on the MALDI-TOF mass measurement. The sugar composition analysis indicated that HRL contained both *N*- and *O*-linked oligosaccharides. The amino acid sequence of HRL indicated the presence of one *N*-glycosylation sites in the molecule (Figure 3). The isoelectric focusing of HRL gave a band with a pI of 6.40 (Figure 1).

The cDNA of HRL was cloned from RNA extracted from the fruiting bodies of *H. russula* and the open reading frame of the cDNA of the protein consisted of 528 bp encoding 176 amino acids (Figure 3). No signal sequence was present in the cDNA. This result suggests that the primary translation product was synthesized as a polypeptide bearing 176 amino acids and its *N*-terminal Met residue was removed after translation. Lectins from the mushroom *Marasmius oreades* (Kruger et al. 2002), *Polyporus squamosus* (Mo et al. 2000; Tateno et al. 2004) and *Pleurocybella porrigens* (Suzuki et al. 2009) also lack the signal peptide. Although the protein sequencing of the lectin indicated that it possessed Gln^{52}or Trp^{52} , and Asp^{111} in the sequence, Trp^{52}, and Asp^{111} or Cys^{111} were observed in the cDNA. Such a phenomenon that two variant residues exist has been also observed in the lectin from the mushroom *Clitocybe nebularis* (Pohleven et al. 2009). Heterogeneity is common for mushroom lectins (Kruger et al. 2002; Tateno et al. 2003; Tateno et al. 2004). It could be the result of gene polymorphism, or the presence of highly conserved multiple gene families encoding the isolectins.

The BLAST search revealed that HRL has sequence homology with the jacalin-related lectin from *G. frondosa* (GFL) (38%) and the mannose-specific jacalin-related lectin from *C. revoluta* (CRLL) (27%). Despite the high degree of sequence homology of HRL with GFL, GFL was not inhibited by any of the tested monosaccharides or disaccharides including Man and Glc (Nagata et al. 2005). Laminaribiose (Glcβ1-3Glc) and Manβ1-2Man were the most potent inhibitors toward CRLL (Yagi et al. 2002). CRLL has affinity toward saccharides with β-linkages, whereas HRL has affinity toward α1-6Man. The common feature of the mannose-binding site of jacalin-related lectins has been determined. The binding site consists of three exposed loops located at the top of the β-prism fold. The

tripeptide, Gly135-Asp136-Val137 and the residue Asp139 belong to the surface-exposed β11-β12 loop. It creates a network of hydrogen bonds with the O6, O5 and O4 hydroxyl groups of mannose (Peumans 2000). However, its consensus sequence was not present in HRL. Probably, HRL has a novel carbohydrate recognition domain.

In the hemagglutination inhibition test, α1-6 mannobiose was the strongest haptenic sugar and the other α-mannobioses, α1-3, α1-2, or α1-4 mannobiose exhibited much weaker binding to the lectin than α1-6 mannobiose (Table III). This result was confirmed by the co-injection in SPR analysis that was developed by us (Figure 5)(Kobayashi et al. 2005; Kobayashi et al. 2004). In the co-injection experiments, these disaccharides must have bound to the immobilized HRL. However, since the molecular masses of the disaccharides are much smaller than that of PSM, effects of the saccharide-binding on the sensorgrams is negligible. The oligosaccharides possessing Glcα1-6Glc were also strong inhibitors compared to α1-2, α1-3, or α1-4 mannobiose in the hemagglutination inhibition test (Table III). The other known Manα specific lectins tested, ConA, PSA, BanLec, GNA and HHA, were not able to distinguish among α1-2, α1-3, α1-4 and α1-6 mannobioses (Table IV). To our knowledge, this is the first lectin that binds to α1-6 linkage of Man or Glc exclusively. Among the glycoproteins, thyroglobulin contains high-mannose-type sugar chains exhibited potent binding affinity (Tsuji et al. 1981). α_1-Acid glycoprotein and transferrin did not have Man at non-reducing end, and were not able to bind to the lectin at all (Yoshima et al.1981; Spik et al. 1975). The sugar-binding specificity of HRL was further analyzed in detail by using FAC (Figure 6). The lectin showed the largest affinity for Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (PA-003). A partial structure of PA-003, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (PA-001) bound to the lectin very weakly, while other partial structure, Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc (PA-002), was not able to bind to the lectin at all. The addition of Fucα1-6 residue (PA-015) to the best haptenic sugar (PA-003) weakened the affinity to the lectin. All the results of the hemagglutination inhibition test, SPR, and FAC allowed us to conclude that 1) HRL recognized Manα1-6 or Glcα1-6 linkage, especially Manα1-6, and has the highest affinity for Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (PA-003) among the sugar chains used, 2) the non-reducing terminal Manα1-6 in sugar chains was essential for the binding, 3)

the branched Manα1-3 residue enhanced the binding of HRL to carbohydrate chains, and 4) the branched Fucα1-6 reduced the affinity of the lectin for sugar chains.

The results of bioassay using rat spleen cells showed that HRL had mitogenic activity through the production of IL-6, IFN-γ and IL-10 (Figure 7 and Supplementary Figure 8). IL-6 is a multi-functional cytokine that is produced by a range of cells including T cells and B cells (Hirano et al. 1990), whereas IFN-γ is produced by T cell (Jonasch et al. 2001). Previous studies demonstrate that IL-10 exhibits key regulatory effects on immune activation, including TH2 cell, mast cell and eosinophil activation (Arock et al. 1996; Royer et al. 2001; Takanaski et al. 1994). IL-10 also serves as a key effecter of regulatory T cell activity (Asseman et al. 1999). The obtained result indicated that HRL activated proliferation of both T cells and B cells. Furthermore, α1-6 mannobiose inhibited the mitogenic activity, suggesting that mannose binding site of HRL was related to the activity.

HRL strongly binds to gp120 protein present on the surface of viruses and surface sugar-chains on host cells, which plays an important role as one of the entry method for virus infection. In the case of HIV, there are two kinds of glycoproteins, gp120 and gp41, in the envelope of the virus, which bind to the receptors on the surface of host cell and lead to the infection of virus to the host. gp120 possesses more than twenty *N*-glycosidically linked sugar chains with varying structural variety. Among the chains, three chains possessing seven to nine mannose residues play a crucial role in the infection. Therefore, drugs or molecules that can specifically bind to these glycans can hinder the interactions between the proteins of the viral envelope and the cells of the host and prevent further interactions with the co-receptors (Balzarini 2006; Botos et al. 2005). Sugar-binding proteins can crosslink glycans on the viral surface (Sacchettini et al. 2001; Shenoy et al. 2002; Ziolkowska et al. 2006). Therefore, lectins that can bind to gp120 are attracting attention recently. Anti-HIV activity of cianovilin-N (CV-N) from the cyanobacterium (blue-green algae) *Nostoc ellipsosporum* has been reported and the lectin showed strong affinity for Man8- and Man9-type glycans (Botos et al. 2002; Helle et al. 2006; Ziolkowska et al. 2006). The binding probably was due to the recognition of Manα1-2 residues in the glycans by the lectin. The anti-HIV activities of Manα1-2Man specific lectins, actinohivine (AH), and *Oscillatoria agardhii* agglutinin (OAA), have been also reported (Hoorelbeke et al. 2010; Matoba et al. 2010; Sato et al. 2007; Takahashi et al. 2010). Recently, anti-HIV activity of a Man-specific Jacalin-related lectin, banana lectin (BanLec), was also reported (Swanson et al. 2009). BanLec is unique in its specificity for internal α1-3 linkages of Man/Glc as

well as β1-3 linkages Man/Glc at the reducing termini (Koshte et al. 1990; Winter et al. 2005). The binding strength of HRL to gp120 was the same as or more than BanLec. FAC analysis revealed that HRL also bound to Man7 (PA-008, 009, 010), Man8 (PA-011, 012, 013), and Man9 (PA-014, 016) (Figure 6).

The mushroom *Hygrophorus russula* from which HRL was isolated is edible. This mushroom might become a candidate for a functional food that can prevent HIV infection in future. Furthermore, HRL has a promising potential for a probe that is able to distinguish Manα1-6 linkage from the other linkages of Man.

Materials and methods

Materials

Fruiting bodies of *H. russula* were collected at Narusawa village, Yamanashi Prefecture, Japan, frozen upon collection and stored at –20ºC. Bananas were purchased from a local grocery store. Snowdrop, amaryllis and tulip bulbs were purchased from local markets. *Canavalia ensiformis* agglutinin (Concanavalin A, ConA) and *Pisum sativum* agglutinin (PSA) were products of J-Oil mills, INC, (Tokyo, Japan). Sephadex G-50 was a product of GE Healthcare UK-Ltd.. Mannose-agarose was purchased from Sigma (St. Louis, MO). BioAssist S and TSK-GEL G3000SW_{XL} were products of Tosoh (Tokyo, Japan). The MALDI-TOF-MS spectrum was acquired on an AutoFlex (Bruker Daltonics, Billerica, MA). The partial *N*-terminal sequence of HRL has been determined by a PPSQ-21A protein sequencer (Shimazu, Kyoto, Japan). Lysyl endopeptidase, endopeptidase Asp-C and Arg-C were products of Wako Pure Chemicals (Osaka, Japan), Sigma (St. Louis, MO) and Takara Bio Inc. (Shiga, Japan), respectively. ABEE reagent and Wakosil-II column were obtained from Wako Pure Chemicals. All the sugars and glycoproteins for the hemagglutinating inhibition tests and the SPR analyses were purchased from Nacalai tesque (Tokyo, Japan), Wako Pure Chemicals, Calbiochem (La Jolla, CA) or Sigma. BIAcore 2000 was a product of GE Healthcare UK-Ltd. PA-oligosaccharides for FAC analysis were purchased from Takara Bio Inc.. HiTrap NHS-activated Sepharose (activated agarose gel) was purchased from GE Healthcare UK-Ltd. Stainless steel empty miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μL) was obtained from Shimadzu.

Animals

Male Fisher 344 rats were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were housed in individual cages of screen-bottomed stainless in room with controlled temperature (23 ± 2°C) and lighting (light on from 8:00 to 20:00).

