Biochemical and Biophysical Research Communications 377 (2008) 303–306

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A C-type lectin of *Caenorhabditis elegans*: Its sugar-binding property revealed by glycoconjugate microarray analysis

Tomoharu Takeuchi [a,*], Remi Sennari [a], Ken-ichi Sugiura [a], Hiroaki Tateno [b], Jun Hirabayashi [b], Ken-ichi Kasai [a]

[a] *Department of Biological Chemistry, School of Pharmaceutical Sciences, Teikyo University, 1091-1 Suarashi, Sagamiko, Sagamihara, Kanagawa 229-0195, Japan*
[b] *Lectin Application and Analysis Team, Research Center for Medical Glycoscience, AIST, Central 2, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan*

ARTICLE INFO

Article history:
Received 22 September 2008
Available online 9 October 2008

Keywords:
Lectin
C-type lectin
C-type lectin-like domain
Glycoconjugate microarray
Caenorhabditis elegans
Galb1-3GalNAc

ABSTRACT

C-type lectins are a family of proteins with an affinity to carbohydrates in the presence of Ca^{2+}. In the genome of *Caenorhabditis elegans*, almost 300 genes encoding proteins containing C-type lectin-like domains (CTLDs) have been assigned. However, none of their products has ever been shown to have carbohydrate-binding activity. In the present study, we selected 6 potential C-type lectin genes and prepared corresponding recombinant proteins. One of them encoded by clec-79 was found to have sugar-binding activity by using a newly developed glycoconjugate microarray based on evanescent-field excited fluorescence. CLEC-79 exhibited affinity to sugars containing galactose at the non-reducing terminal, especially to the Galβ1-3GalNAc structure, in the presence of Ca^{2+}. Combined with structural information of the glycans of *C. elegans*, these results suggest that CLEC-79 preferentially binds to *O*-glycans *in vivo*.

Lectins are a group of proteins that recognize complex carbohydrates present in cells and tissues. They are involved in various regulatory phenomena, including cell–cell and cell–matrix interactions, based on their recognition of the carbohydrates. Animal lectins are classified into several families such as C-type lectin, galectin, and I-type lectin families [1,2]. The C-type lectins contain C-type carbohydrate recognition domains (C-type CRDs) and require Ca^{2+} for the binding of carbohydrates. In the genome of *Caenorhabditis elegans*, one of useful model animals to study the functions of lectins from the molecular level to the whole body level, almost 300 genes encoding proteins containing C-type lectin-like domain (CTLD) have been assigned. CTLDs do not necessarily correspond to C-type CRDs. A portion of CTLDs that exhibit Ca^{2+}-dependent carbohydrate-binding activity are termed as C-type CRDs [3,4]. Some *C. elegans* CTLD proteins have been reported to be involved in immunity and axon guidance [3,5]. Drickamer and Dodd suggested that 19 *C. elegans* CTLD-containing proteins may have carbohydrate-binding activity based on the deduced primary structures [6]. However, to the best of our knowledge, no report has appeared that confirms the carbohydrate-binding activity of any one of the CTLD-containing proteins in *C. elegans*.

In the present study, we successfully prepared the recombinant protein CLEC-79, a CTLD-containing protein in *C. elegans*, and demonstrated its carbohydrate-binding activity using a recently developed glycoconjugate microarray, an extremely sensitive and high-throughput tool that provide binding data for a wide variety of oligosaccharides [7]. CLEC-79 exhibited preferential binding to sugars containing β-galactoside, especially Galβ1-3GalNAc in the presence of Ca^{2+}.

Materials and methods

Construction of a recombinant CLEC-79 expression plasmid: The open-reading frame of CLEC-79 containing the EcoRI and XhoI sites, except for the region corresponding to the signal peptide predicted by SMART (http://smart.embl-heidelberg.de/), was amplified by PCR from the cDNA mixture prepared from mixed-stage culture of *C. elegans* N2 strain. The primers 5′-GAATTCCTCTTGCTCAATTCTG ATG-3′ and 5′-CTCGAGCTAGTCACAATTTTCCTC-3′ were used as forward and reverse primers, respectively. The PCR fragment thus obtained was cloned into the pGEM-T plasmid (Promega). The DNA fragment that had been produced from the above pGEM-T-CLEC-79 vector by digestion with the EcoRI and XhoI was inserted into the EcoRI and XhoI sites of the pET-Flag vector. The pET-Flag vector had been generated by inserting a fragment encoding the Flag-tag sequence into the NdeI and BamHI sites of pET-21a *Escherichia coli* expression vector (Novagen).

