

# Functional production of human antibody by the filamentous fungus *Aspergillus oryzae*

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## Research

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# Abstract

Background: Monoclonal antibodies (mAbs) as one of the biopharmaceuticals take a pivotal role in the current therapeutic application. Generally, mammalian cell lines such as Chinese hamster ovary (CHO) cell lines are used to produce the recombinant antibody. However, there are still concerns about the high cost and the risk of pathogenic contamination when using mammalian cells. *Aspergillus oryzae*, the filamentous fungus recognized as a GRAS (Generally Regarded As Safe) organism, has an ability to secrete a large amount of proteins into the culture supernatant, and thus the fungus has been used as one of the cost-effective microbial hosts for heterologous protein production. Pursuing this strategy, human anti-TNF $\alpha$  antibody adalimumab, one of the world's best-selling antibodies for the treatment of immune-mediated inflammatory diseases including rheumatoid arthritis, was chosen to attempt for producing the full length of mAbs by *A. oryzae*. Generally, *N*-glycosylation in antibody affects the immune effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) via binding to the Fc receptor (Fc $\gamma$ R) on immune cells. The CRISPR/Cas9 system was used to firstly delete the *Aooch1* encoding a key enzyme for the hyper-mannosylation process in fungi to investigate the binding ability of antibody with Fc $\gamma$ RIIIa.

Results: The adalimumab was expressed in *A. oryzae* by the fusion protein system with  $\alpha$ -amylase AmyB. The full-length adalimumab consisting of two heavy and two light chains was successfully produced in the culture supernatants. Among the producing strains, the highest amount of antibody was obtained from the ten-protease deletion strain (39.7 mg/L). Two-step purifications by Protein A and size-exclusion chromatography were applied to obtain the high purity sample for further analysis. The antigen-binding and TNF $\alpha$  neutralizing activities of the adalimumab produced by *A. oryzae* were comparable with those of the commercial product – Humira. No apparent binding with the Fc $\gamma$ RIIIa was detected with the recombinant adalimumab even by altering the *N*-glycan structure under the *Aooch1* deletion, which suggests only a little additional activity of immune effector functions.

Conclusion: These results demonstrated an alternative low-cost platform for human antibody production by using *A. oryzae*, possibly offering a reasonable expenditure for patient's welfare.

## Background

Antibody (or immunoglobulin) is a macro-molecule consisting of four polypeptides. Two pairs of identical heavy chains and light chains form a "Y" shape structure through the disulfide bonds (Fig. S1). Two tops of the "Y" shape contain the fragment antigen-binding region (Fab) including the variable domain (Fv) of the light and heavy chains. The amino acid sequence of this variable region varies greatly among different antibodies, which gives the antibody its specificity for binding to the antigen. The remaining of "Y" is called the fragment crystallizable region (Fc) and can be bonded to the surface of lymphocytes by the endogenous Fc receptors [1]. The antibody plays a crucial role in the immune system to protect the

body from the infection. In the global biopharmaceutical market, the antibodies especially immunoglobulin G (IgGs) have held the largest contribution currently and even in the future. They are recombinantly produced for therapeutic treatment such as cancer and autoimmune diseases [2]. For manufacturing the antibody, among the mammalian platforms, the Chinese hamster ovary (CHO) cell line is the most widely used [3]. However, there are some concerns about the high cost and the risky contamination of human pathogens. The high demand and expensive payment of antibodies have encouraged the development of a biosimilar produced by different hosts, which possesses comparable characteristics to the already approved biopharmaceutical [4]. Thus, biosimilar is predicted to play a vital role in biomedical market in the coming years and to significantly boost the biological therapy by extending more options for treatment processes [5].

Microbial expression system such as bacteria and fungi have been investigated due to their capacity to produce recombinant proteins with low-cost, simple culturing process and easy genetic modification [6]. *Escherichia coli* is the most popular system for recombinant protein production among bacteria. However, the recombinant protein is typically accumulated in the bacterial cytoplasmic compartment, resulting mostly in inclusion bodies. The recovery step to obtain functional proteins by complete denaturation and refolding is not highly efficient. The lack of ability to generate the disulfide bond in the full-length antibody structure is also a drawback of bacteria platform. Thus, bacteria are preferred to produce the antibody fragments such as dAb (single domain antibody) and scFv (single chain fragment variable) [6]. Yeasts have a better proficiency to secrete and to process the disulfide bond of recombinant proteins, and thus they have been used for producing various recombinant proteins for food and industrial application [7, 8]. Yeast species such as *Saccharomyces cerevisiae*, *Pichia pastoris* and *Ogataea minuta* were reported for recombinant production not only of antibody fragments [9] but also of full-length antibodies [10–12]. Besides yeasts, filamentous fungi such as *Aspergillus* and *Trichoderma* species are also expected to be favored hosts for the recombinant production of antibodies due to their high abilities to secrete a large amount of proteins. However, the antibody production by filamentous fungi is less investigated, since the full-length antibody production has been reported only with one report using *Aspergillus niger* var. *awamori* [13] in spite of a number of reports of antibody fragment production [14]. Hence, more efforts need to be paid to unlock the true potential of filamentous fungi.

One of the most important activities of IgG is neutralization, the specific binding to neutralize the antigen, which depends on the structure of Fab region [15]. Another important activity of IgG is the effector functions in antibody-dependent cellular cytotoxicity (ADCC) to recruit the cytotoxic cells such as natural killer cells through the Fc receptor – FcγRIIIa [16]. This feature is taken by the Fc moiety of IgG, which appears in most of therapeutic approved antibodies [17]. In addition, the Fc region helps to protect the IgG from lysosomal degradation by endothelial cells and prolongs the IgG residence time in the vessel circulation to 2–4 weeks, while the antibody fragments only containing the Fab region have the retention time less than 24 hours [18]. Consequently, the longer period of antigen binding in the antibody with Fc region helps to improve the efficiency of antibody in each therapeutic treatment [19]. Therefore, the production of the full-length IgG may be more preferred in the production of biopharmaceutical antibody. The full-length antibody IgG contains an N-linked glycosylation site in the Fc region at Asn297 position.

The N-glycan structure contributes to the effector functions in ADCC through Fc receptor on the surface of immune cells [20]. For therapeutic antibody production, it is necessary to genetically modify the host cells for mimicking the mammalian N-glycosylation to increase the efficiency and avoid unwanted immunological response [21]. Yeasts and filamentous fungi possess N-glycan with the high-mannose structure initiated by the  $\alpha$ -1,6-mannosyltransferase (Och1) in the Golgi [22], which differs from the complex-type structure containing sialic acid, galactose, fucose and N-acetylglucosamine in human IgG [23]. After the addition of the mannose unit by Och1, N-glycan can be elongated by multi-enzymes of Mnn1, Mnn2, Mnn4, Mnn5 and Mnn6 [22]. Therefore, the deletion of och1 gene is often the first step in glycoengineering strategies [24–26]. Further modification of glycosylation by heterologously expressing glycosidases, glycosyltransferases and sugar transporters allowed for producing IgGs with the humanized N-glycan structure in yeasts [27], while glycoengineering has not yet been performed for IgG production in filamentous fungi.

The filamentous fungus *Aspergillus oryzae* is listed as Generally Regarded As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) due to more than a thousand years of use in Japanese traditional food fermentation [28]. The fungus has been used as a favored host for heterologous protein production due to the ability to secrete large amounts of proteins into the culture medium [29], and the production of a hetero-oligomeric protein neoculin with the disulfide bond was reported [30]. Thus, *A. oryzae* is expected to be a high potential host for industrial antibody production. Aiming to introduce an adequate platform for industrial antibody production, herein for the first time, we produced the adalimumab by *A. oryzae*. Adalimumab is an antibody (IgG) that binds specifically to the inflammatory cytokine, human TNF $\alpha$ , and the antibody has been used in the therapy of the chronic inflammatory diseases including rheumatoid arthritis. Among the biotherapeutic antibodies, Humira, a commercial product of adalimumab, has led the list of top-selling pharmaceutical products since 2012 and achieved \$19.9 billion of global sales in 2018 [31]. Moreover, in this study, the initiate attempt for modifying the N-glycosylation by deleting the och1 orthologous gene in *A. oryzae* was performed to analyze the effect of the N-glycan to Fc receptor Fc $\gamma$ RIIIa. These results provided the basis for the development of therapeutic antibodies with cost reduction and easy manufacturing by *A. oryzae*.

## Results

Adalimumab production in the culture supernatant of *A. oryzae*

In this study, the full-length antibody – adalimumab was expressed by *A. oryzae* strains: NSIDv10, AUT1-ID-v10-sD and NSID- $\Delta$ P10, the hyper-producing strains that were successfully used for producing a large amount of foreign proteins in the previous reports. The NSIDv10 strain contains the deletion of Aovps10 for vacuolar protein sorting receptor to improve the protein secretion by reducing the trafficking pathway from Golgi to vacuoles [32]. In the AUT1-ID-v10-sD strain, beside Aovps10, the tripeptidyl peptidase gene AosedD was deleted in the hyper-producing mutant (AUT1) strain to further increase the protein production [29]. Additionally, the production loss in the culture medium was avoided by the deletion of ten-protease genes in NSID- $\Delta$ P10 strain [33]. The production of the full-length antibody requires two

oligopeptides of the heavy chain and light chain. They were fused with the  $\alpha$ -amylase AmyB and expressed under the control of amyB promoter, which is highly induced by culturing in the DPY medium. A short sequence coding for KRGGG for the cleavage site of Kex2-like protease was included in the linker to efficiently separate the fusion protein (Figs. 1 and S1). For comparison between the strains, codon-optimized genes for heavy chain and light chain of adalimumab (Fig. S2) were integrated with a single copy into the niaD and sC loci, respectively (Fig. 1). As expected, the antibody production by these strains was better than the control strain (NSID1) with the highest accumulation 39.7 mg/L at day 6 in the culture supernatant of the strain of ten-protease genes deletion (Fig. 2). The detectable amount of antibody could be seen after 2 days of incubation. In the control strain, the antibody concentration remained stable or slightly reduced after culturing 3 days, while in other strains the productivity continuously increased until day 6.

