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Change in glycosylation pattern with extension of endoplasmic reticulum retention signal sequence of mouse antibody produced by suspension-cultured tobacco BY2 cells

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The production of antibodies using plants as bioreactors has been realized. Because sugar chain structures on recombinant proteins are a cause of concern, remodeling technology is highly promising. Localizing recombinant proteins in the endoplasmic reticulum (ER) affects the glycosylation pattern in transgenic plants. Previously, a mouse antibody produced by suspension-cultured tobacco BY2 cells has sugar chains with possible glycoepitopes as the predominant structures. In this study, we extended the Lys-Asp-Glu-Leu (KDEL) ER retention signal sequence over the heavy (H) and light (L) chains of the antibody and expressed the altered antibody in tobacco BY2 cells to study the effect of the KDEL sequence on glycosylation. For the antibody with the KDEL-extended H-chains, glycans with β(1,2)-xylose or α(1,3)-fucose residues accounted for 49% of the total glycans. Meanwhile, for the antibody with the KDEL-extended H- and L-chains, glycans with xylose or fucose accounted for 38% of the total glycans. Although the addition of an ER retention signal shifted the dominant glycan structures of the KDEL-extended antibody to high-mannose-type structures, some of the antibodies escaped the retrieval system during intracellular traffic and were then modified by xylosylation or fucosylation.

[**Key words:** Antibody; Tobacco; Glycosylation; KDEL; Endoplasmic reticulum (ER)]

The introduction of a gene encoding a pharmaceutical protein of interest into host cells enables such cells to produce a recombinant protein. However, for pharmaceutical proteins, posttranslational modifications, such as glycosylation, are sometimes critical to their stability and biological activity (1). If the protein of interest requires posttranslational modification, its expression in bacterial systems is not desired. Recently, plants have been gaining attention as green bioreactors owing to their low cost, safe production process, and posttranslational modification capability (2–4). Plant systems carry out protein glycosylation that differs from that in mammalian systems (5, 6). The plant-specific feature of the *N*-linked glycan structure is β(1,2)-xylose (Xyl) and α(1,3)-fucose (Fuc) residues being linked to the core $Man_3GlcNAc_2$ structure; mammalian sugar chains generally have α(1,6)-Fuc residues linked to $Man_3GlcNAc_2$ (Man, mannose; GlcNAc, *N*-acetylglucosamine) and do not have β(1,2)-Xyl. These plant-specific sugar residues have been a cause of concern because of their potential immunogenicity or allergenicity (6–10).

The biological functions of the sugar chains of glycoproteins have been shown and discussed for various pharmaceutical proteins. For antibodies, the heavy (H-) chain has an *N*-linked glycosylation site, whereas the light (L-) chain does not. The glycosylation of antibodies has been shown to be necessary for their stability and recognition (11–13).

Recently, mouse antibodies have been produced using suspension-cultured tobacco cells (4, 14, 15). In our previous study, we produced a mouse antibody in suspension-cultured tobacco BY2 cells (4) and determined its glycan structures (Table 1). Glycan structures with α(1,3)-Fuc or β(1,2)-Xyl, such as $GlcNAcMan_3FucXylGlcNAc_2$, account for approximately 74% of the total number of sugar chains, whereas high-mannose-type structures account for only 22%. As described above, α(1,3)-Fuc and β(1,2)-Xyl residues are glycoepitopes and have been a cause of concern for use in human clinical trials. To decrease the number of potential glycoepitopes, protein targetting in the endoplasmic reticulum (ER) has been applied, extending the *C*-termini of proteins by four amino acids residues, namely, Lys-Asp-Glu-Leu (KDEL) (16–21). This ER retention signal sequence aids in keeping the proteins in the ER or returning the proteins to the ER by an interaction between the ER retention signal sequence and the KDEL receptor. In the *N*-linked glycosylation scheme in plants (6), $Glc_3Man_9GlcNAc_2$ on dolichol is transferred cotranslationally to proteins and trimmed by glycosidases, resulting in the high-mannose-type structure $Man_8GlcNAc_2$. Proteins without KDEL are transported to the *cis*-Golgi and modified by mannosidase (Man-ase) I, followed sequentially by *N*-acetylglucosaminyltransferase (GnT) I, Man-ase II, GnT II, and plant-specific glycosyltransferases, such as α(1,3)-fucosyltransferase (FucT) and β(1,2)-xylosyltransferase (XylT). On the other

