Metabolic Engineering 85 (2024) 105–115

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Ala-Cys-Cys-Ala dipeptide dimer alleviates problematic cysteine and cystine levels in media formulations and enhances CHO cell growth and metabolism

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ARTICLE INFO

Keywords:
Chinese hamster ovary
Cysteine
Cystine
Cell culture media
^{13}C-metabolic flux analysis
Fed-batch bioprocessing

ABSTRACT

Cysteine and cystine are essential amino acids present in mammalian cell cultures. While contributing to biomass synthesis, recombinant protein production, and antioxidant defense mechanisms, cysteine poses a major challenge in media formulations owing to its poor stability and oxidation to cystine, a cysteine dimer. Due to its poor solubility, cystine can cause precipitation of feed media, formation of undesired products, and consequently, reduce cysteine bioavailability. In this study, a highly soluble cysteine containing dipeptide dimer, Ala-Cys-Cys-Ala (ACCA), was evaluated as a suitable alternative to cysteine and cystine in CHO cell cultures. Replacing cysteine and cystine in basal medium with ACCA did not sustain cell growth. However, addition of ACCA at 4 mM and 8 mM to basal medium containing cysteine and cystine boosted cell growth up to 15% and 27% in CHO-GS and CHO–K1 batch cell cultures respectively and led to a proportionate increase in IgG titer. ^{13}C-Metabolic flux analysis revealed that supplementation of ACCA reduced glycolytic fluxes by 20% leading to more efficient glucose metabolism in CHO–K1 cells. In fed-batch cultures, ACCA was able to replace cysteine and cystine in feed medium. Furthermore, supplementation of ACCA at high concentrations in basal medium eliminated the need for any cysteine equivalents in feed medium and increased cell densities and viabilities in fed-batch cultures without any significant impact on IgG charge variants. Taken together, this study demonstrates the potential of ACCA to improve CHO cell growth, productivity, and metabolism while also facilitating the formulation of cysteine- and cystine-free feed media. Such alternatives to cysteine and cystine will pave the way for enhanced biomanufacturing by increasing cell densities in culture and extending the storage of highly concentrated feed media as part of achieving intensified bioproduction processes.

1. Introduction

The application of biotherapeutics has been transformative for the treatment of numerous diseases including cancer and autoimmune disorders (Grilo and Mantalaris, 2019). Over the last two decades more than 400 biotherapeutics including monoclonal antibodies (mAbs), fusion proteins, vaccines, and more recently bispecific antibodies (bsAbs) have been approved by the US FDA (Food and Drug Administration) (Walsh and Walsh, 2022). Sales of mAbs alone are expected to soar over US$250 billion this year. Chinese Hamster Ovary (CHO) cells are the most widely used host systems to produce these recombinant biotherapeutics due to their ability to produce proteins with human-like glycosylation patterns and scalability in large-scale suspension cell culture processes among other advantages (Dhara et al., 2018; Kim et al., 2012). Nonetheless, there is a continuing effort to enhance growth capabilities and product titers for these critical cell factories given their widespread use and importance to biomanufacturing. These efforts include cell line engineering, adding PAT capabilities, increasing control

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https://doi.org/10.1016/j.ymben.2024.07.008
Received 24 February 2024; Received in revised form 18 June 2024; Accepted 22 July 2024
Available online 22 July 2024

of bioprocessing parameters, and enhancing basal and feed media compositions (Naik et al., 2018; Templeton et al., 2013; Mohan et al., 2008; Hiller et al., 2017).

Basal and feed media typically contain glucose, vitamins, lipids, trace metals, and critically, up to 20 different amino acids. Some of these amino acids are essential including phenylalanine, methionine, threonine, tryptophan, tyrosine, glutamine (except in CHO-GS cells), asparagine, arginine, histidine, cysteine, isoleucine, leucine, valine, lysine, and proline (in proline auxotrophs), and must therefore be supplied through culture media. As important carbon and nitrogen sources for cells, amino acids are directly utilized for the synthesis of biomass and recombinant proteins, metabolized for energy production, and converted to other key metabolites. As a result, amino acids serve as precursors in a wide array of metabolic pathways including redox control, synthesis of nucleic acid precursors through the tetrahydrofolate (THF) cycle, and activation of citric acid cycle (tricarboxylic acid cycle or TCA cycle) (Carrillo-Cocom et al., 2015; Duarte et al., 2014).

Cysteine, in particular, is an essential amino acid and the major source of sulfur in cell culture media. Besides contributing to biomass synthesis, and recombinant protein production, cysteine also contributes to the intracellular redox state as a key thiol-containing molecule. It serves as a precursor for antioxidants including glutathione and contributes to synthesis of coenzyme A (Stipanuk et al., 2009). Glutathione (GSH) is a cysteine-containing tripeptide (Glu-Cys-Gly) that acts as an intracellular antioxidant to relieve oxidative stress by scavenging highly reactive oxygen species (ROS) during its conversion to its disulfide linked dimer (GSSG) (Aquilano et al., 2014; Schafer and Buettner, 2001). This major function of GSH reinforces the importance of cysteine supply and availability in cell culture basal and feed media. Limitations in the amounts of cysteine can result in insufficient production of GSH which subsequently results in oxidative damage by ROS, poor cell viability, and lowered productivity (Ali et al., 2019). Cysteine along with its thiol side chains participates in disulfide bond formation in recombinant proteins (Chevallier et al., 2020). Furthermore, catabolism of cysteine to hydrogen sulfide (H_2S) has been linked to undesirable trisulfide modifications in cell culture including variations on recombinant monoclonal antibodies (Gu et al., 2010; Kshirsagar et al., 2012). This sulfide bond heterogeneity can also impact the formation of antibody drug conjugates (ADCs) and can be critical to maintaining constant drug to antibody ratio (Cumnock et al., 2013). Thus, supply and availability of cysteine can have a significant impact on cell culture performance.

Cysteine is also one of the most challenging components to maintain in basal cell culture media and highly concentrated feed media. While soluble in water, cysteine is highly unstable and rapidly oxidizes to cystine in cell culture environments in the presence of air as well as metal ion catalysts such as copper (Rigo et al., 2004). Cystine, in contrast, has a very low solubility of 0.112 g/L in water at 25 °C and often precipitates at neutral pH (O'Neil, 2006). Furthermore, cystine can be further oxidized to cystine monoxide, cystine dioxide or cysteic acid in ambient air conditions (Kissi et al., 2017). Longer storage durations can also lead to formation of cystine-metal complexes (Mamun et al., 2011). As a result, the generation and storage of highly concentrated feed media for fed-batch processes are often challenged by the poor stability of cysteine and low solubility of cystine. Inadequate supplies of cysteine and cystine can have adverse effects on cellular metabolism, negatively impacting cell growth and productivity, and resulting in product micro-heterogeneities (Chevallier et al., 2020).