The appearance of the adalimumab in the culture supernatant was confirmed by Western blot analysis (Fig. 3A). The full-length antibody was exhibited in the non-reducing SDS-PAGE around 150 kDa. Another major band around 100 kDa might be the heavy-chain dimer, which was also seen in the previous report of *A. awamori* [13]. Moreover, in the reducing condition, the bands of the heavy chain and light chain were observed around 50 kDa and 26 kDa, respectively (Fig. 3B). Two forms of the IgG heavy chain were seen, which may involve the differential N-glycosylation and will be described in the latter part. There is no sign of the higher band corresponding to the unprocessed-fusion protein with AmyB protein in the culture supernatant of day 6. Since the AmyB has the molecular weight around 55 kDa, the fusion protein of heavy chain and light chain with AmyB protein were anticipated around 105 kDa or 81 kDa, respectively. Therefore, the cleavage between AmyB protein and antibody chains was successful.

#### Purification of the Adalimumab Produced by *A. oryzae*

To determine the characteristics of produced adalimumab, Protein A affinity chromatography was applied to capture the antibodies from the culture supernatant. As shown in the Fig. 4A, the target antibody was successfully obtained in the eluted fraction around 150 kDa. However, some minor bands of lower molecular masses were also visible in the purified sample. The comparison with the commercial product – Humira clearly confirmed the appearance of the target IgG and the unnecessary lower bands (Fig. 4B). The similar results were also reported in case of the antibody – trastuzumab produced by *A. awamori*, in which the bands around 100 kDa and 75 kDa were present for a heavy chain dimer and heavy-light chain dimer, respectively [13]. At this point, additional purification was required for obtaining a good antibody sample for further analysis, and thus the size-exclusion chromatography (SEC) was selected. The Protein A-purified sample was firstly concentrated to a proper volume by Vivaspin concentrator (50,000 MWCO). This step can also help to remove other proteins with a molecular weight equal or less than 50 kDa. Then, the concentrated sample was applied to SEC system as described in Methods. After running through the SEC column, the protein was separated in the range of fractions from 48 to 72 (Fig. 5). As expected, the target antibody was obtained firstly, while other lower molecular weight protein was separated in the later fraction (Fig. 5 and Fig. S3). The fractions containing the target adalimumab were collected and used in the following experiments.

## Analysis of the N-glycosylation in Adalimumab Produced by *A. oryzae*

It is generally accepted that the Och1 plays a crucial role in the divergence of N-glycosylation in yeast and filamentous fungi comparing to mammals [34]. In filamentous fungi, its function was demonstrated in *Aspergillus fumigatus* and *Neurospora crassa* [35, 36]. An Och1 ortholog, hereafter referred to as AoOch1, is also found in *A. oryzae* and has the highest identities around 77% with Och1 from *A. fumigatus* (Fig. S4). Thus, it is expected that AoOch1 also has a similar role in N-glycosylation. The Aooch1 gene was deleted by the CRISPR/Cas9 (Fig. 6) as described in Methods, and the  $\Delta$ Aooch1 strain produced the adalimumab in the culture supernatant as confirmed by Western blot analysis (Fig. 7). Then, the N-glycan pattern was analyzed by Western blot analysis. The adalimumab of Wild-type (WT) glycosylation displayed two bands of the heavy chain (Fig. 8), a similar result of trastuzumab produced by *A. awamori* in which the lower band presents for non-glycosylation and the upper band indicates the high-mannosylation in the range from 6 to 15 mannose units per mannose-type N-glycan [13]. The commercial product – Humira showed only a single band for the heavy chain with a low molecular complex-type N-glycan (Fig. 8). In the *A. oryzae*  $\Delta$ Aooch1 strain, the adalimumab heavy chain was produced only with a smaller N-glycan structure as compared to the high-mannose band of WT, but the unglycosylated heavy chain was not detected (Fig. 8). The different N-glycan sizes were confirmed by the cleavage of N-glycan with Glycopeptidase F, leading to the same migration in acrylamide gel (Fig. 8). Therefore, it is suggested that AoOch1 is involved in the generation of high-mannose N-glycan in *A. oryzae*.

## Antigen-binding assay by ELISA

The antigen-binding ability of adalimumab produced by *A. oryzae* WT and  $\Delta$ Aooch1 strains was tested by ELISA in comparison with the commercial product – Humira. The result showed that no significant difference between the affinity of the produced adalimumab and Humira to the human TNF $\alpha$  (Fig. 9). The similar values of EC50 further confirmed this result at 0.119, 0.108 and 0.095, respectively. Thus, it is concluded that *A. oryzae* can produce the adalimumab with a similar specific antigen-binding activity to that of the commercial product.

## Neutralization of human TNF $\alpha$ -induced cytotoxicity assay

To further confirm the effect of the antigen binding of adalimumab produced by *A. oryzae* toward the living cells, the neutralization activity to inhibit soluble TNF $\alpha$  was investigated and compared to that of the commercial product – Humira. Each antibody was co-incubated with 20 ng/ml recombinant Human TNF $\alpha$  in dose-dependent manner and applied to test the MDA-MB-468 cell viability by MTT assay. The graph as shown in Fig. 10 displayed a quite similar efficiency of neutralization between the IgG samples. These results illustrated the intensive level of similarity in bio-functions of adalimumab produced by *A. oryzae* and the approved product (Humira) in TNF $\alpha$ -mediated cytotoxicity.

## Fc $\gamma$ RIIIa binding assay

Beside the antigen-binding activity, the effector function through the interaction with immune cells at the Fc region is also an important activity to enhance the effectiveness of the therapeutic antibody. Since the N-glycan of antibody locates within the interaction zone with Fc $\gamma$  receptor, the N-glycan structure is expected to affect the Fc receptor binding [20]. To examine this issue, Fc $\gamma$ RIIIa binding ability of the adalimumab produced by WT and  $\Delta$ Aooch1 strains were determined in parallel with Humira (Figs. 11 and S5). Although the binding activity was displayed in Humira, the adalimumab produced by WT and  $\Delta$ Aooch1 did not show any significant binding with Fc $\gamma$ RIIIa. This result suggested the N-glycan structures containing mannose units but lacking other sugar units such as galactose and N-acetylglucosamine could decrease the interaction between adalimumab and Fc $\gamma$ RIIIa.

## Discussion

In the molecular approach, the codon-optimization and expressing the heterologous protein with the homologous secreted protein as a fusion protein have been proven as the most efficient strategy for filamentous fungal platform [37–39]. The full-length antibody production has been only reported in trastuzumab by *A. awamori*, which used the fusion protein system with glucoamylase and a linker containing the cleavage site (Lys-Arg) for Kex2-like protease [13]. In *A. oryzae*, AmyB protein has been used to express the fusion protein for producing heterologous proteins such as chymosin, human lysozyme [29] and especially neoculin, which was produced by expressing 2 different polypeptides to form the heterodimeric protein [30]. Similar to yeast Kex2 enzyme [40], the amino acid sequence after KR cleavage site plays an important role for efficient cutting by Kex2-like protease in fungi to release the extracellular protein [41]. Collectively, to produce the recombinant antibody for the first time in *A. oryzae*, the heavy and light chains of adalimumab were expressed as the fusion protein with AmyB protein. Moreover, the appearance of KRGGG sequence at the end of the linker could help to separate the fusion protein as mentioned above in the Results.

To select the producing strain for antibody, based on our previous reports, three genetically modified *A. oryzae* strains, which abundantly produce the heterologous proteins, were used in this study. Among these hyper-producing strains, the highest amount of adalimumab in the culture supernatant was obtained by NSID- $\Delta$ P10 strain with ten-protease genes deletion (Fig. 2), which indicated the major role of the endogenous proteases in the proteolytic degradation of heterologous proteins, especially the complex-structure protein such as antibodies.

The interaction with effector components is required for therapeutic antibody, and most of the approved antibodies are the full-length IgGs [14]. Thus, the full-length IgG is more preferred in biopharmaceutical. The amount of antibody production is affected by the culture method, and it seems to be quite different among the antibodies even using the same host. Recently, the IgG production by CHO cells can generally reach 5 g/L through optimizing producer cell lines, culture media, and long incubation with high cell densities [42]. In addition, a screening of novel transfected CHO cells from 4345 clones has shown the adalimumab concentration on day 12 ranging from 124–594 mg/L by using the batch culture with glucose supplement [43]. In yeast, the high-throughput screening was applied to select the *P. pastoris*

strain producing IgGs up to 1 g/L in 0.5 L bioreactor [44]. The anti-Her2 IgG was produced by *P. pastoris* with the level of 227 mg/L using 3 L bioreactor [45]. In filamentous fungi, there is one study of trastuzumab production in *A. awamori* using 14 L-fermenter with the amount of 900 mg/L [13]. In this study, *A. oryzae* produced the full-length adalimumab with the productivity of 39.7 mg/L in the culture supernatant. Although this may not be directly suitable for industrial application, the antibody productivity by *A. oryzae* can be further improved by several rounds of mutagenesis and further genetic modifications such as deletion of other protease genes, optimizations involving protein secretion and the increasing of copy-number for heavy and light chains.

The full-length antibody normally contains two N-glycans on the Fc region of pair heavy chains at Asn297 [46], and adalimumab also has the N-glycosylation sites in Fc region at Asn301 in the sequence Asn-Ser-Thr of the typical motif (Asn)-X-Ser/Thr (where X is any amino acid except a Pro) [47]. The two bands of adalimumab heavy chain produced by *A. oryzae* WT strain in Fig. 8 may correspond to high mannose-type glycan (more than or around 9 mannose units) and non-glycosylation (lower) as reported in *A. awamori* [13]. The complex-type glycan in Humira with a low molecular weight placed the corresponding band in the middle position between the two bands of adalimumab produced by *A. oryzae* WT (Fig. 8). In the  $\Delta Aooch1$ , only a single band was found at a lower position of the upper band of WT (Fig. 8), which suggests the ratio reductions of the heavy chain with high mannose-type glycan. Interestingly, the unglycosylated heavy chain was not detected in the  $\Delta Aooch1$  (Fig. 8), raising the possibility that the heavy chain would be susceptible to the proteolytic degradation in the absence of high mannose-type glycan.

