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ARTICLE

Stable isotope labeling of glycoprotein expressed in silkworms using immunoglobulin G as a test molecule

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Abstract Silkworms serve as promising bioreactors for the production of recombinant proteins, including glycoproteins and membrane proteins, for structural and functional protein analyses. However, lack of methodology for stable isotope labeling has been a major deterrent to using this expression system for nuclear magnetic resonance (NMR) structural biology. Here we developed a metabolic isotope labeling technique using commercially available silkworm larvae. The fifth instar larvae were infected with baculoviruses for co-expression of recombinant human immunoglobulin G (IgG) as a test molecule, with calnexin as a chaperone. They were subsequently reared on an artificial diet containing ^{15}N-labeled yeast crude protein extract. We harvested 0.1 mg of IgG from larva with a ^{15}N-enrichment ratio of approximately 80 %. This allowed us to compare NMR spectral data of the Fc fragment cleaved from the silkworm-produced IgG with those of an authentic Fc glycoprotein derived from mammalian cells. Therefore, we successfully demonstrated that our method enables production of isotopically labeled glycoproteins for NMR studies.

Keywords Silkworm · Isotope labeling · Glycoprotein · Artificial diet · Immunoglobulin G · Fc

Abbreviations

BmNPV	*Bombyx mori* nuclleopolyhedrovirus
EDTA	Ethylenediaminetetraacetic acid
Fuc	Fucose
Gal	Galactose
GlcNAc	*N*-Acetylglucosamine

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HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single-quantum coherence
IgG	Immunoglobulin G
LC/MS	Liquid chromatography/mass spectroscopy
Man	Mannose
NMR	Nuclear magnetic resonance
PTFE	Polytetrafluoroethylene
TCA	Trichloroacetic acid

Introduction

In structural biology and structural proteomics, choosing a protein expression system is one of the most crucial factors. Although bacterial expression of recombinant proteins is one of the most widely used methods because of its convenience in terms of low cost, ease of manipulation, and high expression level, this system cannot deal with post-translational modifications such as glycosylation. Consequently, most of the protein structures deposited in the Protein Data Bank lack information on glycosylation, although more than half of the proteins in nature are actually glycosylated (Kamiya et al. 2014). In addition, correct disulfide bond formation is often hampered in *Escherichia coli*, although an ameliorated expression system has been developed for facilitating disulfide bridge formation by protein transportation to the periplasmic space or by expression with a folding catalyst (de Marco 2009). Furthermore, numerous human recombinant proteins form inclusion bodies in *E. coli*. To overcome these issues, protein expression has been attempted using various production vehicles including those derived from yeasts, insects, mammals, and plants (Demain and Vaishnav 2009; Jarvis 2009; Kamiya et al. 2014; Yagi et al. 2015; Zhu 2012). However, these expression systems generally show low productivity at high cost.

Baculovirus-infected silkworm larvae and pupae serve as highly efficient and cost-effective bioreactors for the production of heterologous proteins with posttranslational modifications, including glycosylation (Hiyoshi et al. 2007; Kato et al. 2010b; Motohashi et al. 2005). In this system, the genes of target proteins are inserted into the *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid as a shuttle vector under the control of polyhedrin promoter and then injected into silkworm larvae and pupae for protein production. The proteins are expressed in their hemolymph and fat bodies. The expression of multi-subunit complexes can be achieved simply by injection of a mixture of BmNPV bacmids into silkworms, without prior virus amplification using cultured cells. Silkworms as protein-production factories are extremely useful because of their ability to produce large quantities of high-molecular-weight membrane and soluble proteins with glycosylation in a cost- and effort-saving manner. There have been successful, promising examples of expression of glycoproteins in silkworms including that of immunoglobulins (Igs) (Dojima et al. 2010; Park et al. 2009), mammalian Golgi enzymes (Dojima et al. 2009), and cell surface receptors (Sasaki et al. 2009), which has opened the door for structural analyses of proteins that had been difficult to produce in bacterial expression systems. However, a major drawback of using silkworm-based protein expression systems is the lack of methodology for stable isotope labeling, which is crucial for nuclear magnetic resonance (NMR) protein analyses.

Thus far, metabolic stable isotope labeling has been developed for NMR applications in eukaryotic expression systems including yeast cells, mammalian cells, *Dictyostelium discoideum*, and baculovirus-infected insect Sf9 cells (Dutta et al. 2012; Kamiya et al. 2013; Ohki et al. 2008; Saxena et al. 2012; Skrisovska et al. 2010; Takahashi and Shimada 2010). We have also contributed to this development by primarily using IgG glycoproteins as a model molecule (Kato and Yamaguchi 2011; Kato et al. 2010a; Yagi et al. 2014; Yamaguchi and Kato 2010; Yamaguchi et al. 2006). In this study, we developed a method for stable isotope labeling of IgG using a silkworm expression system with the intent of expanding the application of this system for use in biomolecular NMR research fields.

