

Biochemical and biophysical characterization of humanized IgG1 produced in *Pichia pastoris*

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Abbreviations: IgG, immunoglobulin; NGHC, non-glycosylated heavy chain; HC, heavy chain; LC, light chain; CEX, cation exchange chromatography; SEC, size exclusion chromatography; cIEF, capillary isoelectric focusing; DSC, differential scanning calorimetry; CE-SDS, capillary electrophoresis sodium dodecyl sulfate; ADCC, antibody-dependent cellular cytotoxicity; FcγR, human Fcγ receptor; FcRn, neonatal Fc receptor

The first full length IgG produced in *Pichia pastoris* was reported in late 1980. However, use of a wild-type *Pichia* expression system to produce IgGs with human-like N-linked glycans was not possible until recently. Advances in glycoengineering have enabled organisms such as *Pichia* to mimic human N-glycan biosynthesis and produce IgGs with human glycans on an industrial scale. Since there are only a few reports of the analytical characterization of *Pichia*-produced IgG, we summarize the results known in this field, and provide additional characterization data generated in our laboratories. The data suggest that *Pichia*-produced IgG has the same stability as that produced in Chinese hamster ovary (CHO) cells. It has similar aggregation profiles, charge variant distribution and oxidation levels as those for a CHO IgG. It contains human N-linked glycans and O-linked single mannose. Because of the comparable biophysical and biochemical characteristics, glycoengineered *Pichia pastoris* is an attractive expression system for therapeutic IgG productions.

Introduction

The first IgG was produced in yeast *Saccharomyces cerevisiae* by two independent groups in the 1980s.^{1,2} However, it was not until 1999 that Ogunjimi et al. reported the first fully functional IgG produced in methylotrophic yeast *Pichia pastoris*,³ although there were many reports in the 1990s and later of single chain IgG fragment (scFv), fusion scFc and Fab fragments produced in *Pichia pastoris*.⁴⁻²³

Wild-type yeast glycoproteins contain N-linked high mannose glycans which are immunogenic and have poor pharmacokinetic/pharmacodynamic properties in primates and humans. In the 2000s, both GlycoSwitch and GlycoFi technologies have made tremendous progress such that it is possible for *Pichia* to produce therapeutic IgG with human-like N-linked glycans.²⁴⁻³³

In both technologies, the secretory pathway of *Pichia pastoris* is genetically engineered to replicate human-like N-linked glycan biosynthesis. Genes responsible for yeast high mannose glycans, e.g., *och1*, are disrupted, and a series of glycosidases and glycosyltransferases are introduced (Fig. 1). The first step of the humanized N-linked glycan synthesis involves an early Golgi mannosidase that trims Man8 to Man5 core structure (Step I). Man5 is then glycosylated to a hybrid structure GlcNAcMan5 by a Golgi-residing fusion protein N-acetylglucosaminyltransferase I (Step II). Next, a mannosidase II (MnsII) is introduced in Golgi and quantitatively converts the hybrid structure to GlcNAcMan3 (Step III). GlcNAcMan3 is then further glycosylated to G0 structure by N-acetylglucosaminyltransferase II (GnTII) (Step IV). The last step involves the addition of Gal sugars to the non-reducing end of terminal GlcNAc and is achieved by the introduction of galactosyltransferase (GalT), as well as ways that increase the cellular pool of UDP-galactose substrate (Step V).

Because of the extensive genetic engineering in *Pichia*, one of the perceived challenges for industrial scale *Pichia* IgG production is genetic stability. In the last two years a robust and scalable fermentation process for glycoengineered *Pichia* with titers of more than 1 g/L of fully assembled IgG1 with uniform N-linked glycosylation was reported.^{34,35} Potgieter et al. showed that the N-linked glycan fidelity can be maintained for up to 64 generations, which is double the passage numbers required for a 2,000 L fermentation scale.³⁴ In addition, the authors have demonstrated that both the productivity and N-linked glycan quality can be maintained across a range of fermentation conditions. The genetic stability of this *Pichia* strain has laid a solid foundation for industrial scale IgG production.