One of the most important activities of the IgG is the binding ability to the target antigen. To determine the potency of the adalimumab produced by *A. oryzae* WT and  $\Delta Aooch1$ , their binding to TNF $\alpha$  was analyzed. It is suggested that the N-glycan structure does not affect the antigen affinity due to the far distance between the N-glycosylation site and the Fv domain [48]. Hence, in case of the adalimumab produced by *A. oryzae*, the antigen-binding affinity with TNF $\alpha$  was expected to be equivalent between the WT and  $\Delta Aooch1$  samples. In the antigen affinity measurement (Fig. 9), both adalimumab produced by WT and  $\Delta Aooch1$  exhibited the binding activity with a similar level to that of the commercial product – Humira. Moreover, the ability to neutralize cell toxicity mediated by TNF $\alpha$  also confirmed the high similarity of their bio-functional activity (Fig. 10), which clearly demonstrates the potential of *A. oryzae* in the functional antibody production.

Fc $\gamma$  receptors play critical roles in phagocytosis, endocytosis and antibody-dependent cellular cytotoxicity (ADCC) [49]. One of the receptors, Fc $\gamma$ RIIIa, plays a significant role in the resistance to infection and cancer by activating the immune cells especially natural killer (NK) cells, in which immune complexes with Fc region of antibody induce ADCC [50]. The conformation of the Fc region is changed after the antigen binding, which influences the effector function of IgG [51]. Formation of antibody-antigen complex recruits adalimumab and stimulates its interaction possibility with Fc $\gamma$ RIIIa [52]. Since the Fc region contains N-glycan, the addition of TNF $\alpha$  could lead to the orientation of Fc and N-glycan to become more preference for Fc $\gamma$ RIIIa. The N-glycan structure in Fc region of antibody affects the

interaction between the antibody and Fc receptor. The fucosylated antibody was reported to reduce the binding affinity to FcγRIIIa [53]. In Humira, the fucosylated N-glycan is contained with the ratio of 10% [54], and thus relatively low affinity to FcγRIIIa was detected when high concentrations of Humira (15 µg/ml) and TNFα (40 ng/ml) were used (Fig. 11). In contrast, no apparent binding was found with the adalimumab produced by *A. oryzae*, and the N-glycan alteration by Aooch1 deletion did not improve the FcγRIIIa binding (Fig. 11). Based on the crystal structure of the N-glycan attached to Asn297 of IgG Fc region together with FcγRIIIa (PDB entry code 4CDH), the N-glycan is close to the binding surface of FcγRIIIa [48]; alteration in the N-glycan structure involving ion charge and steric allocation would shift the Fc region-FcγRIIIa interaction to the inappropriate position. On the other hand, the bulky mannose structure of N-glycan in the adalimumab produced by *A. oryzae* WT strain may interfere the approach of FcγRIIIa to bind with the Fc region due to stereometric occupation. The previous study demonstrated that the mannose-type glycan of rituximab produced by *P. pastoris* has a higher FcγRIIIa affinity as compared to the fucosylated complex-type glycan from CHO cell line [55]. The similar comparative analysis revealed the highest FcγRIIIa affinity with the afucosylated complex-type glycan [56], and thus this enhancement is likely due to the afucosylated glycan [57]. In case of the adalimumab, the afucosylated complex-type glycan of Humira could have a dominant effect on the FcγRIIIa binding, while the mannose-type glycan of adalimumab produced by *A. oryzae* WT and Aooch1 deletion strains are inadequate. In addition, the adalimumab of Humira shows some FcγRIIIa binding activity to lyse the TNF-expressing cell in ADCC test; but the mechanism has not yet been clarified and may differ from the response to various TNF-related diseases [52]. Hence, further research is required to determine the effect of the N-glycan structure on the binding activity between the adalimumab and the Fc receptor.

The biosimilar of world's best-selling IgGs – Humira was produced by *A. oryzae* in this study. The full-length of adalimumab was obtained with the equivalent abilities in antigen binding and neutralization. The long history of food and research has provided a strong background for *A. oryzae* in optimized cultivation and genetic manipulation [39]. These features would strengthen the industrial application of *A. oryzae* as a low-cost platform for not only adalimumab or other IgGs but also novel biopharmaceutical products in diagnostic and therapy. In adalimumab, the ADCC activity corresponding to Fc receptor binding has not been established clearly yet [58]. To avoid any possible impact and support other biopharmaceutical production, mimicking the mammalian N-glycan structure will be necessary in *A. oryzae*. The N-glycosylation modification in filamentous fungi is still in the initial step to produce the glycan core, which needs to be catalyzed by additional enzymes for converting to the mammalian-like glycan structure [22]. The effect of the Aooch1 gene on the N-glycan structure and bio-function of adalimumab from *A. oryzae* was demonstrated in this study. To achieve the ultimate goal of mimicking the mammalian N-glycan structure, CRISPR/Cas9 system would be used to knock-out the genes in the high-mannose pathway and to introduce the genes for adding correct sugar monomer to the glycan core-structure, which may be considered from the glycan-engineering process in *P. pastoris* [27]. Further qualitative and quantitative improvement in *A. oryzae* as a host would lead to the establishment of a promising production platform of biopharmaceutical products.

# Conclusion

The great demand together with the requirement for reducing cost of the trading antibodies have drawn more attention to finding a suitable expression platform for producing recombinant antibodies. Our study demonstrated that the adalimumab was successfully produced in the culture supernatant of *A. oryzae* transformants with similar affinities and biological activities to its commercial form - Humira. This study would stimulate the application of *A. oryzae* or filamentous fungi, in general, to be used for the industrial production of pharmaceutical proteins.

# Methods

## Materials and chemical

Humira® - AbbVie and Eisai

## Strains and growth media

*Escherichia coli* DH5 $\alpha$  (Takara Bio, Shiga, Japan) was cultured in LB medium (1% peptone, 0.5% yeast extract, and 0.5% NaCl). The *A. oryzae* wild-type strain, RIB40 [59] and a strain with a highly efficient gene-targeting background (*niaD*<sup>-</sup> *sC*<sup>-</sup>  $\Delta$ ligD), NSID1 [32], were used as a DNA donor. The *A. oryzae* strains used in this study for antibody production are listed in the Table 1. The conidia of *A. oryzae* were collected by growth on the PDA agar medium (Potato Dextrose Agar; Nissui Pharmaceutical, Tokyo, Japan). DPY medium containing 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH<sub>2</sub>PO<sub>4</sub>, and 0.05% MgSO<sub>4</sub> was used for the pre-culture of transformants. Czapek-Dox (CD) medium (2% glucose, 0.3% NaNO<sub>3</sub>, 0.2% KCl, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.002% FeSO<sub>4</sub>·7H<sub>2</sub>O, pH 5.5) was used for selection using *niaD* and *sC*-based plasmid integration. To producing antibody, 5xDPY medium containing 10% dextrin, 5% polypeptone, 2.5% yeast extract, 0.5% K<sub>2</sub>HPO<sub>4</sub>, and 0.05% MgSO<sub>4</sub> was used.

## Plasmid construction

First, the DNA sequences of heavy chain and light chain of adalimumab were obtained from the Drugbank database (<https://www.drugbank.ca/>) and optimized by the codon usage table of *A. oryzae*. Then they were synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific, Waltham, MA, USA). The open reading frame encoded a fusion protein consisting of the  $\alpha$ -amylase gene (*amyB*), a short linker including the sequence encoding for KRGGG (cleavage site for Kex2-like protease) and the mature heavy or light chain of adalimumab. Vectors pUtNAN [60] and *pisCIIA* [61] were used for the transformation of *A. oryzae* to introduce expression cassettes containing adalimumab's heavy chain or light chain into the *niaD* and *sC* loci, respectively. These vectors contain the dextrin-inducible *amyB* promoter and *amyB* terminator, and the expression cassette was inserted at the *Sma*I site located at the downstream of the *amyB* promoter.

## Transformation and expression in *A. oryzae*

Transformation of *A. oryzae* was performed as described previously [62], and transformants were selected by growth on CD medium. Transformants were transferred to a new selective medium twice, and the colony PCR using KOD FX Neo (Toyobo, Tokyo, Japan) was applied to confirm the correct transformants.

To induce antibody production, the conidia of *A. oryzae* transformants were inoculated in 5 × DPY liquid medium (pH 8.0) with an approximate number of  $1 \times 10^7$  conidia per 100 ml. After incubation at 30 °C for 1–7 days at 150 rpm, the culture supernatant was collected by filtrating with Miracloth for further analysis.

### Quantification of antibody

The IgG concentration in the sample was measured by standard ELISA process using goat anti-Human IgG (Southern Biotech, Birmingham, AL, USA) for the capture step. The standard curve was built with the Human IgG isotype control (Genway, San Diego, CA, USA). Goat anti-Human IgG-AP (Southern Biotech) was used for IgG detection. The results were read at the absorbance 405 nm by using TriStar2 LB942 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany)

### Antibody detection

The sample was mixed with an approximate volume of 5 × sample loading buffer (250 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.025% Bromophenol Blue, 250 mM Dithiothreitol (DTT); without DTT in case of non-reducing condition) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Either the gels were stained for protein with Coomassie Brilliant Blue (Nacalai, Kyoto, Japan) or the proteins were transferred to membrane Immobilon-P membranes (0.45 µm; Merck Millipore, Tokyo, Japan) by Western blotting. Anti-IgG (H + L chain) (Human) pAb-HRP (Medical & Biological Laboratories CO., LTD, Nagoya, Japan) was used for detection by Western Lightning Plus system (PerkinElmer, Waltham, MA, USA). The target protein bands were visualized through luminescent image analyzer (LAS-4000 mini; Fujifilm, Tokyo, Japan).

