HHS Public Access

Author manuscript

J Biomol NMR. Author manuscript; available in PMC 2024 April 18.

Published in final edited form as:

J Biomol NMR. 2024 March ; 78(1): 9–18. doi:10.1007/s10858-023-00428-1.

Uniform [^{13}C,^{15}N]-labeled and glycosylated IgG1 Fc expressed in *Saccharomyces cerevisiae*

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Abstract

Despite the prevalence and importance of glycoproteins in human biology, methods for isotope labeling suffer significant limitations. Common prokaryotic platforms do not produce mammalian post-translation modifications that are essential to the function of many human glycoproteins, including immunoglobulin G1 (IgG1). Mammalian expression systems require complex medium and thus introduce significant costs to achieve uniform labeling. Expression with Pichia is available, though expertise and equipment requirements surpass *E. coli* culture. We developed a system utilizing *Saccharomyces cerevisiae*, [^{13}C]-glucose, and [^{15}N]-ammonium chloride with complexity comparable to *E. coli*. Here we report two vectors for expressing the crystallizable fragment (Fc) of IgG1 for secretion into the culture medium, utilizing the ADH2 or DDI2 promoters. We also report a strategy to optimize the expression yield using orthogonal Taguchi arrays. Lastly, we developed two different media formulations, a standard medium which provides 86-92% ^{15}N and 30% ^{13}C incorporation into the polypeptide, or a rich medium which provides 98% ^{15}N and 95% ^{13}C incorporation as determined by mass spectrometry. This advance represents an expression and optimization strategy accessible to experimenters with the capability to grow and produce proteins for NMR-based experiments using *E. coli*.

Keywords

glycoprotein; N-glycan; isotope labeling; yeast; antibody

Introduction

NMR spectroscopy is a powerful tool for probing protein structure with the distinct ability to evaluate highly mobile elements in contrast to x-ray crystallography and cryogenic electron

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Authorship contributions

AWB, ETR, IJA and ARD designed research; ARD, ETR and AWB performed research; AWB, ETR, IJA and ARD analyzed data; ARD, ETR, IJA and AWB wrote the manuscript.

Disclosure statement

The authors declare no potential conflicts of interest.

Supporting materials include five tables and two data figures.

microscopy [1]. Traditionally, proteins for NMR are expressed in bacterial systems utilizing *Escherichia coli* that only utilize [^{13}C]-glucose and [^{15}N]-ammonium chloride, as the sole carbon and nitrogen sources for uniform ^{13}C- and ^{15}N-labeling [2]. However, *E. coli* and other prokaryotic expression hosts generally lack the machinery to aid eukaryotic protein folding and add post-translational modifications (ex. glycosylation and phosphorylation)[3]. It is believed that more than half of proteins in nature are glycoproteins whose glycans play a role in structural stability and function [4]. In fact, 22% of human proteins contain one common modification: an N-glycan. N-glycans are highly mobile elements that enhance multiple aspects of protein function including ligand binding, folding, stability, and resistance to proteases among others [5,6]. Thus, defining the structure/function relationships for these proteins requires glycosylation.

Isotope labeled glycoproteins for NMR are often expressed in insect, plant, and mammalian cells [2]. These systems generally require complex media formulations, and are thus cost-prohibitive to introduce uniform isotope labeling [1,3,7,8]. Furthermore, higher eukaryotes including mammalian cells do not tolerate high $^{2}H_2O$ concentrations [9]. Yeasts offer an expression system comparable to bacteria in that yeasts can grow on minimal media and in $^{2}H_2O$, with glucose and ammonium chloride as the sole carbon and nitrogen sources respectively, with better growth rates than higher order eukaryotic cell systems [3]. Isotopically labeled proteins intended for NMR-based studies are often expressed in *Komagataella pastoris* (Pichia) [10]. Pichia grows to very high OD (~100) and can express protein to high yield utilizing methanol as a carbon source. One drawback of Pichia is the requirement to generate stable chromosome insertions, and select for high insertion numbers. *Saccharomyces cerevisiae* is a commercially important yeast but is minimally used for protein expression. In contrast to Pichia, *S. cerevisiae* can express proteins from plasmids thus avoiding the time and labor to develop stable transformants. Furthermore, *S. cerevisiae* will grow in shake flasks in minimal media formulations, requiring the same level of microbiological expertise and equipment required for expressing proteins using *E. coli*.

We chose the crystallizable fragment (Fc) of immunoglobulin G1 (IgG1) to generate uniform ^{13}C labeling. IgG1 Fc is an essential component of endogenous and therapeutic antibodies, mediating interactions between antibody-coated targets and various immune system proteins. Uniform ^{13}C labeling is highly desirable to evaluate IgG1 Fc structural consequences following interactions with receptors, many of which require Fc N-glycosylation to bind [11]. Thus, ^{13}C labeling would enable high sensitivity methyl-based experiments that are not currently available due to a lack of suitable expression systems.

In order to develop *S. cerevisiae* for glycoprotein expression, our group recently reported the expression of uniform [^{15}N]-immunoglobulin G1 Fc. However, carbon and deuterium labeling have not yet been reported. We aimed to develop a relatively low cost ^{13}C- and ^{15}N-labeling system for probing protein structure, motion, and ligand interactions by NMR using engineered *S. cerevisiae*.

Results

Our previous plasmid utilized the Gal1 promoter and a high galactose concentration to express IgG1 Fc [12]. Though yeast grow efficiently on galactose, it is an unsuitable carbon source for uniform ^{13}C labeling due to the high cost and the Gal1 promoter is strongly repressed by glucose. Thus, we replaced the Gal1 promoter with the DDI2 promoter which is induced with cyanamide, permitting expression using [^{13}C]-glucose as a relatively inexpensive carbon source. Furthermore, the DDI2 promoter allows highly tunable expression and is strongly repressed when cyanamide is absent [13,14].

We evaluated IgG1 Fc expression under the DDI2 promoter using our expression-enhanced *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain in both YPD and a minimal medium (Supplemental Figure 1). Protein expression was undetectable without cyanamide and lysed cell pellets showed the presence of both the unprocessed (app8 tagged) and processed IgG1 Fc.

