



# Metabolic $^{15}\text{N}$ labeling of the N-glycosylated immunoglobulin G1 Fc with an engineered *Saccharomyces cerevisiae* strain

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## Abstract

The predominant protein expression host for NMR spectroscopy is *Escherichia coli*, however, it does not synthesize appropriate post-translation modifications required for mammalian protein function and is not ideal for expressing naturally secreted proteins that occupy an oxidative environment. Mammalian expression platforms can address these limitations; however, these are not amenable to cost-effective uniform  $^{15}\text{N}$  labeling resulting from highly complex growth media requirements. Yeast expression platforms combine the simplicity of bacterial expression with the capabilities of mammalian platforms, however yeasts require optimization prior to isotope labeling. Yeast expression will benefit from methods to boost protein expression levels and developing labeling conditions to facilitate growth and high isotope incorporation within the target protein. In this work, we describe a novel platform based on the yeast *Saccharomyces cerevisiae* that simultaneously expresses the Kar2p chaperone and protein disulfide isomerase in the ER to facilitate the expression of secreted proteins. Furthermore, we developed a growth medium for uniform  $^{15}\text{N}$  labeling. We recovered 2.2 mg/L of uniformly  $^{15}\text{N}$ -labeled human immunoglobulin (Ig)G1 Fc domain with 90.6%  $^{15}\text{N}$  labeling. NMR spectroscopy revealed a high degree of similarity between the yeast and mammalian-expressed IgG1 Fc domains. Furthermore, we were able to map the binding interaction between IgG1 Fc and the Z domain through chemical shift perturbations. This platform represents a novel cost-effective strategy for  $^{15}\text{N}$ -labeled immunoglobulin fragments.

**Keywords** Fragment crystallizable · Kar2p · PDI ·  $^{15}\text{N}$ -labeling · Ribosome skip site

## Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique to characterize protein function, structure and motion that provides atomic-level resolution (Boehr et al. 2006; Neudecker et al. 2009; Kleckner and Foster 2011). High efficiency incorporation of stable isotopes is essential for assigning individual atoms to peaks in spectra. Protein expression for NMR experiments has mostly been limited to prokaryotic or mammalian expression systems (Yanaka et al. 2018). Prokaryotic systems offer several advantages

including rapid growth, low cost, and isotope incorporation using metabolic precursors added to the growth medium. However, prokaryotes are not suitable for expressing many eukaryotic proteins, leading to misfolding and formation of insoluble aggregates (inclusion bodies). Furthermore, standard prokaryotic expression systems are not capable of adding mammalian post-translational modifications which confer many functional properties to the target protein. In contrast, mammalian expression platforms synthesize appropriate modifications, however they have substantial limitations including additional equipment requirements, complex medium formulations that preclude metabolic incorporation of protein labeling precursors from a single nitrogen or carbon source, and a BSL2 requirement (Yao et al. 2002; Yamaguchi et al. 2006; Yagi et al. 2015; Yanaka et al. 2017). Unicellular yeasts are microbes with the advantages of both prokaryotic and mammalian expression platforms including simple growth media, rapid growth, an oxidizing ER lumen for disulfide bond formation, machinery for eukaryotic

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post-translational modifications (N-glycosylation) and chaperones (Yao et al. 2002, Cereghino et al. 2000).

The majority of yeast labeling protocols, however, have been developed in the methylotrophic yeast *Pichia pastoris*, which has widely been used to express heterologous proteins in contrast to other commercially relevant species including *Kluyveromyces lactis* and *Saccharomyces cerevisiae* (Rodriguez and Rama Krishna 2001; Yao et al. 2002; Sugiki et al. 2012; Fan et al. 2015; Elkins et al. 2018; Munro et al. 2020). A number of techniques for expressing proteins using *P. pastoris* are available. In most cases, expression of heterologous proteins in *P. pastoris* was driven by the strong AOX1 promoter which is induced by methanol (Cereghino et al. 2000). The amount of methanol in the medium must be optimized to allow for sufficient protein expression but prevent cell lysis (Fan et al. 2015). Genetic manipulation in *P. pastoris* is typically achieved through integration of a plasmid into the genome through homologous recombination (Cereghino et al. 2000), therefore limiting DNA copy number that would be expected to increase protein expression levels (Yao et al. 2002; Fan et al. 2015). Several groups also addressed medium optimization through supplementation during labeling to boost cell growth and protein expression without negatively affecting isotope-incorporation efficiency (Wood and Komives 1999; Wood et al. 2000; Rodriguez and Rama Krishna 2001; Yao et al. 2002; Sugiki et al. 2012; Fan et al. 2015). Despite the availability of several deletion strains, proteolysis, improper folding, and protein degradation limit labeling and purifying protein from *P. pastoris*. This appears to be a greater problem when using minimal medium to supply a single carbon and nitrogen source for cost-effective isotope labeling (Munro et al. 2020). Optimizing the construct design to allow for proper secretion from *P. pastoris* and addition of sorbitol in the labeling medium were observed to reduce the amount of proteolytic degradation without depleting gene expression (Yao et al. 2002; Munro et al. 2020). Multiple studies have successfully purified several milligrams of isotope labeled target proteins from *Pichia pastoris* by using fermenters and other specialized equipment which boosted the yield above the 3–4 mg/L found using shake flasks (Kjaergaard et al. 2007; Ali et al. 2019).

As an alternative, *S. cerevisiae* is heavily utilized for bread and beer making, as a model organism in the laboratory (Xie et al. 2018; Parapouli et al. 2020) and has been used for the expression of several therapeutics (Kulagina et al. 2021). However, despite the wide application of *Saccharomyces cerevisiae*, there have been few reports of isotope labeling. Hong and coworkers genetically engineered *S. cerevisiae* to produce cholesterol with selective  $^{13}\text{C}$  labeling to study interactions with an exogenous viral protein (Elkins et al. 2018). Similarly, adding [ $^{13}\text{C}$ ]-glucose to the growth medium labeled N-glycans. In this example, a knockout

strain accumulated glycoproteins in the E.R., and N-glycans were hydrolyzed following protein purification for further study (Chiba et al. 1998; Kamiya et al. 2011). Recently, Wall and Hough reported  $^{15}\text{N}$  labeling of a cytosolic *S. cerevisiae* protein fragment Nsp1 using a mixture of yeast nitrogen base, supplemented with [ $^{15}\text{N}$ ]-ammonium chloride to study hydrophobic interactions using in-cell NMR (Wall and Hough 2018). A *S. cerevisiae* growth medium formulation for  $^{15}\text{N}$ -labeling secreted glycoproteins using a simple metabolic precursor is not available to our knowledge.

In this work, we describe an approach utilizing a novel engineered *S. cerevisiae* strain to express [ $^{15}\text{N}$ ]-labeled proteins. We developed an expression and labeling strategy to produce proteins that are naturally secreted by the native host cell. Many proteins are targeted to the lumen of the E.R. during translation, whereupon E.R.-resident modification machinery and chaperones fold and modify proteins in an oxidative environment. Of particular interest are antibodies secreted into the blood that are a critical product of the adaptive immune system. Fully active antibodies, particularly of the immunoglobulin G (IgG) class, require disulfide bond formation and N-glycosylation for activity (Fig. 1a) (Wigzell 1983; Subedi and Barb 2015). Here we apply novel expression and labeling technologies to IgG1 and the IgG1 Fc domain from humans.