Since *Pichia* technology for IgG production is relatively new, there are only a few publications on biochemical and biophysical characterization of *Pichia*-produced IgG. In early reports of IgG fragments expressed in *Pichia pastoris*, most groups used simple biochemical methods, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot and ELISA, for product characterization, although some used size-exclusion chromatography (SEC),⁹ Biacore,^{9,21} nuclear magnetic resonance,¹³ peptide mapping, mass spectrometry (MS) and N-terminal sequencing methods.¹⁵ More in-depth

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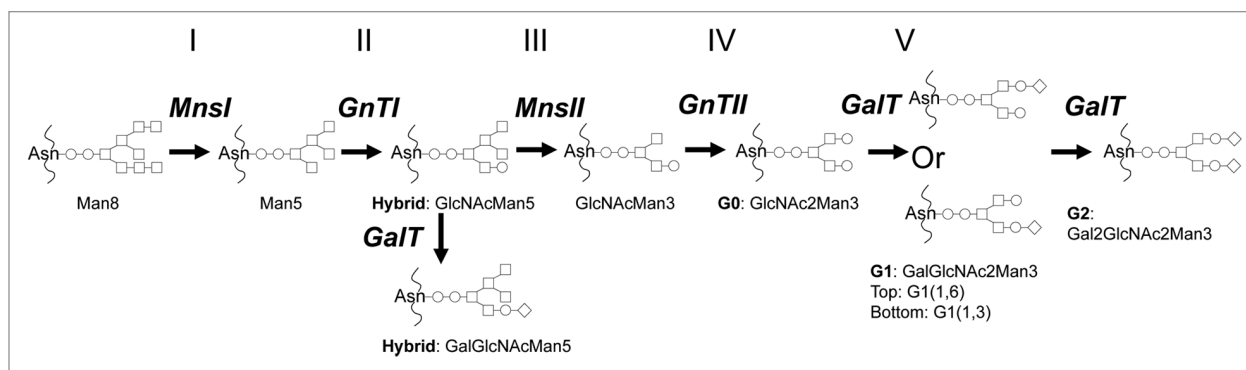


Figure 1. The schematic outline of N-glycan biosynthesis pathway in glycoengineered *Pichia pastoris* that mimics human N-glycan synthesis. MnsI: α -1,2-mannosidase; GnTI: β -1,2-N-acetylglucosaminyltransferase I; MnsII: mannosidase II; GnTII: β -1,2-N-acetylglucosaminyltransferase II; GaIT: β -1,4-galactosyltransferase. O: GlcNAc; □: Man; ◇: Gal. To simplify nomenclature, the two GlcNAc sugars at the reducing end of all glycans are omitted.

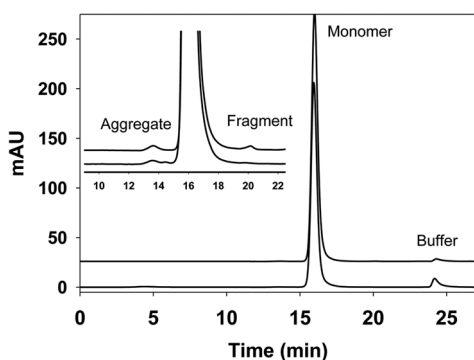


Figure 2. Size exclusion chromatography (SEC) profiles of IgG1 produced from CHO cell lines (upper trace) and from *Pichia* (lower trace). Insert is a zoomed view of the SEC profiles. Peak eluting at 16 min represents the IgG monomer, while peak at 13.5 min represents the aggregate and peak at 20 min represents the fragment. In both cases, the percentage of the monomer is above 99%, and the aggregate and fragment are below 0.5% individually. SEC was performed using a TSK-gelG3000SW column with UV detection at 280 nm and flow rate of 0.5 mL/min. The mobile phase contained 25 mM sodium phosphate, 300 mM sodium chloride and 0.05% sodium azide at pH 6.8.

characterization of *Pichia*-derived IgG has been reported recently in reference 36. In this review, we summarize the current knowledge of *Pichia*-derived IgG and present additional characterization data of a *Pichia*-derived IgG1 in direct comparison with the Chinese hamster ovary (CHO) cell-derived counterpart.