Once expression was confirmed, we utilized a four parameter, three level Taguchi Orthogonal Array to identify optimal inoculation density, cyanamide concentration, glucose concentration and expression temperature for protein expression (Figure 1A). Following quantification of each individual expression reaction by western blot and densitometry, intensity measurements for each individual parameter were averaged to identify the best value for each parameter (Figure 1B). This experiment indicated the highest yield per gram of glucose was growth at 30 °C, 1 g/L glucose, 2 mM cyanamide, and an inoculation OD of 1.0. We conducted a following experiment with a smaller range of glucose, cyanamide, and starting ODs using a second Taguchi Orthogonal Array that defined the following parameters: 30°C with 1 g/L glucose, 1.5 mM cyanamide, and a starting OD of 1.25 (data not shown).

When applying the optimized conditions to larger volumes, we noticed that as we increased culture and flask size our expression level decreased. Believing this was due to an increase in forces exerted on the cells in the larger flasks, we evaluated expression with various shaking speeds and vessel sizes. We determined that the larger culture expressed slightly better at lower speed than the smaller culture while the opposite was observed at higher shaking speed (Figure 1C). These results indicated an optimal culture volume of 100 mL with shaking at 100 RPM (Figure 1D).

The DDI2 promoter tightly controlled protein expression as no protein was visible without cyanamide (Supplemental Figure 1). However, the overall yields fell below our goal for efficient expression for NMR-based studies. We next evaluated the ADH2 promoter using the optimized conditions identified above. The ADH2 gene is expressed when carbon sources for growth become limiting [15]. We compared the yield with the DDI2 and ADH2 promoters, and found greater expression of secreted IgG1 Fc with the ADH2 promoter at all conditions tested (Figure 2A). At 16 hours, we observed a 61% higher expression level in the ADH2 than the DDI2p at 527 ±47 μg Fc/(L*g glucose) and 326 ±26 μg Fc/(L*g glucose) respectively. At 40 hours, we observed a 72% higher expression in the ADH2 compared to the DDI2p at 708 ±16 μg Fc/(L*g glucose) and 411 ±20 μg Fc/(L*g glucose) respectively.

Because the ADH2 promoter led to greater recovery of secreted IgG1 Fc and yield per g glucose, we chose the ADH2 promoter for isotope labeling. We also determine that yield per gram glucose was increased at 1.5 g/L glucose, but not at higher concentrations using the ADH2 plasmid. Lastly, we observed minimal reduction in yield with larger culture volumes using the ADH2 promoter in contrast to DDI2.

We next evaluated expression yield using $^{15}N^{13}C$ Labeling Minimal Expression Medium and purified the secreted IgG1 Fc with a protein A column (Figure 2C). The IgG1 Fc peak was concentrated and showed a lower molecular weight band and a higher molecular weight smear on an SDS-PAGE gel that can be attributed to hyper-mannosylation (Figure 2D). Treating the purified IgG1 Fc with endoglycosidase F1 to remove the N-glycan demonstrated a high level of IgG1 glycosylation, and that the predominant species contains a relatively small N-glycan (Figure 2D).

Three different expressions provided an average yield of 622 ±117 μg Fc/L following purification. Mass spectrometry analysis of Fc peptides indicated 92% ^{15}N incorporation and 30 ±10% ^{13}C incorporation (Figure 3 and Supplemental Table 3). Incorporations <100% likely result from the addition of 5% rich medium that we previously determined was required for protein expression [12]. An analysis of ^{13}C glycan labeling demonstrated a greater incorporation of 91 ±3% (Figure 4 and Supplemental Table 4), likely resulting from efficient incorporation of [^{13}C]-glucose into the glycan from the medium, in contrast to amino acids synthesized de novo from glucose that compete with unlabeled amino acids from the peptone and yeast extract additives.

These conditions utilizing a [^{13}C,^{15}N]-labeled minimal medium supplemented with unlabeled components provide a simple method to generate isotope-labeled proteins for NMR spectroscopy, in particular 2d correlations between a proton and a ^{13}C or ^{15}N nucleus. Furthermore, the glycan was labeled to a high extent. Unfortunately, many higher-dimensionality experiments, including protein backbone assignment experiments, require interactions between multiple ^{13}C and ^{15}N nuclei and thus would be impaired by low incorporation. To increase ^{13}C incorporation we modified the medium to utilize [^{13}C,^{15}N]-labeled yeast extract prepared in the lab, noting previously that protein expression with *Saccharomyces* required supplementation with at least 2-5% rich, unlabeled medium [12]. Yeast extract preparation is simple, growing yeast in a simple chemically-defined medium then recovering and drying the supernatant after autoclaving the cell pellet. Using an expression medium supplemented with [^{13}C,^{15}N]-labeled yeast extract, but lacking both a multivitamin and peptone, we expressed IgG1 Fc with a yield of 273 ±41.5 μg Fc/L. Mass spectrometry analysis of tryptic peptides demonstrated a high degree of isotope incorporation (Figure 3). Because these MS data do not clearly distinguish between ^{13}C or ^{15}N incorporation, we first defined the ^{15}N incorporation to fix this variable during the calculation of ^{13}C labeling. We found that the ^{15}N incorporation using this method, from a sample prepared with using [^{15}N]-labeled medium including [^{15}N]-labeled yeast extract, was 98 ±3% compared to 86 ±7% using the previous method (Supplemental Figure 2, Supplemental Tables 1 and 2). It is interesting to note the broader isotopologue distributions using the suboptimal labeling media, likely due to the lowered incorporation and unequal utilization of labeled compounds during the expression causing broader peaks in the MS

data (Figure 3B). Using this ^{15}N incorporation data, we determined the ^{13}C incorporation to be 95 ±4% (Supplemental Table 5). Thus, the improved labeling medium provided a high percentage of isotope incorporation into the IgG1 Fc polypeptide that is suitable for multi-dimensional NMR experiments.