Purification of the lectin

All of the procedures were carried out at 4°C. After defrosting, the fruiting bodies of *H. russula* were homogenized and extracted with 10 mM phosphate-buffered saline, pH 7.4 (PBS) overnight. The homogenate was centrifuged at 8,500 × *g* for 15 min and the resulting supernatant was applied to a column of Sephadex G-50 (5 × 15 cm) equilibrated with the buffer. After unbound materials were washed with the buffer, the bound fraction was desorbed with 0.3 M Man in the buffer. The eluates were concentrated and equilibrated with 0.1 M acetate buffer, pH 4.7, by ultrafiltration, and further separated by cation-exchange chromatography on a BioAssist S column (1.0 × 10 cm) equilibrated with the buffer. The column was exhaustively washed with the same buffer, and the adsorbed lectin was eluted by a linear gradient of NaCl (0-1 M) in the buffer. The eluant was dialyzed extensively against distilled water, ultrafiltered, and lyophilized. Approximately 2.7 mg of HRL was obtained from 100 g of the fresh fruiting bodies.

SDS-PAGE

SDS-PAGE was done by the method of Laemmli (Laemmli 1970). Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°C. Gels were stained with Coomassie Brilliant Blue. The molecular masses on SDS-PAGE were estimated using recombinant ladder markers (10 to 250 kDa; XL-Ladder Broad; APRO Life Science Institute, Tokushima, Japan).

Isoelectric Focusing

Isoelectric focusing on a gel (PhastGel IEF, pH 3-9) was done in a Phastsystem (GE Healthcare Bio-Sciences). The pI standards (GE Healthcare Bio-Sciences) used were trypsinogen (pI 9.30), lentil lectin basic band (8.65), lentil lectin middle band (8.45), lentil lectin acidic band (8.15), myoglobin basic band (7.35), myoglobin acidic band (6.85), human carbonic anhydrase B (6.55), bovine carbonic

anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50).

Gel Filtration for Estimation of Molecular Mass

Gel filtration by HPLC was carried out on a TSK-gel G3000SW$_{XL}$ column (7.8 × 300 mm) operating at room temperature in PBS at a flow rate of 0.5 mL/min. Fractions were collected by monitoring absorbance at 280 nm. The molecular mass was calibrated with the following standard proteins (Sigma); bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectra were acquired on an AutoFlex (Bruker Daltonics). The spectrum was measured in linear mode using 20 kV ion acceleration without post acceleration. The spectrum was recorded at a detector voltage of 1.65 kV, and was expressed as the averaged results of at least 300 laser shots. Sinapinic acid was used as the matrix. Each sample was dissolved in 0.1% TFA-CH_3CN (2:1 v/v) and mixed with the matrix solution (1:1 or 1:4 v/v). The mixture (1 μL) was put on a stainless target and crystallized at room temperature. A mass calibration procedure was employed prior to the analysis of a sample using protein calibration standards (Bruker Daltonics).

Protein content estimation

Protein concentrations were determined by the method of Lowry. (Lowry et al. 1951), using immunoglobulin G as standard.

Amino Acid Composition Analysis

Each sample was hydrolyzed with 6 M HCl at 110°C for 24 h in a sealed evacuated tube and analyzed on a Hitachi L-8900 amino acid analyzer. The cysteine content was determined by carboxymethylation of the protein with iodoacetic acid followed by hydrolysis under the same conditions as that of the intact protein (Gurd et al. 1967). The content of tryptophan was estimated by the spectrometric method of Edelhoch (Edelhoch 1967).

N-Terminal Sequence Analysis

The *N*-terminal amino acid of the intact protein was analyzed on a PPSQ-21A protein sequencer (Shimadzu).

Enzymatic Digestion and Peptide Sequence Analysis

Each sample (0.5 mg) was reduced with dithiothreitol (0.5 mg) at room temperature for 5 h, *S*-carboxymethylated with ICH_2COOH (1.25 mg) at room temperature for 30 min, dialyzed against 0.1% trifluoroacetic acid (TFA) in H_2O, and digested with an endoproteinase Asp-N from a *Pseudomonas fragi* mutant (Roche Applied Science, Mannheim, Germany) (Enzyme(E)/Substrate(S) = 1:150 w/w), in 50 mM sodium phosphate buffer (pH 7.8) for 6 h at 37°C. The *S*-carboxymethylated HRL was also digested with an endoproteinase Arg-C from *Clostridium histolyticum* (Roche) (E/S = 1:100 w/w), in 90 mM Tris-HCl buffer (pH 7.6) containing 8.5 mM $CaCl_2$, 5 mM dithiothreitol, and 0.5 mM EDTA at 37°C for 6 h, or an endoproteinase Lys-C, *Staphylococcus aureus* V8 (E/S = 1:60 w/w), in 25 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA for 6 h at 37°C. The resulting peptides were separated by reverse-phase HPLC using a Cadenza CD-C18 column (250 × 4.6 mm) with a linear gradient of 10-90% acetonitrile/0.1% TFA in H_2O at a flow rate of 1 mL/min. The effluent was monitored at 215 nm. After the isolation, each peptide was analyzed with an AutoFlex MALDI-TOF Mass Spectrometer (Bruker Daltonics) and a PPSQ-21A sequencer. The homology of the sequences with other proteins was searched by BLAST service.

Isolation of RNA

The fruiting bodies collected as mentioned above were frozen and stored at -80°C. The frozen tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. Total RNA from the frozen powder was purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany).

Cloning of HRL cDNA

RT-PCR was performed with a PrimeScript™ RT-PCR Kit (Takara). The total RNA was used as the template. Two degenerate oligonucleotide primers (sense, 5'- TGGCCNGCNAARYTNGARAT-3'; antisense, 5'-TCNCCRTTNCCRAADATRTT-3') were designed from the amino acid sequence of

HRL. The PCR product (314 bp) was purified and cloned with a Mighty TA-cloning Kit (Takara) for sequencing. The PCR product was extended by RACE using 5'-Full RACE Core set (Takara) and 3'-Full RACE Core set (Takara) with gene specific primer (5'-RACE specific primers, 5'-CGTTCACCATCCTT-3', 5'-TCACTAGTGTTCAGGGTAGG-3', 5'-ACAGGTCTTATAACAGGCCC-3', 5'-CGCCACGGATGGATCATTAA-3', 5'-CCCTATTACTGGGATCACTG-3'; 3'-RACE specific primer, 5'-AAGACTTCCCTATTACTGGG-3') following the manufacturer's instructions. The full-length cDNA sequence was obtained through RT-PCR reaction using primer F1 (5'-ATGACTATTGGAACCGCAAAG-3') and R1 (5'- TATGCATTGGTGATCACCTTG -3'). The cDNA fragments obtained was cloned and sequenced as described above.

Bioinformatics analysis

The peptide sequence was characterized using the Compute pI/Mw and ProtParam algorithms (http://www.expasy.ch). The sequence was also analyzed for homologues using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Neutral Sugar Content Estimation

The sugar content of HRL was measured by the phenol-sulfuric acid method with reference to Glc (Hodge et al. 1962).

Sugar Composition Analysis

Sugar compositions were determined as described previously (Yasuno et al. 1997). Briefly, HRL (0.2 mg) was dissolved in 20 µL of distilled water in a test tube to which 4 M TFA (20 µL for neutral sugars) or 8 M HCl (20 µL for amino sugars) was added. The test tube was incubated at 100°C in a hot block bath. After 4 h (neutral sugars) or 6 h (amino sugars), the tube was cooled to room temperature, and the acid was removed using a centrifugal concentrator at 35°C. The dried sample was derivatized with ABEE in the presence of borane-pyridine complex at 80°C. After 1 h, the reaction mixture was cooled to room temperature. Distilled water (200 µL) and an equal volume of chloroform were added to the reaction mixture. After vigorous vortexing, the sample was centrifuged (6,000 × *g*, 1 min). The

upper aqueous layer was analyzed by reversed-phase HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 × 150 mm); solvent A, 0.02% TFA:acetonitrile (90:10), solvent B, 0.02% TFA:acetonitrile (50:50); program, 0-45 min (solvent B concentration 0%), 45-55 min (solvent B concentration 100%), 55-70 min (solvent B concentration 0%); flow rate, 1 mL/min; temperature, 45°C; detection, absorbance at 305 nm. GlcNAc, GalNAc, Glc, Gal, Man, Xyl, and L-Fuc were used as the monosaccharide and amino monosaccharide standards.

Sialic acid composition was determined according to the method of Hara et al. (Hara et al. 1986). Briefly, the protein (10 μg) was dissolved in 10 μL distilled water in a test tube to which 25 mM HCl (400 μL) was added. The test tube was incubated at 80°C in a hot block bath. After 1 h, the tube was cooled to room temperature and the acid was removed by using a centrifugal concentrator at 35°C. The dried sample was derivatized with 1,2-diamino-4,5-methylenedioxybenzene at 65°C. After 2.5 h, the reaction mixture was cooled to room temperature. The sample was analyzed by reversed-phase HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 × 150 mm); solvent, A MeOH/CH_3CN/H_2O (3/1/10 v/v/v), B MeOH/CH_3CN/H_2O (1/1/1 v/v/v); program, 0-35 min (B conc. 0%), 35-45 min (B conc. 100%), 45-60 min (B conc. 0%); flow rate, 1 mL/min; temperature, 35°C; detection, fluorescence at 373 nm (excitation) and 448 nm (emission). The sialic acid standards used were NeuAc and NeuGc.

Thermostability, pH Stability and Metal Cation Requirements

The thermostability and pH stability of the lectin were examined as described previously (Kawagishi et al. 1994). Briefly, samples in PBS were heated for 30 min at the temperatures indicated, cooled on ice, and titrated. The pH stability of the lectin was measured by incubating the samples in the following buffers for 12 h at 4°C, dialyzing against PBS, and titrating in PBS: 50 mM glycine-HCl buffer (pH 2.0-3.0), 50 mM sodium acetate buffer (pH 4.0-5.5), 50 mM sodium phosphate buffer (pH 6.0-7.5), 50 mM Tris-HCl buffer (pH 8.0-8.5), and 50 mM glycine-NaOH buffer (pH 9.0-11.0). To examine metal cation requirements of the hemagglutination by the lectin, the sample (0.1 mg/mL) was incubated in 10 mM EDTA for 1 h at room temperature, dialyzed against PBS, and titrated. To the demetalized lectin, 0.1 M metal cation ($CaCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$) was added, and the solution was incubated for 1 h at room temperature and titrated.