Consequently, cysteine and cystine derivatives have been considered as alternatives for addition to basal and feed media. For example, Hecklau et al. replaced cysteine with S-sulfocysteine (SSC) in basal and feed media, obtaining comparable peak viable cell density, prolonged viabilities, and an increase in titer and specific productivity with no change in glycosylation or charge variant patterns. This study also confirmed intracellular enzymatic cleavage of SSC to form sulfate and cysteine (Hecklau et al., 2016). In another study, replacing cysteine with SSC in feed medium led to an increase in antibody specific productivity for multiple cell lines with different process conditions (Seibel et al., 2017). SSC supplementation also lowered product heterogeneity by minimizing trisulfide linkages in mAbs and lowering the percentage of antibody fragments. However, in other studies, replacing cysteine with SSC in feed medium led to an increase in cell death and decline in final culture titers (Chevallier et al., 2021). Thus, the effectiveness of substituting cysteine with SSC can depend on the type of cell line used, basal and feed media compositions, and processing conditions.

Alternatively, another cysteine analog, N-acetyl-cysteine (NAC) has been used to replace 50% of cysteine in feed medium, leading to slightly elevated viable cell densities, but this increase was accompanied by a 10% decline in protein titer, lower mAb specific productivities, and changes in the acidic charge variant species. Furthermore, replacing 90% of cysteine in feed medium with NAC or N,N′-diacetyl-l-cystine, a cystine analog, proved to be highly toxic to cell growth (Chevallier et al., 2021). However, the use of NAC as an antioxidant rather than a replacement for cysteine increased culture duration and protein titer in sodium butyrate supplemented cultures. Yet another substitute for 50% cystine, N,N′-diacetyl-l-cystine dimethylester (DACDM) led to a reduction in product microheterogeneity and protein coloration without any reported detrimental impacts on cell growth and final titer (Chevallier et al., 2021).

Cysteine or cystine containing peptides represent another potential alternative substitute for basal and feed media. N,N′-di-L-Alanyl-L-Cystine (Ala-Cys-Cys-Ala, ACCA, Fig. 1) is a dipeptide dimer (solubility greater than 30 mM at pH 7) which is far more soluble than L-cystine (solubility around 1 mM at pH 7). In the only previous study to date, supplementation of ACCA as an additive to basal medium rather than a substitute for cysteine or cystine led to a slight reduction in maximal specific growth rate, final mAb titer, and specific productivity in a batch process (Sánchez-Kopper et al., 2016). Interestingly, slower uptake and consumption rates of ACCA compared to other dipeptides, non-degraded ACCA in the intracellular matrix, and elevated levels of alanine and cystine in spent media and intracellular matrix indicating release of free amino acids from ACCA were observed in this study (Sánchez-Kopper et al., 2016). Given the potential of ACCA to supply high levels of cysteine and cystine in cell culture, a challenge often encountered in bioprocessing due to precipitation of these components in cell culture basal and feed media, there is a need to further examine the impact of ACCA on the performance of traditional fed-batch processes used in biomanufacturing, CHO cell metabolism, and product quality. Interestingly, ACCA has not been considered as a direct substitute for cysteine and cystine and it would be worthwhile to see if it can support cell culture performance in the absence of free cysteine and cystine. Furthermore, its impact on cell culture performance of different cell lines needs to be investigated when used as a direct substitute as well as an additive.

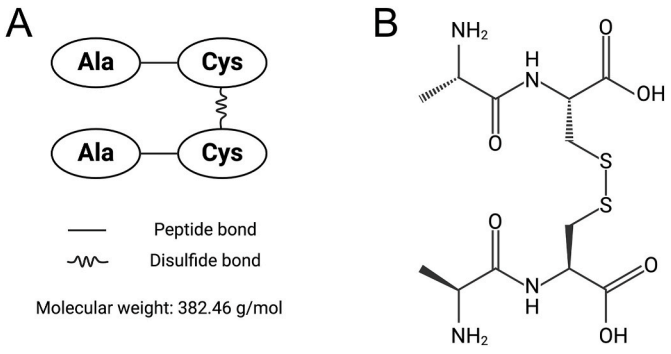

Fig. 1. Chemical formula of Ala-Cys-Cys-Ala (ACCA) dipeptide dimer. (A) Schematic representation and (B) structural formula of ACCA. Created with https://www.biorender.com.

In the current study, we evaluated the impact of ACCA on the cell culture performance of two different CHO cell lines in both batch and fed-batch cultures. We tested ACCA both as an additive and a substitute for cysteine and cystine in basal and feed media. We found that ACCA did not support cell growth in the absence of cysteine and cystine in the basal medium. However, when used as an additive to basal medium containing cysteine and cystine, we observed up to 15% and 27% increase in VCD and 12% and 20% increase in IgG titer with CHO-GS and CHO–K1 cell lines, respectively. ^{13}C-Metabolic flux analysis performed in this study indicated that the addition of ACCA improved the efficiency of glucose metabolism in CHO–K1 cells. Less glucose was metabolized by CHO cells for ATP production, while simultaneously enhancing growth rate and IgG production. ACCA could also replace cysteine and cystine from feed medium in fed-batch processes. Furthermore, we demonstrated that supplementation of ACCA in basal medium containing cysteine and cystine instead of feed medium enabled the complete elimination of cysteine and cystine from feed medium. At high concentrations, ACCA significantly boosted cell growth in fed-batch cultures. ACCA supplementation did not have any impact on the distribution of charge variant species of IgG obtained from CHO-GS and CHO–K1 fed-batch cultures. In short, we show that ACCA has the potential to mitigate the challenges posed in production and storage of highly concentrated feed media containing cysteine and cystine without significantly impacting product quality. With the increasing adoption of intensified fed-batch processes to manufacture biotherapeutics, alternatives to cysteine and cystine will pave the way for improved media formulations, enhanced storage stability of basal and feed media, increased cell densities, and satisfy demands of critical amino acids while maintaining bioprocess performance.

2. Results

2.1. Replacement of cysteine and cystine with ACCA in basal medium does not support robust CHO cell growth in batch cultures

First, we tested if ACCA can completely replace cysteine and cysteine in basal cell culture medium. For this, ACCA was added at 0.68 mM to a medium that was depleted of cysteine and cystine. Control medium contained both cysteine and cystine at a total concentration of 0.68 mM. As seen in Fig. 2, replacing cysteine and cystine with ACCA in basal medium did not support cell growth in batch cultures of CHO-GS and CHO–K1 cells. CHO-GS cells reached a peak VCD of only 2.2 million cells/mL after 10 days compared to the control that reached a peak VCD of 8.2 million cells/mL in 5 days (Fig. 2A). Similarly, CHO–K1 cells reached a peak VCD of 6.5 million cells/mL after 9 days compared to control that reached a peak VCD of 9.0 million cells/mL in 5 days (Fig. 2B).