$^{15}\text{N}$ -labeling of the IgG Fc domain was previously reported by Kato and co-workers using mammalian cells. IgG1 Fc is a 50 kDa homodimer formed by two C-terminal halves of the IgG1 two heavy chains, is glycosylated at Asn297, and mediates interactions between the antibody and cell surface receptors (Huber et al. 1976). This truly heroic expression and labeling effort was labor intensive and required supplementing the growth medium with multiple isotope-labeled compounds including  $^{15}\text{N}$ -labeled algal lysate and seven amino acids (Sawada et al., 1987; Yamaguchi et al. 1995, 2006; Yagi et al. 2015). A very recent contribution reports a modified mammalian cell line and a somewhat simplified medium for this same purpose (Yanaka et al. 2022). Here we describe a novel approach to achieve [ $^{15}\text{N}$ ]-labeling from a single metabolic precursor using an engineered *S. cerevisiae* strain.

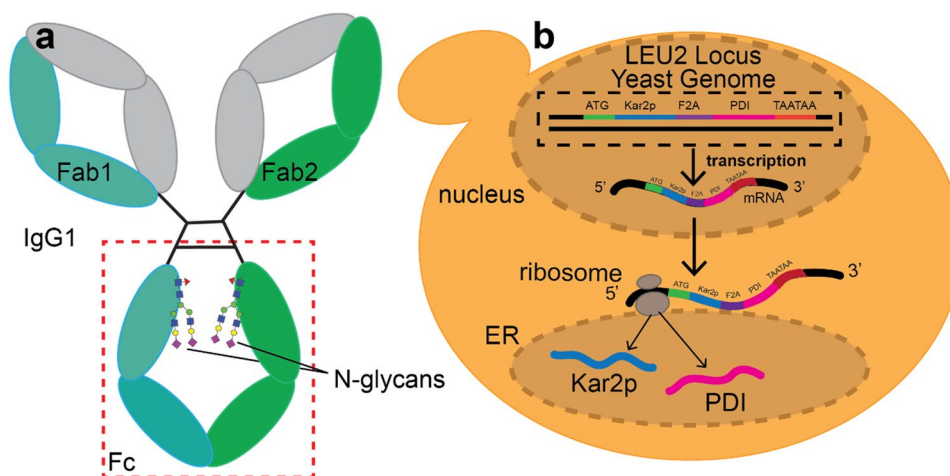
## Materials and methods

All materials were purchased from Millipore Sigma unless otherwise noted.

### Strain

The *S. cerevisiae* strain EBY100 (GAL1-AGA1:URA3 ura3-52 trp1 leu2 $\Delta$ 1 his3  $\Delta$ 200 pep4: HIS2 prb1  $\Delta$ 1.6R can1 GAL) was purchased from the American Type

**Fig. 1 a.** Immunoglobulin G1  
**b.** The EBY100-Kar2p-F2A-PDI yeast strain developed herein allows for the simultaneous expression of both yeast chaperone Kar2p and yeast Protein Disulphide Isomerase (PDI) from a single transcript. These proteins aid protein folding within the endoplasmic reticulum



Culture Collection (ATCC-MYA4941). DNA cloning experiments used *Escherichia coli* DH5 $\alpha$  cells.

### Construct design

Gene fragments for app8 and human IgG1 Fc were codon optimized for expression in *Saccharomyces cerevisiae* and synthesized by IDT (Rakestraw et al. 2009). These two fragments were amplified by PCR. The PCR fragments were gel extracted and used for a fusion PCR reaction to append the app8 fragment at the 5' end of the Fc-encoding fragment. DNA encoding app8-Fc was then cloned into the Sal1 and Nhe1 site of the pESC-TRP1 vector (Agilent). The app8-Fc expression was driven by the GAL1 promoter. The pESC-RTX plasmid was described previously (Shenoy et al. 2021).

Codon-optimized gene fragments encoding Kar2p, PDI and Kar2p-F2A-PDI were synthesized by IDT. These fragments were then cloned into the XbaI and HindIII restriction sites of pAG305-GPD vector (Addgene) to produce vectors: pAG305-PDI, pAG305-Kar2p and pAG305-Kar2p-F2A-PDI. DNA sequence was verified by Sanger sequencing (Eurofins).

### Creating the EBY100-Kar2p, EBY100-PDI and EBY100-F2A-PDI yeast strains

Each vector was linearized with a BstEII digestion (Promega). The linearized plasmid was purified on a 1% agarose gel and the DNA fragment was gel extracted using a Qiagen Gel Extraction kit. Roughly 200 ng of the linearized DNA was used to transform EBY100 yeast cells using the Lithium Acetate (LiAc) method to create EBY100-PDI; EBY100-Kar2p and EBY100-Kar2p-F2A-PDI strains, respectively (Gietz and Woods 2002). Transformants were selected on YNB-Dropout + Trp plates. The integrations were verified with PCR. We then transformed each of these strains with the pESC-TRP1-RTX plasmid or the pESC-TRP1-app8-Fc plasmid, selected transformants with YNB-Dropout medium + agar and identified for positive transformants using yeast colony PCR.

### Rituximab expression from the EBY100-PDI/Kar2p/Kar2p-F2A-PDI strains

Media conditions used for growth and induction of different yeast strains described in Table 1. Overnight cultures (5 mL) were used to start a 50 mL induction culture at an  $OD_{600}$  = 0.5, followed by incubation with shaking for 24 h at 30 °C. Cells were pelleted at 3000 g for 10 min.

**Table 1** Media conditions for rituximab production

| Yeast strain                     | Growth media (Prior to transformation) | Media conditions (Post transformation with pESC-TRP1) | Induction media for RTX production             |
|----------------------------------|--|---|--|
| EBY100-(PDI/Kar2p/Kar2p-F2A-PDI) | YNB-Dropout + Trp + 2% Glucose         | YNB + Dropout + 2% Glucose                            | 0-24 h: YNB-Dropout + 2% Gal<br>24-96 h: YPGal |
| EBY100                           | YPD                                    | YNB-CAA + 2% Glucose                                  | 0-24 h: YNB-CAA + 2% Gal<br>24-96 h: YPGal     |

YNB-Dropout = 6.7 g/L Yeast Nitrogen Base (YNB; without Amino Acids and Ammonium Sulphate) and 1.5 g/L Yeast Synthetic Drop Out Medium Supplements (without uracil, tryptophan, and leucine); YNB-CAA: 6.7 g/L Yeast Nitrogen Base (YNB; without Amino Acids) and 5 g/L Casamino acids; YPD/YPGal: 10 g/L yeast extract + 20 g/L peptone + 2% (glucose or galactose; w/v); Trp: 2 mg/mL

The supernatant was discarded and cells were used to start another induction culture in 50 mL in YPGal. We incubated this culture for an additional 72 h at 20 °C. The culture was centrifuged twice at 3000 g for 10 min. The supernatant was collected, and its pH was adjusted to 6.5 to allow for binding with Protein A. The entire supernatant was applied to a Protein A column. The column was then washed with 5 mL Buffer A (20 mM MOPS and 100 mM sodium chloride, pH 7.4). The column was eluted with 100 mM Glycine pH 3 as 1 mL fractions into tubes containing 500  $\mu$ l 1 M TRIS pH 8.0. All elution fractions were pooled, frozen, then lyophilized. The protein was then resuspended in 200  $\mu$ l 1 M Tris pH 8. 10  $\mu$ l of each sample was then resuspended in 10  $\mu$ l 2 $\times$ SDS Sample buffer, boiled at 95 °C for 5 min and centrifuged in a microcentrifuge at 3000 rpm for 1 min. This sample was then loaded onto a 12% SDS polyacrylamide gel. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane using an Invitrogen Power Blotter (PB0010). After transfer, the membrane was blocked in TBS + Tween20 (TBST) with 5% powdered milk (w/v) for 1 h at RT on an orbital shaker. This was followed by incubation with rabbit anti-hIgG primary antibody (RRID: AB\_228410, ThermoFisher) in 5% dry milk in TBST overnight at 4 °C on an orbital shaker. The membrane was washed three times for 10 min with TBST buffer. Followed by incubation with secondary Goat Anti-Rabbit IgG (H + L) Alexa Fluor Plus 680 (RRID: AB\_2633283, Fisher Scientific, dilution: 1:2000) in 5% powdered milk in 1 $\times$ TBST buffer for 2 h at RT on an orbital shaker. The membrane was washed three times with 1 $\times$ TBST buffer for 10 min and then imaged using LiCOR (LiCor Odyssey CLx). Western blot intensities were quantified using ImageJ.