Preparation of recombinant CLEC-79 protein: The expression of the recombinant CLEC-79 protein was induced as described [8], with the exception that *E. coli* Rosetta-gami 2(DE3) cells (Novagen) transformed with pET-Flag-CLEC-79 plasmid were used in this study. The cells were then harvested and suspended in Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl), and

* Corresponding author. Fax: +81 42 685 3742.
E-mail address: t-take@pharm.teikyo-u.ac.jp (T. Takeuchi).

Abbreviations: CRD, carbohydrate recognition domain; CTLD, C-type lectin-like domain; TBS, Tris-buffered saline.

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.10.001

disrupted by sonication. After centrifugation, the precipitate containing inclusion body was washed with TBS containing 4% Triton X-100 and deionized water. The soluble form of Flag-CLEC-79 was obtained from the inclusion body after refolding process basically as described [9,10]. In brief, the prepared inclusion body was solubilized with a buffer consisting of 8 M urea, 10 mM dithiothreitol, and 50 mM Tris–HCl, pH 8.0. The concentration of solubilized protein was determined by using a Bio-Rad Protein Assay (Bio-Rad) using BSA as a standard. The protein concentration was then reduced to 7.5 μM by diluting with 8 M urea in 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 5 mM $CaCl_2$, and subjected to the refolding process. Urea was gradually removed by dialysis over 3 days at 4 °C during which the urea concentration of the dialysis buffers was reduced stepwise from 8 to 4, 2, 1, 0.5 M, and finally 0 M. At the 1 M and 0.5 M urea stages, 0.4 M L-arginine and 375 μM oxidized form of glutathione were added to the dialysis buffers.

Affinity chromatography on an asialofetuin-sepharose column: Affinity purification of Flag-CLEC-79 was performed on an asialofetuin-sepharose column prepared as described previously [8,11]. The solution after the refolding process was applied to an immobilized asialofetuin column. After extensive washing of the column with TBS-Ca (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM $CaCl_2$), the adsorbed protein was eluted with TBS-Ca containing 0.1 M lactose. The combined eluted fraction was subjected to ultrafiltration using Amicon® filter device (Millipore) to concentrate the protein and remove lactose. The Flag-CLEC-79 thus purified was used for subsequent analyses.

SDS–PAGE and Western blotting: The refolded Flag-CLEC-79 protein was subjected to SDS–PAGE and stained with Bio-Safe Coomassie (Bio-Rad). Silver staining was performed using a Silver Stain II Kit (Wako). Western blotting was performed as described previously [12].

Glycoconjugate microarray analysis: Preparation of glycoconjugate microarray and analysis of the sugar-binding specificity of CLEC-79 were performed basically as described previously [7]. Structures of the glycoproteins and glycoside-polyacrylamide (PAA) spotted on the microarray are shown in Supplementary Table I. Flag-CLEC-79, at a final concentration of 2.5 ng/μl, was precomplexed with anti-Flag-tag M2 antibody and Cy3-labeled anti-mouse IgG antibody in probing buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% Triton-X100) containing 1 mM $CaCl_2$ or 5 mM ethylenediamine tetraacetic acid (EDTA). For the sugar inhibition assay, probing buffer containing 1 mM $CaCl_2$ and 25 mM sugar was used. The precomplex solution (100 μl) was applied to each chamber of the glass slide where dozens of glycans and oligosaccharides had been spotted and the slide was incubated at 20 °C for 16 h. The slide was then scanned using an evanescent-field activated fluorescence scanner (SC-Profiler; Moritex). Data were analyzed by Array Pro analyzer Ver. 4.5 (Media Cybernetics). The net intensity value for each spot was determined by subtracting the background value from the signal intensity. Data are shown as averages of triplicate determinations. Error bars represent standard deviations.