2.2. Supplementation of ACCA to basal medium containing cysteine and cystine boosts CHO cell growth and IgG titer in batch cultures

We next tested if ACCA can be used as a medium additive. Here, we added ACCA at two different concentrations to the basal medium containing cysteine and cystine. The control cultures were not supplemented with equivalent alanine due to its non-essential role as an amino acid (Salazar et al., 2016). When CHO-GS cultures were supplemented with 4 mM and 8 mM ACCA, we observed a modest 12% and 15% increase in peak VCD, respectively (Fig. 3A). All CHO-GS cultures maintained similar cell viability (Fig. 3A). In the case of CHO–K1 cell cultures, the enhancement in VCD was greater, with a 24 and 27% increase in peak VCD at 4 mM and 8 mM ACCA supplementation respectively compared to control cultures (Fig. 3B). All CHO–K1 cultures maintained similar cell viabilities (Fig. 3B). We also measured the extracellular levels of alanine in our cultures (Supplementary Fig. S1). We observed that alanine accumulated in all the cultures with its levels increasing with increased supplementation of ACCA. Previously, it has been observed that alanine can act as a sink for excess intracellular nitrogen in CHO cell cultures (Kirsch et al., 2022; Dean and Reddy, 2013; Synoground et al., 2021). Indeed, the increasing levels of alanine in ACCA supplemented cultures indicated that most alanine obtained from cleavage of ACCA was secreted into the culture media.

We also examined the impact of ACCA supplementation on IgG titers for these cultures. The ACCA supplemented CHO-GS cultures yielded consistently higher overall titers from day 4 to day 6 ranging from 4% to

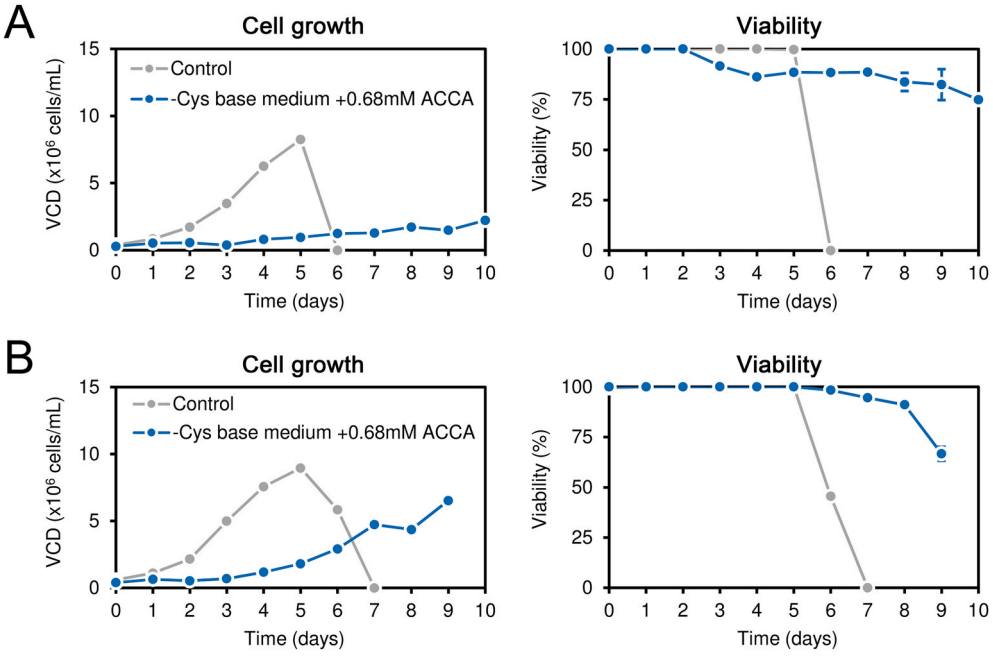

Fig. 2. Substituting cysteine and cystine with ACCA in basal medium does not facilitate growth of CHO cells in batch cultures. Cell growth and viability of CHO cells in batch mode when cysteine and cystine in basal medium is replaced with ACCA for (A) CHO-GS and (B) CHO–K1 cell line. Error bars represent standard deviations (n = 2, biological replicates).

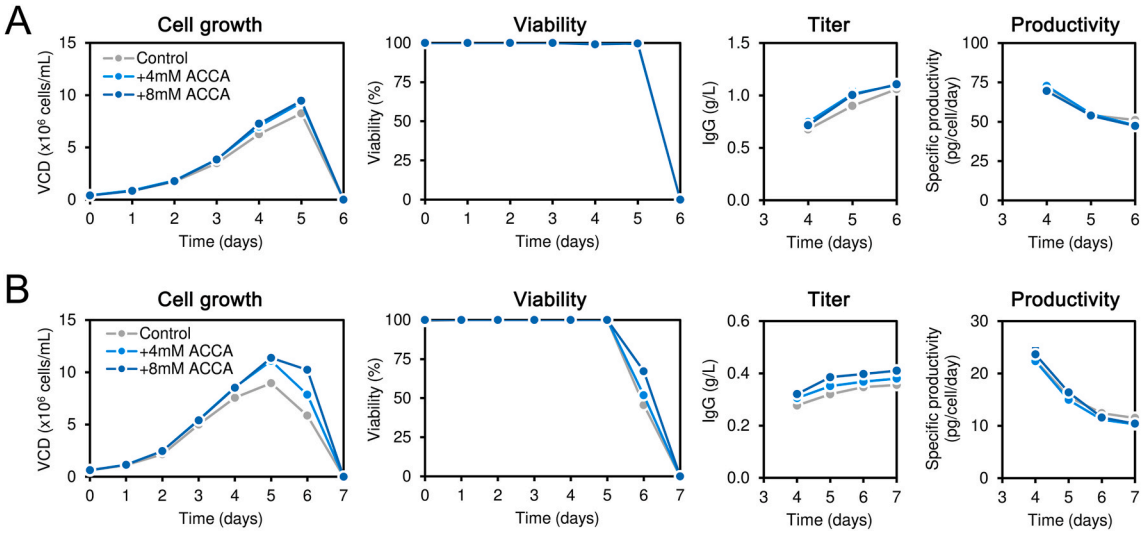

Fig. 3. Supplementation of ACCA to basal medium containing cysteine and cystine enhances both CHO cell growth and IgG titer in batch cultures. Cell growth, viability, IgG titer, and specific IgG productivity of CHO cells in batch mode when ACCA is supplemented to the basal medium as an additive for (A) CHO-GS and (B) CHO–K1 cell line. Error bars represent standard deviations (n = 2, biological replicates).

as high as 12% improvement (Fig. 3A). In CHO–K1 cultures, ACCA supplementation also led to a consistent increase in overall titer ranging from 6% to more than 20% from day 4 to day 7 (Fig. 3B), with the batch cultures supplemented with 8 mM ACCA yielding consistently higher overall titers than cultures with 4 mM ACCA. The increase in titer was primarily due to enhanced viable cell densities achieved in the ACCA supplemented cultures as the specific productivity did not change significantly (Fig. 3). The relative increase in titer with ACCA was less pronounced on day 6 in CHO-GS and on day 7 in CHO–K1, due to loss in cell viability that occurred in batch cultures at these time points resulting in protein degradation that typically accompanies cell lysis (Zustiak et al., 2012).

Overall, these results indicate that while replacement of cysteine and cystine with ACCA is not effective, addition of ACCA to basal media containing cysteine and cystine leads to an increase in overall VCD and titer for both CHO-GS and CHO–K1 cell lines.