Erythrocytes

Intact, Pronase-treated, trypsin-treated and neuraminidase-treated erythrocytes were prepared as described previously (Kawagishi et al. 2001).

Preparation of PSM and asialo-PSM

PSM or asialo-PSM was treated with phenol in order to remove impurities as follows. 50% phenol was added to aqueous solution of each sample (5 g) , and the resulting mixture was stirred overnight. The mixture was centrifuged (15,000 × *g*, 30 min) and the phenol was removed. After this procedure was repeated twice, the water layer was dialyzed extensively against distilled water and lyophilized.

Hemagglutination and Inhibition Assay

The hemagglutinating activity of the lectin was determined by a two-fold serial dilution procedure using intact, Pronase-treated, trypsin-treated, and neuraminidase-treated erythrocytes. The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of titer 4 of the lectin.

SPR Analysis

Real time detection of the lectin binding to glycoproteins was recorded by using a BIAcore 2000. Intact lectin was immobilized covalently via its primary amines to carboxyl groups within a dextran layer on the sensor chip CM-5 according to the manufacture's specifications. After chip activation with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 10 mM *N*-hydroxysuccinimide, the lectin (in 10 mM sodium acetate buffer, pH 5.0) at a concentration of 10 μg/mL was passed through the flow cell at a rate of 5 μL/min. After immobilization, the chip was capped by exposure to 1 M ethanolamine. All analyses were performed at a flow rate of 20 μL/min. Before loading of analytes, the chip was equilibrated with 10 mM Hepes containing 0.15 M NaCl, 3 mM EDTA and 0.005% surfactant P-20, pH 7.4 (HBS-EP). Each analyte at various concentrations in the same buffer was injected over the immobilized ligand. After injection of the analyte, HBS-EP was introduced onto the sensor surface to start dissociation. In the case of the co-injection method, each

analyte in HBS-EP was injected over immobilized ligand, and then the other analyte in the buffer was introduced onto the sensor surface. After each measurement, the sensor chip was regenerated by flowing 3 M NaCl, for 2 min.

The experimental sensorgrams were fitted to various kinetic models in BIAevaluation 3.2 software. Association and dissociation rate constants (*ka* and *kd*) were calculated by using BIAevaluation 3.2 software. The affinity constant (K_D) was calculated from the *ka* and *kd*. For the calculation of rate constants, samples were appropriately diluted in HBS-EP at various concentrations.

FAC Analysis

Frontal affinity chromatography (FAC) using fluorescence detection of the lectin binding to fluorescent-labeled glycans was performed by using FAC-1 (Tateno et al. 2007). The lectin was dissolved in 0.2 M $NaHCO_3$ containing 0.5 M NaCl (pH 8.3) and coupled to HiTrap NHS-activated Sepharose by following the manufacturer's instructions. After washing and deactivation of excess active groups by 0.5 M Tris containing 0.5 M NaCl (pH 8.3), the lectin-immobilized Sepharose beads were suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 0.8% NaCl (TBS) and the slurry was packed into a stainless steel column (2.0 × 10 mm) and connected to the FAC-1 machine, which had been specially designed and manufactured by Shimadzu. The amount of immobilized protein was determined by measuring the amount of uncoupled protein in the washing solutions by the method of Bradford (Bradford MM 1976). The flow rate and the column temperature were kept at 125 μL/min and 25°C, respectively. After equilibration with TBS, an excess volume (0.5 - 0.8 mL) of PA-glycans (2.5 or 5.0 nM) was successively injected into the columns by an auto-sampling system. Elution of PA-glycans was monitored by measuring fluorescence (excitation and emission wave lengths, 310 and 380 nm, respectively). The elution front relative to that of a standard oligosaccharide (PA-701, PA-lactose), i.e., V-V_0, was then determined. V is elution volume of each PA sugar. For the determination of V_0, PA-701, which has no affinity to the lectin, was used.

Splenocyte Isolation and Culture Condition

Spleens from rats (n = 2) were harvested into ice-cold RPMI-1640 containing 0.5% heat-inactivated fetal calf serum (FCS), mashed between frost ends of the glass slides and filtered through a sterile 70 μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ). After centrifugation at 300 × *g* for 5 min, the pellets were suspended in IOtest3 lysing solution (Beckman Coulter, Brea, CA) for lysis of red

blood cells followed by one wash with PBS (pH 7.2) containing 2% FCS. After centrifugation at 300 × *g* for 5 min, the pellets were resuspended in complete medium (RPMI-1640 containing 10% FCS, 50 μM 2-mercaptethanol, 100 unit/mL penicillin and 100 μg/mL streptomycin) and viable mononuclear cells were counted using trypan blue dye exclusion. Then, the cells (5×10^6 cells/mL) were added to flat-bottom 96-well plates at a volume of 200 μL and incubated at 37°C in 5% CO_2 with either various concentration (0.5, 1, 2, 4, 8 or 25 μg/mL) of HRL, 4 μg/mL of Concanavalin A (ConA) or medium as a control. After 38 h, the cell culture supernatants were collected and stored at -80°C until assayed for cytokine analysis.

Cytokine Analysis

Cytokines (IFN-γ, IL-4, IL-6 and IL-10) were analyzed by commercial ELISA set (OptEIA™, BD biosciences).

Biotinylation of lectins and Enzyme-linked immunosorbent assay

BanLec, GNA, HHA and TxLcI were isolated from banana, snowdrop, amaryllis and Tulipa by modification of previously described methods using mannose-agarose (Kaku et al. 1990; Koshte et al. 1990; Oda et al. 1986; Shibuya et al. 1988; Van Damme et al. 1988; Van Damme et al. 1987)

Each lectin was incubated with biotin amidocaproate *N*-hydroxysuccinimide ester (Sigma) in 0.1 M $NaHCO_3$ with its haptenic sugar for 12 h at 4^{O}C, desalted with Sephadex G-25 (GE Healthcare UK Ltd.) and lyophilized. The degree of biotinylation was determined by using the 4-hydroxyazobenzene-2-carboxylic acid (HABA) assay (Green et al. 1970). Briefly, the avidin/HABA reagent was prepared by adding 10 mg of avidin (Funakoshi, Tokyo, Japan) and 600 μl of 10 mM HABA (Sigma) to 19.4 ml PBS (pH 7.4). One hundred μl of serially diluted lectin solution was added to 900 μl of the avidin/HABA solution, and the absorbance at 500 nm was measured. The conjugation ratios of biotin to HRL, BanLec, ConA, PSA, GNA, HHA and TxLc-I were estimated to be 1.7:1, 2.7:1, 3.9:1, 2.0:1, 2.6:1, 1.1:1and 2.0:1, respectively.

Interaction between glycosylated HIV-1 gp120 and the biotinylated Man-specific lectins was detected by ELISA. 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated by adding 25 μL of gp120 at a concentration of 10 nM containing 0.1 M carbonate buffer (pH 9.5) per well and

incubated overnight at 4°C. The plates were blocked for 1.5 h at room temperature with PBS containing 1% bovine serum albumin, and then rinsed with wash buffer (PBS containing 0.05% Tween 20, pH 7.4) three times before the addition of each biotinylated lectins at various concentrations ranging from 0 to 100 nM in blocking buffer. After incubation for 1 h at room temperature, the plates were washed three times before the addition of the Horseradish Peroxidase streptavidin (Vector, Burlingame, CA). After the plate was washed, TMB (3,3',5,5'-tetramethylbenzidine) microwell peroxidase substrate system (KPL, Baltimore, MD) was used for colorimetric analysis, and the absorbance was measured at 450 nm.

Funding

This work was partially supported by a grant-in-aid for scientific research on priority areas, ''Creation of Biologically Functional Molecules'' (no. 17035037) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Acknowledgements

We thank Dr. V. K. Deo (Shizuoka University) for valuable discussion.

Conflict of interest statement

None declared.

Abbreviations

HRL, *Hygrophorus russula* lectin; ConA, *Canavalia ensiformis* agglutinin (Concanavalin A); PSA, *Pisum sativum* agglutinin; BanLec, *Musa acuminate* lectin (banana lectin), GNA, *Galanthus nivalis* agglutinin (snowdrop lectin); HHA,; *Hippeastrum* hybrid agglutinin (amaryllis lectin); TxLc-I, *Tulipa gesneriana* lectin; Man, mannose; Glc, glucose; PBS, 10 mM phosphate-buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate-polyaclylamidegel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; EDTA, ethylenediaminetetraacetic acid; ABEE, *p*-aminobenzoic ethyl ether; TFA, trifluoroacetic acid; PA, pyridylaminated; FAC, frontal affinity

chromatography; SPR, surface plasmon resonance; HEP, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS-EP, 10 mM Hepes containing 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20, pH 7.4; MIC, minimum inhibitory concentration; MTX, methotrexate; ELISA, enzyme-linked immunosorbent assay; BSM, bovine submaxillary mucin; PSM, porcine stomach mucin; HABA, 4-hydroxyazobenzene-2-carboxylic acid; HIV-1, human immunodeficiency virus type1; All sugars are of D-configuration unless otherwise stated.

References

Arock M, Zuany-Amorim C, Singer M, Benhamou M, Pretolani M. 1996. Interleukin-10 inhibits cytokine generation from mast cells. *Eur J Immunol*. 26: 166-170.

Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med*. 190: 995-1004.

Balzarini J. 2006. Inhibition of HIV entry by carbohydrate-binding proteins. *Antiviral Res*. 71: 237-247.

Botos I, O'Keefe BR, Shenoy SR, Cartner LK, Ratner DM, Seeberger PH, Boyd MR, Wlodawer A. 2002. Structures of the complexes of a potent anti-HIV protein cyanovirin-N and high mannose oligosaccharides. *J Biol Chem*. 277: 34336-34342.