### **<sup>15</sup>N labeling and purifying app8-Fc**

The *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain was transformed with the pESC-TRP1-app8-Fc plasmid and grown overnight in a 5 ml culture in YNB-Dropout medium at 30 °C. This culture was used to inoculate a 100 mL culture, in medium containing 1.7 g/L YNB (without amino acids and ammonium sulphate), 91.8 mM <sup>15</sup>NH<sub>4</sub>Cl, 38 mM Na<sub>2</sub>HPO<sub>4</sub>, 71.7 mM NaH<sub>2</sub>PO<sub>4</sub> and 2% glucose. The medium was sterilized by autoclaving prior to use. This culture was incubated at 30 °C for 24 h with shaking. A portion of these cells were centrifuged to remove spent medium and used to inoculate a 1 l culture at a final OD<sub>600</sub>=0.5 in NMR induction medium (with 2% YPGal) containing 1.7 g/L YNB (w/o amino acids and ammonium sulphate), 37.2 mM <sup>15</sup>NH<sub>4</sub>Cl, 38 mM Na<sub>2</sub>HPO<sub>4</sub>, 71.7 mM NaH<sub>2</sub>PO<sub>4</sub>, 2% galactose, 0.2 g/L Yeast Extract, 0.4 g/L Peptone, 1 g/L of a powdered multivitamin (Green Wise Men's Multivitamin, Publix Grocery). Similarly, to induce cells in an NMR induction media with 5% YPGal (same as the previous medium except with 0.5 g/L

Yeast Extract, 1 g/L Peptone). Media were mixed under gentle warming to dissolve soluble components, then centrifuged at 3000 g for 10 min to remove insoluble debris, and sterilized by passage through a 0.2  $\mu$ m filter prior to use. The induction cultures were incubated at 30 °C for 48 h with orbital shaking. Culture supernatant was clarified by centrifugation (2 $\times$ 3000g for 10 min), then adjusted to pH 6.5 with dropwise addition of 10 M sodium hydroxide.

The pH-adjusted culture supernatant was diluted 1:1 with 25 mM sodium phosphate, 150 mM sodium chloride pH 7.2, then applied to a 5 mL Protein A column (Cytiva) preequilibrated with 15 mL of the same buffer. The flow rate was 4.5 mL/min. The protein was eluted with Elution buffer (100 mM glycine, pH 3). Elution fractions (2 mL) were collected in tubes containing 1 mL of 1 M Tris pH 8.0. The column was then washed once again with Buffer A.

### **Endo F1 digestion**

EndoF1 was expressed and purified as described (Meng et al. 2013). EndoF1 was then coupled to AminoLink Coupling Resin according to the manufacturer protocol (Thermo Scientific) at a final concentration at 6 mg/mL. The resin was then exchanged into 25 mM MOPS, 100 mM sodium chloride, pH 7.4. The resin was then used to digest the IgG Fc N-glycans. For this digestion, we used approximately 150  $\mu$ l of the 50% EndoF1-resin slurry (v/v) with approximately 2 mg of Fc. The EndoF1 protein resin and Fc were incubated overnight at RT on an inverting rotator. The resin was removed with a MicroBio spin column (BioRad).

### **Mass spectrometry of <sup>15</sup>N-labeled app8-Fc**

Lyophilized protein samples were reconstituted in HPLC-grade water at 1 mg/mL. Each sample (1  $\mu$ L) was diluted to 500  $\mu$ L in 50:50:0.1 methanol: water: formic acid. To observe the IgG1 Fc monomers, 5  $\mu$ L of each solution was treated with 380  $\mu$ M TCEP at 37 °C for 30 min. Then, the reduced Fc solutions (1  $\mu$ L) were diluted to 500  $\mu$ L in 50:50:0.1 methanol: water: formic acid.

Mass spectra were collected using a Solarix XR 12 T Fourier transform ion cyclotron resonance mass spectrometer (Bruker). Samples were introduced by direct infusion into an electrospray ionization source at a rate of 120  $\mu$ L/h using a capillary inlet voltage of 4500 V. Mass spectra were collected in positive mode in the range of m/z 250–3000. The transient length was 1.3 s and 2 M points were collected. For every spectrum, 100 scans were averaged. In some cases, the large number of ions present in the cell made it difficult to obtain a signal transient of sufficient quality, and so a 300–400 m/z quadrupole isolation window was set to only allow 5–8 charge states of the protein into the analyzer cell. Mass spectra were processed using Data Analysis (Bruker



Scientific), and protein monoisotopic masses were determined using the SNAP™ algorithm.

The SNAP™ algorithm is designed to determine the monoisotopic masses for natural abundance proteins, and therefore was unable to determine the monoisotopic molecular weight of the <sup>15</sup>N labeled Fc. Instead, the average mass of the <sup>15</sup>N labeled was used to determine the percent <sup>15</sup>N incorporation. A smoothing function was applied to the high-resolution mass spectrum for the labeled and unlabeled protein. The average mass for both proteins was determined using the charge deconvolution software in Data Analysis. The difference in average mass between the unlabeled and labeled proteins provided the degree of <sup>15</sup>N enrichment.

### Solution NMR spectroscopy

The Z domain was expressed in LB medium as previously described (Barb and Subedi 2016). Protein concentrations were determined by absorbency at 280 nm using a NanoDrop spectrophotometer. EndoF1-digested IgG1 Fc was exchanged into a buffer containing 20 mM sodium phosphate, 100 mM potassium chloride, 50 μM trimethylsilylpropanesulfonate (DSS), pH 7.2 using a 10 kDa MWCO centrifugal filter. NMR spectra were collected at a 50 °C sample temperature on a 21.1 T spectrometer equipped with a Bruker NEO console and 5 mm TXO cryoprobe. The Bruker troyetf3gpsi pulse sequence was used with 512 and 128 complex points in the direct and indirect dimensions, respectively, and 8–64 scans. Spectra were processed with a sine-squared line broadening function applied in the direct dimension and a sine function with linear prediction to 128 points in the indirect dimension. Spectra were processed in NMRPipe (Delaglio et al., 1995) and analyzed in NMRViewJ (Johnson BA 2004).