2.3. *^{13}C-metabolic flux analysis reveals that supplementation of ACCA to basal medium containing cysteine and cystine improves efficiency of glucose metabolism in CHO–K1 cells*

To gain more detailed insights into possible mechanisms of ACCA's action, ^{13}C-metabolic flux analysis (^{13}C-MFA) was performed using batch culture of CHO–K1 cells since we observed more pronounced benefits of ACCA supplementation in this cell line. Specifically, parallel labeling experiments were performed with CHO–K1 cells grown in standard basal medium with or without 4 mM ACCA. Cysteine and cystine were present in the standard basal medium. Two parallel experiments were performed for each condition, one with [1,2–^{13}C] glucose and one with [U–^{13}C]glutamine as the isotopic tracer. In these tracer experiments, the growth rate during the exponential growth phase was 11% higher in cultures supplemented with ACCA compared to cultures without ACCA (0.81 ± 0.01 vs. 0.73 ± 0.02 day^{-1}, n = 2 biological replicates). In the presence of ACCA, glucose consumption and lactate production were significantly lower during the exponential growth phase (Table 1), while glutamine consumption was not affected by ACCA addition (Table 1). Given that glucose was the limiting nutrient in these batch cultures, i.e. glucose was fully consumed by day 7, suggests that the addition of ACCA to basal medium mainly improved the efficiency of glucose metabolism for cell growth. This was also evident by analyzing total amount of glucose and amino acids consumed at the end of these cultures (Fig. 4A). In cultures supplemented with 4 mM ACCA, we observed 23% higher net cell growth (i.e. higher maximum VCD), 8% less glucose consumed, and 28% higher amount of amino acids consumed. The increased consumption of essential amino acids matched well with the higher maximum VCD observed in cultures supplemented with ACCA (Chen et al., 2019).

Table 1
Growth rate and biomass specific uptake and production rates of extracellular metabolites (nmol/10^6 cells/h) during the exponential growth phase (day 2) in parallel labeling experiments (mean ± stdev; n = 2, biological replicates).

	Complete medium	Complete medium +4 mM ACCA
Growth rate (day^{-1})	0.73 ± 0.02	0.81 ± 0.01
Glucose	−123 ± 8	−96 ± 8
Glutamine	−63 ± 3	−59 ± 3
Lactate	180 ± 10	164 ± 9
Glutamate	11 ± 1	8 ± 1

To determine intracellular metabolic fluxes, ^{13}C labeling of extracted intracellular metabolites was measured during the mid-exponential growth phase (day 2) and the measured uptake and secretion rates (Table 1) were fit to a comprehensive, compartmentalized model of CHO cell metabolism using established methods (Antoniewicz, 2015, 2018; Long and Antoniewicz, 2019). The estimated metabolic fluxes for both conditions, i.e. with and without ACCA supplementation, are shown schematically in Fig. 4B and C. The detailed flux analysis and GC-MS results are provided in Supplementary Tables S1 and S2 respectively. Overall, the metabolic fluxes were very similar for both conditions. For both conditions, metabolism was characterized by a high flux through glycolysis, moderate flux through the TCA cycle, and glutaminolysis as the only significant anaplerotic pathway. Several pathways were inactive, or nearly inactive, in both conditions, including the oxidative pentose phosphate pathway, cytosolic and mitochondrial malic enzymes, and pyruvate carboxylase. The only pathway flux that was significantly different between the two conditions was glycolysis, which was about 20% lower for CHO–K1 cells cultured in medium supplemented with 4 mM ACCA.

To determine how ACCA supplementation impacted cofactor metabolism, we quantified the production and consumption rates of NADH and ATP from the ^{13}C-MFA results (Fig. 4D). We found that ACCA supplementation slightly decreased NADH production and consumption rates, although the relative contributions of glycolysis (60%) and citric acid cycle (40%) to NADH production were not significantly affected.

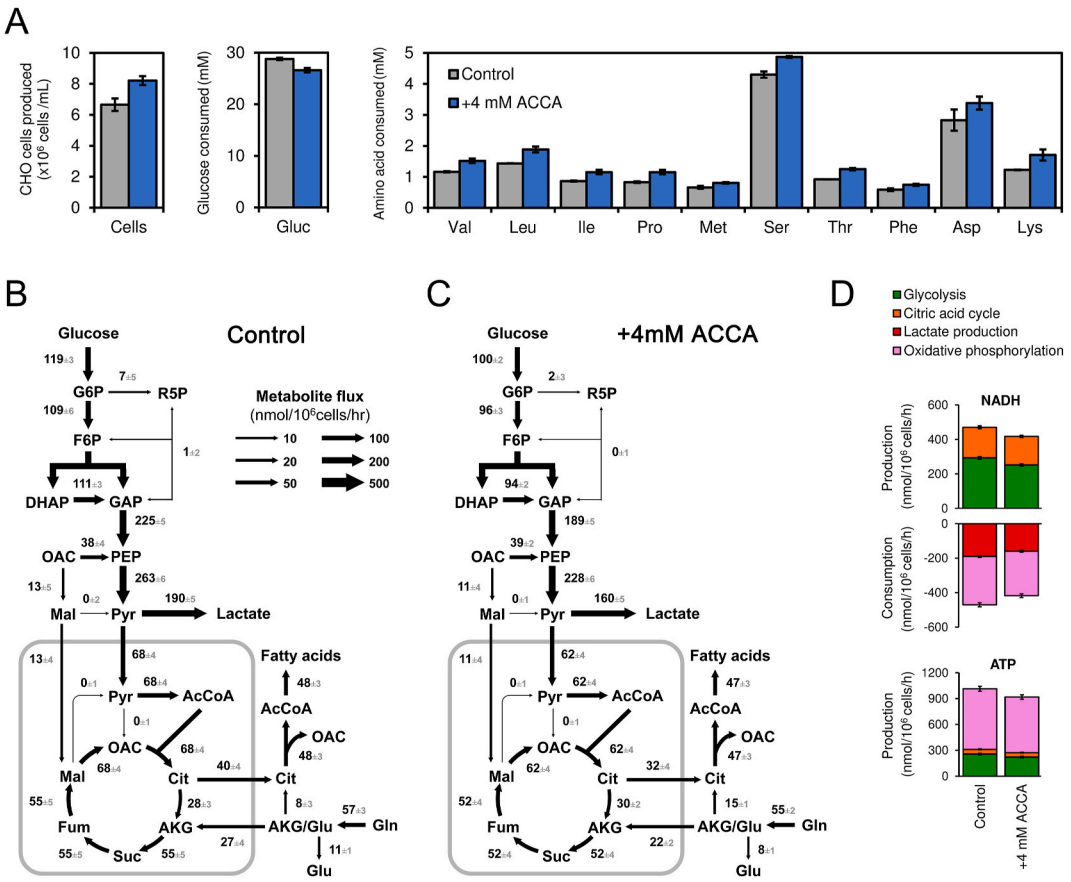

Fig. 4. ^{13}C-MFA elucidates improved efficiency of glucose metabolism in CHO cells grown in medium supplemented with ACCA. (A) Net growth of CHO cells and total consumption of glucose and amino acids at the end of batch cultures of CHO–K1 cells grown in basal medium (control), or basal medium supplemented with 4 mM ACCA (mean ± stdev, n = 2, biological replicates). (B, C) Metabolic flux distributions during exponential growth phase (day 2) were determined by combining uptake and secretion rates and isotopic labeling data from parallel labeling experiments performed with [1,2–^{13}C]glucose and [U–^{13}C]glutamine. Metabolic flux distributions of CHO–K1 cells in batch mode when cultured in basal medium without ACCA (B), or with 4 mM ACCA supplemented (C). Arrow widths indicate absolute magnitudes of net fluxes (nmol/10^6 cells/h, mean ± stdev). The complete flux results are provided in Supplementary Materials (Supplementary Tables S1 and S2). (D) Production and consumption rates of NADH and ATP, as quantified from the ^{13}C-MFA results (nmol/10^6 cells/h, mean ± stdev). To calculate ATP production from oxidative phosphorylation, a P/O ratio of 2.5 was assumed.