Botos I, Wlodawer A. 2005. Proteins that bind high-mannose sugars of the HIV envelope. *Prog Biophys Mol Biol*. 88: 233-282.

Edelhoch H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry*. 6: 1948-1954.

Green NM, Donald BM, Lemuel DW. 1970. Spectrophotometric determination of avidin and biotin. *Methods Enzymol*. 18-A: 418-424.

Gurd FRN, Hirs CHW. 1967. Carboxymethylation. *Methods Enzymol.* 11: 532-541.

Hara S, Yamaguchi M, Takemori Y, Nakamura M, Ohkura Y. 1986. Highly sensitive determination of *N*-acetyl- and *N*-glycolylneuraminic acids in human serum and urine and rat serum by reversed-phase liquid chromatography with fluorescence detection. *J Chromatogr*. 377: 111-119.

Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J. 2006. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. *J Biol Chem*. 281: 25177-25183.

Hirabayashi J. 2004. Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconj J*. 21: 35-40.

Hirano T, Akira S, Taga T, Kishimoto T. 1990. Biological and clinical aspects of interleukin 6. *Immunology Today*. 11: 443-449.

Hodge JE, and Hofreiter BT. 1962. Determination of reducing sugars and carbohydrates. *Methods*

Carbohydr Chem. 1: 338.
Hoorelbeke B, Huskens D, Ferir G, Francois KO, Takahashi A, Van Laethem K, Schols D, Tanaka H, Balzarini J. 2010. Actinohivin, a broadly neutralizing prokaryotic lectin, inhibits HIV-1 infection by specifically targeting high-mannose-type glycans on the gp120 envelope. *Antimicrob Agents Chemother*. 54: 3287-3301.
Howard IK, Sage HJ. 1969. Isolation and characterization of a phytohemagglutinin from the lentil. *Biochemistry*. 8: 2436-2441.
Jonasch E, Haluska FG. 2001. Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities. *Oncologist*. 6: 34-55.
Kaku H, Van Damme EJ, Peumans WJ, Goldstein IJ. 1990. Carbohydrate-binding specificity of the daffodil (*Narcissus pseudonarcissus*) and amaryllis (*Hippeastrum hybr.*) bulb lectins. *Arch Biochem Biophys*. 279: 298-304.
Kawagishi H, Yamawaki M, Isobe S, Usui T, Kimura A, Chiba S. 1994. Two lectins from the marine sponge *Halichondria okadai*. An *N*-acetyl- sugar-specific lectin (HOL-I) and an *N*-acetyllactosamine-specific lectin (HOL-II). *J Biol Chem.* 269: 1375-1379.
Kawagishi H, Yasui M, Uno A, Murata T, Usui T, Furukawa S. 2001. Purification and Characterization of Two Lectins from a Toxic Moray, *Gymnothrax javanicus*. *Biosci Biotechnol Biochem.* 65: 2437-2442.
Kobayashi Y, Nakamura H, Sekiguchi T, Takanami R, Murata T, Usui T, Kawagishi H. 2005. Analysis of the carbohydrate binding specificity of the mushroom *Pleurotus ostreatus* lectin by surface plasmon resonance. *Anal. Biochem*. 336: 87-93.
Kobayashi Y, Kobayashi K, Umehara K, Dohra H, Murata T, Usui T, Kawagishi H. 2004. Purification, characterization, and sugar binding specificity of an *N*-Glycolylneuraminic acid-specific lectin from the mushroom *Chlorophyllum molybdites*. *J Biol Chem*. 279: 53048-53055.
Koshte VL, van Dijk W, van der Stelt ME, Aalberse RC. 1990. Isolation and characterization of BanLec-I, a mannoside-binding lectin from *Musa paradisiac* (banana). *Biochem J*. 272: 721-726.
Krogfelt KA, Bergmans H, Klemm P. 1990. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect Immun*. 58: 1995-1998.
Kruger RP, Winter HC, Simonson-Leff N, Stuckey JA, Goldstein IJ, Dixon JE. 2002. Cloning, Expression, and Characterization of the Galα 1,3Gal High Affinity Lectin from the Mushroom *Marasmius oreades*. *J Biol Chem.* 277: 15002-15005.
Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, Hirabayashi J. 2005. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nature Methods*. 227: 680-685.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227: 680-685.
Loris R, Hamelryck T, Bouckaert J, Wyns L. 1998. Legume lectin structure. *Biochim Biophys.Acta.* 193: 265-275.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol

reagent. *J. Biol. Chem.* 193: 265-275.
Matoba N, Husk AS, Barnett BW, Pickel MM, Arntzen CJ, Montefiori DC, Takahashi A, Tanno K, Omura S, Cao H, Mooney JP, Hanson CV, Tanaka H. 2010. HIV-1 neutralization profile and plant-based recombinant expression of actinohivin, an Env glycan-specific lectin devoid of T-cell mitogenic activity. *PLoS One*. 5: e11143.
Mo H, Winter HC, Goldstein IJ. 2000. Purification and Characterization of a Neu5Acα 2-6Galβ1-4Glc/GlcNAc-specific Lectin from the Fruiting Body of the Polypore Mushroom *Polyporus squamosus*. *J Biol Chem.* 275: 10623-10629..
Nagata Y, Yamashita M, Honda H, Akabane J, Uehara K, Saito A, Sumisa F, Nishibori K, Oodaira Y. 2005. Characterization, occurrence, and molecular cloning of a lectin from *Grifola frondosa*: jacalin-related lectin of fungal origin. *Biosci Biotechnol Biochem.* 69: 2374-2380.
Nakamura S, Yagi F, Totani K, Ito Y, Hirabayashi J. 2005. Comparative analysis of carbohydrate-binding properties of two tandem repeat-type Jacalin-related lectins, *Castanea crenata* agglutinin and *Cycas revoluta* leaf lectin. *FEBS J*. 272: 2784-2799.
Oda Y, Minami K. 1986. Isolation and characterization of a lectin from tulip bulbs, *Tulipa gesneriana*. *Eur J Biochem*. 159: 239-245.
Peumans WJ, Barre A, Hao Q, Rougé P, Van Damme EJM. 2000. Higher Plants Developed Structurally Different Motifs to Recognize Foreign Glycans. *Trends Glycosci Glycotech.* 12: 83-101.
Pohleven J, Obermajer N, Sabotic J, Anzlovar S, Sepcic K, Kos J, Kralj B, Strukelj B, Brzin J. 2009. Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells. *Biochim Biophys Acta*. 1790: 173-181.
Royer B, Varadaradjalou S, Saas P, Guillosson JJ, Kantelip JP, Arock M. 2001. Inhibition of IgE-induced activation of human mast cells by IL-10. *Clin Exp Allergy*. 31: 694-704.
Sacchettini JC, Baum LG, Brewer CF. 2001. Multivalent protein-carbohydrate interactions. A new paradigm for supermolecular assembly and signal transduction. *Biochemistry*. 40: 3009-3015.
Sato Y, Okuyama S, Hori K. 2007. Primary structure and carbohydrate binding specificity of a potent anti-HIV lectin isolated from the filamentous cyanobacterium *Oscillatoria agardhii*. *J Biol Chem.* 282: 11021-11029.
Shenoy SR, Barrientos LG, Ratner DM, O'Keefe BR, Seeberger PH, Gronenborn AM, Boyd MR. 2002. Multisite and multivalent binding between cyanovirin-N and branched oligomannosides: calorimetric and NMR characterization. *Chem Biol*. 9: 1109-1118.
Shibuya N, Goldstein IJ, Van Damme EJM, Peumans WJ. 1988. Binding properties of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) bulb. *J Biol Chem*. 263: 728-734.
Spik G, Bayard B, Fournet B, Strecker G, Bouquelet S, Montreuil J. 1975. Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett*. 50: 296-299.
Suzuki T, Amano Y, Fujita M, Kobayashi Y, Dohra H, Hirai H, Murata T, Usui T, Morita T, Kawagishi H. 2009. Purification, characterization, and cDNA cloning of a lectin from the mushroom *Pleurocybella porrigens*. *Biosci Biotechnol Biochem*. 73: 702-709.

Swanson MD, Winter HC, Goldstein IJ, Markovitz DM. 2009. A lectin isolated from bananas is a potent inhibitor of HIV replication. *J Biol Chem*. 285: 8646-8655.

Takahashi A, Inokoshi J, Tsunoda M, Suzuki K, Takenaka A, Sekiguchi T, Omura S, Tanaka H. 2010. Actinohivin: specific amino acid residues essential for anti-HIV activity. *J Antibiot*. 63: 661-665.

Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, Jordana M. 1994. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med*. 180: 711-715.

Tateno H, Goldstein IJ. 2003. Molecular cloning, expression, and characterization of novel hemolytic lectins from the mushroom *Laetiporus sulphureus*, which show homology to bacterial toxins. *J Biol Chem*. 278: 40455-40463.

Tateno H, Nakamura-Tsuruta S, Hirabayashi J. 2007. Frontal affinity chromatography: sugar-protein interactions. *Nature Protoc*. 2: 2529-2537.

Tateno H, Nakamura-Tsuruta S, Hirabayashi J. 2009. Comparative analysis of core-fucose-binding lectins from *Lens culinaris* and *Pisum sativum* using frontal affinity chromatography. *Glycobiology*. 19: 527-536.

Tateno H, Winter HC, Goldstein IJ. 2004. Cloning, expression in *Escherichia coli* and characterization of the recombinant Neu5Acα2,6Galβ1,4GlcNAc-specific high-affinity lectin and its mutants from the mushroom *Polyporus squamosus*. *Biochem. J.* 382: 667-675.

Tsuji T, Yamamoto K, Irimura T, Osawa T. 1981. Structure of carbohydrate unit A or porcine thyroglobulin. *Biochem J*. 195: 691-699.

Van Damme EJ, Peumans WJ. 1988. Biosynthesis of the Snowdrop (*Galanthus nivalis*) Lectin in Ripening Ovaries. *Plant Physiol*. 86: 922-926.

