



# Effects of various disaccharide adaptations on recombinant IgA1 production in CHO-K1 suspension cells

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**Abstract** Immunoglobulin A (IgA) has been showing potential as a new therapeutic antibody. However, recombinant IgA suffers from low yield. Supplementation of the medium is an effective approach to improving the production and quality of recombinant proteins. In this study, we adapted IgA1-producing CHO-K1 suspension cells to a high concentration (150 mM) of different disaccharides, namely sucrose, maltose, lactose, and trehalose, to improve the production and quality of recombinant IgA1. The disaccharide-adapted cell lines had slower cell growth rates, but their cell viability was extended compared to the nonadapted IgA1-producing cell line. Glucose consumption was exhausted in all cell lines except for the maltose-adapted one, which still contained

glucose even after the 9th day of culturing. Lactate production was higher among the disaccharide-adapted cell lines. The specific productivity of the maltose-adapted IgA1-producing line was 4.5-fold that of the nonadapted line. In addition, this specific productivity was higher than in previous productions of recombinant IgA1 with a lambda chain. Lastly, secreted IgA1 aggregated in all cell lines, which may have been caused by self-aggregation. This aggregation was also found to begin inside the cells for maltose-adapted cell line. These results suggest that a high concentration of disaccharide-supplemented induced hyperosmolarity in the IgA1-producing CHO-K1 cell lines. In addition, the maltose-adapted CHO-K1 cell line benefited from having an additional source of carbohydrate.

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## Introduction

Currently the global biopharmaceutical industry is estimated to be USD \$299 billion in market value and it is expected to increase. The majority of market share comes from therapeutic proteins that are produced mostly by mammalian cell lines. Among these cell lines, Chinese hamster ovary (CHO) cells are used to produce 84% of the therapeutic proteins, and most of them are recombinant antibodies (Walsh 2018). CHO cells are the predominant workhorse mainly because post-translational modifications are closely related to humans; therefore, recombinant proteins produced by CHO cells are bioactive and compatible. In addition, recombinant protein production can be upscaled to produce substantial amounts of therapeutic proteins (Kim et al. 2012a). In 2020, 12 out of 13 therapeutic proteins approved by the US FDA were either monoclonal antibodies or antibody–drug conjugates (Mullard 2021), showing that strong demand still exists for therapeutic antibodies.

Immunoglobulin G (IgG) is the standard isotype in antibody-based therapies. However, among the recent advances in other immunoglobulin isotypes, immunoglobulin A (IgA) shows potential as a therapeutic antibody. One reason for this is IgA's ability to form dimers in the presence of the joining chain, which can improve its tumor killing activity (Lohse et al. 2011). Another advantage of IgA is its transportability to the mucosal membranes as secretory IgA (sIgA) by recognition of polymeric Ig receptor. Hence, sIgA can provide protection against diseases infecting the mucosal surface, such as influenza, tuberculosis, and polio (Balu et al. 2011; Seibert et al. 2013; Devudu Puligedda et al. 2020). In addition, a few studies suggested that it can also bind to toxins and allergens (Sun et al. 1995; Nakanishi et al. 2017). Recently, monoclonal IgA antibody was able to block SARS-CoV-2 spike angiotensin-converting enzyme interaction in the respiratory system (Ejemel et al. 2020).

Improvement on the production and quality of therapeutic antibody has always been a necessity in the biopharmaceutical industry. Recombinant IgAs have potential as effective therapeutic antibodies, but they suffer from low productivity (Reinhart and Kunert

2015). Carbohydrates are a necessary energy source for cell growth and survival. Glucose is the typical source of energy for CHO cells because of the ease of transporting it inside the cell via a sodium–glucose linked transporter and a glucose transporter (Leong et al. 2017). Consequently, a high concentration of glucose in the medium can increase lactate production, which is detrimental to the growth and viability of CHO cells (Leong et al. 2018). Previous studies have shown that other hexoses, such as galactose, mannose, and fructose, improved cell growth and cell viability (Ritacco et al. 2018). Aside from these, a few studies have used other carbohydrates, such as maltose (up to 100 mM) and trehalose (150 mM), to improve recombinant IgG and bi-specific antibody production in CHO cells, respectively (Onitsuka et al. 2014; Leong et al. 2018). Thus, the aims of the present study are to improve recombinant IgA1 production by adapting the stably producing IgA1 CHO-K1 suspension cell line to 150 mM of disaccharide supplementation, namely with sucrose, maltose, lactose, and trehalose, and to evaluate its cell growth, cell viability, glucose consumption, lactate production, specific productivity, and aggregation.