Also not affected was the relative utilization of NADH, i.e. 61% for oxidative phosphorylation and 39% for lactate production. We observed a lower ATP production rate for CHO cells with ACCA supplementation compared to control (919 ± 26 vs. 1014 ± 29 nmol/10^6 cells/h). In both cases, the majority of ATP was produced via oxidative phosphorylation (70%) and glycolysis (25%), with only a small contribution from the citric acid cycle (5%).

Taken together, these results suggest that supplementation of ACCA

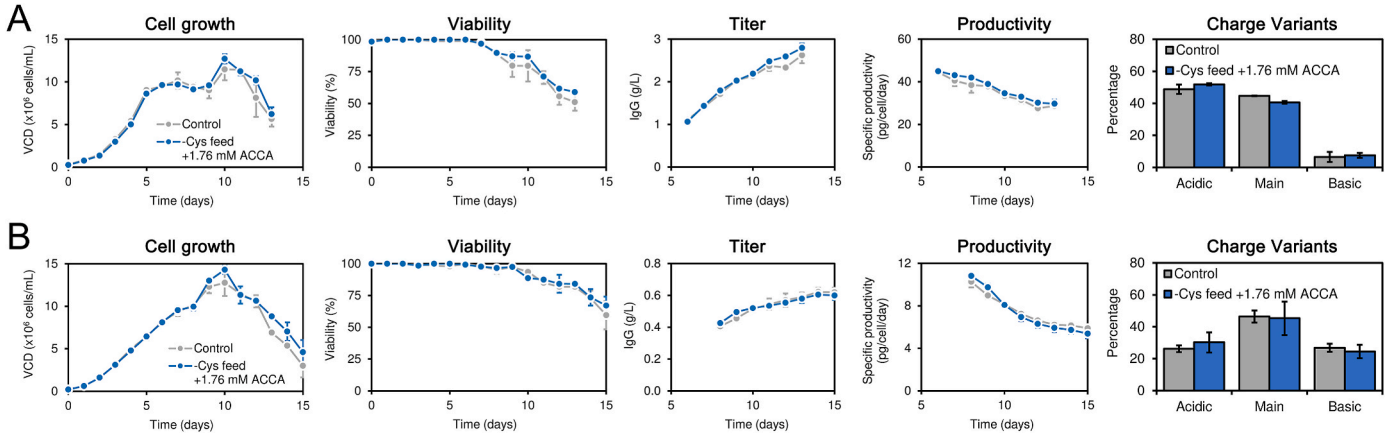

Fig. 5. ACCA can replace cysteine and cystine in highly concentrated feed medium to deliver similar cell culture performance. Cell growth, viability, IgG titer, specific IgG productivity, and IgG charge variants distribution in fed-batch mode when cysteine and cystine in feed medium is replaced with ACCA for (A) CHO-GS and (B) CHO–K1 cell line. Error bars represent standard deviations (n = 2, biological replicates). The glucose and lactate concentrations in spent media are provided in Supplementary Materials (Supplementary Fig. S2).

improved the efficiency of glucose metabolism in CHO–K1 cells, that is, the cells were able to maintain a higher growth rate while metabolizing less glucose for ATP production through glycolysis and oxidative phosphorylation.

2.4. ACCA is a suitable alternative for cysteine and cystine in highly concentrated feed medium

Next, we tested whether cysteine and cystine in feed media can be replaced with an equivalent amount of ACCA. For these experiments, the feed medium contained either cysteine and cystine at a total concentration of 1.76 mM, or 1.76 mM ACCA and no cysteine and cysteine. As shown in Fig. 5, we did not observe any significant differences in cell culture performance between the two feed media for both CHO–K1 and CHO-GS cells. For CHO-GS cell line, the control cultures attained a peak VCD of 11.5 million cells/mL whereas cultures with 1.76 mM ACCA reached a maximum VCD of 12.7 million cells/mL (Fig. 5A). No differences were observed in the cell viability profiles across all CHO-GS cultures (Fig. 5A). Both feed media also produced similar titers, with the control culture producing 2.6 g/L IgG compared to 2.8 g/L IgG with 1.76 mM ACCA and no cysteine and cystine in the feed medium (Fig. 5A). The cell specific productivity was similar for the two feed media (Fig. 5A). The addition of ACCA also did not yield any significant changes in charge variants distribution of the purified IgG across all CHO-GS cultures (Fig. 5A).

In the case of the CHO–K1 cell line, the control culture reached a slightly lower peak VCD compared to the cultures with ACCA in the feed medium, 12.8 million cells/mL vs. 14.3 million cells/mL, respectively (Fig. 5B). The IgG titers were similar for the two feed media, i.e. 0.62 g/L and 0.60 g/L, respectively, on the day of harvest (Fig. 5B). No differences were observed in the cell viability profiles as well as cell specific productivity across all CHO–K1 cultures (Fig. 5B). The addition of ACCA did not yield any significant changes in charge variants distribution of the purified IgG across all CHO–K1 cultures (Fig. 5B). Taken together, these results indicate that ACCA can be used to replace cysteine and cystine in feed media to support growth of CHO-GS and CHO–K1 cells in fed-batch processes.

2.5. Supplementation of ACCA at high concentrations in basal medium enables elimination of cysteine and cystine from feed medium

Finally, we tested if cysteine and cystine can be eliminated from feed medium altogether by supplementing ACCA at high concentrations in the basal medium, given its high solubility. For this, the minimum concentration of ACCA to be supplemented in basal medium was determined by accounting for the frequency and quantity of cysteine and cystine feeding to cell cultures over their total duration (see Materials and Methods). Specifically, 0.44 mM and 0.88 mM ACCA were added to basal medium of CHO-GS and CHO–K1 cultures respectively. In this way, we leveraged the high solubility of ACCA to supply high amounts of cysteine equivalents to CHO cells which otherwise would not be possible due to the low solubility of free cysteine and low stability of free cysteine. We also tested the impact of supplementing ACCA to the basal medium at approximately 2X concentrations, specifically, 1 mM and 2 mM ACCA for CHO-GS and CHO–K1 cultures respectively.