Van Damme EJ, Allen AK, Peumans WJ. 1987. Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivalis*) bulbs. *FEBS Letters*. 215: 140-144

Winter HC, Oscarson S, Slattegard R, Tian M, Goldstein IJ. 2005. Banana lectin is unique in its recognition of the reducing unit of 3-O-β-glucosyl/mannosyl disaccharides: a calorimetric study. *Glycobiology*. 15: 1043-1050.

Worthley DL, Bardy PG, Mullighan CG. 2005. Mannose-binding lectin: biology and clinical implications. *Intern Med J*. 35: 548-555.

Yagi F, Iwaya T, Haraguchi T, Goldstein IJ. 2002. The lectin from leaves of Japanese cycad, *Cycas revoluta* Thunb. (gymnosperm) is a member of the jacalin-related family. *Eur J Biochem*. 269: 4335-4341.

Yasuno S, Murata, T., Kokubo, K., Yamaguchi, T., and Kamei, M. 1997. Two-mode analysis by high-performance liquid chromatography of p-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci Biotechnol Biochem.* 61: 1944–1946.

Yoshima H, Matsumoto A, Mizuochi T, Kawasaki T, Kobata A. 1981. Comparative study of the carbohydrate moieties of rat and human plasma alpha 1-acid glycoproteins. *J Biol Chem*. 256: 8476-8484.

Ziolkowska NE, Wlodawer A. 2006. Structural studies of algal lectins with anti-HIV activity. *Acta Biochim Pol*. 53: 617-626.

FIGURE LEGENDS

Fig. 1. The molecular structure of HRL.

(A) SDS-PAGE of HRL. Lane M, marker proteins; lane 1, HRL, non-reduced; lane 2, HRL, reduced with 2-mercaptoethanol. (**B)** Isoelectric Focusing of HRL. Lane M, marker proteins; lane 1, HRL. **(C)** MALDI-TOF mass spectrometry of HRL.

Fig. 2. Summary of sequence determination of HRL.

Lane 8 shows the complete sequence of HRL. The sequence in lane 1 was determined by Edman degradation of intact HRL. The sequences of the peptides in lanes 2-7 were determined by amino acid sequence analysis and MALDI-TOF mass spectrometry. The peptides obtained with *Lysobacter enzymogenes* proteinase, *Clostridium histolyticum* proteinase, and *Pseudomonas fragi* mutant protease were designated Lys, Arg, and Asp respectively. Isoforms are boxed in black.

Fig. 3. cDNA sequence and deduced amino acid sequence of HRL**.**

Nucleotide numbers are shown above the nucleotide sequence, and the deduced amino acid sequence is shown using one-letter symbols below the nucleotide sequence. The asterisk denotes the termination codon. A possible *N*-glycosilation site is boxed. Isoforms are boxed in black.

Fig. 4. Alignment of the amino acid sequences of HRL, GFL and CRLL.

The amino acid sequence of HRL was compared with those of GFL and CRLL. Identical (:) and similar (.) residues are indicated.

Fig. 5. Sensorgrams showing interaction between immobilized HRL and PSM by co-injection with various mannobioses.

(A) α1-6 mannobiose at various concentrations. (B) various α-mannobioses at 5 mM.

Fig. 6. FAC analysis of affinity of HRL for high mannose type glycans.

PA-003, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, PA-004: Manα1-6(Manα1-3) Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc:

PA-005, Manα1-6(Manα1-3) Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-006, Manα1-6(Manα1-3) Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-056, Manα1-6(Manα1-3) Manα1-6(Galβ1-4GlcNac β1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-009, Manα1-6(Manα1-3) Manα1-6(Manα1-2Man α1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-007, Manα1-6(Manα1-2Manα1-3) Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-015, Manα1-6(Manα1-3) Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc:

PA-053, Manα1-6(Manα1-3) Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-017, Manα1-6(Manα1-3)(Xyl β1-2)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-010, Manα1-6(Manα1-2Manα1-3) Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-013, Manα1-6(Manα1-2Manα1-3) Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-008, Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-012, Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-011, Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-001, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc:

PA-014, Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAc:

PA-016,

Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Glcα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc:

PA-002, Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc:

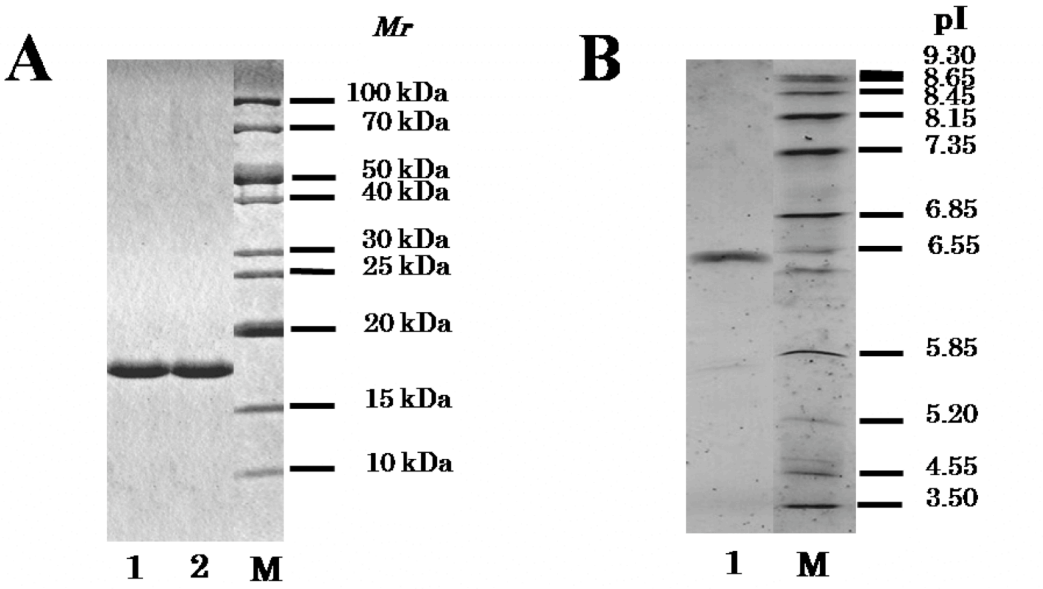
PA-058, Manα1-6Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc.

Fig. 7. Mitogen activity of HRL.

Spleens were added to flat-bottom 96-well plates and incubated at 37°C in 5% CO_2 with either various samples. Cont, medium as a control; ConA, 0.15 μM of Concanavalin A; C+H, 0.15 μM of ConA containing 0.15 μM of HRL; HRL, 0.075, 0.015, 0.3, 0.6, 1.2, 2.4 and 4.8 μM of HRL; HRL+α1-6 mannobiose, 0.3, 0.6, 1.2 and 2.4 μM HRL containing 0.075 μM of α1-6 mannobiose; α1-6 mannobiose, 0.038, 0.075, 0.15 and 0.29 μM of α1-6 mannobiose.

Fig. 8. Binding of HRL and other lectins to gp 120.

HRL (♦), Banlec (■), ConA(▲), PSA (×),GNA (∗), HHA(-), and TxLc-I (|) were interacted to immobilized gp120 (10 nM) on 96-well ELISA plates. Serial dilutions of biotinylated lectins were added to the wells.


A
Mr
100 kDa
70 kDa
50 kDa
40 kDa
30 kDa
25 kDa
20 kDa
15 kDa
10 kDa
1
2
M
B
pI
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50
1
M

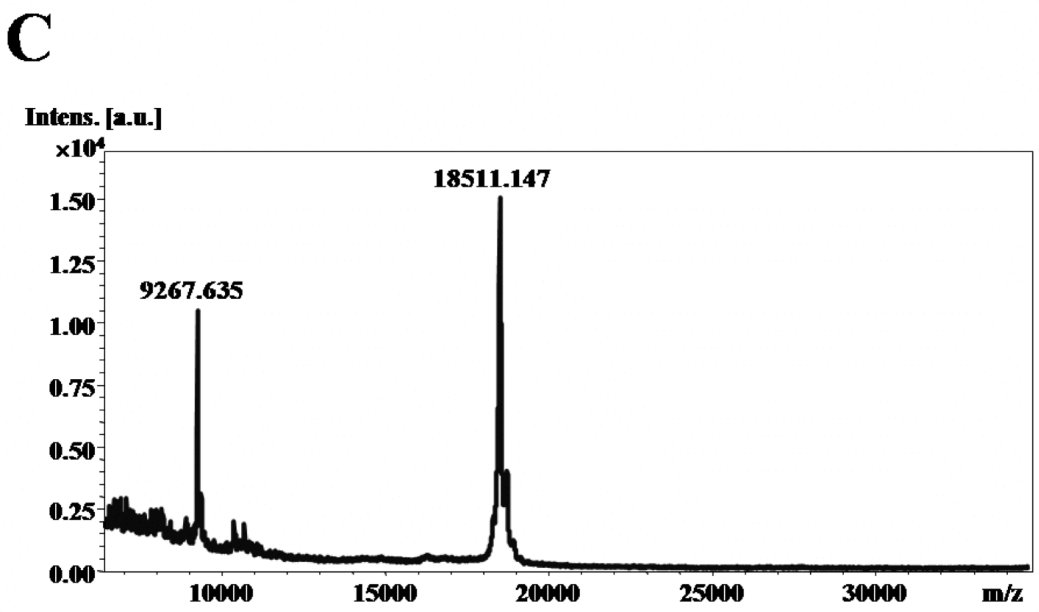

C
Intens. [a.u.]
×10⁴
1.50
1.25
1.00
0.75
0.50
0.25
0.00
9267.635
18511.147
10000
15000
20000
25000
30000
m/z

Figure 1

Peptide		Sequence
1	N-terminal	TIGTAKPILAQTAIVGGPSVPFDDAREVASWPAKLEIAQD
2	Arg-2	EVASWPAKLEIAQDFPITGITVR
3	Lys-3	LEIAQDFPITGITVRHGQIINNLTIIYRTV
4	Arg-3	HGWIINNLTIIYR
5	Arg-5	TVNGNSATVSHGGDSGGIVDKVALNENE
6	Lys-12	
7	Asp-8	
8	1	TIGTAKPILAQTAIVGGPSVPFDDAREVASWPAKLEIAQDFPITGITVRHGQIINNLTIIYRTVNGNSATVSHGGDSGGIVDKVALNENE 90
		W