## Materials and methods

### Plasmid construction

The DNA sequence of the IgA variable region was obtained from a hybridoma cell line infused with immune cells from dengue-infected volunteers in Thailand was established by SPYMEG technology in a previous work (Setthapramote et al. 2012). The following primers were used to clone the variable region of the IgA: 5'-ATGAAACACCTGTGGTTCTTCCTCCT-3' (sense primer for amplification of the heavy chain), 5'-TGCACGTGAGGTTTCGCTTCTGAAC C-3' (antisense primer for amplification of the heavy chain), 5'-ATGGCCTGGWYYCCTCTCYTYCTS-3' (sense primer for amplification of the lambda chain), and 5'-TGGCAGCTGTAGCTTCTGTGGGACT-3' (antisense primer for amplification of the lambda chain). The amplified variable regions were ligated into pGEM®-T Easy vector (Promega, Madison, WI, USA). The IgA1 heavy chain and lambda chain constant region were synthesized by the GeneArt™ gene synthesis service (Thermo Fisher Scientific, Waltham,

MA, USA) based on DNA sequence from GenBank Accession IDs AH007035.2 and JF806287.1, respectively. The heavy chains and lambda chains of variable regions and constant regions were inserted in pQCXIP (Takara Bio, Shiga, Japan) and pQCXIH (Takara Bio), respectively, to construct IgA1 expression vectors, pQCXIP heavy chain and pQCXIH light chain.

#### Construction of IgA1 stably producing CHO-K1 cells

Stable transfection was done by electroporation using the Neon® transfection system (Thermo Fisher Scientific) following the manufacturer's instructions. In brief, cells were seeded in a 6-well plate 24 h before electroporation. During the transfection day, cell density was calculated, and  $1 \times 10^6$  cells/mL were collected. Cells in the tube were centrifuged at  $400 \times g$  for 5 min at room temperature and washed with phosphate-buffered saline (PBS) and centrifuged again at  $400 \times g$  for 5 min at room temperature. Cells were resuspended with resuspension buffer R with a final cell density of  $2 \times 10^7$  cells/mL and mixed with 1.5  $\mu\text{g}$  of pQCXIP heavy chain and 1.5  $\mu\text{g}$  of pQCXIH light chain. The mixture was then aspirated and electroporated under the following conditions: voltage 1150 V, width 5 ms, pulses 10. After electroporation, transfected cells were incubated at 37 °C with 5% CO<sub>2</sub>. After 24 h post-transfection, 100  $\mu\text{g}/\text{mL}$  hygromycin B (Fujifilm Wako Pure Chemical, Osaka, Japan) and 1  $\mu\text{g}/\text{mL}$  puromycin dihydrochloride dihydrate (Fujifilm Wako Pure Chemical) were added. Medium and antibiotics were changed every 3–4 days for 21 days. Once the cells reached confluence, antibiotics were increased by a twofold concentration until reaching 2 mg/mL hygromycin B and 20  $\mu\text{g}/\text{mL}$  puromycin dihydrochloride dihydrate.

#### Cell lines and cultivation

Serum-free adapted CHO-K1 cells were cultured using BMPPro-W medium (Funakoshi, Tokyo, Japan) supplemented with 6 mM L-glutamine (Nacalai Tesque, Kyoto, Japan) and incubated at 37 °C with 5% CO<sub>2</sub>. The recombinant IgA1 stably producing CHO-K1 cell line was cultured using BMPPro-W medium supplemented with 6 mM L-glutamine, 2 mg/mL hygromycin B, and 20  $\mu\text{g}/\text{mL}$  puromycin dihydrochloride dihydrate. IgA1 stably producing

CHO-K1 cells were adapted to 150 mM of each of different disaccharides: sucrose, maltose, lactose, and trehalose (Fujifilm Wako Pure Chemical). In brief, IgA1 stably producing CHO-K1 cells were adapted to 50 mM of each disaccharide stepwise until the disaccharide concentration reached 150 mM. Cell viability and cell growth were determined visually.

Shake flask batch culture sampling and evaluation.

Approximately  $3 \times 10^5$  cells/mL of disaccharide-adapted IgA1 CHO-K1 SF cells were seeded in a 50 mL baffled shaker flask filled with 30 mL of medium supplemented with 150 mM of disaccharide and incubated at 37 °C with 5% CO<sub>2</sub> at 105 rpm. Samples were collected every 24 h and evaluated for total cell count and cell viability by trypan blue exclusion assay using TC20™ Automated Cell Counter (Bio-Rad, Hercules, CA, USA). The specific growth rate was calculated using this formula,

$$\ln \left( \frac{VCD}{VCD_0} \right) = \mu t, \quad (1)$$

where  $VCD$  = final viable cell density,  $VCD_0$  = initial viable cell density,  $\mu$  = specific growth rate, and  $t$  = time.