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TABLE 1. Comparison of sugar chain structures

		Abbreviation in this study	BY2				Plant			
			Endogenous proteins	mIgG0000 (IgG)	mIgG0100 H-KDEL	mIgG0101 H-KDEL L-KDEL	IgG	IgG	IgG H-KDEL	IgG H-KDEL L-KDEL
High-mannose-type	$Man_9GlcNAc_2$	–	–	–	–		–		0.72	0.9
	$Man_8GlcNAc_2$	M8A	–	–	35.6	7.4	6	2.61	11.25	4.8
	$Man_7GlcNAc_2$	M7B	–	–	9.4	3.1	12	4.90	73.24	59.5
	$Man_6GlcNAc_2$	M6B	–	15.4	5.7	5.3	9	2.40	7.13	10.1
	$Man_5GlcNAc_2$	M5A	7.5	6.9	–	46.2	13	1.09	–	2.5
	$GlcNAcMan_5GlcNAc_2$	–	–	3.1	–	–	–	–	–	–
	$GlcNAc_2Man_3GlcNAc_2$	GN2M3	–	–	–	–	–	53.87	2.31	1.8
	$GlcNAcMan_3GlcNAc_2$	GNM3	–	–	–	–	–	2.39	–	–
	$GlcNAcMan_3XylGlcNAc_2$	GNM3X	–	17.8	–	–	–	–	–	6.0
	$GlcNAc_2Man_3XylGlcNAc_2$	GN2M3X	–	–	–	–	–	32.74	–	9.2
	$GlcNAc_2Man_3FucXylGlcNAc_2$	GN2M3FX	26.5	–	14.1	–	18	–	–	2.8
	$GlcNAcMan_3FucXylGlcNAc_2$	GN^1M3FX or GN_1M3FX	21.7	24.4	–	11.0	28	–	–	0.5
	$Man_3FucXylGlcNAc_2$	M3FX	41.0	24.3	35.2	27.0	14	–	–	–
	$Man_3XylGlcNAc_2$	M3X	3.3	8.1	–	–	–	–	5.34	–
	Ref. no.		28	4	This study		29	14		16

hand, proteins with KDEL at their *C*-termini are transported to the *cis*-Golgi, modified by Man-ase I, and returned to the ER, resulting in more high-mannose-type structures (16–21). In transgenic tobacco plants, the extension of the ER retention signal sequence at the *C*-terminus of the antibody H-chain has resulted in the antibody H-chain having an increased number of high-mannose-type structures (16). Although suspension-cultured plant cells are alternative systems, the ER retention technology has not yet been applied to antibody production in the suspension-cultured cell system. In this study, we constructed H- and L-chains with the ER retention signal sequence KDEL, expressed KDEL-extended antibodies in suspension-cultured tobacco cells, and investigated how the ER retention signal affects the glycan structures of antibodies produced by plant cell culture.

MATERIALS AND METHODS

Construction of plant expression vector Two plasmids, namely, pBI221-H and pBI221-L-SalI (4), were used in this study. pBI221-H and pBI221-L-SalI contain the H- and L-chains, respectively. As the ER retention signal sequence, the hexapeptide Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) sequence was used, where the dipeptide SE was a linker between the *C*-terminus of each chain and the KDEL sequence. To insert the hexapeptide SEKDEL sequence at the *C*-terminus of the H-chain, we used pBI221-H carrying the cDNA for the H-chain as a template for PCR. The primers used were mIgGH-SEKDEL (5′- GCGGGAATGAGCTCTTATTACAGTTCGTCTTTCTCTGACTTACCAGGAGAGTGGGAGAG -3′; the SacI site is underlined) and XbaI-mIgGH-SEKDEL (5′- GACTCTAGAATGAGAGTTCTTATTCTCTTT -3′; the XbaI site is underlined). The resulting fragment encoding the H-chain with the SEKDEL sequence was digested with XbaI and SacI, and ligated to XbaI- and SacI-digested pBI221-H. The resulting plasmid was sequenced using an ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer), and designated pBI221-H-SEKDEL. The SalI–EcoRI fragment from pBI221-L was ligated to SalI- and EcoRI-digested pGPTV-HPT (22) to yield the pGPTV-L-chain. The HindIII–SalI fragment from pBI221-H-SEKDEL was ligated to HindIII- and SalI-digested pGPTV-L chain, resulting in pGPTV-mIgG0100 (Fig. 1).