Results and discussion

Preparation of recombinant Flag-CLEC-79

We selected 6 candidate CTLD genes for producing recombinant proteins (clec-50, clec-51, clec-79, clec-97, clec-103, and clec-126) considering the sequences corresponding to their putative sugar-binding sites. However, we failed to obtain correctly folded active proteins for 5 of the 6 candidates used. We successfully obtained active protein product from clec-79 alone. CLEC-79 has a calculated molecular mass of about 65 kDa, and contains a signal sequence at the N-terminal portion and 2 CTLDs, one of which is considered to have carbohydrate-binding ability (Supplemental Fig. 1) [6]. The recombinant

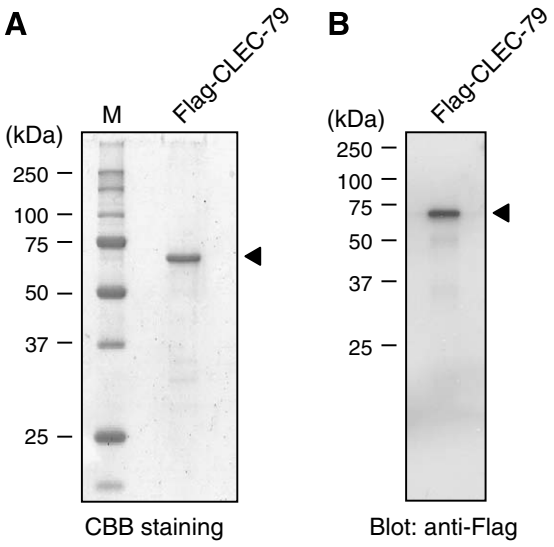

Fig. 1. SDS–PAGE and Western blotting of recombinant Flag-CLEC-79. Recombinant Flag-CLEC-79 was expressed in *E. coli*. After refolding of the inclusion body, the sample was subjected to SDS–PAGE and detected using Coomassie brilliant blue (CBB) (A) or anti-Flag-tag antibody (B). Arrowheads indicate the position of Flag-CLEC-79. M, molecular mass markers.

CLEC-79 protein was expressed as a fusion protein with a Flag-tag in *E. coli*. Since most portion of the recombinant proteins were mainly obtained in the insoluble fraction, the inclusion body was collected and solubilized under denaturing conditions. The solubilized Flag-CLEC-79 protein was subjected to the refolding process, and the product was analyzed by SDS–PAGE and Western blotting (Fig. 1). A single band was detected in both experiments, indicating that the homogenous recombinant Flag-CLEC-79 protein was obtained.

Affinity purification of Flag-CLEC-79

The monosaccharide preference of C-type lectins can be predicted from their primary structure [4]. If the sequence includes

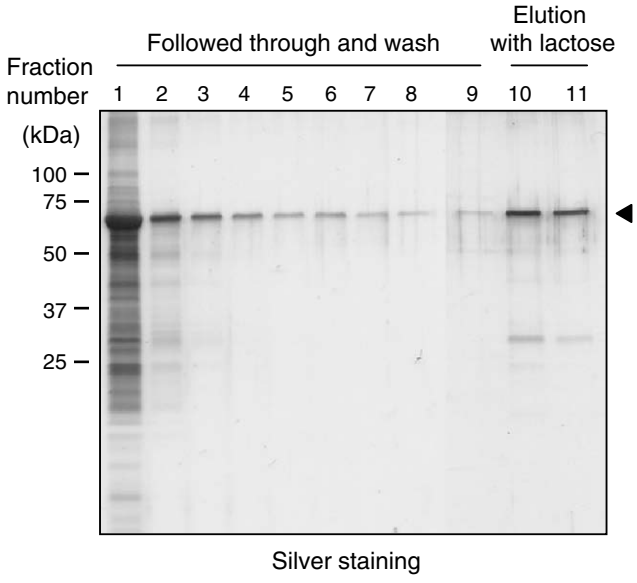

Fig. 2. Affinity purification of Flag-CLEC-79. The refolded, soluble Flag-CLEC-79 was applied to an immobilized asialofetuin column. Following extensive washing, the bound material was eluted with 0.1 M lactose. Of each fraction, 5 ml was collected, subjected to SDS–PAGE, and detected by silver staining. The arrowhead indicates the position of Flag-CLEC-79. The numbers on the left of the panel are the molecular masses of standard proteins.

EPN at the specificity determining motif, the protein will be specific for mannose, and if the tripeptide sequence is substituted by QPD, the protein will be specific for galactose. Since CLEC-79 does not contain neither of these motif, but contains SPN at that position, it was not possible to predict its monosaccharide-binding specificity [6]. Since a preliminary experiment using a glycoconjugate microarray suggested that Flag-CLEC-79 preferentially binds to sugars containing β-galactoside at the non-reducing terminal, we performed affinity purification in order to obtain its active form. The refolded protein was applied to an immobilized asialofetuin column, and the bound molecules were specifically eluted with lactose (Galβ1-4Glc) (Fig. 2). Although a portion of protein was detected in the flow-through and washed fractions, a certain amount of protein was bound to the affinity adsorbent and specifically eluted (fractions 10 and 11). The refolded active Flag-CLEC-79 protein thus prepared was used for subsequent analysis.