Finally, we evaluated NMR spectra of the [^{13}C,^{15}N]-IgG1 Fc. A 2d ^{1}H-^{15}N HSQC-TROSY spectrum is of high quality and indistinguishable from a spectrum previously collected with [^{15}N]-IgG1 Fc (Figure 5A). A 2d ^{1}H-^{13}C HSQC shows intense signals for the methyl and methylene regions, with the expected weaker intensity for the alpha carbons (Figure 5B). This spectrum also clearly shows a high amount of signal from the N-glycans. A high-resolution spectrum shows one-bond ^{13}C-^{13}C scalar couplings in the methyl region, without evidence for a clear central peak that would be evident if the ^{13}C methyl were adjacent to a ^{12}C carbon atom (Figure 5C). This spectrum indicates ^{13}C methyl atoms in the [^{13}C,^{15}N]-IgG1 Fc are adjacent to ^{13}C atoms. The physical connection between ^{13}C and ^{15}N atoms is also formed, as is evident in a 2d HNCO experiment that requires one-bond distances between these atoms to develop coherences and observe the ^{1}HN and ^{13}CO chemical shifts (Figure 5D).

Discussion

These experiments demonstrate the recombinant expression of a uniformly ^{13}C and ^{15}N-labeled and glycosylated IgG1 Fc from *S. cerevisiae* using glucose and ammonium chloride as the predominant carbon and nitrogen sources, respectively. Through optimizing IgG1 Fc expression, we identified key variables that impact expression yields and labeling efficiency. Optimal expression conditions for each protein likely differ, and this report identifies variables to evaluate for expressing different glycoproteins, including:

1. Promoter:

Both the DDI2 and ADH2 promoter led to successful expression using glucose as the sole carbon source. Though IgG1 Fc yields using the DDI2 promoter proved lower than using the ADH2 promoter, the DDI2 promoter activity is highly tunable by changing cyanamide concentrations [16]. This control is in contrast to the ADH2 promoter which activates following glucose depletion and more difficult to modulate [17]. We have previously noticed that greater promoter strength can lead to ER stress and reduced yield, thus a tunable induction system may prove valuable. One drawback of the DDI2 promoter is the use of cyanamide, a nitrogen-containing compound, which induces the native DDI2 and DDI3 enzymes that break cyanamide into urea. Urea may then serve as a secondary nitrogen source, and if unlabeled, would reduce ^{15}N incorporation. This undesirable reaction may be prevented by using DDI2/3 knockouts.

2. Optimization strategy:

The use of orthogonal Taguchi arrays proved highly effective in identifying optimal expression conditions simultaneously for multiple culture variables. Originally developed for manufacturing, orthogonal Taguchi arrays provide the ability to reduce sample numbers while simultaneously evaluating the effect and variability imposed by each parameter

value, saving significant time and expense [18]. A strict application of these arrays requires independent variables, and there is a degree of interdependence in these culture variables. However, our results demonstrate that the approach effectively to optimize inoculation OD, inductant concentration, glucose concentration, temperature, culture volume, and shaking rate increased expression yields.

3. Labeling percentage:

We identified two different culture medium compositions that provided different labeling percentages. The simplest approach is the [^{13}C,^{15}N] minimal medium with unlabeled yeast extract and peptone to provide significant ^{13}C labeling (~30% polypeptide and ~90% glycan). This is suitable for titrations and other experiments where high labeling percentages are not needed. We likewise developed a strategy of supplementing with lab-made [^{13}C,^{15}N] yeast extract to increase ^{13}C labeling to ~95% for high-demand applications.

With the increasing recognition that post translational modification including glycans modulate protein function and biological responses, it is anticipated that structure/function studies glycoproteins will become increasingly prevalent. Solution NMR spectroscopy provides the ability to study these highly mobile elements, but suitable methods to generate isotopically-enriched samples must be developed. This report defines strategies for glycoprotein expression and labeling using *S. cerevisiae*, and underutilized protein expression host, requiring laboratory capabilities identical to protein expression using *E. coli*. Unlike prokaryotic expression hosts, however, *S. cerevisiae* readily provides eukaryotic post-translational modification including N-glycosylation, and growth in isotope-labeled medium provides isotope-labeled polypeptides and glycans suitable for NMR spectroscopy.

Materials and Methods

Materials:

Reagents were purchased from Millipore Sigma unless otherwise indicated.

Reference Media Compositions:

All media are autoclaved and glucose is added from a 20% w/v stock once the medium is cooled for all formulations unless otherwise stated.

YNB-Dropout: 6.7 g/L Yeast Nitrogen Base without Amino Acids and Ammonium Sulphate (YNB), 1.5 g/L Yeast Synthetic Drop Out Medium Supplements without uracil, tryptophan, and leucine (DO), 2% w/v glucose.

YNB Minimal Growth Medium: 6.7 g/L YNB, 7.5 g/L DO, 38 mM Na_2HPO_4, 71.7 mM NaH2PO4, 2% w/v glucose.

^{15}N Labeling YNB Minimal Growth Medium: 6.7 g/L YNB, 93.5 mM $^{15}NH_4Cl$, 38 mM Na2HPO4, 71.7 mM NaH2PO4, 2% w/v glucose

YPD: 10 g/L Yeast Extract, 20 g BactoPeptone, 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4, 2% w/v glucose

Minimal Expression Medium: 1.7 g/L YNB, 37.8 mM NH_4Cl, 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4, 0.5 g/L Yeast Extract, 1 g/L Peptone, 1 g/L GreenWise Men's multivitamin, 0.1-0.15% w/v glucose. Note: this medium isn't autoclaved. Instead it's heated to ~60-70°C to dissolve soluble components, centrifuged 4000 g for 10min to pellet insoluble components, and filter sterilized.

^{13}C, ^{15}N Labeling Minimal Expression Medium: 1.7 g/L YNB, 37.8 mM $^{15}NH_4Cl$ (Cambridge Isotope Laboratories), 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4, 0.5 g/L Yeast Extract,1 g/L Peptone, 1 g/L GreenWise Men's multivitamin, 0.15% w/v ^{13}C glucose (Cambridge Isotope Laboratories). Note: this medium isn't autoclaved. Instead, it's heated to ~60-70°C to dissolve soluble components, centrifuged 4000 g for 10min to pellet insoluble components, and filter sterilized.