Biochemical and Biophysical Characterization of IgG1 Produced in *Pichia pastoris*

Characterization is typically done to assess product attributes, including aggregation, charge heterogeneities, methionine oxidation, purity, N-linked glycan occupancy and composition, O-linked glycosylation, secondary and tertiary structures, thermal stability and stability during storage.

Aggregation by Size Exclusion Chromatography

IgG is typically formulated for the high-concentration doses required to achieve therapeutic efficacy. However, IgG has a tendency to form aggregates such as dimers, trimers and multimers when concentrated, and the high-order aggregates could have deleterious consequence, e.g., reduced activity, increased immunogenicity,³⁷⁻⁴⁰ poor solubility. The tendency toward formation of aggregates is governed by several factors, including intrinsic properties and external conditions during product development. For example, during process development, IgG can experience temperature, pressure, pH and ionic strength changes that can cause aggregation. A number of methods can be used to measure aggregations,⁴¹⁻⁴³ but SEC is considered the workhorse for detection and quantification of IgG aggregations. The SEC profile of one IgG1 produced in *P. pastoris* is shown in **Figure 2**; SEC method used is described in Cohen et al.⁴⁴ The early elution peak represents aggregations, while the monomer form elutes at about 16 min. The fragment is below the quantitation limit for this IgG1 molecule. The amount of aggregation in *Pichia* expressed IgG is less than 5%, which is similar to typical IgGs produced in either CHO or NS0 cell lines.

Charge Heterogeneities

Charge variant heterogeneities are generated through several pathways such as chemical modification, incomplete enzymatic reaction, and other post-translational modifications. In IgG, these modifications result in charge-based heterogeneities such as deamidation, acetylation, N-terminal cyclization to pyroglutamate, incomplete C-terminal lysine cleavage, glycation, phosphorylation and sialylation. Deamidation, in particular, is of great interest as it is one of the major degradation pathways for IgG, and IgG with deamidation in the complementarity-determining region is shown to have reduced biological activity. Deamidation contributes to the majority of the acidic variants of an IgG and is typically monitored through cation-exchange chromatography