In addition, glucose consumption and lactate production were analyzed using a BF-9 multi-use biosensor (Oji Scientific Instruments, Hyogo, Japan). After 9 days, samples from the media were collected to determine IgA-specific productivity as shown below. The remaining media were used to purify recombinant IgA1.

#### Specific productivity by sandwich enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was done to evaluate the specific productivity. In brief, a microplate was coated with 5  $\mu\text{g}/\text{mL}$  goat anti-human IgA (MBL, Nagoya, Japan) capture antibody in 50  $\mu\text{L}$  of PBS pH 7.4 and incubated at 4 °C overnight. The coating solution was removed, and the wells were washed with 200  $\mu\text{L}$  wash buffer (0.05% Tween 20 in PBS) three times. Wells were blocked with 1% BSA in wash buffer for 1.5 h at room temperature. After washing with wash buffer three times, IgA standard purified from human serum (Bethyl Laboratories, Montgomery, TX, USA) at different concentrations was introduced and cell-free supernatant containing IgA1 was added, and the plate was incubated for

1.5 h at 37 °C. Samples were discarded and washed under the same conditions as above. Horseradish peroxidase (HRP)-conjugated goat anti-human IgA (MBL) was added and incubated for 1.5 h at room temperature. After washing three times in the same manner as above, 200 µL of Sigmafast *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated in the dark at room temperature for 12 min. The reaction was stopped with 100 µL of 3 M HCl. The reaction was measured using an iMARK™ microplate reader (Bio-Rad) at 450 nm wavelength. Data were analyzed using microplate reader manager 6 (Bio-rad) using a linear curve. Specific IgA1 productivity ( $q_p$ ) was calculated using the formula below:

First, the integrated viable cell density (IVCD) was calculated using the trapezium rule:

$$IVCD_t = IVCD_{t-1} + 0.5 \times (VCD_t + VCD_{t-1}) \times \Delta t \quad (2)$$

Specific IgA1 productivity was calculated between two culture times by plotting IgA1 concentration (P) vs IVCD:

$$q_p = (P_{t_2} - P_{t_1}) / (IVCD_{t_2} - IVCD_{t_1}) \quad (3)$$

Tests were done in triplicates and standard error of mean (SEM) were used to calculate for the error bar.

#### Preparation of intracellular and extracellular fractions

The intracellular fractions of nonadapted and maltose-adapted CHO-K1 suspension cells were collected using Cytobuster™ protein extraction reagent (Merck, Darmstadt, Germany) following manufacturer's instructions. In brief, cells were collected and washed with PBS twice. After which, 1 mL of cytobuster protein extraction reagent were added and incubated at room temperature for 5 min. Then, samples were centrifuge at 15,000×g for 5 min. Supernatant were collected as the intracellular fraction and used for SDS-PAGE. Seven-day cultured medium was used as the extracellular fraction.

#### IgA1 purification, western blotting, and silver staining

IgA1 was purified using peptide M agarose (InvivoGen, San Diego, CA, USA) following the

manufacturer's instructions. The purified IgA1s were then separated under 10% polyacrylamide gels. After separation, the IgA1s were transferred to polyvinylidene difluoride membrane (Immobilon-P, Merck, Kenilworth, NJ, USA) at 15 V for 25 min. The membranes were blocked with 1% skim milk for 1 h then washed with PBS with 0.5% Tween 20 three times. The membranes were incubated with HRP-conjugated goat anti-human IgA for 1 h. Silver staining was done using the Silver Stain II Kit (Fujifilm Wako Pure Chemical) following the manufacturer's instructions.

#### Recombinant IgA1 aggregation analysis

Purified recombinant IgA1 was concentrated using the Amicon 10 K Spin Column (Merck). The concentrated samples were separated using size exclusion chromatography—high-performance liquid chromatography (SEC-HPLC). In brief, 1 µg of purified recombinant IgA1 was injected into the HPLC system (LaChrom L-7000; Hitachi, Tokyo, Japan) under the following conditions: column TSKgel Ultra SW Aggregate 7.8 mm internal diameter (ID)×30.0 cm (Tosoh Bioscience, Tokyo, Japan) UV wavelength at 280 nm, mobile phase 40 mM, and sodium phosphate buffer pH 6.7 containing 400 mM sodium perchlorate at 0.3 mL/min for 60 min. The column temperature was 25 °C. The results collected were then interpreted as aggregate percentage and monomeric IgA1 percentage.