^{13}C, ^{15}N Labeling Minimal Expression Medium without Peptone & Multivitamin: 1.7g/L YNB, 37.8 mM $^{15}NH_4Cl$ (Cambridge Isotope Laboratories), 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4, 0.5 g/L $^{13}C^{15}N$ Yeast Extract, 0.15% w/v ^{13}C glucose (Cambridge Isotope Laboratories)

DDI2 and ADH2 Plasmid Generation: The DDI2 and ADH2 promoters were amplified from the *S. cerevisiae* genome by PCR using the following primers; DDI2 forward (5'-CGTTAGAATTCTCTAAGATAAAACACAGATCGGC), DDI2 reverse (5'-TGATCGGATCCGATTGATTCTTTTGAAGAGGAGC), ADH2 forward (5'-AAAACGTAGGGGCAAACAAAC), and ADH2 reverse (5'-GTATTACGATATAGTTAATAGTTGATA) using GoTaq DNA polymerase. The PCR products were gel extracted with a Promega SV gel and PCR cleanup kit, and cloned into the pESC-TRP1-app8 Fc vector in place of the Gal1 promoter using *EcoRI* and *BamHI*, followed by ligation with T4 DNA ligase. Promoter insertion was verified by bacterial colony screening and Sanger sequencing.

Yeast transformation: YNB-Dropout supplemented with 100 μg/mL tryptophan (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is used to inoculate a secondary YNB-Dropout supplemented with 100 μg/mL Tryptophan (5 mL) at OD_{600}=0.2 and grown at the same conditions until the OD_{600} reaches 0.6-1 (~3.5-4 h). The secondary culture is centrifuged at 3000 g for 5 min and cells are resuspended in 1 mL of sterile deionized water. Cells are pelleted by centrifugation at 1500 g for 1 min and resuspended in 800 μL of 100 mM lithium acetate (LiAc). Cells are again pelleted by centrifugation at 1500 g for 1 min and resuspended in the transformation mix consisting of 240 μL 50% w/v PEG 3350, 35 μL 1 M LiAc, 25 μL 2 mg/mL boiled salmon sperm, and 100-200 ng of plasmid. The cells are incubated in the transformation mix at 30°C for 30 min then 42°C for 30 min. Cells are pelleted by centrifugation at 1500 g for 1 min and resuspended in 1 mL YNB-Dropout supplemented with 100 μg/mL Tryptophan. One third of the resuspension is transferred to 1 mL YNB-Dropout supplemented with 100 μg/mL Tryptophan and grown overnight at 30°C. Cells are pelleted by centrifugation at 3000g for 2 min, resuspended in 1 mL sterile

deionized water, and 100 μL is plated on a YNB-Dropout/agar plate. Plated cells are grown at 30°C overnight.

DDI2p-Fc Expression: YNB-Dropout (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain transformed with our pESC-TRP1-DDI2p-app8 Fc vector and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is then used to inoculate a YNB minimal growth media culture, which is then grown for 24 h under the same conditions. Once grown, cells are pelleted by centrifugation at 3000 g for 10 min, and the cells are resuspended in minimal expression medium at the desired OD_{600} and cyanamide concentration. The culture is induced for 48 h at 30°C prior to harvesting by centrifugation 4000 g for 10 min.

ADH2-Fc Expression: YNB-Dropout (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain transformed with our pESC-TRP1-ADH2-app8 Fc vector and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is then used to inoculate a YNB minimal growth media culture, which is then grown for 24 h under the same conditions. Once grown, cells are pelleted by centrifugation at 3000 g for 10 min, and the cells are resuspended in minimal expression media at OD_{600}=1. The culture is induced for 48 h at 30°C with 100 RPM orbital shaking prior to harvesting by centrifugation at 4000 g for 10 min.

Labeled ADH2-Fc Expression: YNB-Dropout (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain transformed with our pESC-TRP1-ADH2-app8 Fc vector and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is then used to inoculate a ^{15}N Labeled YNB minimal growth media culture, which is then grown for 24 h under the same conditions. Once grown, cells are pelleted by centrifugation at 3000 g for 10 min, and the cells are resuspended in $^{15}N^{13}C$ labeling Minimal Expression Media at OD_{600}=1. The culture is induced 48 h at 30°C with 100 RPM orbital shaking prior to harvesting by centrifugation at 4000 g for 10 min.

Labeled ADH2-Fc Expression with Labeled Yeast Extract: YNB-Dropout (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain transformed with our pESC-TRP1-ADH2-app8 Fc vector and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is then used to inoculate a ^{15}N Labeled YNB minimal growth media culture, which is then grown for 24 h under the same conditions. Once grown, cells are pelleted by centrifugation at 3000 g for 10 min, and the cells are resuspended in $^{15}N^{13}C$ labeling Minimal Expression Media without Peptone & Multivitamin, made with $^{15}N^{13}C$ or ^{15}N labeled yeast extract made in-house, at OD_{600}=1. The culture is induced 48 h at 30°C with 100 RPM orbital shaking prior to harvesting by centrifugation at 4000 g for 10 min.

Yeast Extract Preparation: YNB-Dropout (5 mL) is inoculated with the *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain and incubated overnight at 30°C with 190 RPM orbital shaking. This culture is then used to inoculate a ^{15}N Labeled YNB minimal growth media culture, which is then grown for 24 h under the same conditions. Once grown, cells are pelleted by centrifugation at 3000 g for 10 min, and the cells are resuspended in $^{15}N^{13}C$ labeling Minimal Expression Media without Peptone & Multivitamin, made with unlabeled

yeast extract, at OD_{600}=1. The culture is induced 48 h at 30°C with 100 RPM orbital shaking prior to harvesting by centrifugation at 4000 g for 10 min. Post growth the cells are harvested by centrifugation at 4000 g for 10 min and the cell pellet is frozen at −80°C. The frozen pellet is resuspended in deionized water and autoclaved using a 30 min sterilization cycle, minimum temperature of 121°C, without a drying cycle. Following autoclaving the solution is immediately cooled on ice and centrifuged 400 g for 10 min to pellet out insoluble components. The collected supernatant is autoclaved a second time and cooled as described prior. The cooled solution is frozen at −80°C and lyophilized to obtain dry yeast extract [19].