As shown in Fig. 6A for CHO-GS cultures, when 0.44 mM ACCA was added to the basal medium and no cysteine equivalents were used in the feed medium, we did not observe any significant differences in VCD and viabilities compared to the control cultures which did not contain any ACCA in basal medium but contained 1.76 mM of total cysteine and cystine in feed medium. Both conditions yielded a similar maximum VCD of 11.5 million cells/mL and 12.5 million cells/mL, respectively (Fig. 6A). Interestingly, when 1 mM ACCA was added to the basal medium and no cysteine and cystine were present in the feed medium, we observed a greater than 30% increase in VCD from day 7 onward compared to control. Furthermore, cell viabilities were about 10% greater from days 9–13 when 1 mM ACCA was added to basal medium compared to the control with no ACCA (Fig. 6A). As shown in Fig. 6B for CHO–K1 cultures, when 0.88 mM ACCA was present in the basal medium and no cysteine equivalents were present in the feed medium, the cultures consistently maintained higher VCDs from day 11 to day 15 and the culture duration increased by 3 days compared to the control without any ACCA in basal medium but with 1.76 mM cysteine and cystine in the feed medium (Fig. 6B). ACCA supplementation also improved cell viabilities from day 12–15 when 0.88M ACCA was added to the basal medium (Fig. 6B). When 2 mM ACCA was added to the basal medium and no cysteine and cystine in the feed medium, we observed an overall 24% increase in peak VCD with the 2 mM ACCA supplemented culture maintaining higher VCD from day 5 to day 15 when compared to the control (Fig. 6B). We observed that higher VCD levels were accompanied by slightly elevated lactate levels from day 7 to day 15 (Supplementary Fig. S3).

While final titers were not significantly increased for CHO-GS cultures when 0.44 mM ACCA was added to the basal medium and no cysteine equivalents were in the feed medium compared to the control, addition of 1 mM ACCA increased IgG titers by up to 15% from day 8 onward compared to the control (Fig. 6A). However, specific IgG productivity was 10–15% lower, likely due to the enhanced growth

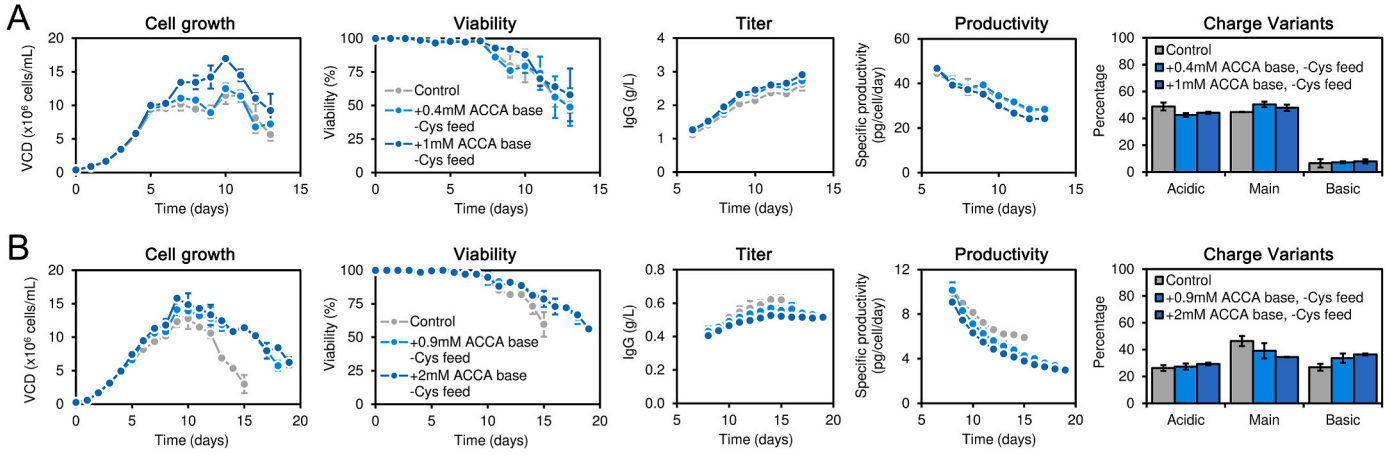

Fig. 6. Supplementation of ACCA at high concentrations in basal medium eliminates the need to supply cysteine and cystine in feed medium. Cell growth, viability, IgG titer, specific IgG productivity, and IgG charge variants distribution in fed-batch mode when cysteine, cystine, and ACCA in feed medium are eliminated by addition of equivalent or higher amount of ACCA in the basal medium for (A) CHO-GS and (B) CHO–K1 cell line. Error bars represent standard deviations (n = 2, biological replicates). The glucose and lactate concentrations in spent media are provided in Supplementary Materials (Supplementary Fig. S3).

(Fig. 6A). The addition of ACCA did not yield any significant changes in charge variants distribution of the purified IgG across all CHO-GS cultures (Fig. 6A). In the case of CHO–K1 cultures, when 0.88 mM ACCA was added to the basal medium and no cysteine equivalents in the feed medium, no significant difference in IgG titer was detected compared to the control (Fig. 6B). When 2 mM ACCA was added to the basal medium and no cysteine equivalents were present in the feed medium, up to a 15% decrease in titer from day 12 to day 15 was detected when compared to control (Fig. 6B). Specific IgG productivity was also lower compared to the control, likely due to the amplified growth (Fig. 6B). The addition of ACCA did not yield any significant changes in charge variants distribution of the purified IgG across all CHO–K1 cultures (Fig. 6B).

Overall, these results suggest that rather than supplementing cysteine equivalents (cystine, cystine, and ACCA) multiple times in cell culture through the feed medium, ACCA can be added directly to basal medium (0.44 mM for CHO-GS and 0.88 mM for CHO–K1) to support cellular cysteine and cystine demands for the entire duration of fed-batch cultures. Such a strategy could eliminate the need to incorporate cysteine equivalents in feed media and potentially ease the process of making highly concentrated feed media by mitigating the challenges of low solubility of cystine and poor stability of cysteine in such feed media.