Materials and methods

Preparation of an artificial diet for silkworm rearing

A protein mixture derived from the yeast strain, *Candida utilis* NBRC 0396, was added to an artificial diet for silkworm rearing. The yeast cells were grown for 22 h in a culture medium that contained ^{15}N-labeled ammonium sulfate as nitrogen source (Table 1). After cultivation, the cells were collected by centrifugal separation, washed twice with ion exchanged water, frozen in liquid nitrogen and subsequently lyophilized in a freeze dryer. Approximately 120 g of ^{15}N-labeled *C. utilis* cells were dried, finely ground, and placed in a 5-L pear-shaped flask; to this, 2 L of 10 % aqueous solution of trichloroacetic acid (TCA) was subsequently added. The mixture was subjected to an extraction with reflux by heating to 100 °C for 1.5 h for extraction/removal of water-soluble, low molecular weight components. Then the mixture was centrifuged at 12,000*g* for 30 min to remove the supernatant. Ethanol was then added to wash the obtained precipitates. The suspension was centrifuged at 12,000*g* for 30 min, and the resulting precipitates were recovered. After repeating this

Table 1 Composition of culture medium for ^{15}N labeling of *C. utilis*

Substances	Substance % (w/v)
D-glucose (or D-glucose-$^{13}C_6$)	0.5
Ammonium-^{15}N sulfate	0.25
Yeast nitrogen base without amino acid & ammonium sulfate (manufactured by Difco Laboratories)	0.17

procedure twice, the precipitates were dried at 30 °C in a vacuum dryer for 2 days.

The dried extract from *C. utilis* was placed in a 5-L pear-shaped flask, to which 2 L of a mixed solvent of methanol and chloroform (2:1) was added. Then, the mixture was subjected to an extraction with reflux by heating for 1.5 h at 90 °C using an oil bath for defatting. Next, the mixture was cooled to room temperature and then filtered using a polytetrafluoroethylene (PTFE) membrane filter (5.0-μm pore size; Merck Millipore). Approximately 40 g of the ^{15}N-labeled crude protein fraction derived from the yeast cells was obtained after washing with methanol and then with diethyl ether, followed by dehydration and drying.

The artificial silkworm diet containing the abovementioned ^{15}N-labeled yeast crude protein extract was prepared using the composition shown in Table 2, uniformly kneaded and then steamed.

Table 2 Composition of the artificial diet used for cultivating silkworms

Substances	% of dry diet
Yeast protein	40.0
Mulberrry leaf powder	10.0
Glucose	12.0
Soybean oil	3.0
Phytosterol	0.3
Ascorbic acid	2.0
Sorbic acid	0.2
Agar	10.0
Salt mixture[a]	4.2
Vitamin B mixture[b]	0.4
Cellulose powder	17.9
(Total)	(100.0)
Antiseptics[c]	Added
Distilled water	3 ml/g diet

[a] See (Hirayama et al. 1996)

[b] See (Horie et al. 1966)

[c] Antiseptics consisted of chloramphenicol (0.015 % in diet) and propionic acid (0.75 % in diet)

Amino acid analysis

For amino acid analysis, the yeast crude protein extract was heated for 24 h at 110 °C to hydrolyze proteins to amino acids. After cooling to room temperature, the hydrolyzate was filtered through a PTFE membrane filter (0.45-μm pore size). The solution was transferred into an egg-plant shaped flask, and unreacted HCl was removed by evaporation under a vacuum at 40 °C. The dried residue was dissolved in Milli-Q water.

The amino acid components derived from yeast crude proteins were quantitatively analyzed according to the Waters AccQ-Tag method (Cohen and Michaud 1993). Ten microliters of sample mixed with 70 μl of borate buffer solution was derivatized with 20 μl of Waters AccQ-Fluor Reagent at 55 °C for 10 min. One microliter of the derivatized solution was injected into a Waters Acquity Ultra Performance Liquid Chromatography (LC) with an ion-exchange column (AccQ-Tag amino acid analysis column, AccQ-Tag Ultra, 2.1 × 100 mm, Waters). The amino acid derivatives were detected using fluorescence at 395 nm. Chromatograms were processed using Empower software (Waters). Concentrations of individual amino acids were calculated from peak area using calibration curves for each amino acid prepared with AccQ-Tag Ultra Standards (Waters).