Sugar-binding specificity of Flag-CLEC-79 analyzed by glycoconjugate microarray technique

We applied a recently developed glycoconjugate microarray analysis [7] to Flag-CLEC-79. Glycoconjugate microarray is an extremely high-throughput device which generates binding data for dozens of oligosaccharides, and it requires extremely small amounts of fluorescence-labeled lectin (less than 100 ng). Furthermore, since the detection principle of this technique is based on evanescent-field activated fluorescence, it is possible to omit the washing steps and detect only the bound lectin molecules. Therefore, it is possible to detect very weak binding that might be missed due to the washing steps. The recombinant Flag-CLEC-79 protein was premixed with anti-Flag-tag antibody and Cy3-labeled anti-mouse IgG antibody in probing buffers containing Ca^{2+} or EDTA with or without various sugars. These solutions were applied to the array and the array was incubated at 20 °C for 16 h. Then, the array was directly applied to a scanner without any washing step. As shown in Fig. 3A, in the presence of Ca^{2+}, Flag-CLEC-79 bound to various glycans. However, no binding was observed in the presence of EDTA, galactose, or lactose. On the other hand, mannose or sucrose exhibited no influence on the binding of Flag-CLEC-79 to glycans. These results clearly indicated that CLEC-79 recognizes the non-reducing terminal galactose residues of glycans in a Ca^{2+}-dependent manner. Fig. 3B is a bar graph expressing intensities of each spot of the image obtained in the presence of Ca^{2+}. Higher amounts of CLEC-79 were found to bind to asialo glycoproteins containing complex-type asialo *N*-glycans (**43–45**), while no binding was observed to their sialylated or agalactosylated forms