Fc Purification by Protein A: Harvested supernatant is pH adjusted to 6.5, passed through a 0.22 μm filter (Fisher), and diluted 1:1 with 20 mM Na_2HPO_4, 150 mM NaCl, pH 7.2. The Fc is purified from the diluted supernatant using a Protein A column as described previously [12].

Peptide Preparation for Mass Spectrometry: Approximately 10 μg of each protein was digested with trypsin (Promega, Trypsin Gold, Mass spectrometry grade) overnight at 37 °C. Following the digestion, the peptides were desalted using C_{18} Zip-Tips. Trifluoroacetic Acid (TFA; Fisher 99.9%) was added to each sample for a final concentration of 1%. The peptides were bound to the C_{18} resin and washed with a 0.1% TFA solution in water. The peptides were eluted into 10 μL 50:50 Acetonitrile (ACN; HPLC grade):H_2O. MALDI spots were prepared by spotting 0.5 μL of protein solution onto an MTP AnchorChip 384 MALDI plate (Bruker) and allowing the solution to dry fully. Then, 0.5 μL of 2,5-Dihydroxybenzoic acid (DHB; Alfa Aesar 99%; 15 mg/mL in 50:50:0.1 MeOH:H_2O: Formic Acid (FA; Sigma Aldrich, 98% - 100% LC-MS grade)) was spotted on top of each peptide spot and allowed to dry fully. If good DHB crystals did not form, a series of dilutions were performed on the MALDI plate to reduce the peptide concentration.

Intact Protein Sample Preparation for Mass Spectrometry: Each protein for intact measurement was buffer exchanged into 50 mM ammonium acetate using 10 kDa MWCO filters (ThermoFisher). The proteins were subjected to up to ten rounds of centrifugation and buffer washing with 50 mM ammonium bicarbonate until the Na^+ or K^+ was reduced to below 1 μM. The proteins were then diluted to 5 μM in 200 mM ammonium acetate. Prior to collecting data, 10 μL of protein solution was loaded into a borosilicate nano-electrospray emitter with a tip ID of < 1 μm. Nano-electrospray emitters were pulled in house using a Sutter P-1000 micropipette puller from filamented glass capillaries (1.2 mm OD, 0.69 mm ID, 10 cm).

FT-ICR Mass Spectrometry Data Collection and Analysis: Mass spectra were collected on a Bruker SolariX XR 12 T Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer equipped with a dual ESI/MALDI source. Calibration used electrospray ionization of a 15 mg/mL solution of Cesium Iodide (Aldrich 99.9999%) in positive mode. Mass spectra for peptides were acquired using the MALDI source equipped with a SmartBeam II laser. The laser power was set to between 50 – 60% and between 5 – 50 laser shots were used. Mass spectra were collected between 500 – 5000 m/z. 512 k data

points were collected with a transient length of 0.70 s which gave a resolution of 155,000 at 500 m/z. For the ^{15}N labeled Fc, 24 – 48 scans were averaged, and for the $^{15}N^{13}C$ Fc peptides 100 scans were averaged.

Mass spectra for the oligomannose glycans were obtained using nanoESI in positive mode. Mass spectra were collected between 500 – 6000 m/z. 4M data points were collected with a transient length of 5.59 s which gave a resolution of 1.3 M at 500 m/z. 100 scans were averaged for each spectrum. Additionally, the instrument was tuned to perform native protein mass spectrometry experiments. The source gas temperature was lowered to 100 C, ion optics were switched to lower frequencies, the time-of-flight was set to 1.5 ms, and CID was applied with energies between 10 – 20 V in order to desolvate the protein and improve ion transmission. The CID energy had the unintended consequence of cleaving the glycans from the protein, which also appeared in the spectrum.

Initial analysis of mass spectra was performed using Bruker Data Analysis 5.3. Isotopic enrichment levels were determined using in house MATLAB code. The MATLAB isotope distribution simulation function, isotopicdist(), was modified to simulate isotopically enriched distributions. Isotope patterns were generated for each peptide across a range of ^{15}N or ^{13}C enrichment, and each pattern was scored against data using an RMSE calculation. The enrichment level with the lowest RMSE score was chosen as the most likely percent enrichment. For the $^{15}N^{13}C$ Fc expressions, the ^{15}N enrichment was set to a fixed enrichment value based on the level determined from the ^{15}N Fc analysis.

NMR Spectroscopy: NMR spectra were collected at a 50 °C sample temperature on one of three instruments: a 21.1 T spectrometer equipped with a Bruker NEO console and 5 mm TXO cryoprobe; an 18.8 T equipped with a Bruker NEO console and 1.7 mm TXO cryoprobe, or a 14.0 T instrument equipped with a Bruker NEO console and 5 mm TXO cryoprobe. Chemical shifts were referenced to DSS. Spectra were processed with a sine-squared line broadening function applied in both dimensions. Spectra were processed in NMRPipe [20] and analyzed in NMRViewJ [21].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Dr. Eric Freeman for the use of his Sutter P-1000 micropipette puller.