Peptide		Sequence
1	N-terminal	
2	Arg-2	
3	Lys-3	
4	Arg-3	
5	Arg-5	IITSVQGRAGQHRSYNRPYLDSISFTILDTK
6	Lys-12	TLVTRTTNIFGNGDGTNQGDPFQVAQPYAF
7	Asp-8	DGTNQGDPFQVAQPYAFAGATYTDGQTGVAGLSFFKVITNA
8	91	IITSVQGRAGQHRSYNRPYLDSISFTILDTKTLVTRTTNIFGNGDGTNQGDPFQVAQPYAFAGATYTDGQTGVAGLSFFKVITNA 175

Figure 2

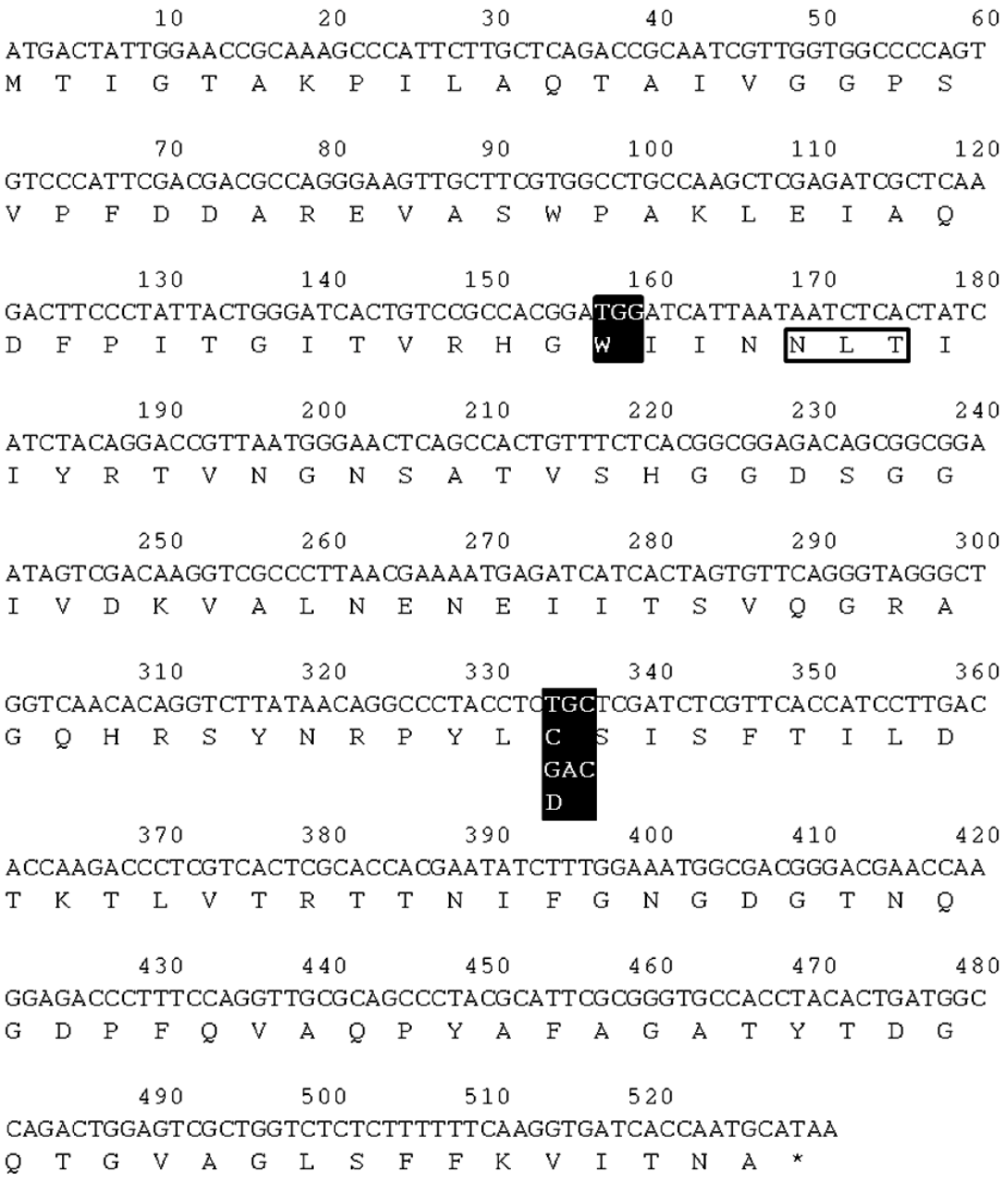

10 20 30 40 50 60
ATGACTATTGGAACCGCAAAGCCCATTCTTGCTCAGACCGCAATCGTTGGTGGCCCCAGT
M T I G T A K P I L A Q T A I V G G P S
70 80 90 100 110 120
GTCCCATTCGACGACGCCAGGGAAGTTGCTTCGTGGCCTGCCAAGCTCGAGATCGCTCAA
V P F D D A R E V A S W P A K L E I A Q
130 140 150 160 170 180
GACTTCCCTATTACTGGGATCACTGTCCGCCACGGATGGATCATTAATAATCTCACTATC
D F P I T G I T V R H G W I I N N L T I
190 200 210 220 230 240
ATCTACAGGACCGTTAATGGGAACTCAGCCACTGTTTCTCACGGCGGAGACAGCGGCGGA
I Y R T V N G N S A T V S H G G D S G G
250 260 270 280 290 300
ATAGTCGACAAGGTCGCCCTTAACGAAAATGAGATCATCACTAGTGTTCAGGGTAGGGCT
I V D K V A L N E N E I I T S V Q G R A
310 320 330 340 350 360
GGTCAACACAGGTCTTATAACAGGCCCTACCTCTGCTCGATCTCGTTCACCATCCTTGAC
G Q H R S Y N R P Y L C S I S F T I L D
GAC
D
370 380 390 400 410 420
ACCAAGACCCTCGTCACTCGCACCACGAATATCTTTGGAAATGGCGACGGGACGAACCAA
T K T L V T R T T N I F G N G D G T N Q
430 440 450 460 470 480
GGAGACCCTTTCCAGGTTGCGCAGCCCTACGCATTCGCGGGTGCCACCTACACTGATGGC
G D P F Q V A Q P Y A F A G A T Y T D G
490 500 510 520
CAGACTGGAGTCGCTGGTCTCTCTTTTTTCAAGGTGATCACCAATGCATAA
Q T G V A G L S F F K V I T N A *

Figure 3

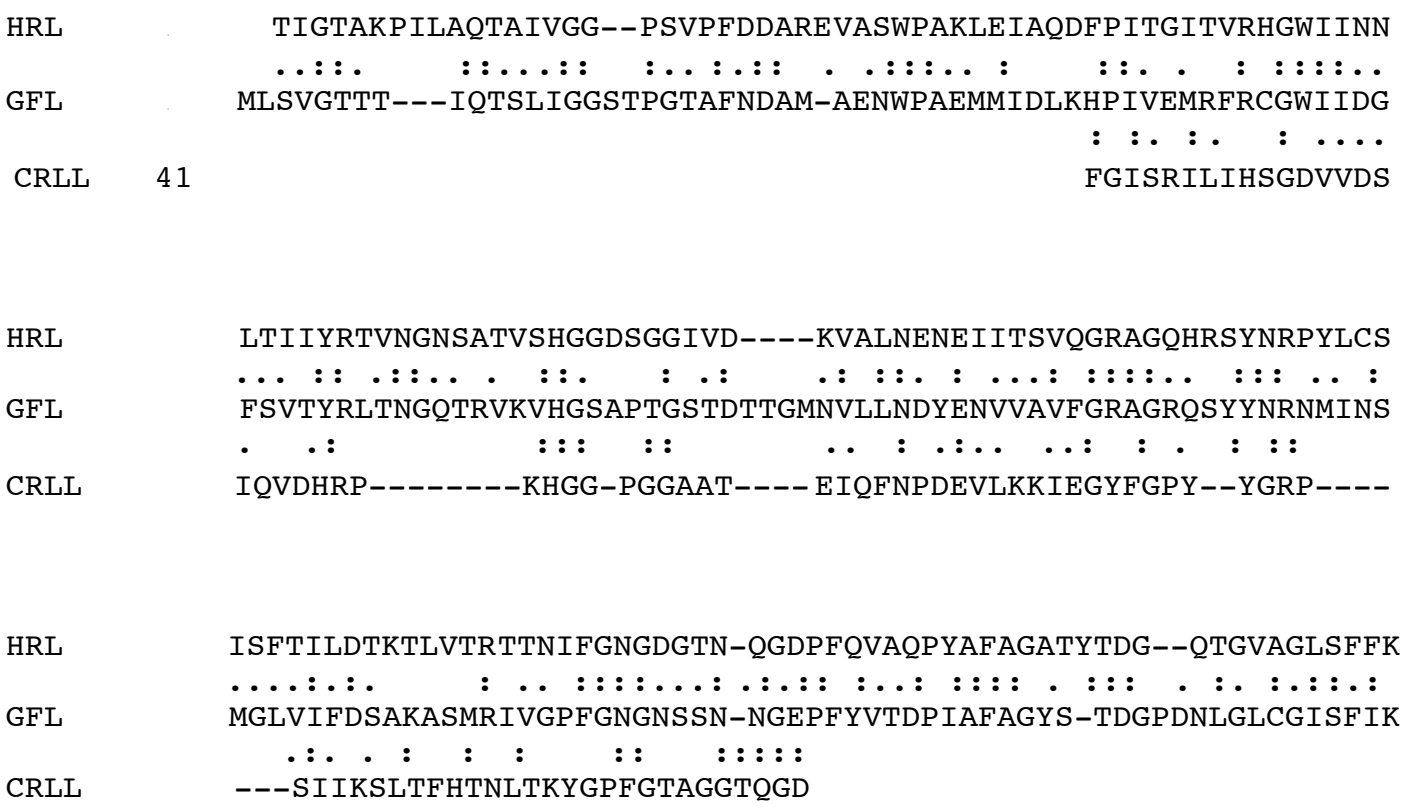
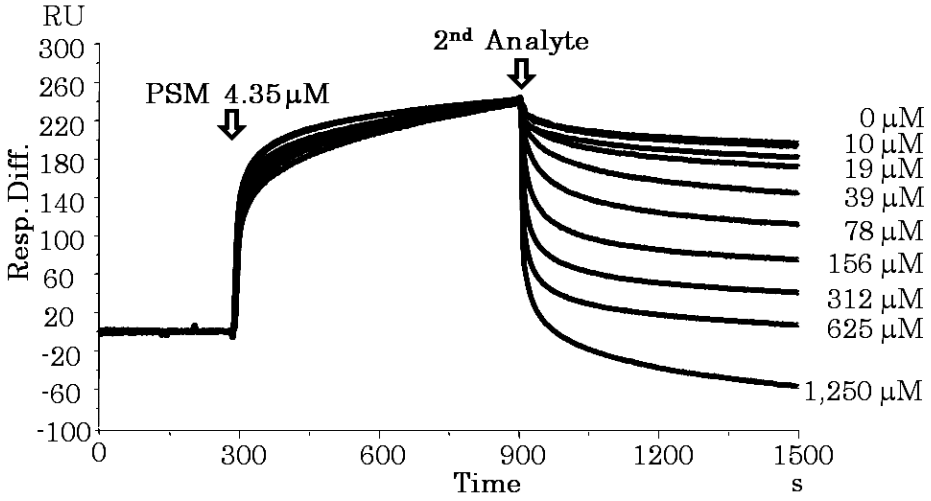