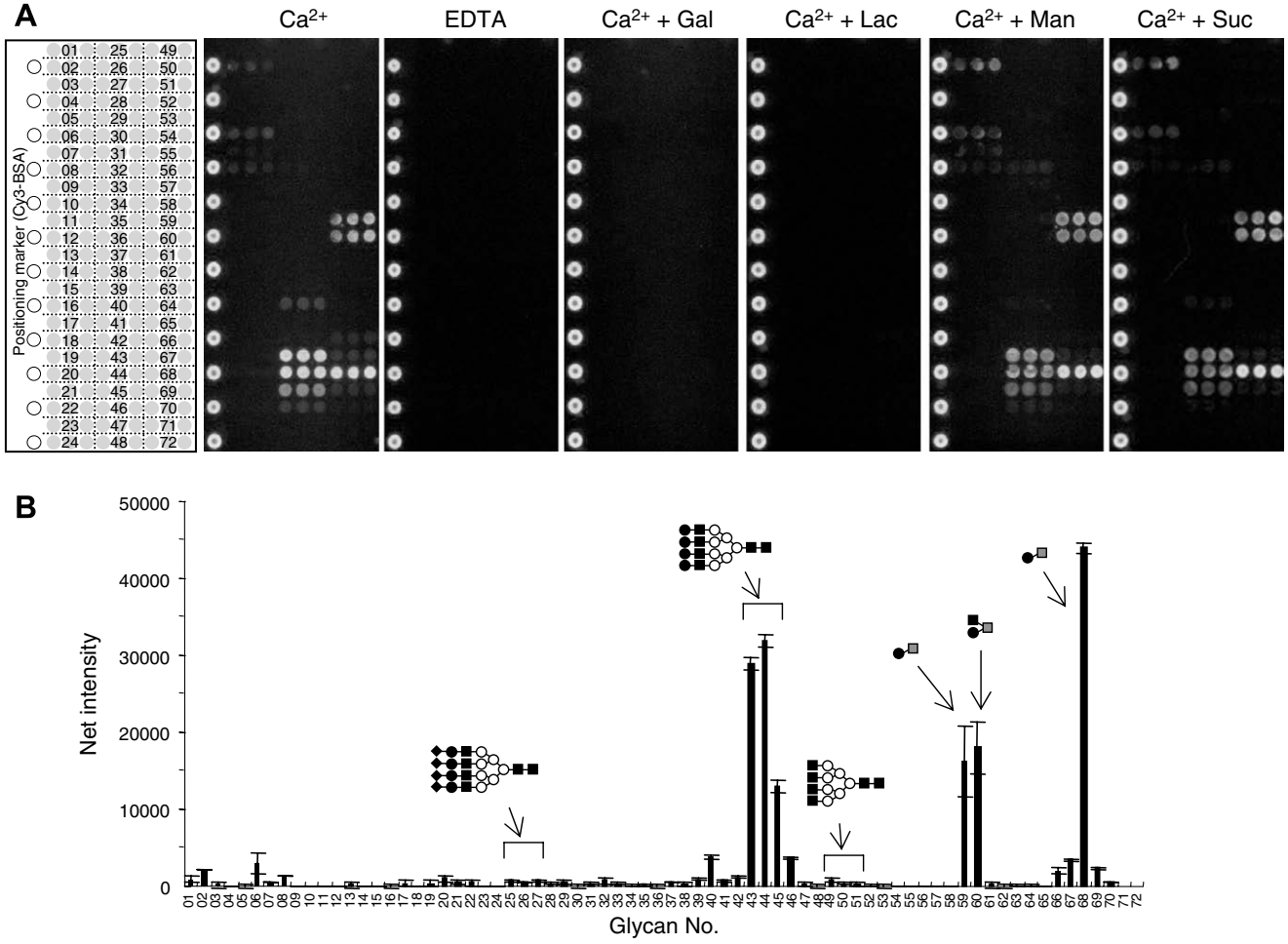

Fig. 3. Glycoconjugate microarray analysis of Flag-CLEC-79. (A) Flag-CLEC-79 precomplexed with anti-Flag-tag antibody and Cy3-labeled anti-mouse IgG antibody was applied to the glycoconjugate microarray spotted with a variety of glycans (Supplementary Table I). The binding experiment was performed using the following probing buffers containing Ca^{2+} (1 mM $CaCl_2$), EDTA (5 mM EDTA), Ca^{2+} + Gal (1 mM $CaCl_2$, and 25 mM galactose), Ca^{2+} + Lac (1 mM $CaCl_2$, and 25 mM lactose), Ca^{2+} + Man (1 mM $CaCl_2$, and 25 mM mannose), and Ca^{2+} + Suc (1 mM $CaCl_2$, and 25 mM Sucrose), respectively. (B) The scan image obtained in the presence of Ca^{2+} was analyzed with the Array Pro analyzer ver. 4.5. The net intensity value for each spot was determined as the signal intensity minus the background value. Data are the averages of triplicate determinations with the error bars representing standard deviation. Some examples of glycan structures are shown in the figure. Open circle, mannose; filled circle, galactose; gray square, *N*-acetylgalactosamine; filled square, *N*-acetylglucosamine; filled diamond, sialic acid. Positive signals: **43**, asialo fetuin; **44**, asialo α1-acid glycoprotein; **45**, asialo transferrin; **59**, core 1; **60**, core 2; **68**, asialo bovine submaxillary mucin.

(**25–27**, **49–51**). Among the glycan spots, CLEC-79 bound markedly to sugars having Galβ1-3GalNAc unit (**59**, **60**). However, the binding was weak for Galβ1-4Glc, Galβ1-3GlcNAc and Galβ1-4GlcNAc (**31**, **33** and **35**). CLEC-79 scarcely bound to GalNAcβ1-3GalNAc and Galα1-3GalNAc (**40** and **65**). The strongest signal was observed in asialo bovine submaxillary mucin (**68**), which is known to contain multiple Galβ1-3GalNAc units such as core 1 (Galβ1-3GalNAc) and core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc) in addition to Tn (GalNAc) and GlcNAcβ1-3GalNAc [13]. These data indicate that CLEC-79 recognizes sugars containing non-reducing terminal β-galactoside, especially those having the Galβ1-3GalNAc unit.

This is the first study that revealed the sugar-binding specificity of a C-type lectin of *C. elegans*. To the best of our knowledge, the Galβ1-3GalNAc structure has been found in *O*-glycans of *C. elegans*, but not in *N*-glycans or glycolipids [8,14-17]. This suggests that CLEC-79 interacts with *O*-glycans *in vivo*, although its significance is yet to be elucidated. A search for the endogenous ligands of CLEC-79 and their subsequent structural analysis will be needed. The present work clearly demonstrated that CLEC-79 binds to galactose residues, suggesting that an uncommon SPN sequence in the specificity-determining motif corresponds to the galactose-binding ability. In this respect, X-ray analysis of the complex between CLEC-79 and Galβ1-3GalNAc is of particular interest.