Expression and purification of ^{15}N-labeled IgG from larval silkworm hemolymph

Recombinant human anti-bovine serum albumin IgG1 was produced in the silkworms using rBmNPV bacmid systems as described previously (Dojima et al. 2010; Park et al. 2009). Their variable regions, which originated from the human single chain Fv 29IJ6, (Aburatani et al. 2002; de Wildt et al. 2000) were linked to genes for the heavy chain contestant region of human IgG1 and the human λ light chain region, which were incorporated into pfastBac Dual vector (Life Technologies). A cysteine protease- and chitinase-deficient *B. mori* multiple nucleopolyhedrovirus (BmNPV-*CP*$^-$-*Chi*$^-$) bacmid was employed in order to suppress possible degradation of the expressed proteins (Park et al. 2008). *E.coli* DH10Bac Bm-*CP*$^-$-*Chi*$^-$ was transformed with the pFastBac Dual/29IJ6 IgG and then cultivated in a LB plate containing kanamycin, gentamycin, tetracycline, 5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside (XGal) and isopropyl-β-$_D$-1-thiogalactopyranoside (IPTG). White colonies were selected for isolation of the positive clones harboring the BmNPV bacmid. Baculovirus-containing hemolymph was collected from the larvae injected with BmNPV bacmid/29IJ6 IgG and BmNPV bacmid/human calnexin, diluted with 50-fold phosphate-buffered saline (PBS), and then injected into

fifth-instar hybrid Kinsyu × Syowa silkworm larvae (Ehime Sansyu, Yahatahama, Japan) on the second day of fifth instar for expressing the IgG glycoprotein. The larvae were reared on an ^{15}N-labeled artificial diet every 12 h at 27 °C during each 12 h light and dark cycle. After the larvae were fed the ^{15}N-labeled artificial diet for 96 h, hemolymph from 60 larvae was collected. The collected hemolymph was immediately added to sodium thiosulfate solution to prevent oxidation and then diluted 10 times using PBS.

Purification of IgG was performed as previously described (Yamaguchi et al. 1995, 2006). In brief, the hemolymph from larvae was centrifuged at 7300*g* for 10 min and diluted tenfold using PBS. The diluted sample was centrifuged at 19,000*g* for 30 min, filtered using a 0.45-μm nitrocellulose membrane filter, and purified using an Affi-Gel protein A column (GE Healthcare Bio-Sciences). After this initial purification, IgG was further purified by gel filtration chromatography using a Superose 12 column (GE Healthcare Bio-Sciences).

Purification of the Fc fragment

The IgG-Fc fragment was prepared as previously described (Yamaguchi et al. 1995, 2006). IgG was cleaved by papain digestion, performed at 37 °C for 12 h in 75 mM sodium phosphate buffer [pH 7.0; containing 75 mM NaCl and 2 mM ethylenediaminetetraacetic acid (EDTA)]. The protein concentration was 10 mg/mL and the papain:IgG ratio was 1:50 (w/w). For purification of the Fc fragment, digestion products were loaded onto an Affi-Gel protein A column and then applied to a Superose 12 gel filtration column.

Nuclear magnetic resonance measurements

For NMR measurements, the Fc fragment was dissolved in 0.5 mL of 5 mM sodium phosphate buffer [pH 6.0; containing 50 mM NaCl and 10 % (v/v) D_2O]. Two-dimensional ^{1}H–^{15}N heteronuclear single-quantum coherence (HSQC) spectral data were acquired at 42 °C using an AVANCE 800 spectrometer equipped with a cryogenic probe (Bruker BioSpin). NMR peak assignments for the residues of IgG1-Fc were obtained from a previous study (Yagi et al. 2014; Yamaguchi et al. 2006). Chemical shifts of ^{1}H were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (0 ppm), and ^{13}C and ^{15}N chemical shifts were referenced indirectly using the gyromagnetic ratios of ^{13}C, ^{15}N, and ^{1}H ($\gamma^{13}C/\gamma^{1}H = 0.25144952$; $\gamma^{15}N/\gamma^{1}H = 0.10132905$). All NMR data were processed using NMRPipe software (Delaglio et al. 1995) and analyzed using SPARKY (Goddard and Kneller 1993) and CCPNMR (Vranken et al. 2005) softwares.

Measuring ^{15}N enrichment using liquid chromatography and mass spectrometry

^{15}N-labeled amino acids in the artificial diet

Chromatographic separation was achieved using reverse phase chromatography with gradient elution. The chromatography system consisted of an LC800 separation module (GL Sciences) coupled with an API2000 mass spectrometer equipped with an electrospray ionization source (ABsciex). The LC system and mass spectrometer were both controlled by Analyst 1.6 software (ABsciex).

Data were acquired in the negative ion mode between *m/z* 50 and 450 (350 °C gas temperature, 4500 V ion spray gas, 50 psi nebulizer gas pressure, 40 psi curtain gas pressure, and 80 psi turbo gas pressure). LC800 separation module with an autosampler was used with sample cooler (set to 4 °C) and column oven (set to 35 °C). For separation, an Intrada Amino Acid column [100 × 2.0 mm (i.d.); 3-μm particle size; Imtakt] was used at a flow rate of 0.2 mL/min. The mobile phase consisted of acetonitrile:tetrahydrofuran:25 mM ammonium formate:formic acid [9:75:16:0.3 (v/v/v/v); A] and acetonitrile:100 mM ammonium formate [20/80 (v/v); B]. A gradient was run from 0 to 17 % B in 12–26 min, 17–100 % B in 26–45 min.