Purified recombinant IgA1 from maltose-adapted cell line was used to assess the effects of different reducing agents to reduce the aggregation caused by disulfide bond formation. Different concentrations of dithiothreitol (DTT) (Nacalai Tesque), Tris (2-carboethyl) phosphine hydrochloride (TCEP HCl) (Fujifilm Wako Pure Chemical), Glutathione (Nacalai Tesque), Cystamine diHCl (Nacalai Tesque) was mixed with recombinant IgA1 at 37 °C for 1 h and run under non-reducing SDS-PAGE.

#### Statistical analysis

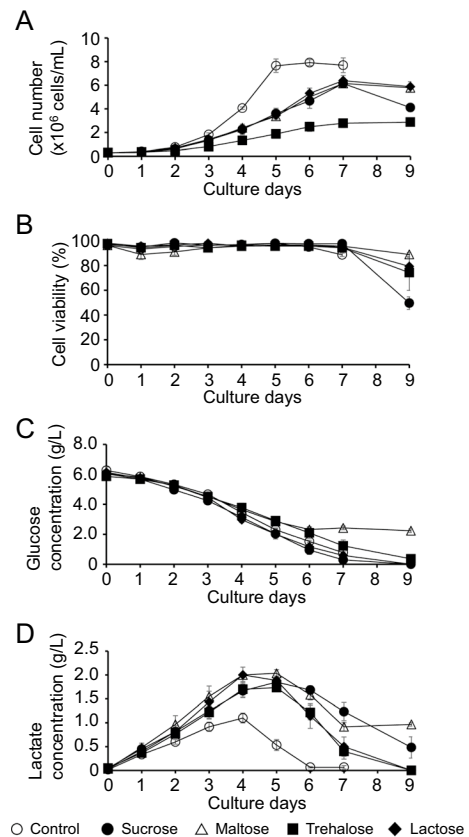
All quantitative data were collected in triplicates. Statistical analysis was done using Microsoft excel. For comparison of the different groups, one-way ANOVA with post-hoc Tukey's HSD test was done to compare which groups have significant difference.

Values  $p < 0.05$  was considered having significant differences.

## Results

### Cell growth, cell viability, glucose consumption, and lactate production

In a previous experiment, IgA1-producing CHO-K1 suspension cells were adapted with 100 mM maltose and trehalose to investigate whether these concentrations will increase the productivity. Indeed, increase in specific productivity was observed in these cell lines (Supplementary Fig. 1). Therefore, increasing the concentration to 150 mM disaccharide might have a greater increase in productivity. In addition, we followed a study by Onitsuka et al. 2014, which used 150 mM of trehalose for the experiment. The IgA1-producing CHO-K1 suspension cell line was adapted with 150 mM of each disaccharide (sucrose, maltose, lactose, and trehalose) to evaluate the effects on cell growth, glucose consumption, lactate production, and recombinant IgA1 production. The viable cell count and the viability of disaccharide-adapted IgA1-producing cell lines were compared with the nonadapted IgA1-producing cell line. As shown in Fig. 1A, the nonadapted cell line reached log phase sooner (5 days) than the disaccharide-adapted IgA1-producing cell lines (7 days). However, the nonadapted IgA1-producing cell line reached the death phase at 7 days, whereas the disaccharide-adapted cell line did so at 9 days. In addition, the maximum cell density for the nonadapted cell line reached  $7.92 \times 10^6$  cells/mL, higher than those of the disaccharide-adapted cell lines; the highest viable cell count among the disaccharide-adapted cell lines was in the lactose-adapted IgA1-producing cell line, reaching  $6.39 \times 10^6$  cells/mL. The trehalose-adapted IgA1-producing cell line tended to have the lowest viable cell count, at  $2.87 \times 10^6$  cells/mL. This result is comparable to that obtained by Onitsuka et al 2014. Cell viability was comparable among the cell lines from day 0 until day 7, after which the nonadapted cell line started to fall. The disaccharide-adapted lines, especially maltose, tended to have extended cell viability, at 89%, even after 9 days of culturing (Fig. 1B). The adapted lines also tended to have slower growth rates than the nonadapted line (Table 1). The cultivation



**Fig. 1** Effect of each disaccharide adaptation on cell culture and IgA1 productivity. Viable cell count (a); Cell viability (b); Glucose consumption (c); Lactate production (d). Control: nonadapted IgA1 producing CHO cell line,  $n = 3$

of mammalian cells relies mostly on glucose as a carbohydrate source because it is easily transported into cells and easily utilized by them for energy. Glucose consumption is an important parameter in biochemical profiling because it is the primary energy source via aerobic glycolysis. The nonadapted and adapted cell lines (except the maltose-adapted one) depleted their glucose concentrations within the cultivation time. Interestingly, the maltose-adapted line still contained 2.24 g/L of glucose on the 9th day of incubation (Fig. 1C). Lactate production is a crucial parameter in cell lines used for recombinant protein production because high lactate production can affect cell growth rate and cell viability (Lao and Toth 1997). In this study, lactate production was measured daily until the 9th day. During the 4th and 5th days of incubation, lactate production increased (Fig. 1D). This can be attributed to the Warburg effect, in which