pBI221-L-SalI was used to extend the L-chain with SEKDEL at the *C*-terminus. The primers used were mIgGL-SEKDEL (5′-ATTGACCTGAGCTCTTATTACAGTTCGTCTTTCTCT-GAACACTCATTCCTGTTGAAGCT-3′; the SacI site is underlined) and XbaI-mIgGL-SEKDEL (5′-GACTCTAGAATGAGAACTCCAGCTCAATTT-3′; the XbaI site is underlined). The PCR fragment corresponding to the L-chain with SEKDEL was digested with XbaI and SacI, and ligated to XbaI- and SacI-digested pBI221-L-SalI. The resulting plasmid, pBI221-H-SEKDEL, was sequenced. The SalI–EcoRI fragment from pBI221-L-SEKDEL was ligated to SalI- and EcoRI-digested pGPTV-HPT to yield pGPTV-L-SEKDEL. The HindIII–SalI fragment from pBI221-H-SEKDEL was ligated to HindIII- and SalI-digested pGPTV-L-SEKDEL chain, resulting in pGPTV-mIgG0101.

The vectors were then introduced into *Agrobacterium tumefaciens* strain LBA4404 via electroporation. Tobacco BY2 cells were maintained in modified Linsmaier and Skoog (mLS) medium (23) and transformed using the *Agrobacterium*-mediated system (24). Transformants were selected and maintained in the mLS medium containing antibiotics (250 mg/l carbenicillin sodium salt and 50 mg/l hygromycin B sulfate).

Purification of antibody from transformed tobacco cells Ten-day-old tobacco cells were harvested by centrifugation at 3000 rpm for 15 min at 4 °C, washed with distilled water, and frozen at −80 °C. The cells were thawed on ice and ground in liquid nitrogen with a mortar and pestle. The resulting cell powder was suspended in a binding buffer (200 mM sodium phosphate buffer, pH 7.0). After centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatant obtained was extensively dialysed against the binding buffer. The dialysed enzyme solution was then loaded onto a 5-ml Protein G sepharose column (Amersham Biosciences). The column was washed extensively with 20 mM sodium phosphate buffer (pH 7.0). Immunoglobulin proteins were eluted with 100 mM glycine–HCl (pH 2.0), neutralized immediately with 0.05 volumes of 1 M Tris–HCl (pH 9.0), dialyzed against MilliQ water, and freeze-dried.

Immunological detection The enzyme-linked immunosorbent assay (ELISA) was carried out as described (25) using the anti-mouse IgG (H+L) antibody (Zymed Laboratories, Inc., CA, USA) as a standard.

***N*-glycan preparation and HPLC** Sugar chains were released from the purified recombinant antibody (2 mg) by hydrazinolysis (100 °C, 10 h). After *N*-acetylation with

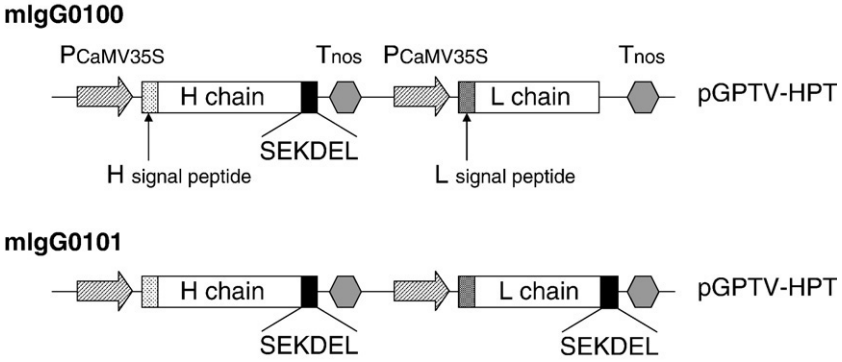

FIG. 1. Plasmid pGPTV-mIgG0100 and pGPTV-mIgG0101 for expression of mouse IgG with KDEL extension of H-chain C-terminus (IgG-hKDEL).