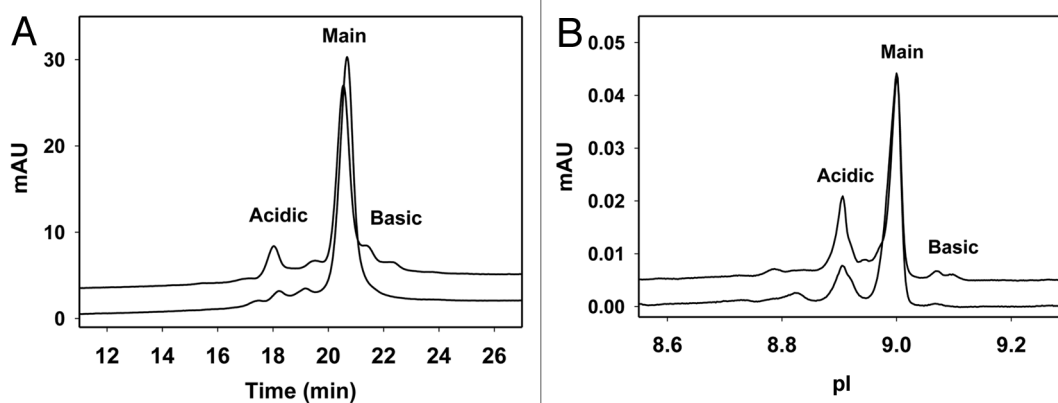


Figure 3. (A) Charge heterogeneity of IgG1 revealed by cation exchange chromatography. In this assay, the Pichia-produced IgG1 (lower trace) has 15% acidic variants, 84% main fraction and 1% basic variants. The CHO produced IgG1 (upper trace) has 23%, 61% and 16% acidic, main and basic fractions, respectively. The CEX separation was performed using Dionex ProPac WCX-10 column (4 x 250 mm) with a salt gradient elution and UV detection at 280 nm wavelength. The mobile phase A contained 25 mM sodium phosphate at pH 6.5 and the mobile phase B contained 25 mM sodium phosphate, 300 mM sodium chloride and 0.05% sodium azide at pH 6.5. The salt gradient elution was 4%–22% mobile phase B in 28 min at 1 mL/min flow rate. (B) Charge heterogeneity of IgG1 revealed by imaged capillary isoelectric focusing. The acidic, main and basic fractions assessed by this assay are 24, 75, 1% for Pichia-produced IgG1 (lower trace) and 33, 62, 5% for CHO-produced IgG1 (upper trace), respectively. Imaged capillary isoelectric focusing was carried out using Convergent Bioscience iCE280 analyzer. Samples were diluted to 0.25 mg/mL in a solution containing 0.35% methylcellulose, a mixture of pH 3–10 and 8–10 carrier ampholytes, and two pI markers of 7.6 and 9.5. The prepared samples were focused in a 5 cm long, 100 μ m ID x 200 μ m OD silica capillary and detected at 280 nm. Focusing time was 1 min at 1.5 kV then 8 min at 3 kV.

(CEX) and capillary isoelectric focusing (cIEF). The two methods are complementary, and each has its own advantages and disadvantages.^{45–47} **Figure 3** illustrates the charge heterogeneity of a pair of IgG1 with the same sequence produced from CHO and *P. pastoris*, respectively, as determined by CEX (**Fig. 3A**) and cIEF (**Fig. 3B**) methods. The overall features between the IgGs produced in the two expression systems are quite similar. For this specific IgG1, Pichia-produced material has less acidic and less basic variants than that of the CHO-produced IgG1 by both analytical techniques. The difference in charge heterogeneity may be due to differences in C-terminal lysine variants, sialylation and process conditions. The CEX method used was similar to that described in Vlasak et al. (gradient was slightly modified),⁴⁸ while the cIEF method used was similar to that described in Li et al.⁴⁹

Methionine Oxidation

Methionine oxidation is another post-translational modification that can occur either during antibody production or storage. Two conserved methionine residues in human IgG1, Met 252 and Met 428, are particularly prone to oxidation.^{50–52} Oxidation of these two methionine residuals has been shown to decrease the binding affinity of IgG to FcRn,⁵³ which consequently reduces its serum half-life.⁵⁴ Methionine oxidation can be quantified by either Protein A high-performance liquid chromatography or peptide mapping.^{53,55} Using the peptide mapping method as described in Bertolotti-Ciarlet et al.⁵³ we demonstrated that Pichia-produced IgG1 has a level of oxidation comparable to the oxidation level typically observed in CHO-produced IgG1.

Purity and N-Linked Glycan Occupancy by Capillary Electrophoresis-SDS Gel

Capillary electrophoresis (CE)-SDS gel is becoming a standard technique to evaluate IgG purity under both reducing and non-reducing conditions, replacing the older, labor-intensive and manual slab SDS-PAGE gel. Similar as SDS-PAGE, the separation principal of the CE-SDS gel is based on molecular weight, but CE-SDS gel has the advantages of automatability, reproducibility, easy quantitation, robustness, high resolution and throughput. Unique to the CE-SDS method under reducing condition, the glycosylated heavy chain can be well separated from the non-glycosylated heavy chain (NGHC). Therefore, this method can provide information regarding N-linked glycan occupancy, in addition to purity and percentage of fragmentation. Furthermore, non-reducing CE-SDS gel provides orthogonal information to SEC regarding covalent aggregations and intact IgG.^{56,57} We have compared CHO- and *P. pastoris*-produced IgGs using CE-SDS gel under both reducing and non-reducing conditions. The electropherograms under reducing conditions show the profile of light chain (LC), heavy chain (HC) and NGHC (**Fig. 4A**). High purity with (LC + HC + NGHC) >99% and no fragmentation are achieved for both IgGs. However, with this particular Pichia strain and the fermentation conditions used, the NGHC from the Pichia-produced IgG is ~10%, higher than the level normally observed for IgG produced in CHO or NS0 (<2%). The majority of the observed NGHC could pair with a glycosylated HC and form hemi-glycosylated IgG. Since hemi-glycosylated IgG has impaired binding affinity to Fc γ Rs, the quantity of NGHC could be important for some applications where effector functionality is required.⁵⁸ As shown in **Figure 4B**, under non-reducing condition the intact IgG percentage for Pichia-produced IgG