**Table 1** Specific growth rate of nonadapted and disaccharide adapted IgA producing CHO-K1 cell lines

| Cell lines           | Specific growth rate (day <sup>-1</sup> ) |
|----------------------|---|
| Nonadapted (Control) | 0.72 ± 0.00                               |
| Sucrose-adapted      | 0.39 ± 0.04                               |
| Maltose-adapted      | 0.38 ± 0.01                               |
| Lactose-adapted      | 0.38 ± 0.02                               |
| Trehalose-adapted    | 0.21 ± 0.04                               |

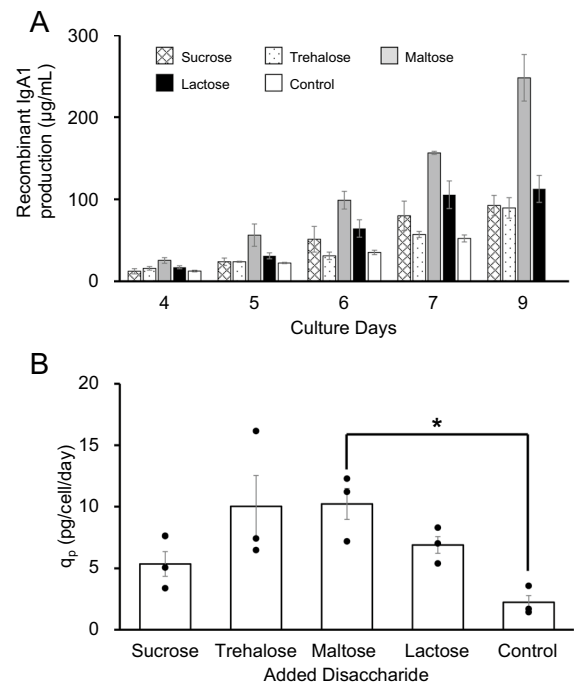
cells prefer glucose as a source of ATP during the exponential phase (Rish et al. 2022). In addition, the disaccharide-adapted cell lines produced more lactate than the nonadapted cell line. Interestingly, the maltose-adapted line contained a higher lactate level even after the 9th day of incubation, which was not seen in the other cell lines.

#### Recombinant IgA1 production and specific productivity

The cumulative recombinant IgA1 production and specific productivity of recombinant IgA1 was measured by sandwich ELISA. The samples were collected starting from 4th day until 9th day of subculturing. All of the disaccharide-adapted cell lines produced more recombinant IgA1 compared to nonadapted CHO-K1 cell line (Fig. 2A). In addition, Maltose-adapted cell lines produced the most recombinant IgA1 reaching 248 µg/mL at 9th day of culture. Next, we calculated the specific productivity ( $q_p$ ) of recombinant IgA1 of all the cell lines. The  $q_p$  was higher among all adapted cell lines compared to nonadapted cell line (Fig. 2B and Supplementary Fig. 2). Maltose-adapted cell line and trehalose-adapted cell line showed a high  $q_p$  at 10.22 pg/cell/day and 10.02 pg/cell/day respectively. In contrast, nonadapted cell line showed the lowest among the cell lines with a  $q_p$  of 2.23 pg/cell/day. However, the high  $q_p$  of trehalose-adapted cell line did not reflect in the amount of recombinant IgA1 produced possibly because of the substantial decrease in the viable cells.