## Results

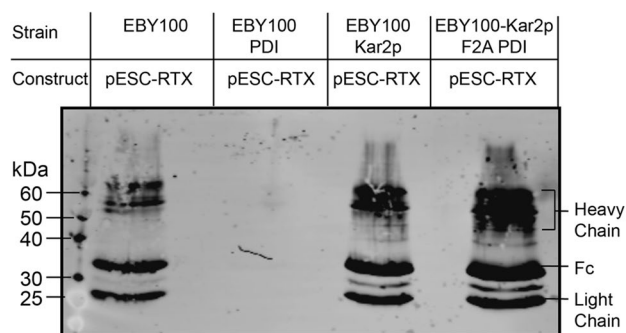
### Engineering the EBY100 strain with ER-targeted chaperones

Though targeted for secretion, we observed a high abundance of protein retained in cells following expression of the antibody rituximab (not shown). To improve secretion, we explored expressing constitutive ER-localized proteins that promote folding. Co-expression of the intrinsic Kar2p chaperone and protein disulphide isomerase (PDI) was previously shown to exhibit variable effects on the level of heterologous protein expression; in some cases there is a significant boost in expression (Shusta et al. 1998), but in some cases there was only a minor increase (Hou et al. 2012). There have also been reports of increased ER stress in yeast cells expressing multiple heterologous proteins simultaneously (Rakestraw

and Wittrup 2006; Mei et al. 2019). We engineered *S. cerevisiae* to simultaneously co-express the Kar2p and PDI in the ER lumen. To reduce ER stress, we incorporated a F2A ribosome skip site and combined the Kar2P and PDI open-reading-frames into a single transcript (de Felipe et al. 2003). F2A was previously used to successfully produce multiple proteins simultaneously in mammalian expression systems (de Felipe et al. 2003; Yu et al. 2012; Cruz-Teran et al. 2017). The cleavage efficiencies of these 2A peptides have been estimated to be around 60–70% in mammalian cells but can be increased by placing two of these peptides in tandem (Liu et al. 2017). The functionality of these peptides can also be modulated through changes in amino acid sequence (Yu et al. 2012). Thus, protein expression with the 2A peptide provides options for further fine-tuning, if needed. Lastly, we integrated the Kar2p-F2A-PDI construct into the yeast genome to allow for better retention of the construct during culture as compared to plasmids which require continual selection (Fig. 1b) (Mei et al. 2019).

### Yeast engineering increased antibody production

We next determined the effect of yeast strain engineering on heterologous antibody production. Antibodies, including IgG1, are challenging targets for heterologous expression in yeast due to the heterotetrameric assembly (two heavy chains, two light chains), formation of ten disulfide bonds and N-glycosylation of each heavy chain. We transformed each yeast strain with the pESC-TRP-RTX plasmid that encodes the heavy and light chains for rituximab expression (Shenoy et al. 2021). We observed increased rituximab protein expression in the yeast strain containing both Kar2p and PDI, compared to the original strain, EBY100, and the strain with only Kar2P (Fig. 2, 100%, 72.6%, 72.6% relative



**Fig. 2** Comparison between antibody production with the engineered *S. cerevisiae* strains. Each of the lanes in the Western blot represent the production of Rituximab (RTX). Rituximab is a heterotetramer composed of two (N-glycosylated) heavy chains > 50 kDa and two light chains (~25 kDa). Cleavage of the heavy chain is evident by the appearance of the Fc domain and is expected for an antibody secreted by yeast

intensity, respectively). Curiously, the EBY100-PDI strain failed to produce protein in multiple repeated experiments despite showing comparable culture growth.

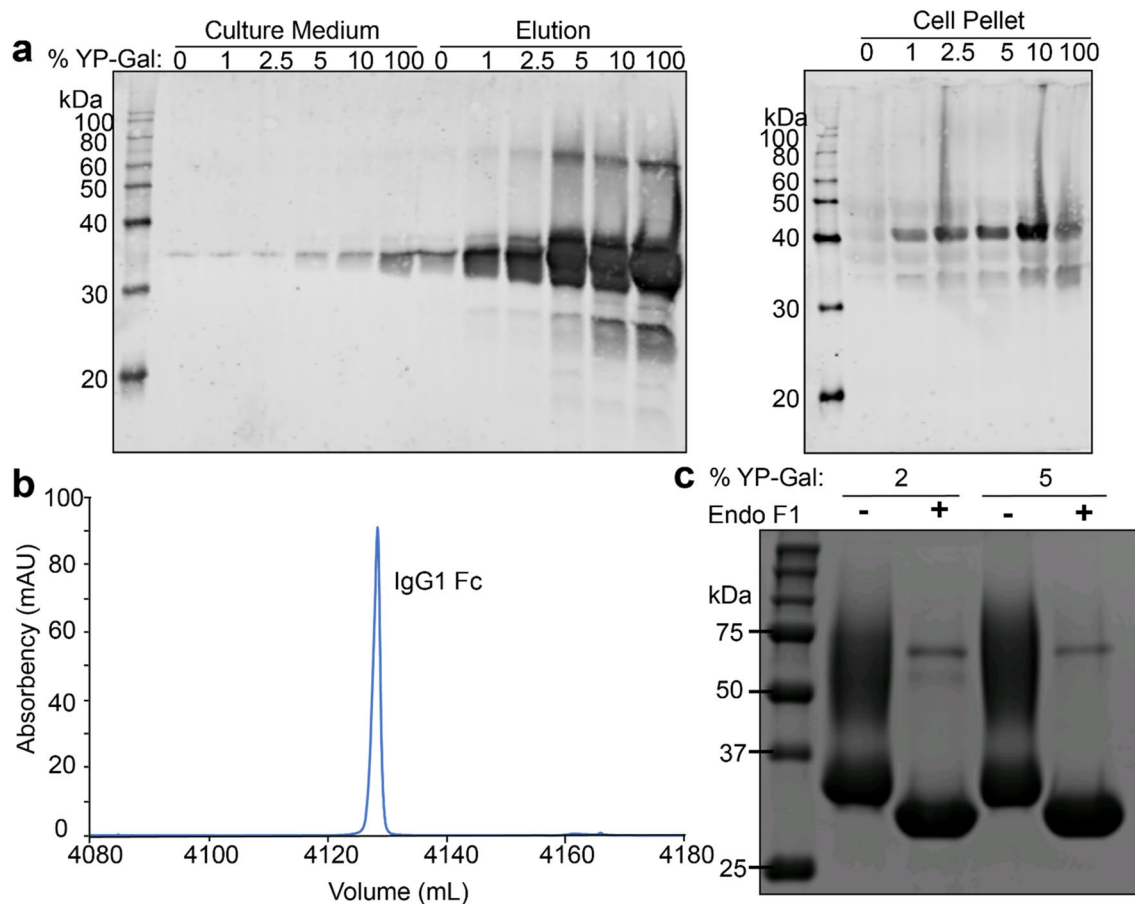
The pattern of IgG1 bands observed in the anti-IgG1 western blot are similar in all cases, indicating that the secreted antibody, purified from the culture supernatant, is comparable in all samples. However, the amount of protein differed between these cultures. In all cases, we observed a smearing in the heavy chain likely due to varying levels of N-glycan remodeling. We also observed IgG1 Fc, likely resulting from heavy chain proteolysis in the hinge region following secretion. We were encouraged by the EBY100-Kar2p-F2A-PDI strain to express and label app8-Fc for NMR.

### <sup>15</sup>N-labeling of Fc using an engineered yeast strain

We applied the engineered yeast strain to express the IgG1 crystallizable fragment (Fc). In contrast to prior reports utilizing mammalian expression and highly complex media

formulations, we explored expressing uniform [<sup>15</sup>N]-labeled IgG1 Fc in yeast using a minimal growth medium.