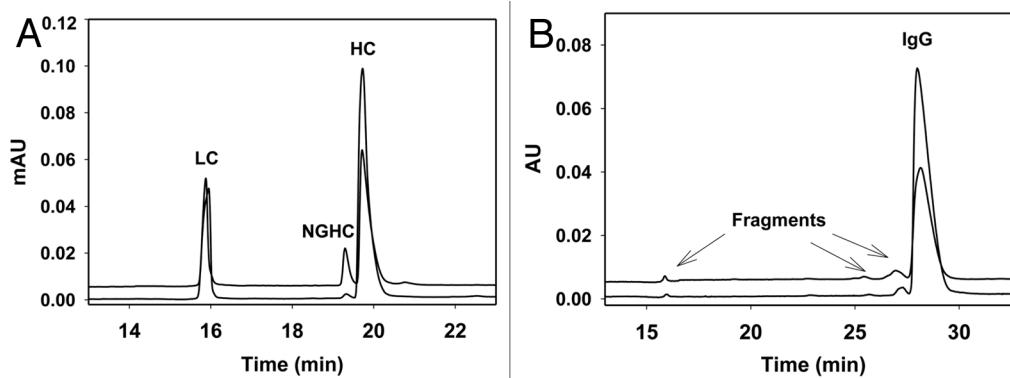


Figure 4. (A) CE-SDS gel electropherograms of IgG1 produced in CHO (lower trace) and Pichia (upper trace) under reducing condition. Both have typical IgG pattern with LC, HC and NGHC. This particular Pichia-produced IgG1 contains about 10% NGHC compared to 1% of NGHC in CHO IgG1. Samples (1 mg/mL) were reduced in beta mercaptoethanol (2-ME) containing 0.5% SDS and heated at 70°C for 10 min. CE-SDS gel was performed using Beckman PA800 CE system (220 nm detection) in bare-fused silica capillary. Sample was injected at anode with reverse polarity using -5 kV for 25 s. The separation was performed at -15 kV reverse polarity with 20 psi at both ends of capillary for 30 min. (B) CE-SDS gel electropherograms of IgG1 produced in CHO (lower trace) and Pichia (upper trace) under non-reducing condition. Both have >96% intact IgG with no covalently linked aggregates. Non-reducing samples (1 mg/mL) were treated with 25 mM iodoacetamide in the presence of 0.5% SDS and heated at 70°C for 10 min. CE separation was the same as described in reducing samples above.

is >96%, comparable with IgG produced in CHO or NS0. There are no covalently linked aggregations observed for either of the IgGs. The CE-SDS method used was similar to the one described in Rustandi et al.⁵⁷

N-Linked Glycan Composition

Human IgG is glycosylated at heavy chain Asn297. The N-linked glycan of human serum IgGs contains predominantly biantennary complex-type structures, with the majority being core-fucosylated—G0F/G1F/G2F. A small percentage of IgG can have sialylated termini—A2F/A1F or contain a bisecting GlcNAc,^{59,60} and N-linked glycosylation can also be found in the Fab region.⁶¹ Current commercial IgGs produced in CHO or NS0 cell lines contain mostly core-fucosylated glycan structures on Asn297, similar to glycoforms present in human IgGs. However, CHO or NS0 can add abnormal sugars to IgGs, which may lead to immunogenic reactions. A recent report by Chung et al. on the hypersensitivity reaction towards cetuximab (Erbix[®]) has highlighted the importance of glycan structures.⁶² Thus, industry guidelines for the thorough characterization of the glycan profile of IgG products have been provided by regulatory agencies.

The N-linked glycan composition can be analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI)-MS using either intact or PNGase digested IgG.^{63,64} N-glycan profiles for both Pichia- and CHO-derived anti-Her2 mAb have been compared.^{36,65} While CHO-derived IgG contains predominantly fucosylated G0F and G1F glycans, Pichia-derived IgG is 100% afucosylated and contains mainly G0 and G1 glycans. N-glycan composition can also be analyzed by capillary electrophoresis with laser-induced fluorescence detection.^{66,67} The ESI-TOF spectra of reduced HCs for both CHO and Pichia-produced IgGs are shown in Figure 5, which illustrates that the Pichia-produced IgG contains mainly

G0 and small amount of G1, G2 and Man5. Man5 is also present in the commercially-available, CHO-produced IgG.