#### Recombinant IgA1 aggregation analysis

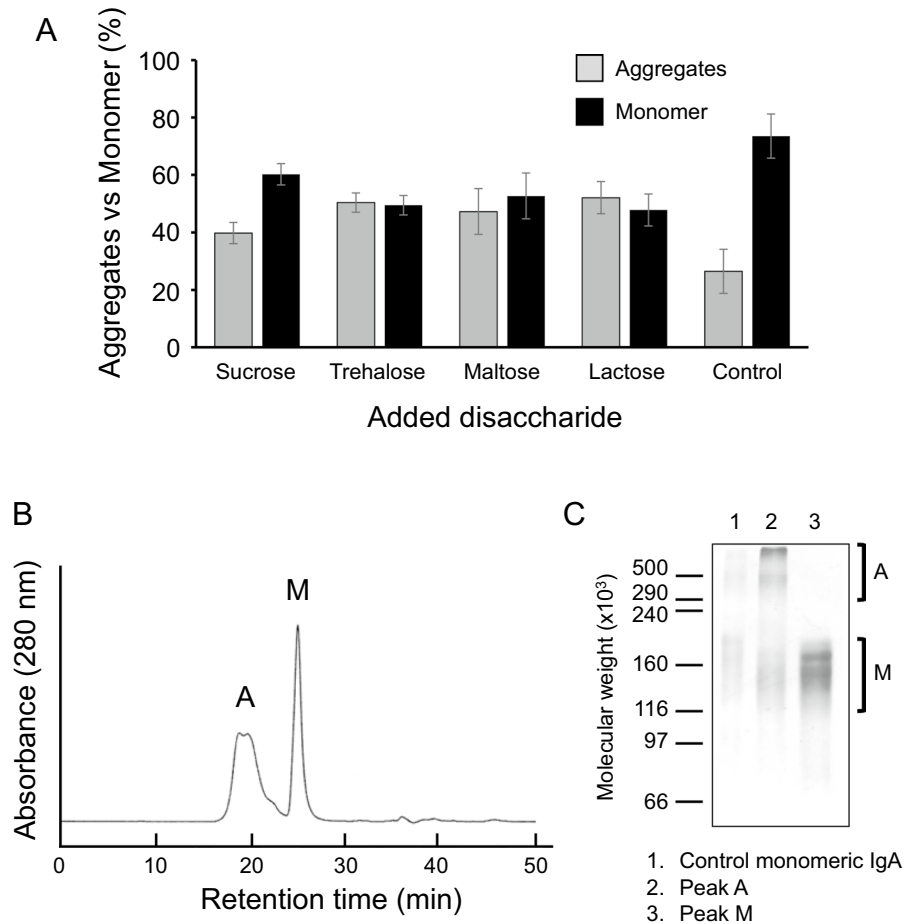
Inspection for the aggregation of recombinant antibody is an important parameter in biopharmaceutical



**Fig. 2** Recombinant IgA1 concentration of disaccharide-adapted and nonadapted IgA1 producing cell lines (a) Specific IgA1 productivity of disaccharide adapted and nonadapted IgA1 producing cell lines (b). Control: nonadapted IgA1 producing CHO-K1 cells, n = 3; \* =  $p < 0.05$

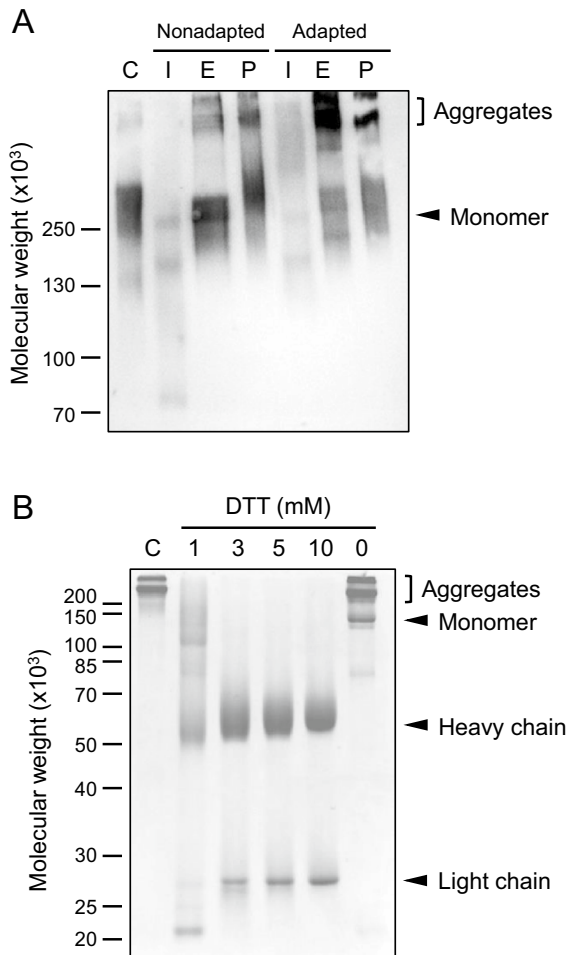
production. In this study, SEC-HPLC was performed to assess the quality of recombinant IgA1 produced in the disaccharide-adapted cell lines; all of the lines showed aggregation. However, aggregation of IgA1 was higher among the adapted cell lines than in the nonadapted cell line (Fig. 3A). The peaks of purified IgA1 were collected and separated using SEC-HPLC. The collected peaks were separated under non-reducing SDS-PAGE to confirm their identities. Peak A and Peak M from Fig. 3B show aggregated IgA1 and monomeric IgA1, respectively (Fig. 3C). Previous studies showed that culturing CHO cells in high osmolarity decreases recombinant antibody aggregation (Onitsuka et al. 2014; Qin et al. 2019). However, our results showed that IgA1 aggregation increased when the cell lines were cultured in medium with increased osmolarity. In order to determine when did the IgA1 aggregation began to occur, the intracellular fraction, extracellular fraction, and purified IgA1 of nonadapted and maltose-adapted cell line were collected. In Fig. 4A, the intracellular fraction