Acknowledgments

We are grateful to Drs. Yoichiro Arata, Yoko Nemoto-sasaki, and Atsushi Yamashita for helpful discussions. We also thank Takanori Igarashi and Atsuko Nagata for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.001.

References

[1] N. Sharon, H. Lis, History of lectins: from hemagglutinins to biological recognition molecules, Glycobiology 14 (2004) 53R–62R.
[2] D.C. Kilpatrick, Animal lectins: a historical introduction and overview, Biochim. Biophys. Acta 1572 (2002) 187–197.
[3] H. Schulenburg, M.P. Hoeppner, J. Weiner 3rd, E. Bornberg-Bauer, Specificity of the innate immune system and diversity of C-type lectin domain (CTLD) proteins in the nematode *Caenorhabditis elegans*, Immunobiology 213 (2008) 237–250.
[4] A.N. Zelensky, J.E. Gready, The C-type lectin-like domain superfamily, FEBS J. 272 (2005) 6179–6217.
[5] G. Kulkarni, H. Li, W.G. Wadsworth, CLEC-38, a transmembrane protein with C-type lectin-like domains, negatively regulates UNC-40-mediated axon outgrowth and promotes presynaptic development in *Caenorhabditis elegans*, J. Neurosci. 28 (2008) 4541–4550.
[6] K. Drickamer, R.B. Dodd, C-Type lectin-like domains in *Caenorhabditis elegans*: predictions from the complete genome sequence, Glycobiology 9 (1999) 1357–1369.
[7] H. Tateno, A. Mori, N. Uchiyama, R. Yabe, J. Iwaki, T. Shikanai, T. Angata, H. Narimatsu, J. Hirabayashi, Glycoconjugate microarray based on an evanescent-field fluorescence-assisted detection principle for investigation of glycan-binding proteins, Glycobiology 18 (2008) 789–798.
[8] T. Takeuchi, K. Hayama, J. Hirabayashi, and K.I. Kasai, *Caenorhabditis elegans* *N*-glycans containing a Gal-Fuc disaccharide unit linked to the innermost GlcNAc residue are recognized by *C. elegans* galectin LEC-6, Glycobiology (2008), doi: 10.1093/glycob/cwn077.
[9] R. Asano, T. Kudo, K. Makabe, K. Tsumoto, I. Kumagai, Antitumor activity of interleukin-21 prepared by novel refolding procedure from inclusion bodies expressed in *Escherichia coli*, FEBS Lett. 528 (2002) 70–76.
[10] M. Umetsu, K. Tsumoto, K. Ashish, S. Nitta, Y. Tanaka, T. Adschiri, I. Kumagai, Structural characteristics and refolding of in vivo aggregated hyperthermophilic archaeon proteins, FEBS Lett. 557 (2004) 49–56.
[11] Y. Arata, J. Hirabayashi, K. Kasai, The two lectin domains of the tandem-repeat 32-kDa galectin of the nematode *Caenorhabditis elegans* have different binding properties. Studies with recombinant protein, J. Biochem. 121 (1997) 1002–1009.
[12] T. Takeuchi, H. Yokosawa, Detection and analysis of protein ISGylation, Methods Mol. Biol. 446 (2008) 139–149.
[13] T. Tsuji, T. Osawa, Carbohydrate structures of bovine submaxillary mucin, Carbohydr. Res. 151 (1986) 391–402.
[14] Y. Guerardel, L. Balanzino, E. Maes, Y. Leroy, B. Coddeville, R. Oriol, G. Strecker, The nematode *Caenorhabditis elegans* synthesizes unusual *O*-linked glycans: identification of glucose-substituted mucin-type *O*-glycans and short chondroitin-like oligosaccharides, Biochem. J. 357 (2001) 167–182.
[15] K. Paschinger, M. Gutternigg, D. Rendic, I.B. Wilson, The *N*-glycosylation pattern of *Caenorhabditis elegans*, Carbohydr. Res. 343 (2008) 2041–2049.
[16] S. Gerdt, R.D. Dennis, G. Borgonie, R. Schnabel, R. Geyer, Isolation, characterization and immunolocalization of phosphorylcholine-substituted glycolipids in developmental stages of *Caenorhabditis elegans*, Eur. J. Biochem. 266 (1999) 952–963.
[17] J.S. Griffitts, S.M. Haslam, T. Yang, S.F. Garczynski, B. Mulloy, H. Morris, P.S. Cremer, A. Dell, M.J. Adang, R.V. Aroian, Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin, Science 307 (2005) 922–925.