^{15}N-labeled glycoprotein produced in silkworms

The ^{15}N-labeled IgG glycoprotein (100 μg) was dissolved in 50 μL of 0.5 M Tris–HCl (pH 8.6) containing 7 M guanidine-HCl and 5 mM EDTA. After adding 2.0 μL of 1 M dithiothreitol, this solution was incubated at 65 °C for 30 min. Subsequently, 1 M sodium monoiodoacetate (4.8 μL) was added to the solution and then the mixture was incubated at room temperature for 40 min in the dark. The reaction mixture was desalted using a PD10 column (GE Healthcare Bio-Sciences, Uppsala, Sweden), and the solution containing proteins was freeze-dried. The carboxymethylated proteins were dissolved in 20 μL of bicarbonate buffer (pH 8.5) and incubated with 1 μg of trypsin (Trypsin Gold, Mass Spec Grade, Promega, Madison, WI, USA) at 37 °C for 16 h. The solution was dried using a SpeedVac, dissolved in 0.1 % formic acid, and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis. LC/MS was performed using an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) connected to a Paradigm MS4 high-performance liquid chromatography (HPLC) system (Michrom BioResources, Auburn, CA, USA), which was equipped with a reversed-phase column (L-column 2 ODS, 150 × 0.075 mm, 3 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase was composed of 0.1 % formic acid containing 2 % acetonitrile

(Buffer A) and 0.1 % formic acid containing 90 % acetonitrile (Buffer B). The peptides were eluted at a flow rate of 300 nL/min with a gradient of 2–65 % Buffer B for 50 min. MS/MS conditions were as follows: 2.5 kV electrospray voltage in positive ion mode, 275 °C capillary temp, and 35 % MS/MS collision energy. The spectral data obtained by MS/MS were subjected to database search analysis with the SEQUEST algorithm (Proteome Discoverer 1.4; Thermo Fisher Scientific) using an inhouse database containing amino acid sequences of anti-BSA antibody. Carboxymethylation (58.0 Da) at cysteine residues was used as the static modification parameter for the database search analysis.

Table 3 Amino acid composition of the yeast-derived protein mixture

Amino acids	mol %
Alanine	9.3
Arginine	4.23
Aspartic acid	11.09
Glutamic acid	11.45
Glycine	7.23
Histidine	2.10
Isoleucine	5.20
Leucine	8.55
Lysine	7.45
Methionine	1.39
Phenylalanine	4.06
Proline	4.21
Serine	7.11
Threonine	6.60
Tyrosine	3.29
Valine	6.77

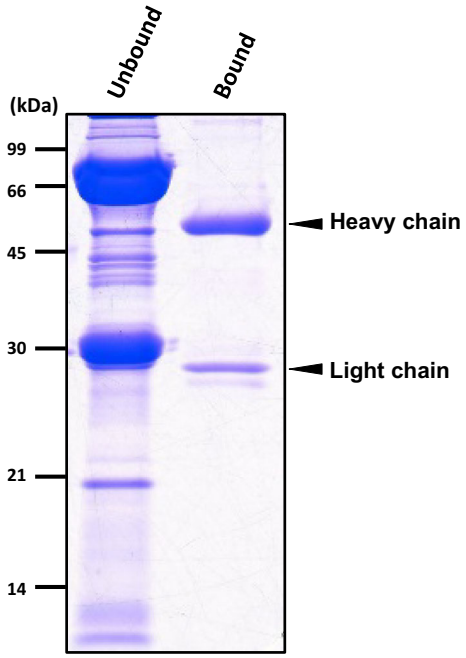

Fig. 1 Purification of IgG1 produced in silkworms reared with a ^{15}N-labeled artificial diet. Bound and unbound fractions of larval silkworm hemolymph to protein A columns were subjected to 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. *Arrowheads* indicate the positions of heavy or light chains

Table 4 MS data used for evaluating the ^{15}N-labeling ratio of amino acids in the artificial diet