### Purification

The supernatant of *A. oryzae* transformant was applied to Protein A Sepharose™ 4 Fast Flow system (GE Healthcare, Chicago, IL, USA) at room temperature. The antibody purification was performed by the instruction of the manufacturer with phosphate-buffered saline (PBS) as the equilibrating and washing buffers. The target protein was eluted with 0.1 M citric acid (pH 3.5) and 1 M Tris-HCl (pH 9.0) was quickly added to neutralize the eluted fraction for preserving the antibody activity.

To achieve higher purity, the antibody sample was subsequently performed by size-exclusion chromatography (SEC). Firstly, the Protein A-purified sample was collected and concentrated to proper volume by using Vivaspın Turbo ultrafiltration spin column 50K (Sartorius Lab, Göttingen, Germany). Secondly, the concentrated sample was loaded to the HiLoad™ 26/60 Superdex™ 200 prep grade (GE

Healthcare) in the ÄKTA purifier chromatography system at 4°C. The flow rate was maintained at 2 ml/min with PBS buffer (pH 7.4).

#### Deletion of Aooch1 gene in the *A. oryzae* by CRISPR/Cas9 system

To delete the target gene, the genome-editing plasmid together with a circular donor DNA plasmid were generated as described by Katayama et al. [60]. The sequence GTGGTTCCAGACGACACCCA in the middle of Aooch1 gene was selected to make the sgRNA cassette with U6 promotor. The genome-editing plasmid was created by introducing the sgRNA expression cassette to the pRGE-gRT6 plasmid [60] at the SmaI cutting site. Meanwhile, the 1 kb fragments of upstream and downstream of Aooch1 gene (gene ID AO090120000208) were amplified and joined together. This flanking sequence was inserted into the pUC19 linearized vector (Takara Bio) to create the donor plasmid. Both of the plasmids were applied to transformation using the NSID-ΔP10-derived strain producing adalimumab as mentioned above.

#### N-glycan analysis

For Glycopeptidase F (Takara Bio) treatment, 10 μl of protein sample was mixed with 2.5 μl of Denatured buffer with 0.2 M 2-mercaptoethanol and heated at 100°C for 3 min. Stabilizer solution (5 μl) was added and then mixed with 5.5 μl of distilled water. The reaction was carried by adding 2 μl (1 mU) of Glycopeptidase F and incubating at 37°C for 15–20 hours

#### Enzyme-linked immunosorbent assay (ELISA)

The binding activity of antibody to its antigen – recombinant human TNFα (BioLegend, San Diego, CA, USA) was measured by using 96-well Nunc MaxiSorp™ Flat-Bottom plate (Thermo Fisher Scientific). First, 100 μl TNFα solution was coated with a concentration of 100 ng/ml in sodium carbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed three times with PBS-Tween solution (PBS buffer with 0.05% Tween 20) and blocked with blocking buffer (PBS-Tween containing 5% skim milk) at room temperature for 1 hour. After washing three times with the PBS-Tween solution, 100 μl of adalimumab sample in serial dilution was added to each well and incubated at room temperature for 1 hour. Then, the wells were washed again three times with PBS-Tween solution and incubated with 1:8000-diluted Anti-IgG (H + L chain) (Human) pAb-HRP antibody at room temperature for 1 hour. The plate was washed four times with PBS-Tween solution and incubated with 100 μl ELISA POD Substrate TMB Solution (Nacalai) at room temperature. The reaction was stopped by adding an equal volume of 1 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance was measured at 450 nm using Multiskan FC microplate reader (Thermo Fisher Scientific).

#### Neutralization of human TNFα-induced cytotoxicity assay

The MDA-MB-468 cells were cultured into 96-well plate at a  $5 \times 10^4$  cells/well density in D-MEM / Ham's F-12 media (Fujifilm Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, MO, USA), 50 μg/mL gentamycin (Nacalai), 0.1 μM 2-mercaptoethanol (Nacalai), 100 ng/ml actinomycin D (Sigma) and 20 ng/ml human TNFα (BioLegend). Subsequently, cells were incubated with

three different types of anti-human TNF $\alpha$  antibody or human IgG isotype antibody in the range from 1 to 1000 ng/ml concentrations. After 24 hours, cell viability was analyzed by Cell proliferation Kit I (Roche, Mannheim, Germany). Briefly, 0.5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) labeling reagent was added in cell culture media. After 3 hours, 100  $\mu$ l of the solubilization solution was added into each well, and the culture plate was incubated for 24 hours at 37°C. The absorbance of the plate was measured at 600 nm by TriStar2 LB942 Multimode Reader (Berthold Technologies).

### Fc $\gamma$ R1IIa binding assay

The human Fc $\gamma$ R1IIa coding sequence was purchased from RIKEN Human cDNA Clones in Japan (clone ID: 5180561). The gene ORF sequence was amplified by PCR and cloned into the pcDNA3.1(+) expression vector. HEK-293T cells were cultured into 12-well plate in D-MEM / Ham's F-12 media supplemented with 10% fetal bovine serum, 50  $\mu$ g/mL gentamycin and 0.1  $\mu$ M 2-mercaptoethanol until 70–80% confluence. The complex of 10  $\mu$ g Polyethylenimine (Sigma) and 1.5  $\mu$ g of pcDNA3.1- human Fc $\gamma$ R1IIa or pcDNA3.1 empty vector in serum-free media was gently added into cell culture media. After 24 hours, expression of human Fc $\gamma$ R1IIa was confirmed by staining of the PE anti-human Fc $\gamma$ R1IIa antibody (BioLegend) in the SA3800 Spectral Analyzer (Sony Biotechnology, San Jose, CA, USA). After aspiration of culture media of transfected cells, three different types of 15  $\mu$ g/ml anti-human TNF $\alpha$  antibody and 40 ng/ml TNF $\alpha$  in serum-free D-MEM / Ham's F-12 media were added into cells. At 3 hours after incubation, these cells were harvested and stained by the APC anti-human light chain kappa antibody (BioLegend). Antibody-binding cells were detected by the SA3800 Spectral Analyzer.

## Abbreviations

Fab

Fragment antigen-binding

Fc

Fragment crystallizable region

CHO

Chinese hamster ovary

ADCC

antibody-dependent cell-mediated cytotoxicity

GRAS

Generally Regarded As Safe

CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats

## Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and materials

All data supporting the results are included within this article and its additional file. Plasmids and strains are available upon request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not Applicable.

Authors' contributions

H.H.H., R.S. and J.M. conceived and designed the research. H.H.H., N.M., T.S., T.M. and Y.C. conducted the experiments. T.M., M.T. and Y.C. analyzed and interpreted the data. H.H.H, N.M., T.S., T.K., R.S. and J.M. wrote the manuscript.

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Not Applicable.

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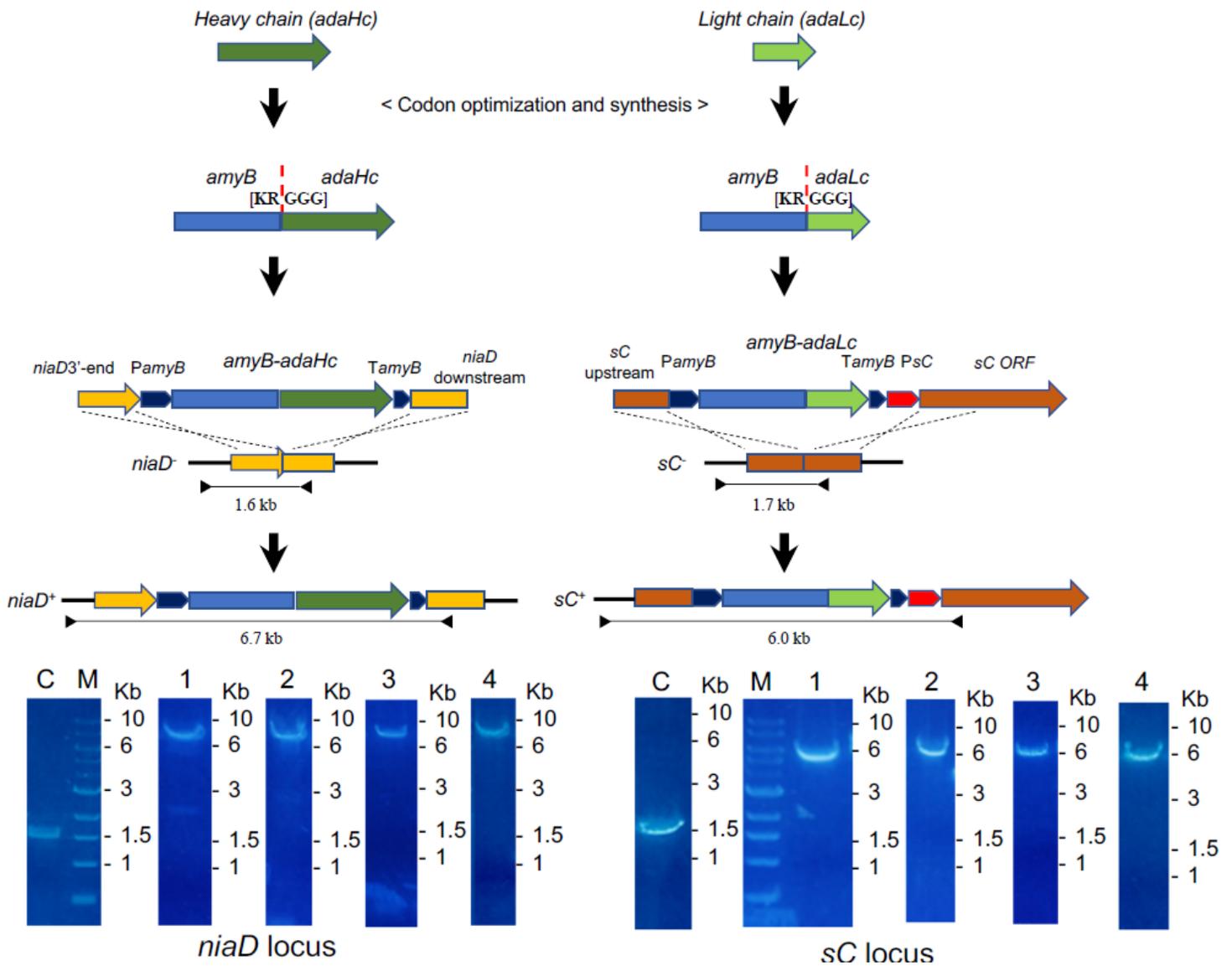
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## Table