HRL TIGTAKPILAQTAIVGG--PSVPFDDAREVASWPAKLEIAQDFPITGITVRHGWIINN
GFL MLSVGTTT---IQTSLIGGSTPGTAFNDAM-AENWPAEMMIDLKHPIVEMRFRCGWIIDG
CRLL 41 FGISRILIHSGDVVDS
HRL LTIIYRTVNGNSATVSHGGDSGGIVD----KVALNENEIITSVQGRAGQHRSYNRPYLCS
GFL FSVTYRLTNGQTRVKVHGSAPTGSTDTTGMNVLLNDYENVVAVFGRAGRQSYYNRNMINS
CRLL IQVDHRP--------KHGG-PGGAAT----EIQFNPDEVLKKIEGYFGPY--YGRP----
HRL ISFTILDTKTLVTRTTNIFGNGDGTN-QGDPFQVAQPYAFAGATYTDG--QTGVAGLSFFK
GFL MGLVIFDSAKASMRIVGPFGNGNSSN-NGEPFYVTDPIAFAGYS-TDGPDNLGLCGISFIK
CRLL ---SIIKSLTFHTNLTKYGPFGTAGGTQGD

Figure 4

A


RU
300
260
220
180
140
100
60
20
-20
-60
-100
Resp. Diff.
PSM 4.35μM
2nd Analyte
0 μM
10 μM
19 μM
39 μM
78 μM
156 μM
312 μM
625 μM
1,250 μM
0
300
600
900
1200
1500
Time
s

B

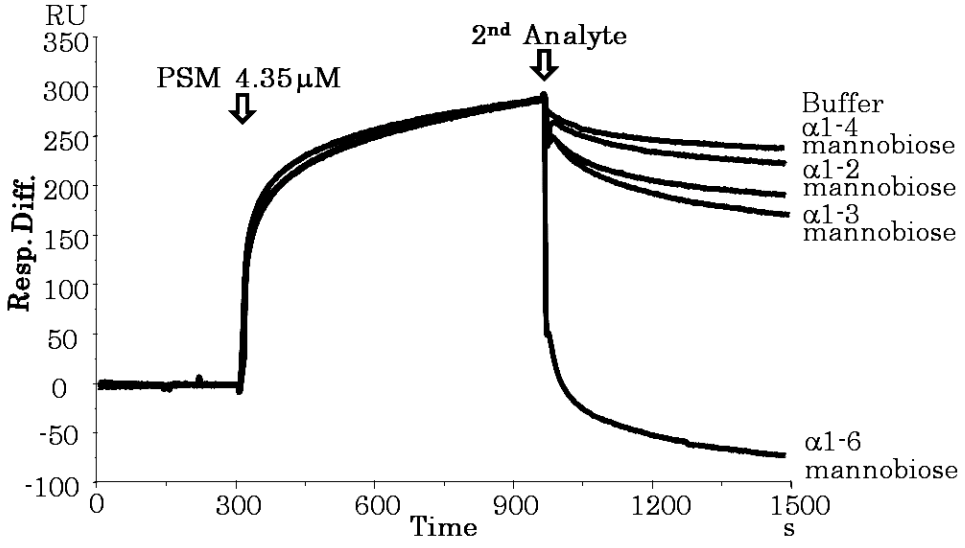

RU
350
300
250
200
150
100
50
0
-50
-100
Resp. Diff.
PSM 4.35μM
2nd Analyte
Buffer
α1-4 mannobiose
α1-2 mannobiose
α1-3 mannobiose
α1-6 mannobiose
0
300
600
900
1200
1500
Time
s

Figure 5

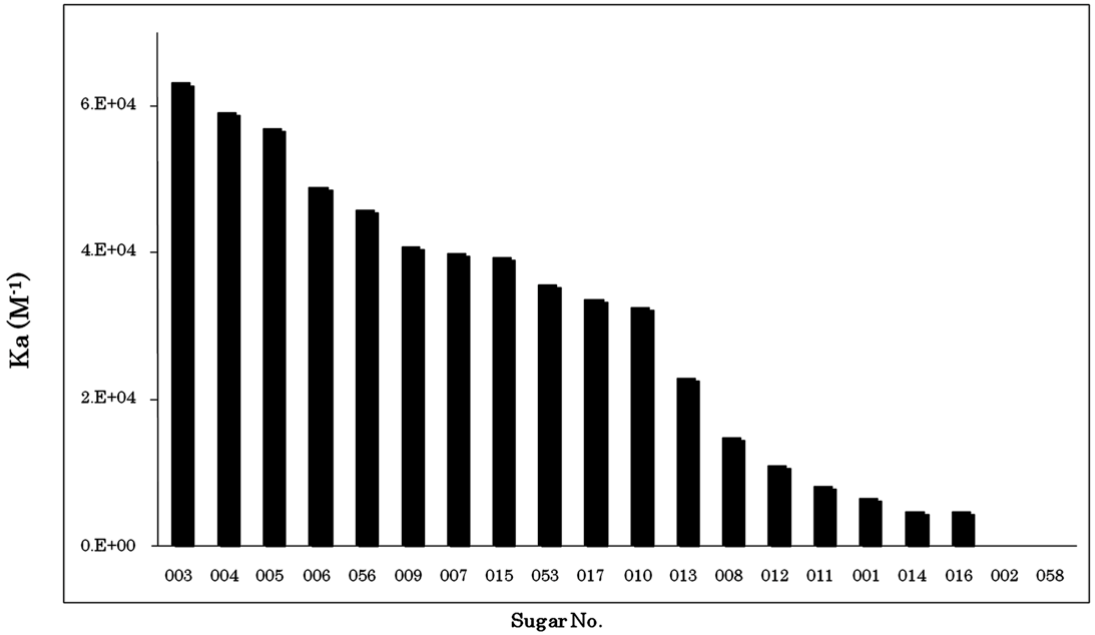

Ka (M-1)
6.E+04
4.E+04
2.E+04
0.E+00
003 004 005 006 056 009 007 015 053 017 010 013 008 012 011 001 014 016 002 058
Sugar No.