Funding

This work was supported by the National Institutes of Health U01 AI148114 (NIAID) to AWB and NIH S10 OD025118 to IJA.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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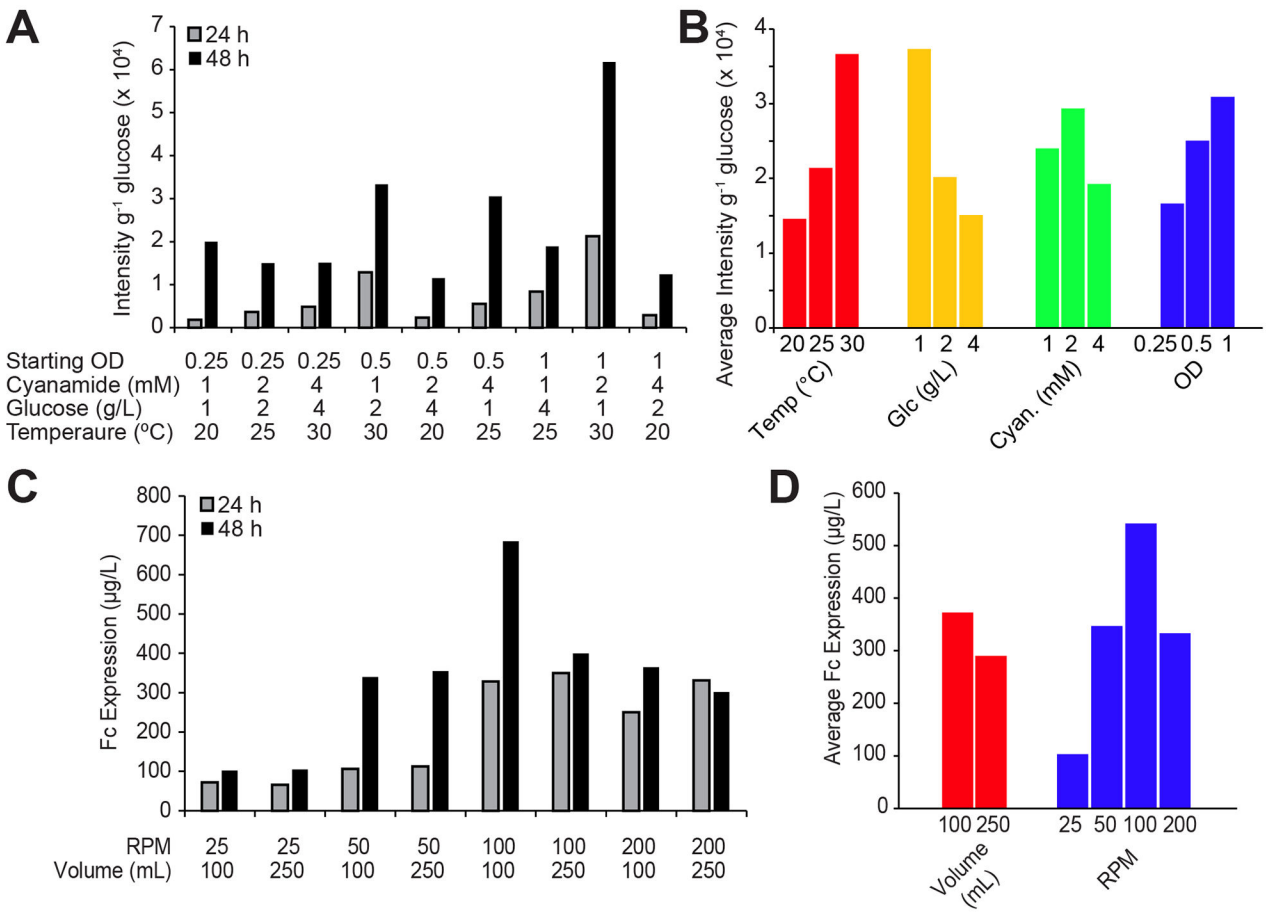

Fig. 1. Improved expression conditions identified through orthogonal Taguchi arrays

A. IgG1 Fc expression from nine individual expression trials were quantified using a western blot, then these were normalized to 1 g/L glucose to determine glucose efficiency. **B**. The mean yields for each parameter are shown following a 48 h expression. **C**. Estimated Fc expression levels using band intensities from a western blot at two different time points with variable volume and orbital shaking speed. **D**. Average estimated Fc expression after expressing 48 h.