**Fig. 3** Effect of disaccharide addition on IgA1 aggregation. Aggregated IgA1 vs Monomeric IgA separated using SEC-HPLC (a); SEC-HPLC Chromatogram of maltose adapted IgA producing cell line (b); Coomassie Brilliant Blue staining of IgA1s separated by SDS-PAGE under nonreducing condition of collected peaks from SEC-HPLC (c). Control: Human IgA standard, 2: Peak A in B, 3: Peak M in B. A: Aggregated IgA1, M: Monomeric IgA1, n=3



of nonadapted cell line contained no aggregated IgA1 while IgA1 aggregation is present in the intracellular fraction of maltose-adapted cell lines. In the extracellular fractions, the nonadapted cell line contains few aggregated IgA1 while the maltose-adapted cell line contains more aggregated IgA1. Lastly, the purified IgA1 fraction of nonadapted cell line still contained less aggregated IgA1 than maltose-adapted cell lines. This result shows that IgA1 aggregation began inside the maltose-adapted cell line. A recent study proposed that Cys-471 found on the tailpiece of IgA has a propensity to form self-aggregates in the absence of the J-chain (Xie et al. 2021). In order to determine if our IgA1 also shows the same behavior, we used different reducing agents such as DTT, glutathione, TCEP hydrochloride, cystamine dihydrochloride to reduce the disulfide bond formed by the Cys-471. Indeed, adding 1 mM DTT to purified IgA1 from maltose-adapted cell line showed reduction in

aggregated IgA1 (Fig. 4B). Adding higher DTT concentration reduced all the disulfide bonds, thus, separating the recombinant IgA1 into heavy chains and light chains (Fig. 4B). In addition, different reducing agents was also tested to assess which is a better reducing agent to reduce the aggregation. In Supplementary Fig. 3A, we can observe that TCEP HCl, a tertiary phosphine that have been known as disulfide reductants, at 1 mM reduced the IgA1 aggregation however, at higher concentrations it also reduced the IgA1 into heavy chain and light chain. Glutathione, a known reducing agent found in living organisms, was able to decrease the amount of aggregated IgA1 at 1 mM and reduced the IgA1 into both heavy chain and light chain with increasing concentrations (Supplementary Fig. 3B). In contrast, cystamine dihydrochloride, an aminothiols that can react with cysteine is formed naturally in mammalian cells and is also utilized in medicine to treat cystinosis (Xie et al. 2021),



**Fig. 4** Investigation on the aggregation of recombinant IgA1 produced in maltose-adapted and nonadapted CHO-K1 cell line. Western blot of intracellular fractions, extracellular fractions, and purified IgA1 from nonadapted cell line and maltose-adapted cell line separated by SDS-PAGE under non-reducing conditions (a); Coomassie Brilliant Blue staining of IgA1 purified from maltose-adapted cell line treated with 1 mM, 3 mM, 5 mM, 10 mM of DTT and non-treated IgA1, and separated by SDS-PAGE under non-reducing conditions (b). *C* human IgA standard, *I* intracellular fraction, *E* extracellular fraction, *P* purified IgA1

did not decrease the aggregation in any concentration (Supplementary Fig. 3C).

## Discussion

Recombinant IgA offers certain advantages that IgG cannot provide; for example, it reaches the mucosal

surface to provide protection and improves tumor-killing activity by forming dimers. To fully reap these benefits, we must first improve productivity. Media supplementation is one of the simplest effective methods. On the other hand, the transient addition of high concentrations of substances may cause rapid osmotic changes in cells, which can have adverse effects on cells (Han et al. 2010). Thus, in this study we adapted recombinant IgA1-producing CHO cell lines for a long time to high concentrations of different disaccharides, namely sucrose, maltose, lactose, and trehalose, and assessed their cell growth, glucose consumption, lactate production, specific productivity, and degree of aggregation.