O-Linked Glycosylation

O-glycosylation is an important post-translational modification on the Ser/Thr residuals. Mammalian proteins have mainly O-linked GalNAc (mucin-type), Fuc and Glu sugars, while those from yeast have distinctive O-linked oligomannose. The majority of yeast cell wall proteins are O-mannosylated, which is essential for maintaining a stable cell wall. In 1998, Duman et al. showed direct evidence that not only the yeast cellular proteins, but also recombinant proteins from the yeast host, contain O-mannosylation.⁶⁸ The O-linked glycans from Pichia-expressed recombinant protein comprise from dimeric to pentameric α -1,2-linked mannose, while dimeric and trimeric mannose are the two major components of the O-linked glycans.⁶⁸

Although O-mannosylation is highly abundant in yeast, it is less frequent in mammals. Protein O-mannosylation in mammals has been identified in brain, nerve and muscular tissues.⁶⁹⁻⁷² The most well-characterized human O-mannosylated protein is dystroglycan.⁷³ It has a highly O-mannosylated extracellular domain, with a high abundance of Neu5Ac-Gal-GlcNAc-Man glycan structure. Its impaired O-glycosylation has been linked to several muscular dystrophies.

Although most marketed therapeutic IgGs do not have O-glycosylation, endogenous IgG is known to contain O-glycosylations. Approximately 40% of mouse IgG2b heavy chain hinge region is O-glycosylated, containing mainly the mucin-type tetrassacharide.⁷⁴ Arnold et al. have shown that human serum IgA and IgD have multiple O-linked glycosylation within their hinge region.⁷⁵ O-mannosylated IgG is uncommon; however, an IgG2 produced in both CHO cell line and COS transient cells has been reported to be O-mannosylated on Ser66 of the LC.⁷⁶

The challenge for the *Pichia* expression system is how to eliminate yeast-like O-mannosylation on the expressed IgGs. Because O-glycosylation is essential to cell survival, complete elimination of O-glycosylation is lethal to yeast.⁷⁷ However, progress has been made in inhibiting O-glycosylation using small molecule protein-O-mannosyltransferase inhibitors (PMTi).⁷⁸

As there is no specific enzyme to cleave O-glycans from IgG, the most common release is via chemical β -elimination reaction catalyzed by base. The reaction is then followed immediately with reduction to prevent peeling reaction. Subsequently, the released O-glycans are analyzed with high-performance anion-exchange chromatography with pulsed amperometric detection.⁷⁹ In general, there is only one type of O-glycan, mannose, observed in *Pichia*-produced IgG, and its total occupancy can be controlled to a low level of less than 2 mole of mannose per mole of IgG.

Secondary and Tertiary Structures

Circular dichroism (CD) has been routinely used to evaluate protein secondary and tertiary structures. We have compared the far-ultraviolet (UV: 200–260 nm) and the near-UV (260–320 nm) CD spectra (Fig. 6A and B, respectively) of CHO- and *Pichia*-produced IgG1. The far-UV CD spectra of the two IgG1 overlap with each other, indicating that the two IgG1 have comparable secondary structures regardless of the expression system. The similarity between the near-UV CD spectra indicates the comparable tertiary structures of the two IgG1. The CD method is similar to that described in Wang et al. with minor modifications.⁸⁰

Thermal Stability by Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is an important tool to characterize the thermal stability of IgGs. Since antigen-binding fragments (Fab), CH₂ and CH₃ domains unfold with increasing temperature, the DSC profile has been used to evaluate the structural integrity and stability of these domains. The DSC thermograms of one IgG1 expressed from both CHO cells and *Pichia pastoris* are shown in Figure 7. The first transition peak (Tm1) represents the unfolding of the CH₂ domain, and the second transition peak (Tm2) represents the unfolding of both the Fab and CH₃ domains. CHO- and *Pichia*-derived IgG1 have the same Tm1 (72°C) and Tm2 (81°C), indicating that *Pichia*-produced antibody has similar thermal stability compared to the CHO-produced counterpart. The DSC method used is described in Ionescu et al.⁸¹