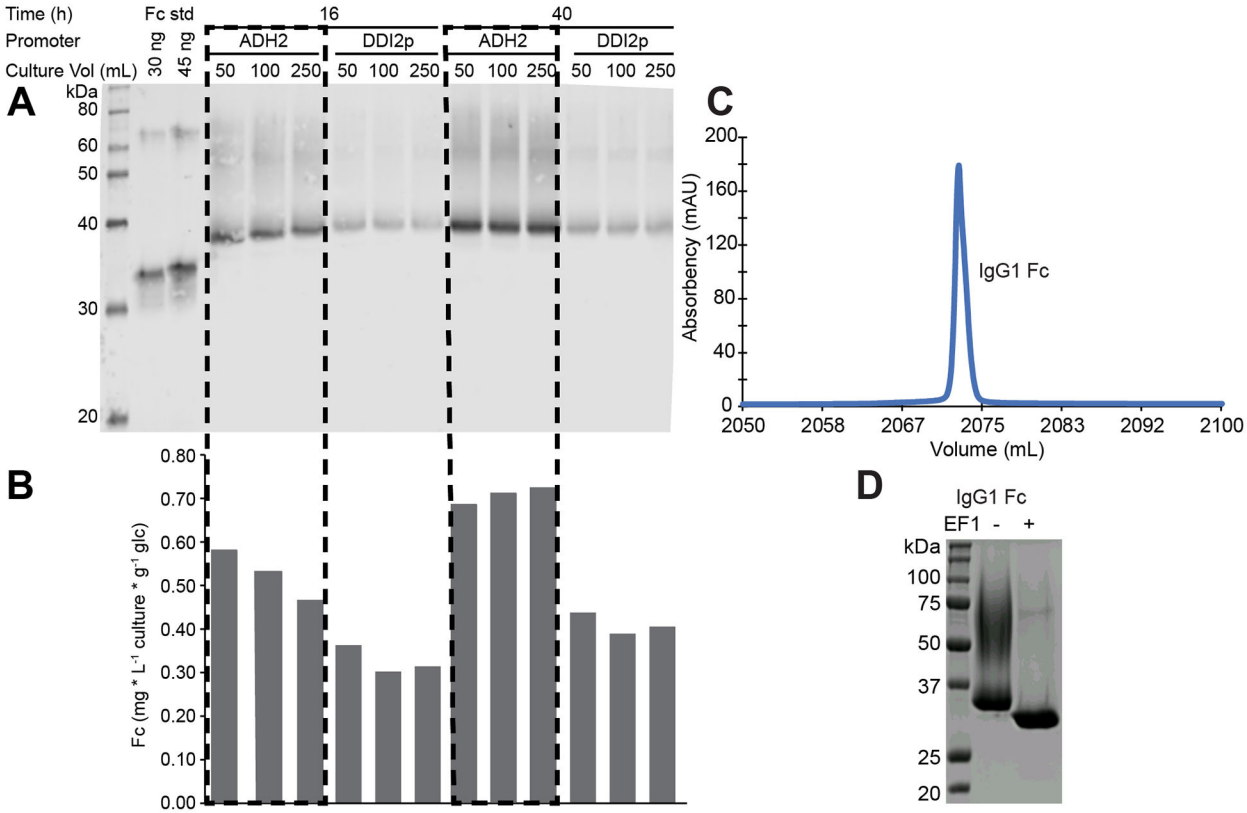

Fig. 2. Comparing the ADH2 and DDI2 promoters

A. Western comparing Fc expression under the ADH2 and DDI2 promoters ran alongside EndoF1 digested Fc standards (Fc std). **B**. Bar graph showing the estimated yields per liter from the intensity of the bands above. **C**. Chromatograph of [^{13}C, ^{15}N]-labeled IgG1 Fc elution from a Protein A column. **D.** Coomassie stained gel of concentrated IgG1 Fc. The upper smear results from hypermannosylation of the N-glycan during expression.

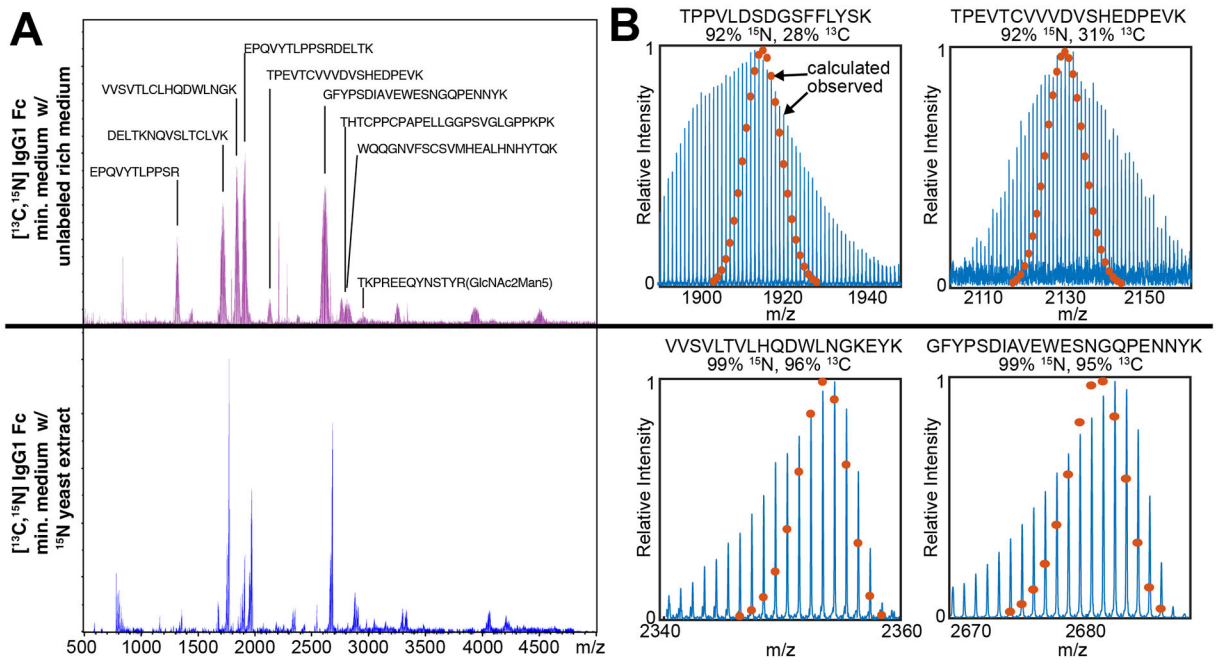

Fig. 3. Mass spectrometry analysis of isotope-labeled IgG1 Fc produced in *Saccharomyces cerevisiae*

A. MALDI-MS spectrum of trypsinized IgG1 Fc. **B.** Observed and best-fitted isotope distributions. Both samples analyzed were expressed in minimal medium containing [^{15}N]-NH_4Cl and [^{13}C]-glucose. The culture above the horizontal line was supplemented with 5% YP, containing unlabeled peptone and yeast extract. The sample below the line was supplemented with [^{13}C,^{15}N]-yeast extract.