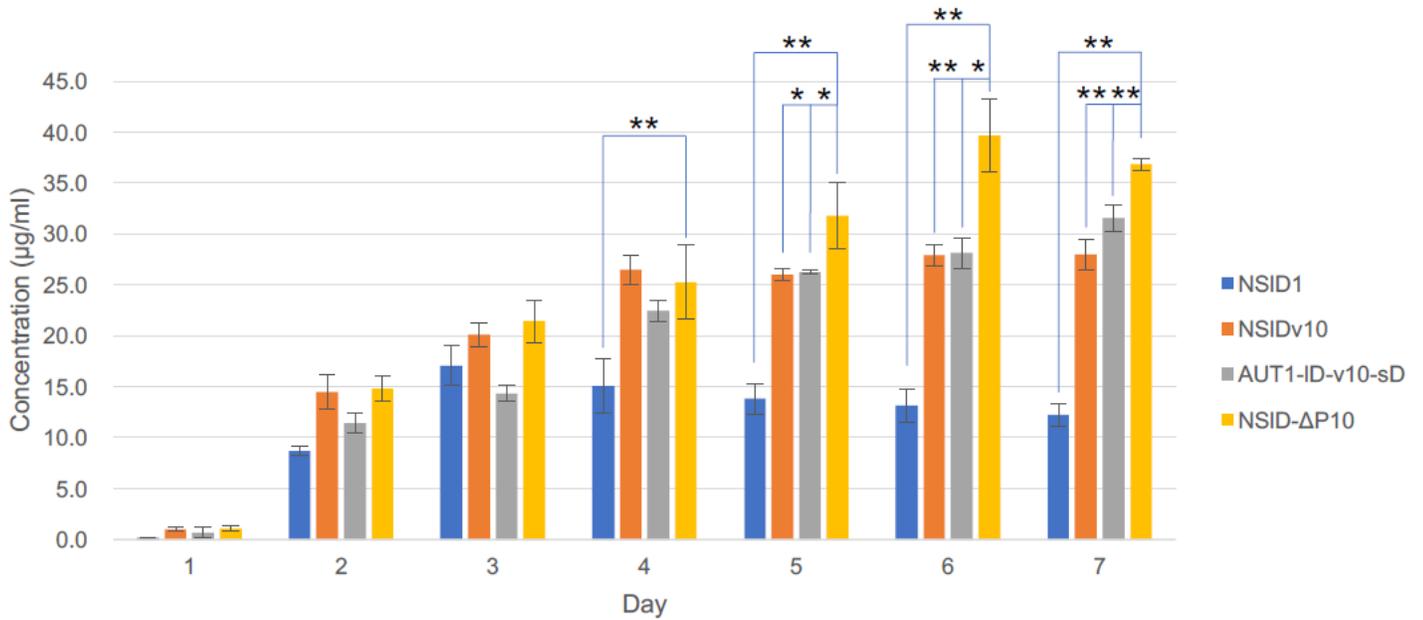
Due to technical limitations, Table 1 is provided in the Supplementary Files section.

## Figures



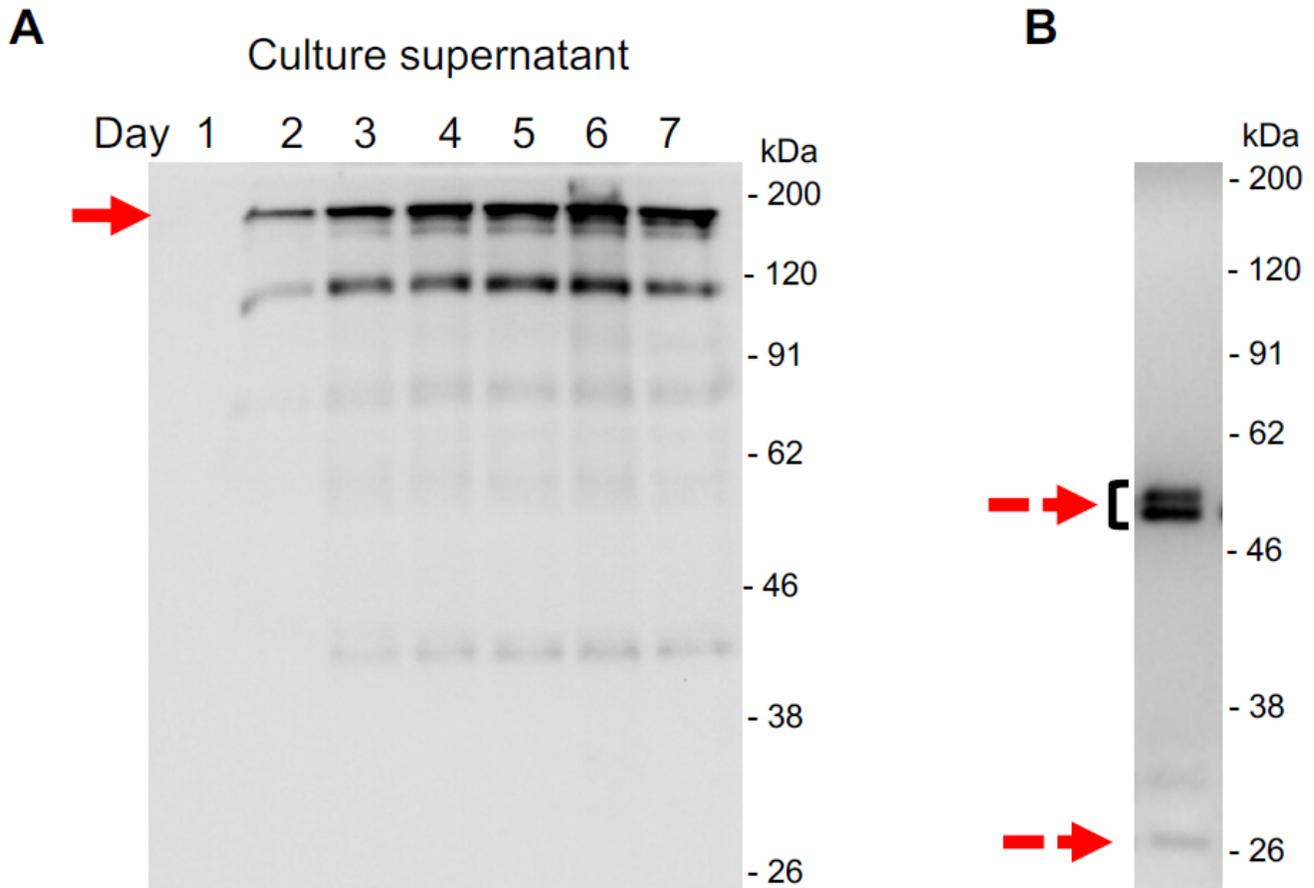
**Figure 1**

Schematic of the expression construct of adalimumab by *A. oryzae* and the genome PCR. Expression cassettes for heavy chain and light chain of adalimumab were introduced to *niaD* and *sC* loci, respectively. The dashed lines indicate the cleavage site of Kex2-like protease to separate the fusion protein. C, parental strains *niaD*- or *sC*-; 1, ID1-ada; 2, IDv10-ada; 3, AUT1-ID-v10-sD-ada; 4, ID- $\Delta$ P10-ada; M, DNA marker