Amino acids	RT (min)	Nonlabeled		^{15}N-labeled		Label (%)
		m/z	Peak area (cps)	*m/z*	Peak area (cps)	
Alanine	14.99	90	14,400	91	3,190,000	99.6
Arginine	44.26	175	ND	179	1,380,000	~99
Aspartic acid	13.23	134	ND	135	1,860,000	~99
Cystine	ND	241	ND	243	ND	ND
Glutamic acid	10.6	148	ND	149	3,350,000	~99
Glycine	19.08	76	ND	77	1,420,000	~99
Histidine	41.05	156	ND	159	139,000	~99
Isoleucine	6.91	132	64,300	133	6,480,000	99.0
Leucine	6.31	132	107,000	133	8,160,000	98.7
Lysine	42.93	147	ND	149	908,000	~99
Methionine	6.57	150	ND	151	2,480,000	~99
Phenylalanine	4.93	166	113,000	167	5,850,000	98.1
Proline	11.27	116	69,700	117	3,470,000	98.0
Serine	17.45	106	20,500	107	2,120,000	99.0
Threonine	12.46	120	6021	121	4,480,000	99.9
Tyrosine	5.42	182	33,100	183	2,060,000	98.4
Valine	8.64	118	31,900	119	4,260,000	99.3

The average molecular masses of peptides were calculated using Eq. 1. The ^{15}N-labeling ratio was calculated as follows: (average molecular mass ^{15}N–average molecular mass ^{14}N)/the number of nitrogen atoms.

$$Average\ molecular\ mass = \frac{\sum_{k=1}^{n} M_k \cdot I_k}{\sum_{k=1}^{n} I_k} \quad (1)$$

M_k: mass calculated from *m/z* of *k*th isotopolog ion, I_k: intensity of *k*th isotopolog ion.

Glycosylation profiling

N-glycosylation profiling of IgG was performed by a previously described HPLC mapping technique (Park et al. 2009; Takahashi and Kato 2003; Yagi et al. 2010, 2012). ^{15}N-labeled IgG (0.5 mg) was used as a starting material.

Results

Production and evaluation of an isotope-labeled artificial diet for silkworms

C. utilis cells were cultured in ^{15}N-labeled medium (Table 1). After TCA precipitation and delipidation of the dried yeast, the ^{15}N-labeled yeast crude protein extract was prepared and subjected to amino acid analysis. The amino acid composition of the yeast proteins is shown in Table 3. Using the ^{15}N-labeled yeast proteins, the artificial diet for silkworms was prepared according to composition shown in Table 2. The ^{15}N-labeled artificial diet was subjected to LC/MS/MS analyses to evaluate the ^{15}N-enrichment ratios of amino acid components. Table 4 summarizes the MS data of amino acid components and their calculated ^{15}N-labeling ratios; the ^{15}N-labeling ratios were approximately 99 %.

Table 5 MS data used for evaluating the ^{15}N-labeling ratio of peptides in the trypsin digest of ^{15}N-labeled IgG produced in silkworms

Sequence	N	Charge	MH^+ [Da]	RT [min]	*m/z*	Δ*m/z*	Δmass	Label (%)
Heavy chain								
THTcPPcPAPELLGGPSVFLFPPKPK	30	3	2846.448	34.439	949.488	7.837	23.512	78
LScAASGFTFSSYAMSWVR	23	2	2128.957	37.470	1064.982	9.051	18.103	79
TPEVTcVVVDVSHEDPEVK	22	2	2140.022	28.40	1070.515	8.801	17.602	80
ScDKTHTcPPcPAPELLGGPSVFLFPPKPK	35	3	3337.607	34.193	1113.207	9.222	27.666	79
GFYPSDIAVEWESNGQPENNYK	28	3	2544.150	31.817	848.722	7.334	22.001	79
TTPPVLDSDGSFFLYSK	18	3	1873.930	32.903	625.315	4.735	14.204	79
VVSVLTVLHQDWLNGK	22	2	1808.018	36.077	904.513	8.532	17.064	78
FNWYVDGVEVHNAK	20	2	1677.812	27.447	839.410	7.949	15.898	79
STSGGTAALGcLVK	15	2	1322.671	25.582	661.839	5.859	11.718	78
AEDTAVYYcAK	12	2	1291.557	20.429	646.282	4.739	9.478	79
NTLYLQMNSLR	17	2	1352.707	28.278	676.857	6.650	13.299	78
GLEWVSHISPYGANTR	23	3	1786.901	28.569	596.305	5.992	17.977	78
EPQVYTLPPSRDELTK	21	2	1872.981	23.282	936.994	8.411	16.821	80
GPSVFPLAPSSK	13	2	1186.655	26.845	593.831	5.100	10.200	78
NQVSLTcLVK	13	2	1162.620	27.635	581.814	5.034	10.069	77
DTLMISR	10	2	835.437	20.956	418.222	3.905	7.810	78
YADSVK	7	1	682.343	9.705	682.343	5.487	5.487	78
ALPAPIEK	9	1	838.508	20.004	838.508	7.202	7.202	80
Light chain								
FSGSGSGTDFTLTISSLQPEDFATYYcQQGR	37	3	3421.532	36.89	1141.182	9.66536	28.9961	78
YAASSYLSLTPEQWK	18	2	1743.868	31.073	872.438	7.02245	14.0449	78
ATLVcLISDFYPGAVTVAWK	22	3	2212.145	43.084	738.053	5.63932	16.918	77
SYScQVTHEGSTVEK	19	2	1712.752	16.796	856.880	7.51431	15.0286	79
ASQSISSYLNWYQQKPGK	25	2	2085.046	29.286	1043.027	9.9657	19.9314	80
ANPTVTLFPPSSEELQANK	23	2	2043.053	30.085	1022.030	8.98426	17.9685	78
ASLLQSGVPSR	15	2	1114.628	22.174	557.818	5.90032	11.8006	79
TVAPTEcS	8	1	865.365	14.510	865.365	6.23754	6.23754	78