Our goal was to identify a minimal medium to facilitate cell growth and protein expression that supported a high level of isotope incorporation. Minimal media is designed to supply simple carbon and nitrogen sources in contrast to complex undefined growth medium based on peptone or yeast extracts. Unfortunately, minimal media often reduces yeast growth and protein production. Our base medium was yeast nitrogen base (YNB), which despite the name contains mostly vitamins and metals, and is combined with ammonium sulfate and a carbon source to make a complete growth medium. We explored using YNB supplementation with [<sup>15</sup>N]-ammonium chloride, additional biotin, an over-the-counter multivitamin, and low percentages of rich medium (YPGal). We determined that YNB with [<sup>15</sup>N]-ammonium chloride, supplemented with a multivitamin and 2–5% rich medium provided suitable yield with predicted low levels of <sup>14</sup>N contamination from the (unlabeled) rich medium (Fig. 3a). Interestingly, expression yields improved



**Fig. 3** Expression and purification of IgG1 Fc from the EBY100-Kar2p-F2A-PDI strain. **a**. Optimization of culture medium conditions by adding rich medium (YP-Gal). IgG1 Fc detected in an anti-IgG western blot of the spent medium, cell pellet and as eluted from a

Protein A column. **b**. IgG1 Fc domain purified using a Protein A column. **c**. Endo F1 digestion of purified Fc, using samples purified from two different media condition and stained with Coomassie

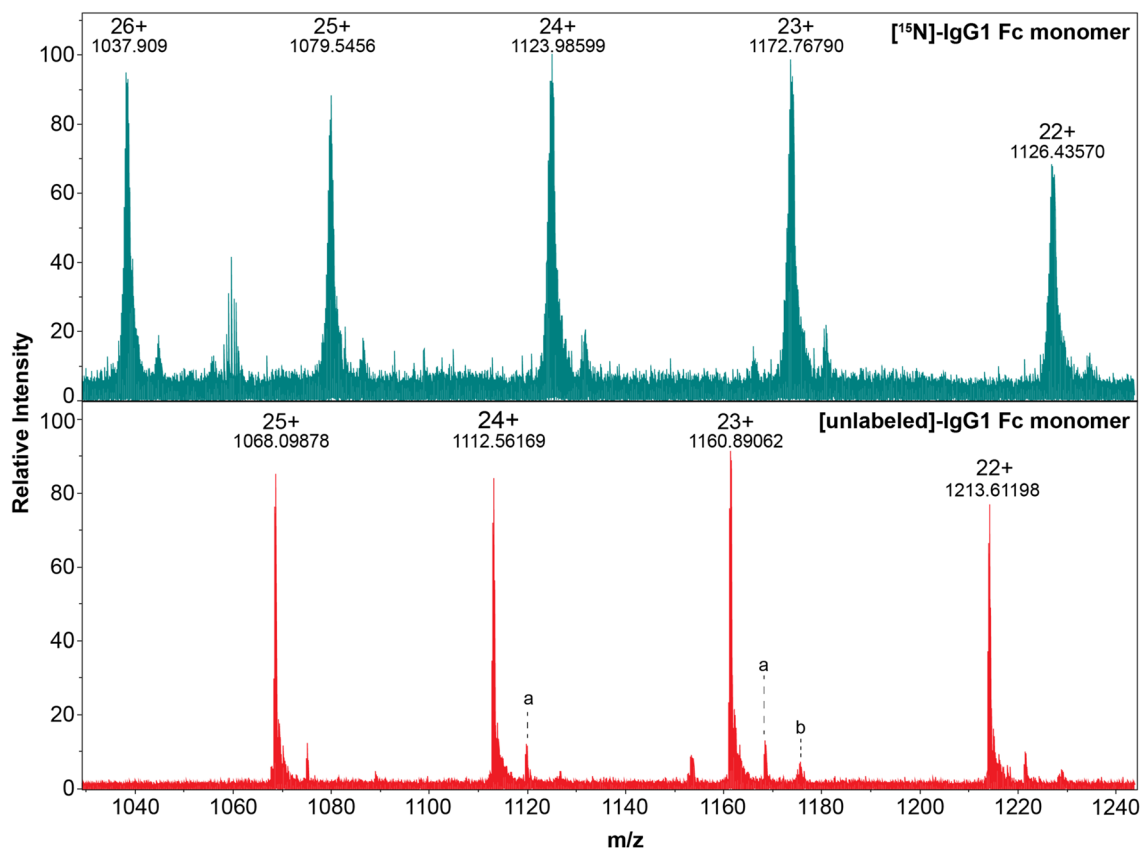
dramatically from 0 to 5% rich medium, indicating that the minimal medium still lacks at least one key component that is in abundance at levels greater than 5%. Also, the cell pellet showed clear retention of the app8-Fc fusion protein, with the secreted protein detected in the spent culture medium and the elution fractions appeared highly processed to remove the app8 secretion tag.

Growth in the minimal medium reduced the pH of the culture supernatant to 2.7–3.0, introducing the additional challenge to protein stability during expression as well as an acidic pH which prevented purification. Adding sodium phosphate to the medium alleviated acidification, resulting in final cultures ranging from pH 5.5–6. These optimization steps improved expression yields and purification. Interestingly, it is important to note that expression was highly repeatable in Erlenmeyer flasks, but never observed in baffled Fernbach flasks.

We recovered 4.5 mg of [ $^{15}$ N]-IgG1 Fc from a 2.0 l culture using the engineered EBY100-Kar2p-F2A-PDI *S. cerevisiae* strain and [ $^{15}$ N]-minimal medium supplemented with 5% rich medium (Fig. 3b). A comparable 2.7 l culture supplemented with 2% rich medium yielded 1.9 mg. One limitation of yeast is the extensive mannosylation of

N-glycans that can extend to hundreds of residues (Hamilton et al., 2006). This extensive modification is visible as a dark, high molecular weight smear in a reducing SDS–polyacrylamide gel with the purified protein (Fig. 3c). We can achieve N-glycan homogeneity by treating the sample with endoglycosidase (Endo)F1 to trim the N-glycan back to a single N-acetylglucosamine residue. This glycoform retains binding affinity for a critical Fc receptor, Fc  $\gamma$  receptor IIIa (Subedi and Barb 2015; Kao et al. 2015). EndoF1 treatment eliminated the smearing, producing a single band. This result also indicated the expressed material is N-glycosylated to a high percentage, as no digested or smaller bands appeared in the untreated samples. Importantly, we also did not observe smaller protein degradation products in this Fc sample indicating negligible proteolysis of the purified protein.

Analysis of [ $^{15}$ N]-IgG1 Fc expressed with 5% rich medium using intact protein MS revealed an increased molecular mass relative to IgG1 Fc expressed in unlabeled medium (Fig. 4). The observed mass for the unlabeled protein differed slightly from the molecular mass calculated using the protein sequence with a mass difference of 0.13–0.20 Da (Table 2). Comparing the labeled and unlabeled proteins revealed that  $^{15}$ N is present at 90.55% of the



**Fig. 4** FT-ICR-MS spectrum of intact IgG1 Fc. Charge states and  $m/z$  values are shown. Average molecular masses are reported in Table 2. “a” corresponds to an increased molecular mass of 163.057 Da, and “b” is an additional 162.164 Da

**Table 2** Average IgG1 Fc masses from FT-ICR-MS

| IgG1 Fc                    | Calc. mass (Da) | Obs. mass (Da) | error (ppm) |
|----------------------------|-----------------|----------------|-------------|
| [unlabeled] monomer        | 26695.91000     | 26695.70355    | 7.73        |
| [ <sup>15</sup> N] monomer | 27008.97899     | 26980.04891    | n/a         |
| [unlabeled] dimer          | 53379.71360     | 53379.84824    | 2.52        |
| [ <sup>15</sup> N] dimer   | 54005.85158     | 53952.17020    | n/a         |

sites. Furthermore, we observed no evidence of proteolysis in either preparation, consistent with the SDS-PAGE analysis (Fig. 3c).