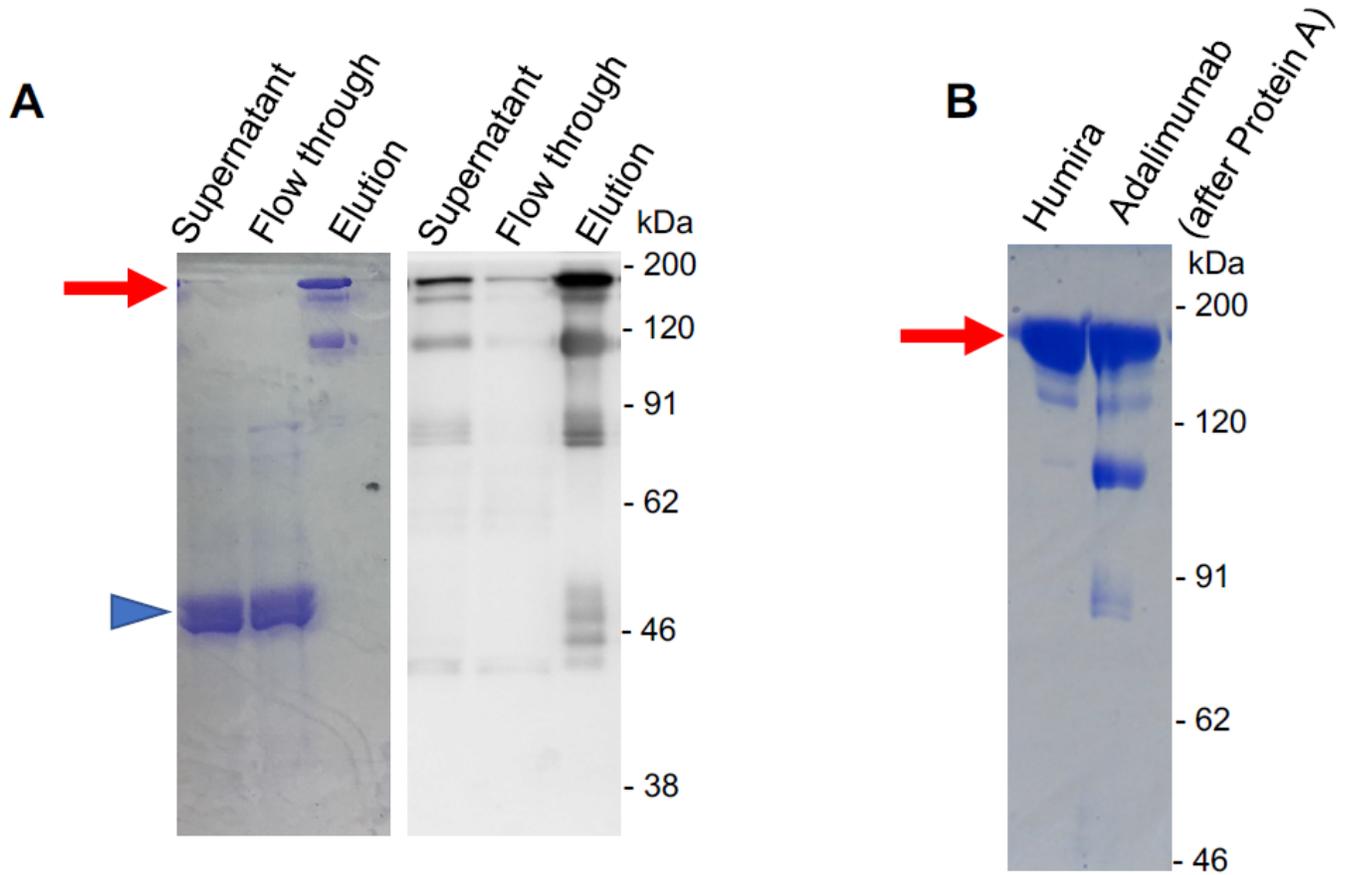
**Figure 2**

Adalimumab production in the culture supernatant of *A. oryzae*. The producing strains were grown in the 5×DPY medium. The host strains used for transformation to produce adalimumab were as follows: NSID1, control strain; NSIDv10, strain with the gene deletion of *Aovps10* for the vacuolar protein sorting receptor; AUT1-ID-v10-sD, hyper-producing mutant (AUT1) strain with double deletion of the tripeptidyl peptidase gene *sedD* and *Aovps10*; NSID-ΔP10, ten-protease genes deletion strain. Data present the mean of three independent experiments with error bars indicating SD. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$  (t-test)



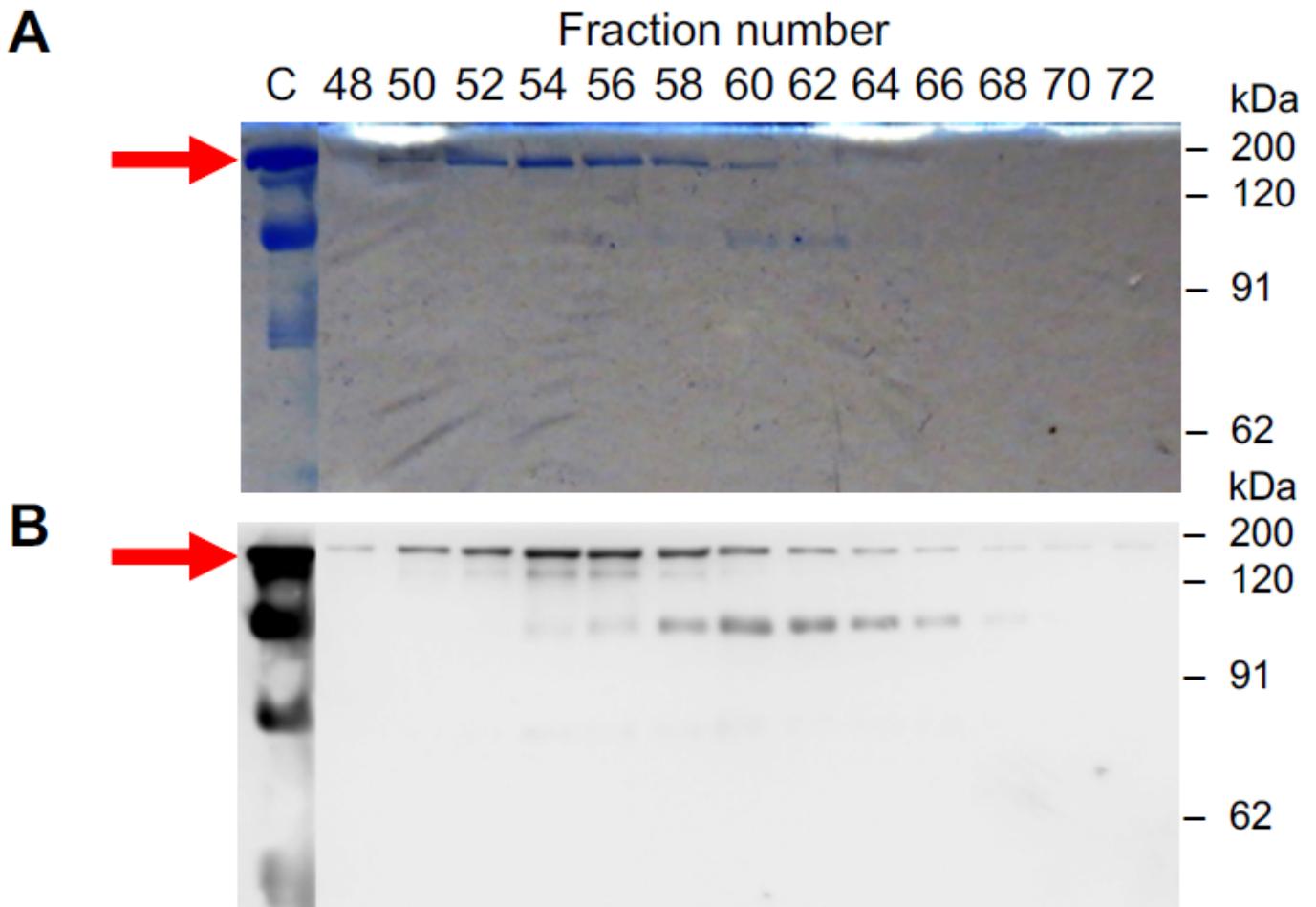
**Figure 3**

Western blot analysis of the adalimumab from *A. oryzae* in the culture supernatant. The NSID- $\Delta$ P10 transformant for producing adalimumab was cultured in 5 $\times$ DPY media, and culture supernatant was collected each day for analysis. A) Time-course analysis in non-reducing condition and B) the sample of day 6 in reducing condition. The arrow indicates the target IgG, and dashed arrows show the heavy chain and light chain around 50 kDa and 26 kDa, respectively



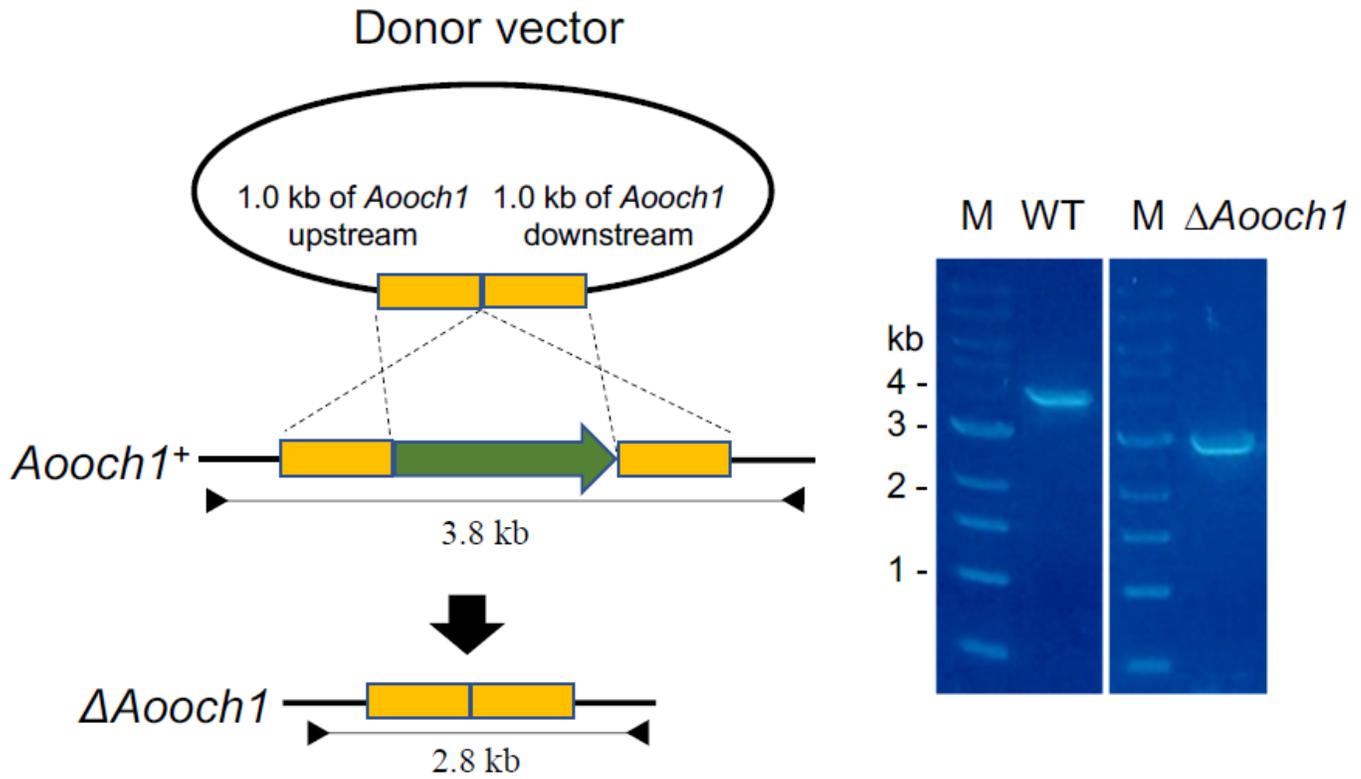
**Figure 4**

SDS-PAGE analysis of the purified antibody by Protein A affinity chromatography. A) CBB staining (left) and Western blot (right) analyses and B) comparison with the commercial product - Humira® (AbbVie and Eisai) by CBB staining. The arrow indicates the target antibody around 150 kDa, and the arrowhead shows the AmyB protein.