Stability Over Storage

Pichia-produced IgGs have demonstrated good physical stability. No significant aggregation or degradation has been detected for the *Pichia*-derived anti-HER2 mAb solution during six-week storage at 4, 25 and 37°C.³⁶ We have compared head-to-head the chemical degradation of CHO- and *Pichia*-produced IgG1 in liquid formulation under different temperatures (4, 25, 37 and

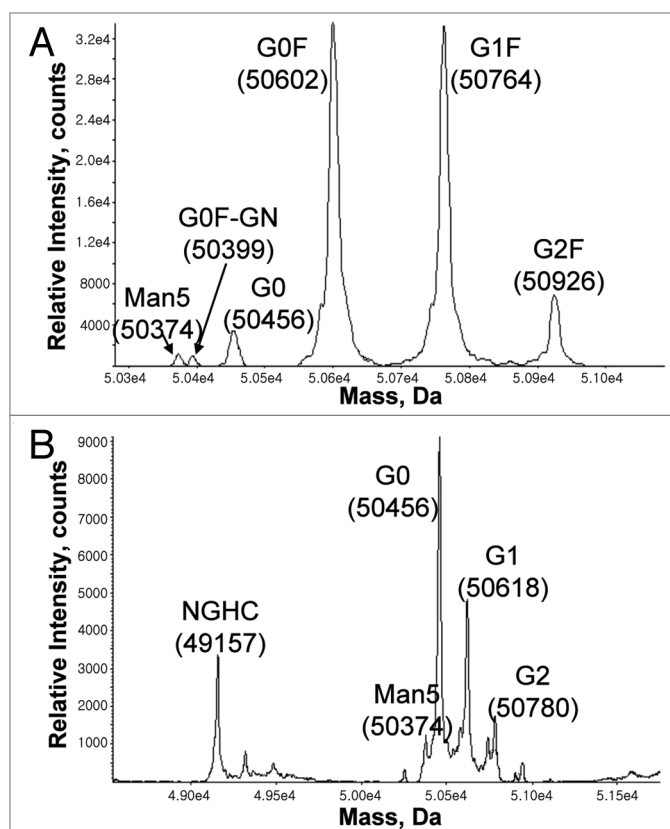


Figure 5. ESI-TOF spectra of the heavy chain in (A) CHO- and (B) *Pichia*-produced IgG1. As expected, *Pichia*-produced IgG1 has no core fucose and contains predominantly G0 glycan. Samples were reduced in the presence of 1 mM DTT at 75°C for 15 minutes, then separated on a PS-DVB column (0.5 mm x 50 mm) at 80°C with a gradient elution of mobile A and B and a flow rate of 20 μ L/min. The mobile phase A contained water with 0.1% formic acid, and the mobile phase B contained acetonitrile with 0.1% formic acid. The liquid chromatography separation was coupled to an Agilent MSD-TOF MS and the mass was determined using TOF Protein Confirmation software.

45°C) for two months. Both IgG1 demonstrate similar chemical degradation profiles, and similar activation energies can be derived from the temperature dependent chemical degradation profiles using the Arrhenius model.

Conclusion

As the market for therapeutic IgGs continues to grow rapidly, there is a high demand for industrial scale IgG production. Currently, all marketed therapeutic IgGs are produced from mammalian cell cultures. However, mammalian cell culture is expensive, lengthy (e.g., typical two-week cultivation), and sensitive to fermentation process parameters. The glycoengineered *Pichia pastoris* expression system can substantially reduce the cultivation time³⁴ and the cost associated with fermentation facility, raw material and viral clearance. We have shown that *Pichia* produces stable IgGs with comparable aggregation, charge variant and oxidation profile compared to the CHO-produced counterpart. Because of the comparable biophysical and biochemical