### NMR of the [<sup>15</sup>N]-IgG1 Fc expressed in *S. cerevisiae*

An <sup>15</sup>N-<sup>1</sup>H HSQC-TROSY spectrum of the [<sup>15</sup>N]-IgG1 Fc showed well dispersed peaks consistent with this mainly beta-sheet and loop protein (Fig. 5). These peaks superimposed very well with a spectrum assigned by Kato and co-workers using mammalian expressed IgG1 Fc (Yagi et al. 2015)(Fig. 5b). We quantified the difference in peak positions by calculating the average of the absolute differences in peak position, resulting in an average difference of 0.02 ppm and 0.15 ppm (<sup>1</sup>H and <sup>15</sup>N, respectively). The similarity between these NMR spectra, combined with the high N-glycosylation of the yeast-expressed IgG1 and the ability to purify through a protein–protein affinity interaction (Protein A) supports the conclusion that yeast expressed properly folded [<sup>15</sup>N]-IgG1 Fc.

We next analyzed Fc binding to the IgG-binding three-helix Z domain (Nilsson et al. 1987). The location of the binding interaction is known; however, it is not known if binding only affects IgG residues proximal to the interaction site or throughout the protein (Tashiro et al.; Idusogie et al. 2000). Adding increasing amounts of the Z domain to [<sup>15</sup>N]-IgG1 Fc showed substantial chemical shift perturbations throughout the 2d NMR spectra (Fig. 6). Most peaks

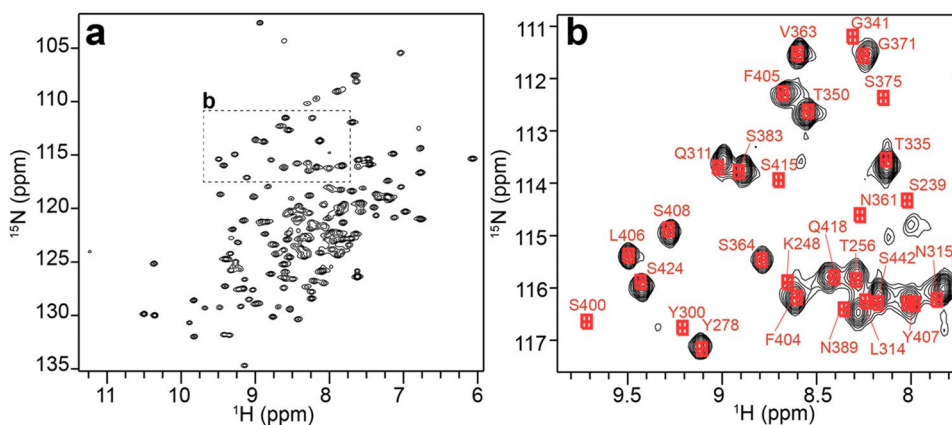
exhibited intermediate to slow chemical exchange kinetics, though a few with small chemical shift perturbations showed intermediate to rapid exchange (Fig. 6b/c), consistent with an expected affinity of 10–50 nM (Cedergren L 1993). Mapping the shifted residues to a structural model of the IgG1 Fc domain revealed the sites most impacted by binding, including those in slow exchange, are found predominantly near the Z domain binding site (Fig. 6d). Interestingly, the A strand was also affected by titration; this strand normally interacts with distal portions of the IgG1 Fc N-glycan (Barb and Prestegard 2011; Subedi et al. 2014). Unaffected peaks are primarily located far from the binding site and at the dimer interface formed by the C γ 3 domains. These spectra demonstrate clear binding of the Z domain and chemical shift perturbations throughout the IgG1 Fc domain.

### Discussion

Here we describe two advances in protein production for NMR spectroscopy. First, we developed a *S. cerevisiae* strain with increased capacity to secrete a complex glycosylated and disulfide-bonded heterooligomeric antibody, rituximab. Although antibodies have been purified in *P. pastoris*, no attempt has focused on isotope labeling antibodies using this expression platform thus far (Schaefer and Plückthun 2012). It is important to note that overexpression of PDI and Kar2p may not always boost expression levels, and the expression of each protein will likely require some optimization to produce maximal yields.

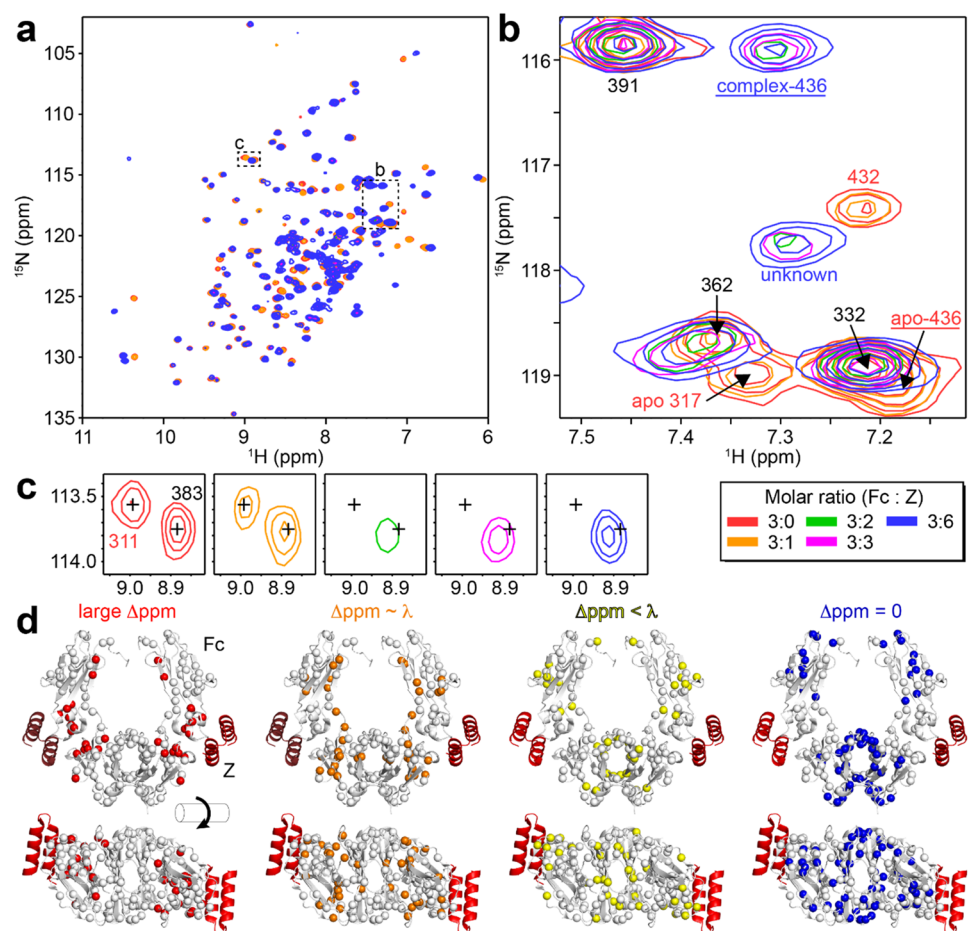
Second, we also report the application of this strain to express IgG1 Fc using a minimal medium formulation that incorporated <sup>15</sup>N from a single metabolic precursor. It is possible that this approach will be applicable to the expression and labeling of other proteins, particularly naturally secreted proteins. It is also notable that the medium formulation does provide the capability to incorporate <sup>13</sup>C labels using [<sup>13</sup>C]-galactose. Unfortunately,