Purification of IgG from larval silkworm hemolymph

After collecting hemolymph from 60 larvae that were fed the ^{15}N-labeled artificial diet, IgG was purified from the fluids using protein A affinity chromatography (Fig. 1), followed by Superose 12 gel filtration. Consequently, 6 mg of IgG was purified from 60 larvae fed the isotope-labeled artificial diet prepared from approximately 120 g of ^{15}N-labeled *C. utilis* cells. The yield of IgG, i.e. 0.1 mg IgG from a single larva, is almost commensurate with normal rearing (Dojima et al. 2010).

Measurement of the ^{15}N-labeling ratio by tandem liquid chromatography/mass spectroscopy

The ^{15}N-labeled and nonlabeled IgG preparations were subjected to LC/MS/MS analyses to evaluate the ^{15}N-labeling ratio of this glycoprotein produced in silkworms fed the ^{15}N-labeled artificial diet. The silkworm-expressed IgGs were carboxymethylated, digested by trypsin, and subjected to LC/MS (Supplemental Figs. 1 and 2). Table 5 summarizes the assignments of detectable peptides based on MS/MS data, and their ^{15}N-labeling ratios. The ^{15}N-enrichment ratios were in the range of 78–80 %.

N-glycosylation profile of the ^{15}N-labeled antibody

N-glycans derived from the ^{15}N-labeled IgG glycoprotein were released using glycoamidase A, tagged with 2-aminopyridine, and subjected to HPLC profiling. Supplemental Fig. 3 shows the *N*-glycosylation profile on ODS column of the ^{15}N-labeled IgG. Table 6 summarizes the structures and incidence of individual *N*-glycans that were identified on the basis of their elution times and mass values using the GALAXY database (Takahashi and Kato 2003).

Approximately 90 % of total *N*-glycans derived from ^{15}N-labeled IgG produced in silkworms were fucosylated paucimannose-type *N*-glycans [Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc]. The data were consistent with previously reported *N*-glycosylation profiles of glycoproteins expressed by silkworms (Dojima et al. 2010; Park et al. 2009; Sasaki et al. 2009).

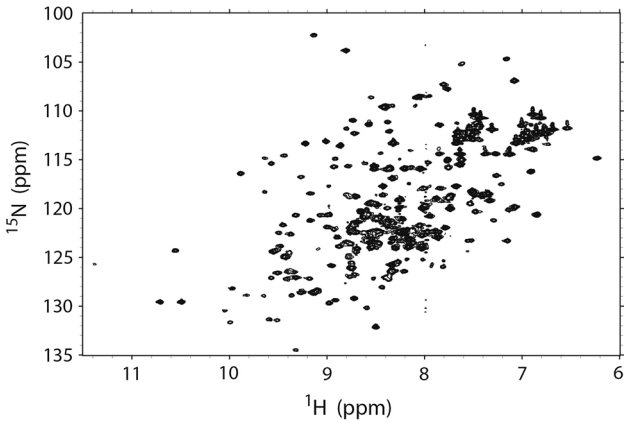

Fig. 2 ^{1}H–^{15}N HSQC spectra of the ^{15}N-labeled Fc fragment of the IgG glycoprotein produced in silkworms fed the isotope-labeled artificial diet

Table 6 The structure and incidence of PA-*N*-glycans derived from IgG produced in silkworms

Peak code no.	%[b]	ODS (GU)	Mass value (Da)	Structures
N1 M5.1	5.1	7.3	1313	Manα1-6 / Manα1-6 / Manα1-3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA / Manα1-3
N2 000.1	5.8	7.6	989	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc-PA / Manα1-3
N3 M2.1	1.0	7.8	827	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc-PA
N4-1[a] 010.1	52.2	10.5	1135	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc-PA / Manα1-3
N4-2[a] MF2.1	35.2	10.5	973	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc-PA
Others	0.7			

[a] Fraction N4 from the ODS column was separated into two subfractions on the amide column. The molar percentage of each glycan was calculated on the basis of peak areas in elution profiles on the amide column

[b] The ratio (mol %) was calculated from the peak areas in Supplemental Fig. 3. The relative quantities were estimated after considering the abundance of the epimeric byproducts of the pyridylamination reaction, which are indicated with double primes in Supplemental Fig. 3