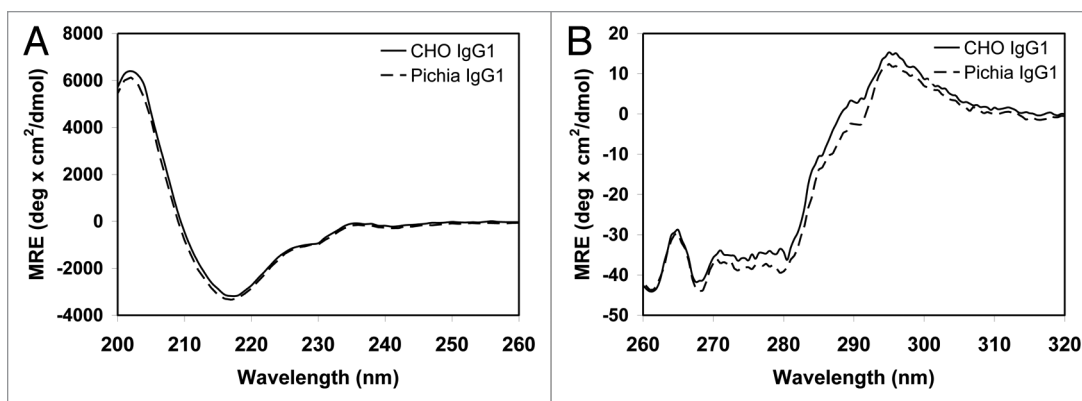


Figure 6. Far-UV (A) and near-UV (B) circular dichroism (CD) spectra of CHO- and Pichia-produced IgG1. The two antibodies have similar spectra, indicating that Pichia-produced IgG1 has similar secondary and tertiary structures compared to the CHO-produced counterpart. The CD measurements were performed on a Jasco J-810 Spectropolarimeter. Samples were dialyzed into PBS buffer and their concentrations were measured by UV. CD measurements were performed using a 1 cm quartz cuvette. A total of five spectral scans were collected at ambient temperature and signals were averaged. The buffer spectrum was collected under the same condition and was subtracted from the sample spectrum prior to the mean residue ellipticity calculation.

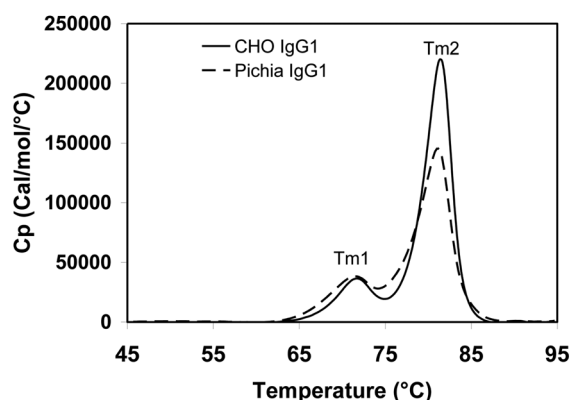


Figure 7. Temperature-induced unfolding of IgG1 produced from CHO and *Pichia pastoris*. CHO- and Pichia-produced IgG1 have identical Tm1 and Tm2 melting temperatures. DSC was conducted on a VP-DSC Capillary Cell Microcalorimeter with a scan rate of 1°C/min from 25°C to 95°C. The thermograms were processed using the Origin 7.0 software and normalized to the molar concentration of the IgG sample.

product characteristics, glycoengineered *Pichia pastoris* is becoming an attractive alternative to the traditional mammalian expression system for therapeutic IgGs.

While the mammalian expression system offers little control of glycosylation, the Pichia expression system has great

flexibility in modulating the N-linked glycan composition. It has been shown that IgG with afucosylated N-linked glycosylation binds to FcγRIIIa about 20- to 40-fold tighter than fucosylated variant^{32,65,82} and elicits higher ADCC response compared with fucosylated IgG.^{65,83} A fucosyltransferase knock-out CHO cell line (Potelligent™ technology) has been explored by several pharmaceutical companies to produce IgGs that require enhanced ADCC function. Glycoengineered Pichia produces 100% afucosylated IgG. A direct comparison of ADCC function has shown that Pichia-produced anti-HER2 antibody elicits 4-fold higher ADCC function with PBMC effector cells than CHO-produced counterpart.⁶⁵ Thus, glycoengineered Pichia offers a promising platform for the production of IgGs with a higher ADCC function.

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