Figure 6

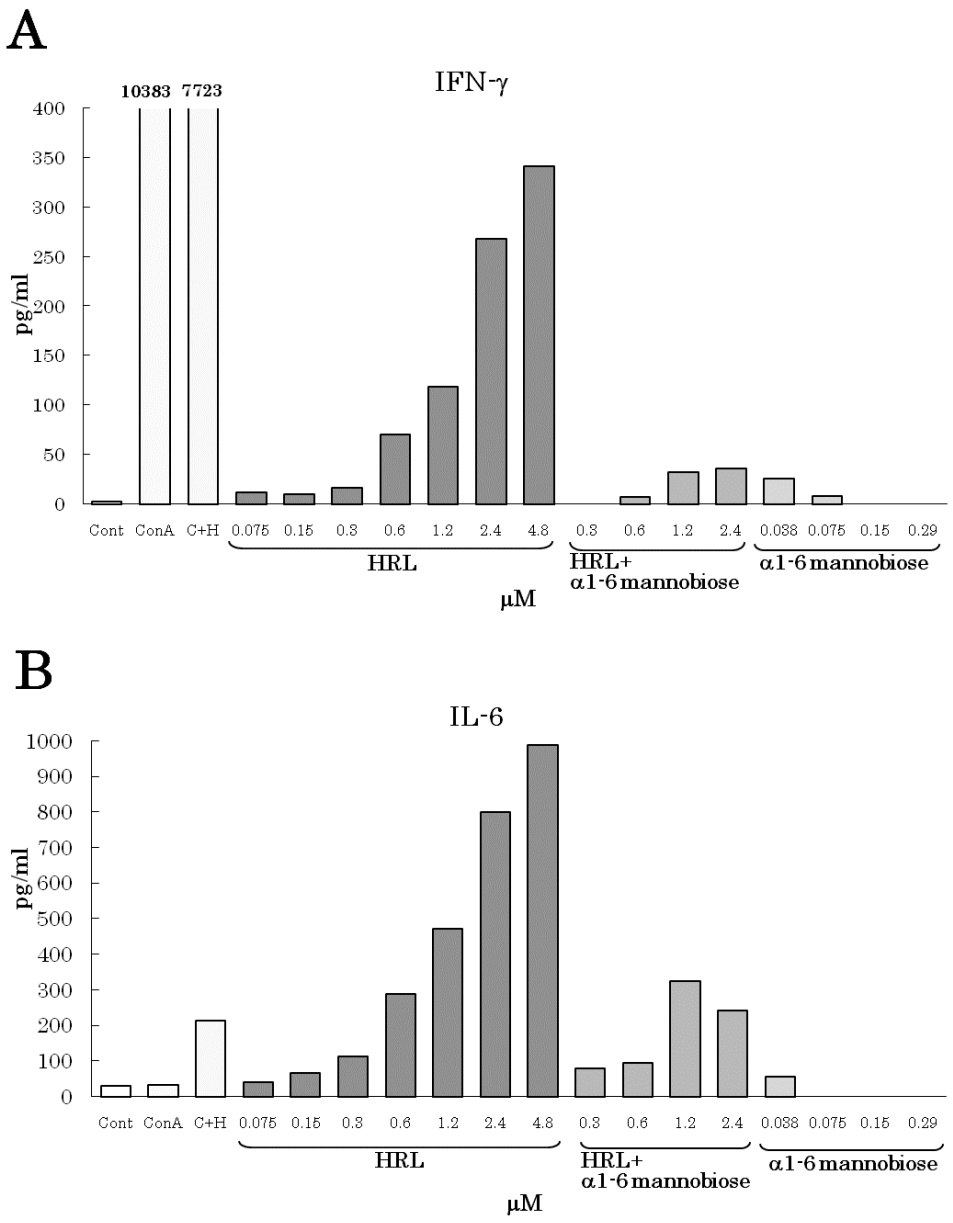

A
IFN-γ
10383
7723
400
350
300
250
200
150
100
50
0
pg/ml
Cont
ConA
C+H
0.075
0.15
0.3
0.6
1.2
2.4
4.8
0.3
0.6
1.2
2.4
0.038
0.075
0.15
0.29
HRL
HRL+
α1-6 mannobiose
α1-6 mannobiose
μM
B
IL-6
1000
900
800
700
600
500
400
300
200
100
0
pg/ml
Cont
ConA
C+H
0.075
0.15
0.3
0.6
1.2
2.4
4.8
0.3
0.6
1.2
2.4
0.038
0.075
0.15
0.29
HRL
HRL+
α1-6 mannobiose
α1-6 mannobiose
μM

Figure 7

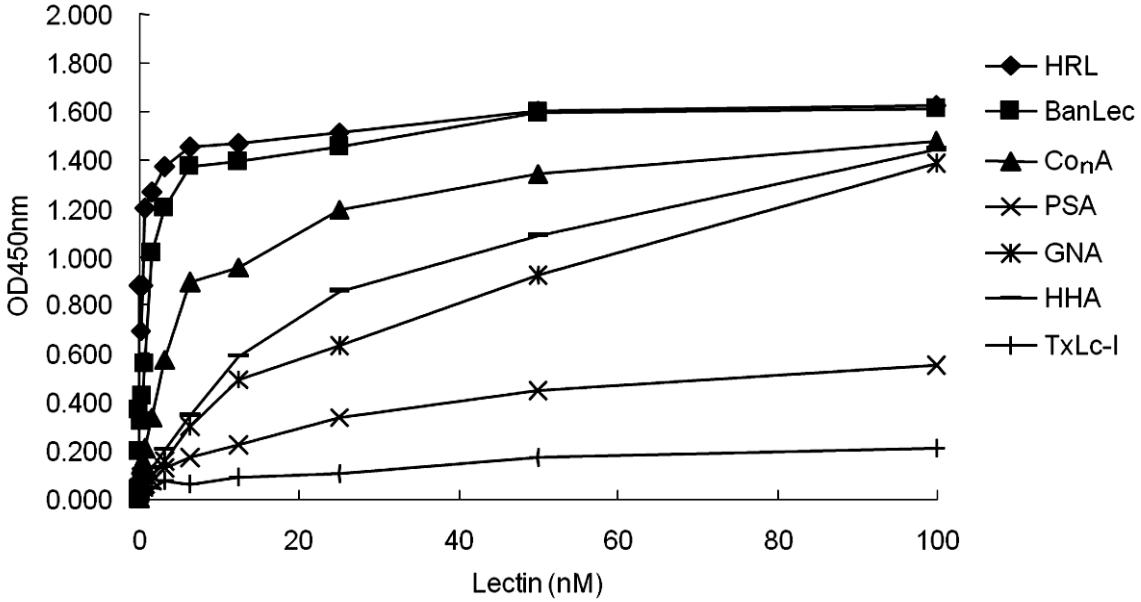

2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
OD450nm
0
20
40
60
80
100
Lectin (nM)
HRL
BanLec
ConA
PSA
GNA
HHA
TxLc-I

Figure 8

Table I. Purification of HRL from 100 g of fruiting bodies of *Hygrophorus russula.*

Fraction	Total protein (mg)	Total agglutination (titer) [a]	Specific agglutination (titer /mg protein)	Recovery of activity (%)
Supernatant after homogenization	8.7×10^2	4.4×10^5	5.1×10^2	100
Sephadex G-50 affinity chromatography	6.0	9.8×10^4	1.6×10^4	22
BioAssist S cation exchange chromatography	2.7	8.8×10^4	3.2×10^4	20

[a] Titer was defined as the reciprocal of the end-point dilution exhibiting hemagglutination.

Table II. Agglutination profiles of HRL

Group of erythrocytes	Titer[a]			
	Untreated	Pronase treated[b]	Trypsin treated[c]	Neuraminidase treated[d]
Human A	0	2^{11}	0	2^{4}
Human B	0	2^{9}	0	0
Human O	0	2^{11}	0	2^{4}
Rabbit	2^{5}	2^{8}	NT[e]	NT

[a] Titer was defined as the reciprocal of the end-point dilution exhibiting the hemagglutination

[b] 10% suspension of erythrocytes in PBS (10 ml) was treated with Pronase (5.0 mg) for 30 min at 45°C.

[c] 10% suspension of erythrocytes in PBS (10 ml) was treated with trypsin (1.0 mg) for 180 min at 37°C.

[d] 10% suspension of erythrocytes in PBS (10 ml) was treated with neuraminidase (1 U/ml) for 60 min at 37°C.

[e] NT; not tested.

Table III. Inhibition of HRL-mediated hemagglutination by mono- and oligosaccharides and glycoproteins

Inhibitor[a]	MIC[b] (mM)
α1-6 mannobiose	0.0391
isomaltose (Glcα1→6Glc)	0.156
isomaltotriose	0.313
isomaltotetraose	0.313
isomaltopentaose	0.313
isomaltohexaose	0.313
methyl α-mannoside	1.56
α1-3 mannobiose	2.50
methyl β-mannoside	3.13
α1-2 mannobiose	10.0
α1-4 mannobiose	10.0
methyl α-glucoside	12.5
mannose	12.5
lacturose	12.5
N-acetylglucosamine	50
fructose	50
2-deoxy-2-fluoro-D-mannose	50
glucose	100
2-deoxy-glucose	100
	(μg/ml)
Thyroglobulin	3.91
Ribonuclease B	7.81
Asialo PSM[c]	7.81
PSM	15.6
IgA	31.3
Mannan	31.3
IgM	62.5
Asialo BSM[d]	62.5
Asialo fetuin	125
BSM	125
IgG	250
Albumin (chicken egg)	250
Fetuin	250
Lactoferrin	250

[a] Galactose, fucose, L-fucose, arabinose, L-arabinose, ribose, L- rhamnose, xylose, raffinose, saccharose, lactose, lactitol, ribulose, *N*-acetylgalactosamine, *N*-acetyllactoosamine, methyl β-glucoside, methyl α-galactoside, melibiose, galacturonic acid, glucosamine hydrochloride, galactosamine hydrochloride, mannosamine hydrochloride, gluconic acid, chitobiose, maltose, 2-deoxy-galactose, 2-deoxy-ribose and 2-deoxy-2-fluoro-D-glucose did not inhibit at all at concentrations up to 00 mM. *N*-Acetylneuraminic acid and *N*-glycolylneuraminic acid did not inhibit at concentrations up to 40 mM. α_1-Acid glycoprotein (human), transferrin (human), hyalronic acid and albumin (bovine) did not inhibit at all at concentrations up to 1 mg/ml.

[b] Minimum inhibitory concentrations required for inhibition 4 hemagglutination doses of the lectin.

[c] PSM : porcine stomach mucin.

[d] BSM : bovine submaxillary glands mucin.

Table IV. Comparison of sugar-binding specificity of HRL with those of other Man/Glc-specific lectins

	MIC[a]						
Inhibitors	HRL	ConA	PSA	BanLec	GNA	HHA	TxLc-I
	(mM)						
Glucose	100	50	25	12.5	>100	>100	>100
mannose	12.5	12.5	12.5	3.13	25	25	>100
methyl α-mannoside	3.13	3.13	12.5	3.13	12.5	12.5	>100
isomaltose	0.156	12.5	25	1.25	>40	>40	>40
isomaltotriose	0.313	25	25	2.5	>40	>40	>40
isomaltotetraose	0.313	25	25	5.0	>40	>40	>40
isomaltohexaose	0.313	25	25	12.5	>40	>40	>40
isomaltoheptaose	0.313	25	25	12.5	>40	>40	>40
α1-2 mannobiose	10.0	1.25	1.25	1.25	10.0	10.0	>30
α1-3 mannobiose	2.50	2.5	5.0	1.25	10.0	10.0	>30
α1-4 mannobiose	10.0	2.5	5.0	5.0	20.0	20.0	>30
α1-6 mannobiose	0.0391	2.5	5.0	2.5	10.0	10.0	>30
2-deoxy-glucose	100	25	50	>100	>100	>100	>100
2-deoxy-2-fluoro-D-glucose	>100	50	100	100	>100	>100	>100
2-deoxy-2-fluoro-D-mannose	50	50	50	>100	100	>100	>100
	(μg/ml)						
Thyroglobulin	3.91	3.91	15.6	7.81	125	125	125
Asialo-PSM	7.81	125	125	>500	62.5	62.5	62.5
PSM	15.6	250	250	>500	250	250	250
IgA	31.3	31.3	125	125	125	125	125
IgG	250	250	>500	>500	>500	>500	>500
Asialo-fetuin	125	250	125	>500	250	250	125
Fetuin	250	250	250	>500	125	125	>500

[a] Minimum inhibitor concentration required for inhibition of 4 hemagglutination dose of the lectins.