Key: Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Man, mannose

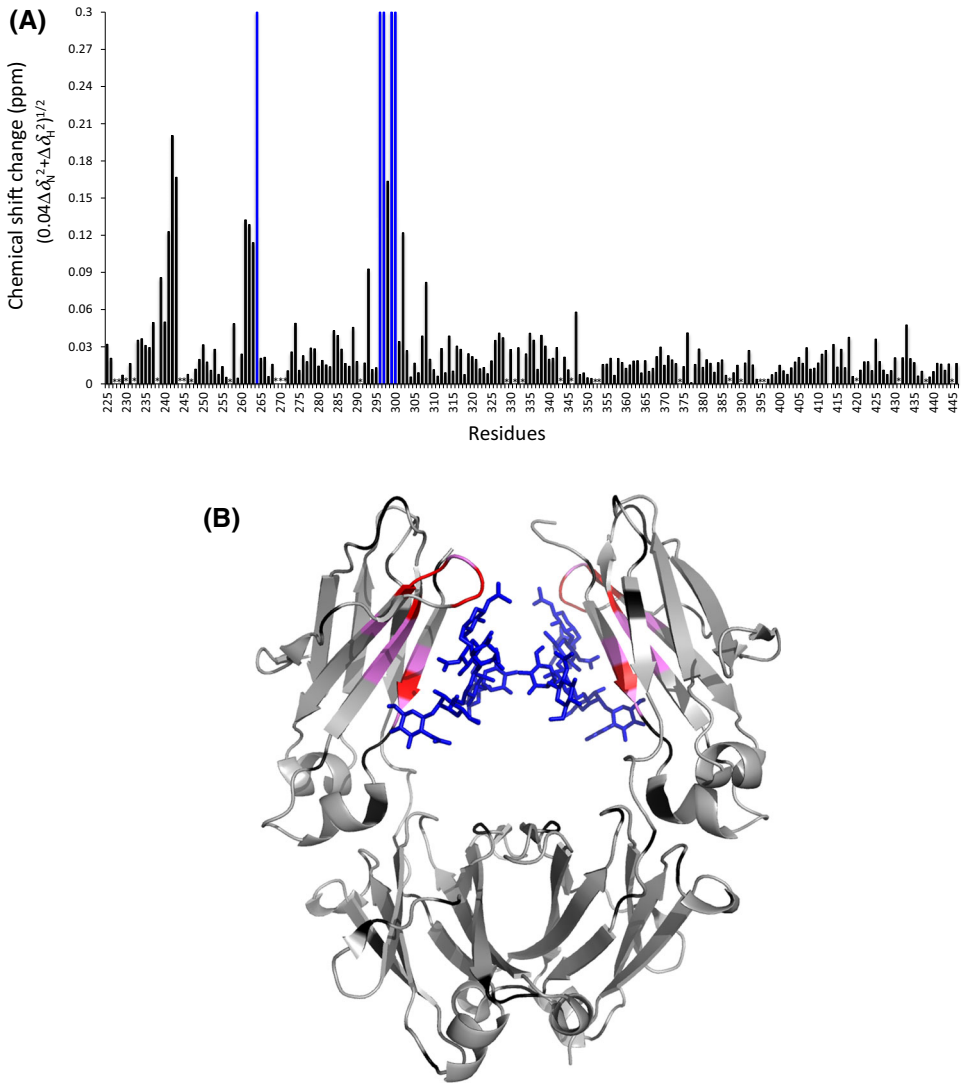

Fig. 3 NMR chemical shift comparison between the IgG-Fc glycoprotein derived from silkworms and that from CHO cells. **a** The data are displayed for each residue according to the equation $[0.04\Delta\delta_N{}^2 + \Delta\delta_H{}^2]^{1/2}$, where $\Delta\delta_N$ and $\Delta\delta_H$ represent the chemical shift difference of the amide nitrogen and proton, respectively. *Blue bars* indicate residues whose NMR peaks were undetectable because of extreme chemical shift differences between silkworm- and CHO-expressed Fc. *Asterisks* indicate proline residues, three unassigned residues, and residues whose chemical shift could not be obtained because of severe peak overlapping. **b** Mapping of the crystal structure of IgG1-Fc residues exhibiting chemical shift differences when IgG1-Fc proteins produced in silkworms were compared with those expressed in CHO cells. The residues exhibiting chemical shift differences >0.1 and >0.2 ppm are shown in *pink* and *red*, respectively. Proline residues, unassigned residues, and residues whose chemical shift perturbation data could not be obtained because of severe peak overlap are shown in *black*. The crystal structure [PDB code: 3AVE (Matsumiya et al. 2007)] is represented with fucosylated biantennary complex-type *N*-glycans with nonreducing terminal GlcNAc residues (*blue*). The molecular graphics were generated using PyMOL (DeLano 2002)

Nuclear magnetic resonance spectroscopy of IgG-Fc

The Fc region of ^{15}N-labeled IgG was prepared for NMR analysis by papain digestion. Figure 2 shows the ^{1}H–^{15}N HSQC spectrum of the IgG-Fc, indicating that the ^{15}N enrichment achieved using the developed protocol was sufficient for heteronuclear 2D NMR measurements. To assess structural differences in IgG1-Fc that arose due to the use of different production vehicles, we compared the spectral data with those already collected for CHO-expressed human IgG-Fc (Supplemental Fig. 4) (Yagi et al. 2014; Yamaguchi et al. 2006).