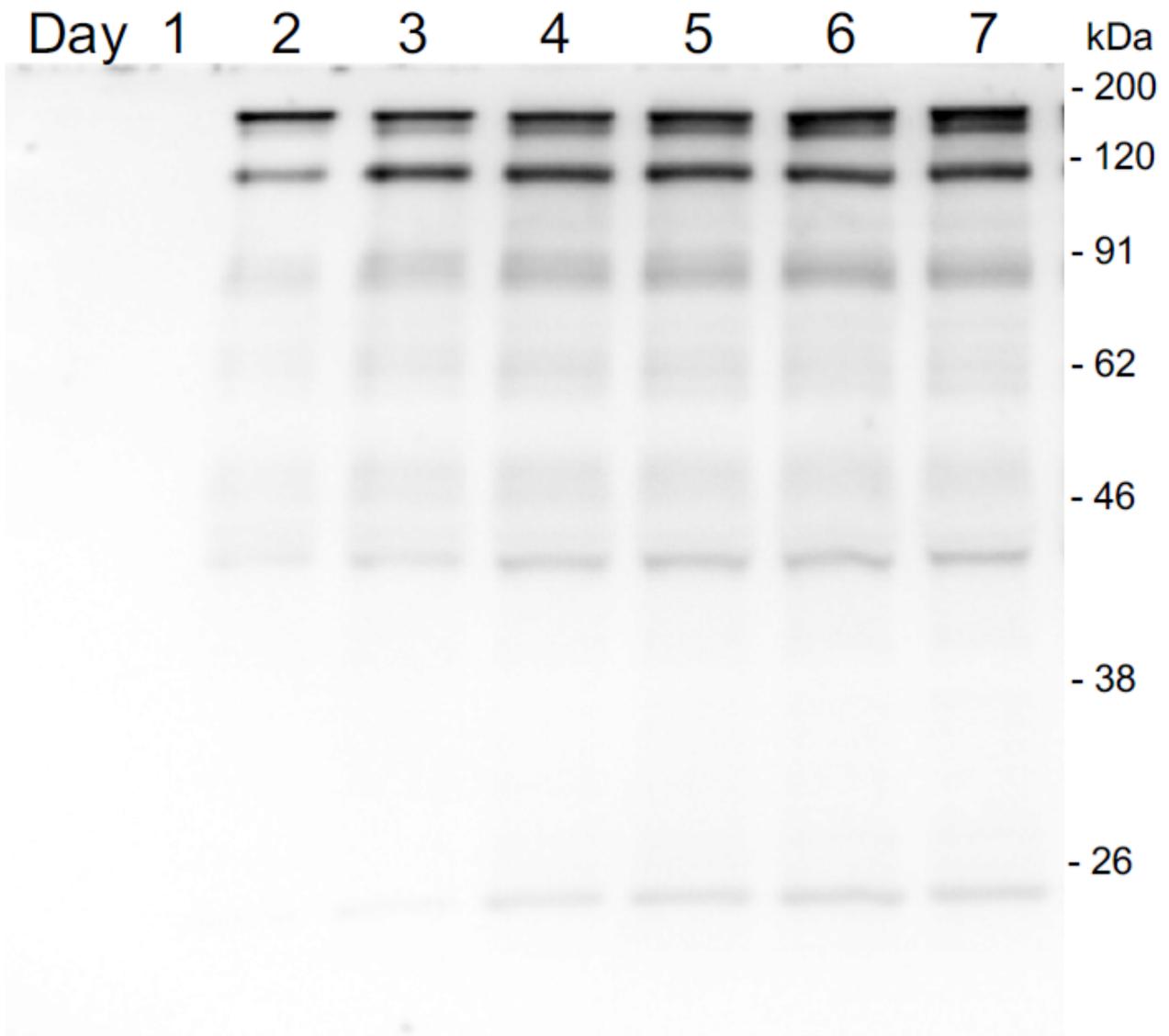
**Figure 5**

SDS-PAGE of adalimumab from *A. oryzae* by size-exclusion chromatography (SEC) in non-reducing condition. The purified adalimumab by Protein A was applied for secondary purification by SEC to isolate the target antibody at 150 kDa (shown by the arrow). A) CBB staining and B) Western blot analysis of the SEC fragments.



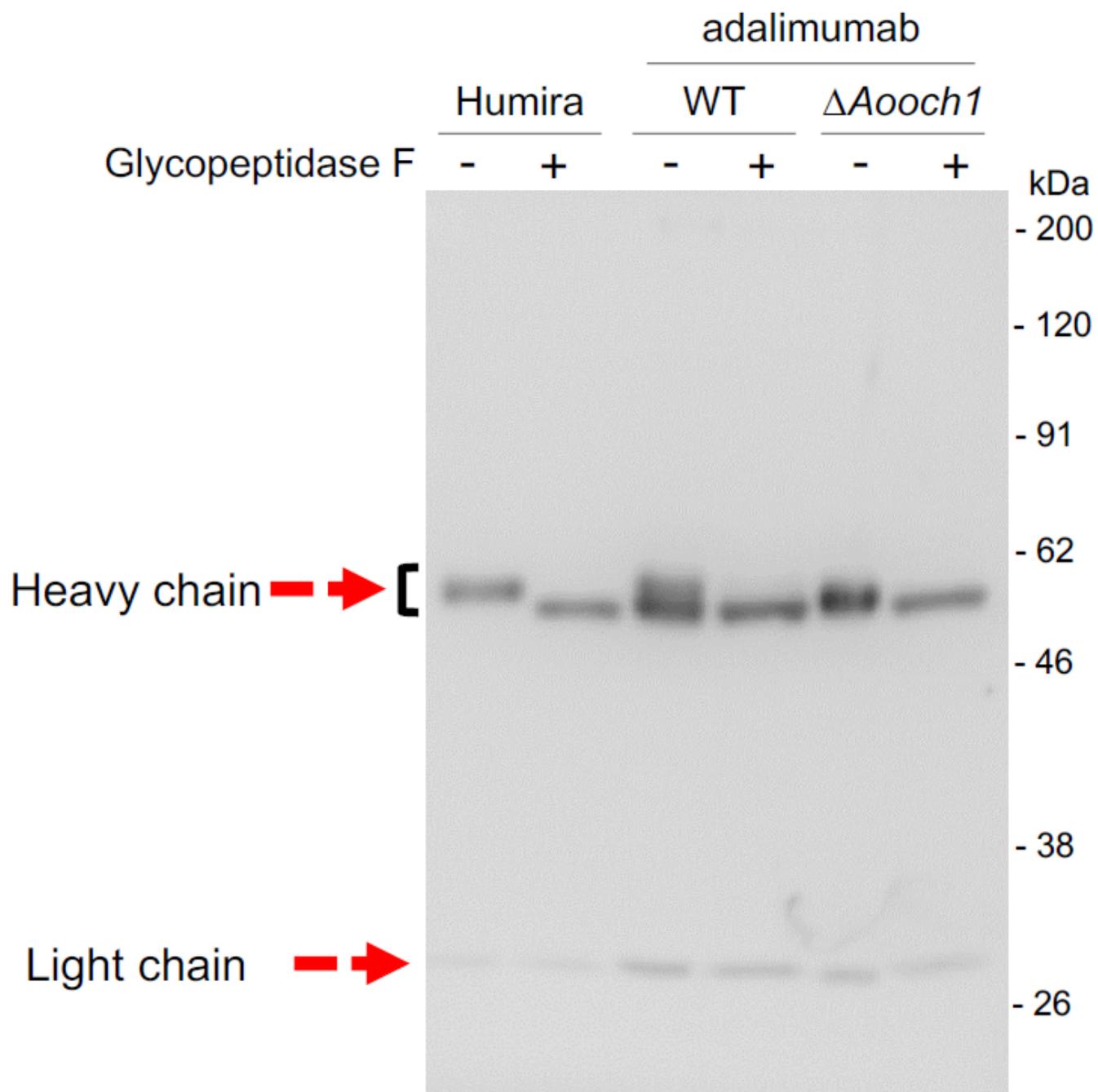
**Figure 6**

Construction of CRISPR/Cas9 system for deleting the *Aooch1* gene and the colony PCR. WT and  $\Delta Aooch1$  represent the wild-type NSID- $\Delta P10$  strain producing adalimumab and *Aooch1* gene deletion strain, respectively. M, DNA marker