**Fig. 5** Solution NMR of the [<sup>15</sup>N]-labeled IgG1 Fc expressed from the engineered EBY100-Kar2p-F2A-PDI yeast strain (5% YP-Gal). **a.** <sup>1</sup>H-<sup>15</sup>N HSQC-TROSY of Fc at 21.1 T and 50 °C. **b.** The enlarged section shows similarity between the observed peaks and those assigned by Kato and coworkers (BMRB – 27,453). Labels indicate amino acid residue position and type





**Fig. 6** Interaction of [ $^{15}\text{N}$ ]-labeled IgG1 Fc (2% YP-Gal) with the Z domain. **a.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC-TROSY spectra collected during an Fc-Z titration; molar ratios are indicated below panel (b). **b.** Section enlarged from panel (a) to show large displacement of the Y436 peak. The assignment for the 436 peak in the complexed spectrum was made based on Barb and Subedi (2016) JBNMR 64(1):75–85. **c.** Section enlarged to show fast exchange for the 383 peak, but slow exchange for the 311 peak. **d.** Peaks with similar chemical shift perturbation regimes mapped onto the IgG1 Fc structure (white ribbon, from pdb 5vu0) showing the location of Z domain binding, (red ribbon, two of three helices shown from pdb 1l6x)



[ $^{13}\text{C}$ ]-galactose is prohibitively expensive for large cultures and other strategies will be required for inexpensive metabolic  $^{13}\text{C}$  labeling in this system.

Heterologous protein expression in yeast usually utilizes *P. pastoris* that can produce large quantities of protein, though limitations as noted above are well described. Accordingly, several groups developed labeling conditions for NMR spectroscopy. In most cases, protein expression levels were significantly boosted by the use of fermenters and other specialized equipment in comparison to the shake flask method which yield amounts comparable to the amount obtained in this study ( $\sim 3\text{--}4\text{ mg/L}$ ) (Kjaergaard et al. 2007; Ali et al. 2019). In comparison, there are fewer labeling protocols and examples using *S. cerevisiae*, despite the wide adoption of this yeast for biological research and relative ease of genetic manipulation. Furthermore, *S. cerevisiae* is easily transformed and readily expresses proteins from plasmids into growth medium with neutral pH, unlike *P. pastoris*. These data suggest *S. cerevisiae* has the potential to be an important expression host for NMR spectroscopy with the capability to perform essential post-translational modifications and an ability to produce properly folded mammalian proteins. This work

represents an advance in an underdeveloped application of *S. cerevisiae*.

In recent years, state legislatures in the United States have considered bills that would block the use of fetal tissue for any purpose, including HEK293 cells that are of fetal origin. HEK293 cells are a vital resource for glycoprotein expression, and though limited, for isotope labeled proteins. The development of novel scientific resources, including yeast platforms to express and label glycoproteins, may provide an important alternative should one of these bills be made into law.

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**Data Availability** All data generated or analyzed during this study are included in this published article.

## Declarations

**Conflict of interest** The authors report no competing interests of financial conflicts of interest.

## References

- Ali R, Clark LD, Zahm JA et al (2019) Improved strategy for isoleucine 1H/13C methyl labeling in *Pichia pastoris*. *J Biomol NMR* 73:687–697. <https://doi.org/10.1007/s10858-019-00281-1>
- Barb AW, Prestegard JH (2011) NMR analysis demonstrates immunoglobulin G N-glycans are accessible and dynamic. *Nat Chem Biol* 7:147–153. <https://doi.org/10.1038/nchembio.511>
- Barb AW, Subedi GP (2016) An encodable lanthanide binding tag with reduced size and flexibility for measuring residual dipolar couplings and pseudocontact shifts in large proteins. *J Biomol NMR* 64:75–85. <https://doi.org/10.1007/s10858-015-0009-6>
- Boehr DD, Dyson HJ, Wright PE (2006) An NMR perspective on enzyme dynamics. *Chem Rev* 106:3055–3079. <https://doi.org/10.1021/cr050312q>
- Cedergren L, Andersson R, Jansson B, Uhlén M, Nilsson B (1993) Mutational analysis of the interaction between staphylococcal protein A and human IgG1. *Protein Eng* 6(4):441–448. <https://doi.org/10.1093/protein/6.4.441>
- Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev* 24(1):45–66. <https://doi.org/10.1007/s10858-015-0009-6>
- Chiba Y, Suzuki M, Yoshida S et al (1998) Production of human compatible high mannose-type (Man 5 GlcNAc 2) sugar Chains in *Saccharomyces cerevisiae*. *J Biological Chem* 273:26298–26304
- Cruz-Teran CA, Tiruthani K, Mischler A, Rao BM (2017) Inefficient ribosomal skipping enables simultaneous secretion and display of proteins in *Saccharomyces cerevisiae*. *ACS Synth Biol* 6:2096–2107
- de Felipe P, Hughes LE, Ryan MD, Brown JD (2003) Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. *J Biol Chem* 278:11441–11448
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6(3):277–293
- Elkins MR, Sergeev I, Hong VM (2018) Determining cholesterol binding to membrane proteins by cholesterol 13C labeling in yeast and dynamic nuclear polarization NMR. *J Am Chem Soc* 140:15437–15449
- Fan Y, Emami S, Munro R et al (2015) Isotope labeling of eukaryotic membrane proteins in yeast for solid-state NMR. *Methods in Enzymology*. Academic Press Inc, Cambridge, pp 193–212
- Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 350:87–96
- Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischniewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* 313(5792):1441–1443
- Hou J, Tyo KEJ, Liu Z et al (2012) Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. *FEMS Yeast Res* 12:491–510
- Huber R, Deisenhofer J, Colman P et al (1976) Crystallographic structure studies of an IgG molecule and an Fc fragment. *Nature* 264:415–420. <https://doi.org/10.1038/264415a0>
- Idusogie EE, Presta LG, Gazzano-Santoro H et al (2000) Mapping of the C1q binding site on Rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164:4178–4184
- Johnson BA (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol*. <https://doi.org/10.1385/1-59259-809-9:313>
- Kamiya Y, Yamamoto S, Chiba Y et al (2011) Overexpression of a homogeneous oligosaccharide with 13C labeling by genetically engineered yeast strain. *J Biomol NMR* 50:397–401
- Kao D, Danzer H, Collin M et al (2015) A monosaccharide residue is sufficient to maintain mouse and human IgG subclass activity and directs IgG effector functions to cellular Fc receptors. *Cell Rep* 13:2376–2385
- Kjaergaard M, Gårdsvoll H, Hirschberg D et al (2007) Solution structure of recombinant somatomedin B domain from vitronectin produced in *Pichia pastoris*. *Protein Sci* 16:1934–1945
- Kleckner IR, Foster MP (2011) An introduction to NMR-based approaches for measuring protein dynamics. *Biochimica Et Biophysica Acta—Proteins and Proteomics* 1814:942–968
- Kulagina N, Besseau S, Godon C et al (2021) Yeasts as biopharmaceutical production platforms. *Front Fungal Biol*. <https://doi.org/10.3389/ffunb.2021.733492>
- Liu Z, Chen O, Wall JBJ et al (2017) Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep*. <https://doi.org/10.1038/s41598-017-02460-2>
- Mei M, Li J, Wang S et al (2019) Prompting fab yeast surface display efficiency by ER retention and molecular chaperon Co-expression. *Front Bioeng Biotechnol* 7:1–11. <https://doi.org/10.3389/fbioe.2019.00362>
- Meng L, Forouhar F, Thieker D et al (2013) Enzymatic basis for N-glycan sialylation: structure of rat  $\alpha$ 2,6-sialyltransferase (ST6GAL1) reveals conserved and unique features for glycan sialylation. *J Biol Chem* 288:34680–34698. <https://doi.org/10.1074/jbc.M113.519041>
- Munro R, de Vlugt J, Ladizhansky V, Brown LS (2020) Improved protocol for the production of the low-expression eukaryotic membrane protein human aquaporin 2 in *pichia pastoris* for solid-state NMR. *Biomolecules*. <https://doi.org/10.3390/biom10030434>
- Neudecker P, Lundström P, Kay LE (2009) Relaxation dispersion NMR spectroscopy as a tool for detailed studies of protein folding. *Biophys J* 96:2045–2054
- Nilsson B, Moks T, Jansson B et al (1987) A synthetic IgG-binding domain based on staphylococcal protein A. *Protein Eng Design Sel*. <https://doi.org/10.3934/microbiol.2020001>
- Parapouli M, Vasileiadis A, Afendra AS, Hatziloukas E (2020) *Saccharomyces cerevisiae* and its industrial applications. *AIMS Microbiol* 6:1–31
- Rakestraw A, Wittrup KD (2006) Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 93:896–905. <https://doi.org/10.1002/bit.20780>
- Rakestraw JA, Sazinsky SL, Piatasi A et al (2009) Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 103:1192–1201. <https://doi.org/10.1002/bit.22338>
- Rodriguez E, Rama Krishna N (2001) An economical method for 15 N/13 C isotopic labeling of proteins expressed in *Pichia pastoris*. *J Biochem*. <https://doi.org/10.1093/oxfordjournals.jbchem.a002957>
- Sawada J, Terao T, Itoh S, Maeda M, Tsuji A, Hosoda H, Nambara T (1987) Production and characterization of monoclonal antibodies to 17 alpha-hydroxyprogesterone. *J Steroid Biochem* 28(4):405–410. [https://doi.org/10.1016/0022-4731\(87\)91058-2](https://doi.org/10.1016/0022-4731(87)91058-2)
- Schaefer J, Plickthun A, v (2012) Engineering aggregation resistance in IgG by two independent mechanisms: lessons from comparison