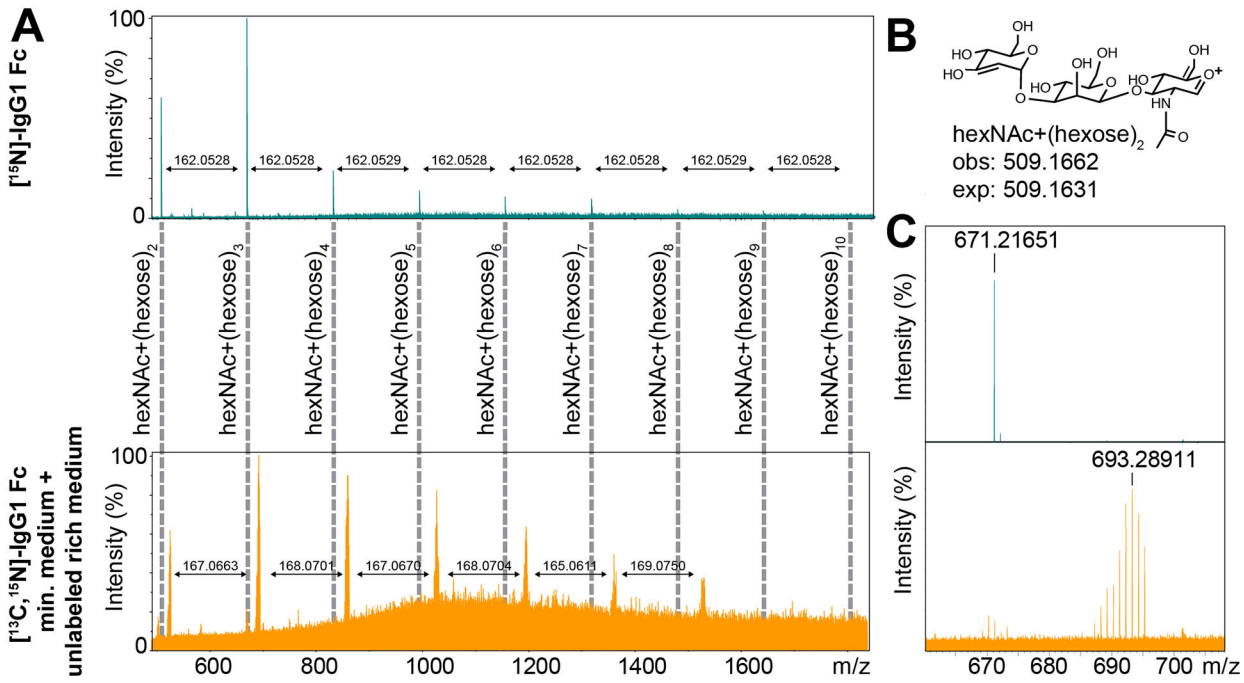

Fig. 4. ^{13}C labeling of the N-glycan.

A. ESI-MS followed by CID revealed glycan degradation species for the [^{15}N]-IgG1 Fc and the [^{13}C,^{15}N]-IgG1 Fc preparations. Mass differences were calculating using the most intense peak in the isotopologue distribution, which were much broader for the ^{13}C-labeled species. **B.** Structural model of one possible N-glycan degradation species observed by ESI-MS; other possible structural models were not distinguished in these studies. **C.** Enlarged region showing the isotopologue distributions for the HexNAc+(hexose)$_3$ species in A.

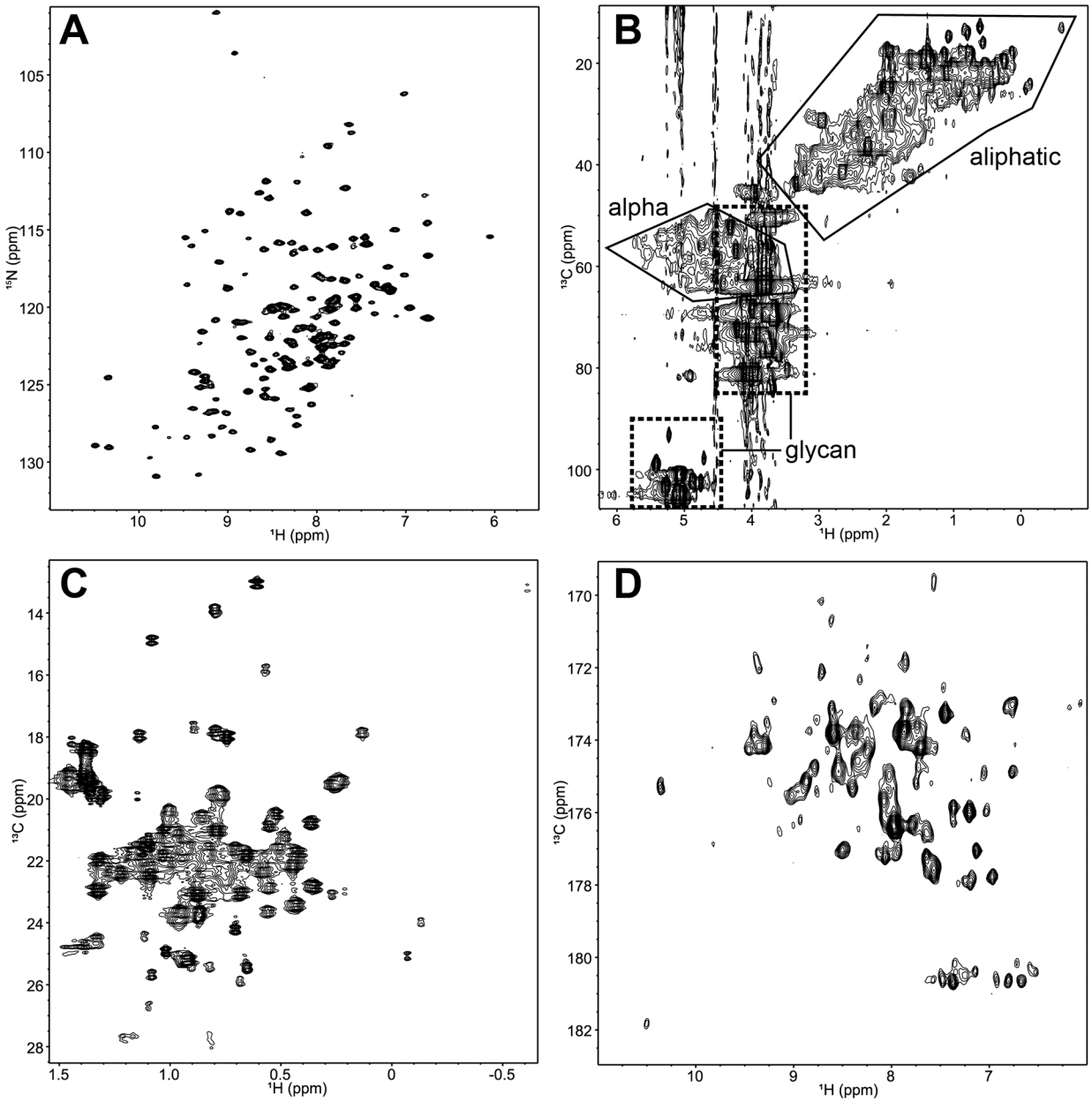

Fig. 5. Solution NMR of [^{13}C,^{15}N]-IgG1 Fc

A. ^{1}H-^{15}N HSQC-TROSY. **B**. ^{1}H-^{13}C HSQC. **C**. High resolution ^{1}H-^{13}C HSQC spectrum to show $^{1}J_{CC}$. **D**. 2d HNCO-TROSY experiment.