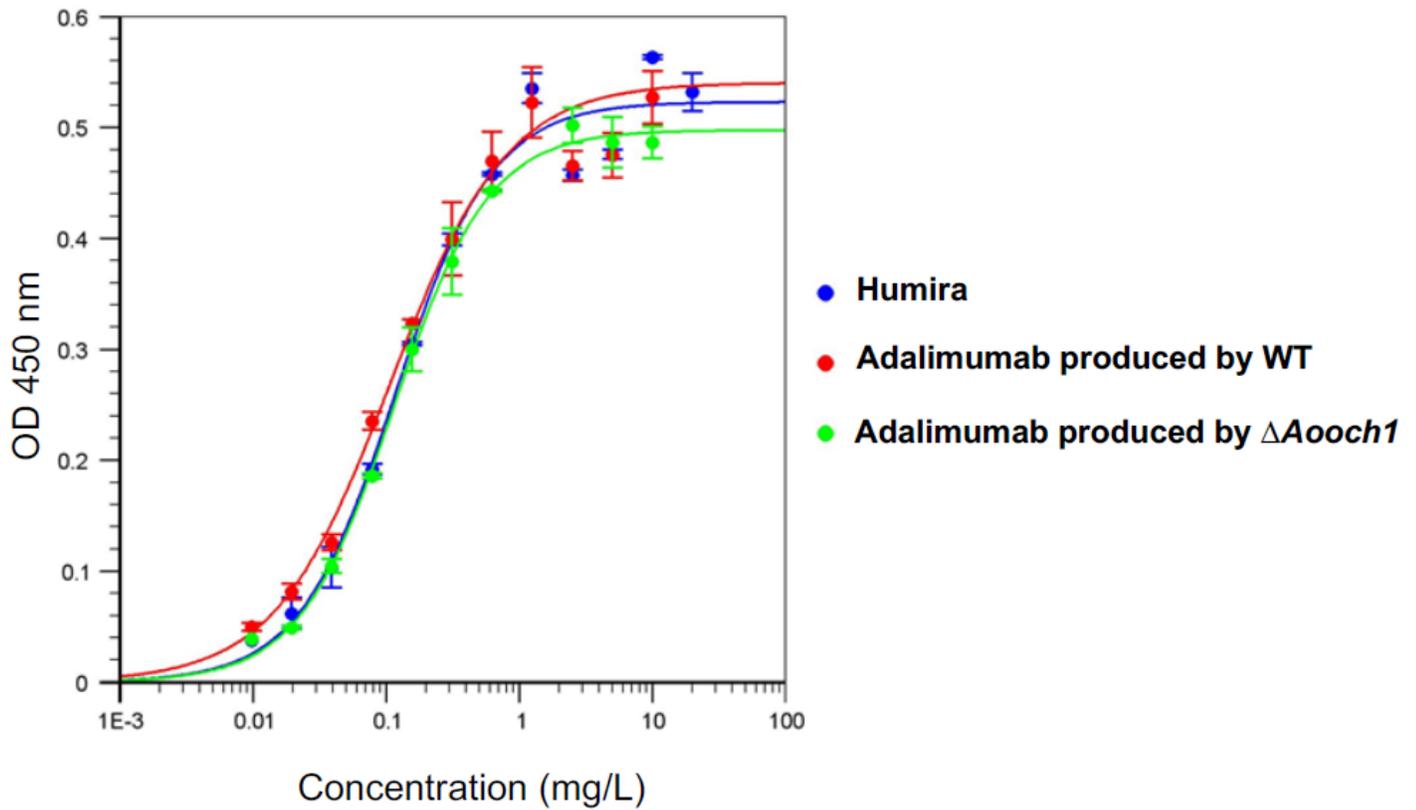
**Figure 7**

Western blot analysis of the adalimumab from the culture supernatant of  $\Delta Aooch1$  strain in non-reducing condition.



**Figure 8**

N-glycan analysis of adalimumab produced by *A. oryzae* WT and  $\Delta Aooch1$ . Humira and purified adalimumab produced by *A. oryzae* WT and  $\Delta Aooch1$  with and without Glycopeptidase F treatment were applied to SDS-PAGE for Western blot analysis in reducing condition.



**Figure 9**

Antigen-binding assay of the adalimumab produced by *A. oryzae*. Different concentrations of adalimumab produced by WT and  $\Delta Aooch1$ , and Humira were incubated in the 96-plate coated with human TNF $\alpha$ . The Anti-IgG (H+L chain) (Human) pAb-HRP antibody was used for detection at OD 450 nm. Each bar represents the standard deviation of the mean. n = 3.

### MTT assay

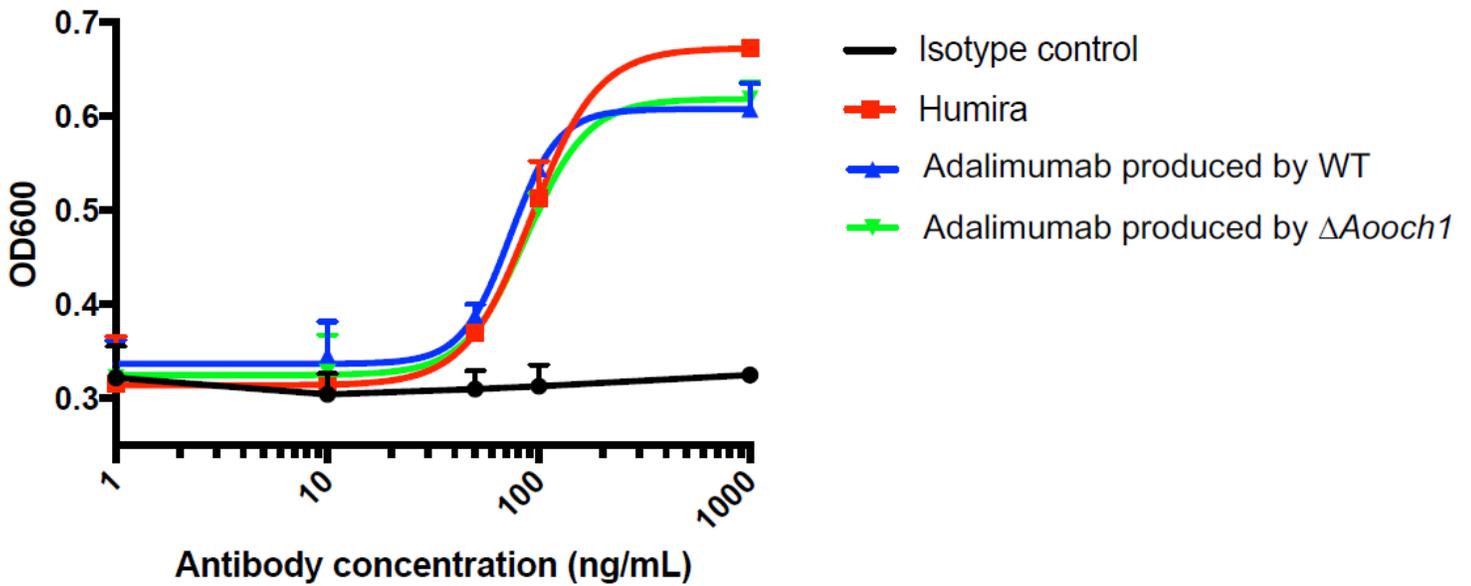


Figure 10

TNF $\alpha$  neutralizing activity of the adalimumab produced by *A. oryzae*. For neutralization, 20 ng/ml human TNF $\alpha$  and 1  $\mu$ g/ml actinomycin D were mixed with different concentration antibodies. Then, 100  $\mu$ l of these mixtures were added to the well containing MDA-MB-468 cells. The cell viability was measured by MTT assay at 600 nm. Each bar represents the standard deviation of the mean. n = 3.

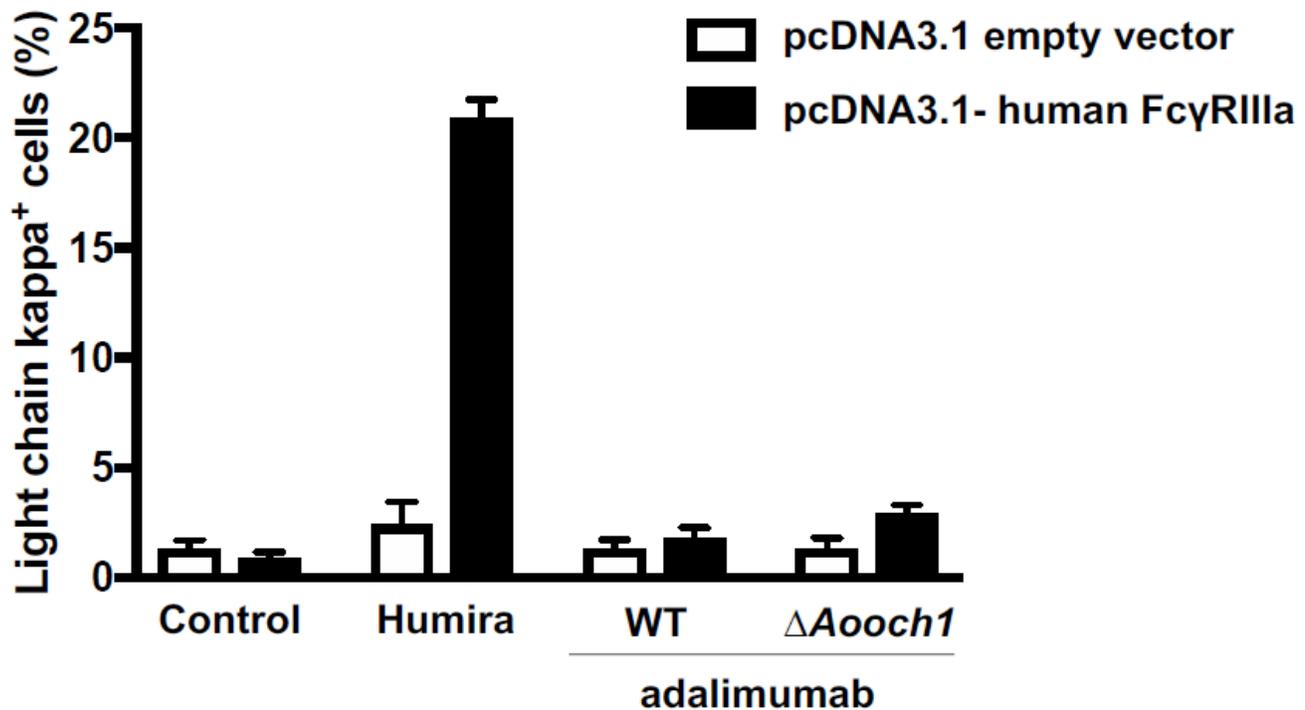


Figure 11

FcγRIIIa binding assay of the adalimumab produced by *A. oryzae*. The transfected HEK-292T cell expressing Fc receptor – FcγRIIIa was incubated with the adalimumab produced by WT or ΔAooch1 (15 μg/ml) mixed 40 ng/ml TNFα. Humira was used as control. APC anti-Human IgG light chain kappa antibody was used for detection with the absorbance at 405 nm. Each bar represents the standard deviation of the mean. n = 3.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.docx](#)
- [HuynhFungalBiologyandBiotechnologySupplemental200214.pdf](#)