The disaccharide-adapted cell lines did not reach viable cell counts as high as that of the nonadapted cell line. In addition, the specific growth rates of the disaccharide-adapted cell lines were lower than those of the nonadapted cell line. Previous studies using NaCl to transiently apply hyperosmolarity also showed fewer viable cells and lower specific growth rates (Kim and Lee 2002; Zhang et al. 2010; Kim et al. 2012b), suggesting that the increase in osmolarity caused by disaccharides might also impose a load on cells similar to that imposed by NaCl. However, the adapted cell lines had better cell viability even at 9 days compared to 7 for the nonadapted line. This is due to the high osmolarity condition that came with supplementing the media with high concentrations of disaccharides. Glucose consumption analysis showed that some glucose remained in the maltose-adapted IgA1-producing cell line, indicating that maltose was utilized as another carbohydrate source. Indeed, a previous study provided evidence that maltose is utilized by CHO cells as a carbohydrate source (Leong et al. 2017, 2018). Lactate production was increased among the disaccharide-adapted cell lines; this can be attributed to the Warburg effect, in which osmotic pressure is increased by the addition of the different disaccharides (Moreira et al. 2021). However, when the cell lines reached the stationary phase, lactate production decreased. This reduction could be related to lactate inhibition by lactate dehydrogenase (Omasa et al. 1992). In this study we did not expect to have aggregated monomeric IgA1 due to it being unreported in other studies. This aggregation is possibly due to self-aggregation, was observed in all recombinant IgA1-producing cell lines. The increase of aggregated IgA1 on the disaccharide-adapted cell



line might be attributable to the higher productivity of recombinant IgA1 compared to the nonadapted cell lines, as it can increase exposure to other IgA1s and form self-aggregates. To improve this, a previous study changed the Cys-471 to Serine which drastically improved the amount of monomeric IgA (Xie et al. 2021). In addition, supplementation of weak disulfide bond reducing agent in the post processing might be able to reduce the aggregation of recombinant IgA1. Lastly, the specific productivity of recombinant IgA1 was improved especially in the maltose-adapted line, which reached specific productivity of 10.22 pg/cell/day of recombinant IgA1, whereas the nonadapted line had only 2.23 pg/cell/day. In comparison with other research, this is the highest IgA1-specific productivity produced in CHO-K1 cells with a lambda light chain (Reinhart and Kunert 2015). Currently, the reason for this increase in productivity is unclear. However, it might be an effect of CHO cells being cultured under high-osmolarity conditions and maltose utilization in the last few days. In addition, a previous study has reported that an increase in recombinant protein production was observed during the stationary phase of the mammalian cell cultures (Templeton et al. 2013). Thus, extension of the stationary phase as seen in the growth curve of the disaccharide-adapted cell lines might have caused the additional increase in recombinant IgA1 production. These observed results agree with other studies relating to cell cultures grown under hyperosmolarity conditions (Oh et al. 1993; Kamachi and Omasa 2018; Romanova et al. 2021). However, care should be taken in increasing monomeric IgA1 productivity which might also increase the chances of aggregation.

Among the disaccharides whose effects on IgA1 production were examined in this study, maltose showed the most promise, as it has the combined effects of hyperosmolarity culture conditions and an additional carbohydrate source for in the last few days of cultivation. In addition, maltose is readily available and economical. It is thought that clarifying how maltose is transported to cells and how it is metabolized will make it possible to further increase the effectiveness of its addition. The exact effects and the detailed mechanism underlying hyperosmolarity in CHO cells remain elusive. Previous studies reported the upregulation of mRNA expression of cell-proliferation and cell-cycle-arrest genes, and that cytosolic and mitochondrial ATP were increased during

cultivation under hyperosmolarity conditions (Pfizenmaier et al. 2016). In addition, proteomics analysis of IgG-expressing CHO cells cultured under hyperosmotic conditions led to the identification of 23 proteins with variable expression, such as increased expression of glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, presumably to raise metabolic energy for antibody production has been reported (Lee et al. 2003). Another study showed that hyperosmolarity-exposed CHO cells have triple the volume and increased mitochondrial activity compared to nonexposed cells (Romanova et al. 2021). Since there is little information on the gene expression of transcription factors and signal transduction proteins under high osmotic pressure, it would be useful to establish host CHO-K1 cells that are tolerant to high osmotic pressure and capable of producing high-target proteins. This would provide expression level control through comprehensive analysis of gene expression levels using RNA-seq and other methods.

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**Author contributions** KF and RM: conceived the idea of this study. JBC: conducted all experiments. TS: contributed to prepare experimental materials. HK: contributed to support this study and revise the manuscript. KI and KF: acquired the funding and revised the manuscript. RM: supervised the conduct of this study. All authors reviewed the manuscript draft and approved the final version for submission.

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**Data availability** The data analyzed in this study is available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors have no conflict of interest to declare.

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