3. Discussion

In this study, we have evaluated the potential of ACCA as a replacement for cysteine and cystine in basal and feed media of CHO cell cultures. ACCA was unable to support robust cell growth in batch culture when basal medium was depleted of cysteine and cysteine. This could be due to insufficient transport of the dipeptide dimer leading to inefficient availability of cysteine inside the cells. Indeed, Sánchez-Kopper et al. reported that due to its bulky nature, the uptake rate of ACCA by CHO cells was slower than other dipeptides such as L-alanyl-L-glutamine, glycyl-L-glutamine, L-alanyl-L-tyrosine, glycyl-L-tyrosine, L-prolinyl-L-tyrosine, and L-alanyl-L-proline (Sánchez-Kopper et al., 2016). Although intracellular and extracellular cleavage of dipeptides has been reported in previous studies (Naik et al., 2024; Franěk et al., 2003), it may be possible that intracellular and/or extracellular cleavage of ACCA was insufficient to sustain CHO cell growth in batch phase in this study. Interestingly, when ACCA was used as an additive to basal medium containing cysteine and cystine, an enhancement in cell growth and IgG titer was observed, with proportional increases in cell growth and IgG titer based on the amount of ACCA supplemented. The free cysteine and cystine originally present in the medium likely jump-started CHO cell growth. Subsequently, ACCA may have rewired CHO cell metabolism to enhance cell growth and IgG production. Indeed, ^{13}C-metabolic flux analysis performed in this study indicated a reduction in glycolytic flux in CHO–K1 cell cultures supplemented with 4 mM ACCA. Additional availability of cysteine through extracellular cleavage of ACCA and/or transport and cleavage of ACCA inside the cells may have boosted the synthesis of glutathione, an intracellular antioxidant, and subsequently lowered ROS levels in culture. Since high ROS levels have been known to upregulate glycolysis due to the Warburg effect, elevated antioxidant levels leading to reduction in ROS could explain the decline in glycolytic flux observed following ACCA supplementation (Shi et al., 2009; Ghanbari Movahed et al., 2019). Also, the reduced glycolysis flux in ACCA supplemented cultures was complemented with reduced lactate production. Previously, lactate has been reported to exert a growth inhibitory impact on cell culture performance (Lao and Toth, 1997; Ozturk et al., 1992). Indeed, prior studies have also reported a positive correlation of cysteine and cystine availability with cell growth, viability, and productivity as well as the adverse impacts of cysteine depletion including redox imbalances, mitochondrial dysfunction, and endoplasmic reticulum stress (Ali et al., 2019, 2020; Yao et al., 2021). However, we cannot disregard the potential impact of additional alanine from ACCA, although the majority appears to be secreted and not utilized, or other peptide signaling effects of ACCA on CHO cell metabolism. Overall, our data suggests that ACCA supplementation enhanced the efficiency of glucose metabolism in CHO–K1 cells as less glucose was metabolized while simultaneously achieving higher cell growth and IgG titers.

Next, we demonstrated that ACCA can be used to replace cysteine and cystine in highly concentrated feed medium. When ACCA was supplemented in feed medium instead of cysteine and cystine, similar cell growth, IgG titers, and charge variants distribution were observed on IgG when compared to feed medium containing cysteine and cystine and no ACCA. Concentrated feed media undergo high amounts of precipitation due to the poor stability of cysteine and low solubility of cystine resulting in sub-optimal feed media and cell culture performance due to a decrease in amount of bioavailable cysteine and cystine plus the formation of undesired products (Rigo et al., 2004; Hecklau et al., 2016). This impact of cysteine makes storage of feed medium highly challenging. Given its high solubility, ACCA can be used to replace cysteine and cystine in concentrated feed media and help alleviate challenges with precipitation, storage, and manufacturing of raw materials for biotherapeutic protein production. We further leveraged the high solubility of ACCA to eliminate any cysteine equivalents (cysteine, cystine, and ACCA) from feed medium by adding ACCA to the basal medium at concentration high enough to support the cellular demands of cysteine and cystine for the entire duration of fed-batch cultures. We observed no significant differences in cell growth, IgG titer, and IgG charge variants for both CHO-GS and CHO–K1 cells cultured in a fed-batch process using a cysteine equivalents-free feed medium and basal medium supplemented with 0.44 mM and 0.88 mM ACCA for CHO-GS and CHO–K1 cultures, respectively. Since other amino acids have higher solubility than cysteine and cystine, the absence of cysteine and cystine from feed medium will enable formulation of more highly concentrated feed media for intensified fed-batch processes without issues of cystine precipitation and storage. We also examined the impact of supplementing ACCA in basal medium at concentrations higher than the amount of cysteine and cystine needed to support our typical fed-batch cultures. Consistent with our observations in batch cultures, we observed that when CHO-GS cells were cultured in a fed-batch process using cysteine equivalents-free feed medium and basal medium supplemented with 1 mM ACCA, cell growth increased by 30% and IgG titers increased by 15%. When CHO–K1 cells were cultured in a fed-batch process using cysteine equivalents-free feed medium and basal medium supplemented with 2 mM ACCA, CHO-GS cell line, cell growth increased by 24% and surprisingly, IgG titer decreased by 15%. It may be possible that in fed-batch processes with such high cell densities, transcriptional and translational bottlenecks in the recombinant protein production capability of the CHO cells limited an increase in IgG production proportionate to the increase in cell growth (Ingolia et al., 2012; Kallehauge et al., 2017). Nevertheless, in this study we show that ACCA can be used to replace or even eliminate cysteine and cystine in feed medium without any adverse impacts on cell culture performance. Leveraging peptides such as ACCA could pave the way for enhanced manufacturing and storage of highly concentrated feed media without encountering the challenges of precipitation and formation of undesired solids, which also reduces nutrient bioavailability. Achieving high cell densities is a major objective of biomanufacturing as we move toward intensified fed-batch bioprocessing and using ACCA will facilitate more rapid deployment of concentrated feed media for advanced bioproduction processes of the future.

4. Materials and Methods

4.1. Materials

The dipeptide dimer, N,N′-di-L-Alanyl-L-Cystine (cQrex® AC), also referred to as Ala-Cys-Cys-Ala (ACCA), was provided by Evonik (Essen,

Table 2
List of cell culture conditions tested for CHO cells in batch mode.

Condition no.	Basal medium	ACCA supplementation in basal medium
1	Complete basal medium containing cysteine and cystine	0 mM ACCA (control)
2	Modified basal medium lacking cysteine and cystine	0.68 mM ACCA (ACCA as replacement for cysteine and cystine)
3	Complete basal medium containing cysteine and cystine	4 mM ACCA (ACCA as an additive)
4	Complete basal medium containing cysteine and cystine	8 mM ACCA (ACCA as an additive)

Germany). Isotopic tracers [1,2–^{13}C]glucose, [U–^{13}C]glutamine, and [U–^{13}C]algal amino acid mixture were purchased from Cambridge Isotope Laboratories (Andover, MA). The [U–^{13}C]algal amino acid mixture was solubilized in 0.1 N HCl at 10 mg/mL and used for quantification of amino acid concentrations in medium samples by GC-MS (Oates and Antoniewicz, 2023). Isotopic purity of all tracers was verified by GC-MS (Long and Antoniewicz, 2019). Chemically defined basal medium, Immediate Advantage® medium (Cat. No. 87093C), and custom media missing cysteine/cystine or glucose were purchased from MilliporeSigma (Saint Louis, USA). Chemically defined feed medium (Cat. No. 87093C, Feed A) and its modified form lacking cysteine and cystine was also obtained from MilliporeSigma (Saint Louis, USA). Feed medium did not contain glucose or glutamine. Except cysteine and cystine which were removed from the custom media formulation, all the other media components were present at the same concentrations in complete medium as well as custom medium. ACCA was added as a powder at the appropriate concentrations to the media. Upon dissolution, all media were filter-sterilized prior to use in cell culture experiments using a 0.22 μm or 0.4 μm pore-size membrane filter.

4.2. CHO cell lines

CHO-GS cell line (CHOZN® GS−/− ZFN-modified CHO cell line) producing IgG was obtained from MilliporeSigma (Saint Louis, USA). CHO–K1 suspension cell line producing IgG (VRC01) was provided by National Institute of Health (Bethesda, MD). Cells were thawed and subcultured every 3 days for two passages at a seeding density of 0.3 million cells/mL in complete basal medium, Immediate Advantage® medium (Cat. No. 87093C), which already contained cysteine and cystine. At the end of the second passage, batch and fed-batch cultures were initiated by transferring cells into appropriate basal medium for testing.