Chemical shift differences $[(0.04\Delta\delta_N{}^2 + \Delta\delta_H{}^2)^{1/2} > 0.1$ ppm] between the IgG1-Fc glycoproteins were observed for Phe241, Leu242, Phe243, Cys261, Val262, Val263, Val264, Y296, Asn297, Ser298, Thr299, Tyr300 and Val302. In Fig. 3, the amino acid residues that exhibited chemical shift differences were mapped onto the crystal structure of human IgG1-Fc. The

^{1}H–^{15}N HSQC spectral data indicate that IgG-Fc expressed in silkworms maintained structural integrity, except in the vicinity of *N*-glycans.

Discussion

In the present study, using IgG as test molecule, we successfully developed a ^{15}N-labeling method for glycoproteins produced by the silkworm expression system. We obtained 0.1 mg of recombinant human IgG with a ^{15}N-enrichment ratio of approximately 80 %, enabling ^{1}H–^{15}N HSQC measurements of its Fc fragment (with a molecular mass of 53 kDa). To the best of our knowledge, the present study is the first description of production of an isotopically labeled protein using silkworms in NMR quantities.

In our approach, choosing the best silkworm strain was critical. In the beginning, we attempted to use polyphagous silkworm strains such as Shinasagiri; however, these strains on infection with baculovirus produced little to no recombinant IgG. Hence, our efforts were primarily devoted to optimizing the composition of the artificial diet containing isotopically labeled metabolic precursors. Finally, we observed that an optimized artificial diet, containing a ^{15}N-labeled protein mixture derived from yeast cells as a nitrogen source, could be used to rear commercially available silkworms. Isotope labeling method employing mammalian cell lines thus far reported require culture media containing isotopically labeled amino acid mixtures, which are typically derived from algae and supplemented with commercially expensive, isotope-labeled amino acids such as glutamine and tryptophan (Yamaguchi and Kato 2010). By contrast, the present isotope-labeling method employing living silkworms could be achieved using artificial diet that contained the crude proteins (not necessary to be hydrolyzed into amino acids) extracted from the yeast cells grown with ^{15}N-ammonium chloride as the primary nitrogen source. Needless to say, the silkworm cultivation does not require any flasks and bottles. Accordingly, the silkworm-based method is low-cost and low-effort in comparison with the mammalian expression approaches.

The present NMR data thus obtained confirmed the structural integrity of the silkworm-produced IgG-Fc glycoprotein in comparison with a CHO-produced, authentic IgG-Fc. The data showed significant chemical shift differences in close proximity to *N*-glycans, suggesting a glycoform-dependent microenvironmental alteration in these regions (Fig. 3). In general, silkworm-derived glycoproteins express paucimannose-type glycans as major components as in the case of recombinant proteins produced in other insect cell-based vehicles (Ailor et al. 2000; Hollister et al. 2002; Park et al. 2009; Sasaki et al. 2009). Our glycosylation profiling confirmed this tendency for the ^{15}N-labeled IgG produced in silkworms. Such glycoform differences might be a shortcoming of the silkworm expression of mammalian glycoproteins. We have, however, previously observed that the terminal *N*-acetylglucosamine and galactose residues were displayed on the *N*-glycans of the recombinant protein, β1,3-*N*-acetylglucosaminyltansferase 2 produced in living silkworm (Dojima et al. 2009), suggesting that this production vehicle is potentially advantageous for expression of mammalian-type glycoforms of recombinant glycoproteins. Several reports have recently demonstrated that co-expression of Golgi glycosyltransferases (e.g., *N*-acetylglucosaminyltransferase, galactosyltransferase, and sialyltransferase) in cultured insect cell lines, enables the production of glycoproteins with mammalianized *N*-glycosylation (Breitbach and Jarvis 2001; Harrison and Jarvis 2006; Hollister and Jarvis 2001; Palmberger et al. 2011). It is highly plausible that applications of similar improvement strategies for living silkworm lead to establishment of more useful vehicles for the expression of mammalian-type glycoproteins.

In summary, we developed a stable isotope labeling method for glycoproteins expressed in silkworms, which offers a potentially powerful tool for preparing recombinant glycoproteins for NMR studies.

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