- of *Pichia pastoris* and mammalian cell expression. *J Mol Biol* 417:309–335
- Shenoy A, Yalamanchili S, Davis AR, Barb AW (2021) Expression and display of glycoengineered antibodies and antibody fragments with an engineered yeast strain. *Antibodies*. <https://doi.org/10.3390/antib10040038>
- Shusta EV, Raines RT, Plückthun A, Wittrup KD (1998) Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat Biotechnol* 16(8):773–777. <https://doi.org/10.1038/nbt0898-773>
- Subedi GP, Barb AW (2015) The structural role of antibody N-glycosylation in receptor interactions. *Structure* 23(9):1573–1583. <https://doi.org/10.1016/j.str.2015.06.015>
- Subedi GP, Hanson QM, Barb AW (2014) Restricted motion of the conserved immunoglobulin G1 N-glycan is essential for efficient FcγRIIIa binding. *Structure* 22:1478–1488. <https://doi.org/10.1016/j.str.2014.08.002>
- Sugiki T, Ichikawa O, Miyazawa-Onami M et al (2012) Isotopic labeling of heterologous proteins in the yeast *Pichia pastoris* and *Kluyveromyces lactis*. *Methods Mol Biol* 831:19–36. <https://doi.org/10.1016/j.jstr.2014.08.002>
- Tashiro M, Tejero R, Zimmerman DE et al (1997) High-resolution solution NMR structure of the Z domain of Staphylococcal protein A. *J Molecular Biol* 272(4):573–590
- Wall KP, Hough LE (2018) In-cell NMR within budding yeast reveals cytoplasmic masking of hydrophobic residues of FG repeats. *Biophys J* 115:1690–1695
- Wigzell H (1983) Biological significance of carbohydrate chains on monoclonal antibodies (tunicamycin/Fc receptor/complement/protein A/immunocomplex)
- Wood MJ, Komives EA (1999) Production of large quantities of isotopically labeled protein in *Pichia pastoris* by fermentation. *J Biomol NMR* 13(2):149–159
- Wood MJ, Sampoli Benitez BA, Komives EA (2000) Solution structure of the smallest cofactor-active fragment of thrombomodulin. *Nat Struct Biol* 7(3):200–204
- Xie Y, Han X, Miao Y (2018) An effective recombinant protein expression and purification system in *Saccharomyces cerevisiae*. *Curr Protoc Mol Biol*. <https://doi.org/10.1002/cpmb.62>
- Yagi H, Zhang Y, Yagi-Utsumi M et al (2015) Backbone 1H, 13C, and 15N resonance assignments of the Fc fragment of human immunoglobulin G glycoprotein. *Biomol NMR Assign* 9:257–260
- Yamaguchi Y, Kim H, Kato K et al (1995) Proteolytic fragmentation with high specificity of mouse immunoglobulin G mapping of proteolytic cleavage sites in the hinge region. *J Immunol Methods*. [https://doi.org/10.1016/0022-1759\(95\)00010-8](https://doi.org/10.1016/0022-1759(95)00010-8)
- Yamaguchi Y, Nishimura M, Nagano M et al (2006) Glycoform-dependent conformational alteration of the Fc region of human immunoglobulin G1 as revealed by NMR spectroscopy. *Biochim Biophys Acta Gen Subj* 1760:693–700. <https://doi.org/10.1016/j.bbagen.2005.10.002>
- Yanaka S, Yamazaki T, Yogo R et al (2017) NMR detection of semi-specific antibody interactions in serum environments. *Molecules*. <https://doi.org/10.3390/molecules22101619>
- Yanaka S, Yagi H, Yogo R et al (2018) Stable isotope labeling approaches for NMR characterization of glycoproteins using eukaryotic expression systems. *J Biomol NMR* 71:193–202
- Yanaka S, Yagi H, Yogo R et al (2022) Glutamine-free mammalian expression of recombinant glycoproteins with uniform isotope labeling: an application for NMR analysis of pharmaceutically relevant Fc glycoforms of human immunoglobulin G1. *J Biomol NMR*. <https://doi.org/10.1007/s10858-021-00387-5>
- Yao Y, Wang J, Viroonchatapan N et al (2002) Yeast expression and NMR analysis of the extracellular domain of muscle nicotinic acetylcholine receptor α subunit. *J Biol Chem* 277:12613–12621
- Yu KK, Aguilar K, Tsai J et al (2012) Use of mutated self-cleaving 2A peptides as a molecular rheostat to direct simultaneous formation of membrane and secreted anti-HIV immunoglobulins. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0050438>

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