4.3. Batch cell culture

For each cell line, we tested 4 conditions in batch cultures as described in Table 2. All conditions were tested in duplicate. CHO cells were seeded at 0.3 million cells/mL in 30 mL medium and cultured in 125-mL shaker flasks (Corning® Erlenmeyer cell culture flasks, Sigma-Aldrich, Cat No. CLS431143) in a humidified incubator operating at 37 °C, 125 RPM and 5% CO_2 level. In the case of CHO–K1 cell line, 6 mM of L-glutamine and 0.4 vol% anti-clumping agent (Gibco, Canada) were supplemented on day 0. Batch cell cultures lasted 6–8 days. Cell culture samples were collected every 24 h. The samples were then centrifuged at 1000 RPM to separate spent medium (supernatant) from cell pellets for further analysis.

4.4. Parallel labeling experiments for ^{13}C-metabolic flux analysis

The CHO–K1 cell line was used for parallel labeling experiments for ^{13}C-metabolic flux analysis. The basal medium, which did not contain glucose or glutamine, was supplemented with 30 mM glucose (either unlabeled glucose or [1,2–^{13}C]glucose), 6 mM glutamine (either unlabeled glutamine or [U–^{13}C]glutamine), 4 mM ACCA, and 0.4 vol% anti-clumping agent (Gibco, Canada). CHO cells were inoculated at 0.3 million cells/mL in 30 mL medium and grown in 125-mL shaker flasks (Corning Erlenmeyer cell culture flasks, Sigma-Aldrich, Cat No. CLS431143) in a humidified incubator operating at 37 °C, 125 RPM, and 5% CO_2. To obtain cell pellets and cell-free spent media, a predetermined amount of cell culture was harvested and centrifuged at 1000 RPM to separate the supernatant from cell pellets. Spent media were filtered and stored at −20 °C. Cell pellets were washed at least once using cold saline solution and stored at −20 °C.

4.5. Fed-batch cell culture

For each cell line, we tested 4 conditions in fed-batch cultures as described in Table 3. All conditions were tested in duplicate. CHO cells were seeded at 0.3 million cells/mL in 30 mL medium and cultured in 125-mL shaker flasks (Corning® Erlenmeyer cell culture flasks, Sigma-Aldrich, Cat No. CLS431143) in a humidified incubator operating at 37 °C, 125 RPM and 5% CO_2 level. In the case of CHO–K1 cell line, 6 mM of L-glutamine was supplemented on day 0.

For CHO-GS fed-batch cultures, feed medium was supplemented at 5 vol% on days 3, 5, 7, 9, and 11. Glucose was supplemented up to 5.5 g/L using 45% D-(+)-Glucose solution in water (MilliporeSigma, Cat. No.: G8769) when the glucose levels dropped below 3 g/L. Cell culture samples were collected every 24 h. The samples were then centrifuged at 1000 RPM to separate spent medium (supernatant) from cell pellets for further analysis. Cysteine and cystine were present in the complete feed medium at a total concentration of 1.76 mM. ACCA added during the entire duration of CHO-GS fed-batch cultures using feed medium was calculated as:

$$= \frac{(5\%\ of\ 30\ mL\ working\ culture\ volume) * (1.76\ mM) * 5\ days\ of\ feed\ addition}{30mL\ (working\ culture\ volume)}$$

$$= \frac{(0.05 * 30) * 1.76 * 5}{30} = 0.44\ \text{mM}$$

For CHO–K1 fed-batch cultures, feed medium was supplemented at 5 vol% daily starting day 3. Glucose was supplemented up to 5.5 g/L using 45% D-(+)-Glucose solution in water (MilliporeSigma, Cat. No.: G8769) when the glucose levels dropped below 3 g/L 6 mM of L-glutamine (Corning®, Cat. No.: 25-005-CI) was fed on days 5 and 10. Cell culture samples were collected every 24 h. The samples were then centrifuged at 1000 RPM to separate spent medium (supernatant) from cell pellets for further analysis. Cysteine and cystine are present in the complete feed medium at a total concentration of 1.76 mM. ACCA added during the entire duration of CHO–K1 fed-batch cultures using feed medium was calculated as:

$$= \frac{(5\%\ of\ 30\ mL\ working\ culture\ volume) * (1.76\ mM) * 10\ days\ of\ feed\ addition}{30mL\ (working\ culture\ volume)}$$

$$= \frac{(0.05 * 30) * 1.76 * 10}{30} = 0.88\ \text{mM}$$

All cultures were harvested when viability dropped below 70% by spinning down the cultures at 5000 RPM for 10 min. Supernatants were filtered using 0.22 μm filters and stored at −80 °C for further analysis.

4.6. Analytical methods

Samples were collected every 24 h. Viable cell density (VCD) and cell viability (%) were calculated from cell counts based on the trypan blue dye exclusion method using a hemocytometer (technical duplicate measurements were performed by filling the slide with sample twice) and/or an automated cell counter (Corning Cytosmart). If cell clumps were visible, culture samples were first mixed with 0.25% trypsin-EDTA (Gibco, Canada) at 1:1 ratio and incubated for 10 min at 37 °C, prior to cell counting. To quantify ^{13}C labeling of intracellular amino acids, 2 million cells were collected and intracellular metabolites were extracted using the methanol/chloroform/water extraction method (Ahn et al., 2016). To quantify concentrations of amino acids in spent media, samples were mixed with an internal standard solution containing labeled or unlabeled amino acids with a known concentration and analyzed by GC-MS (Oates and Antoniewicz, 2023). Glucose and lactate concentrations were measured using a YSI 2950D and/or YSI 2500 biochemical analyzer (Yellow Springs Instrument Inc., OH). IgG levels in spent medium were quantified on an Agilent HPLC using a protein A column (Poros 2 μm, 2.1 × 30 mm, Thermofisher, Waltham, MA).

4.7. Gas chromatography-mass spectrometry

For GC-MS analysis of isotopic labeling and quantification of amino acid concentrations, dried samples were derivatized using the MOX-TBDMS derivatization method (Long and Antoniewicz, 2019). GC-MS analysis was performed on an Agilent 7890A GC system equipped with a HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977B Mass Spectrometer operating under ionization by electron impact (EI) at 70eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230 °C, the MS quad temperature at 150 °C, the interface temperature at 280 °C, and the inlet temperature at 280 °C. Mass spectra were recorded in single ion monitoring (SIM) mode with 4 ms dwell time on each ion. Mass isotopomer distributions were obtained by integration of ion chromatograms (Gomez et al., 2023), and corrected for natural isotope abundances (Fernandez et al., 1996).

Table 3
List of cell culture conditions tested for CHO cells in fed-batch mode.

Condition no.	Basal medium	ACCA supplementation in basal medium	Feed medium	ACCA supplementation in feed medium
1	Complete basal medium containing cysteine and cystine	No ACCA was added	Complete feed medium containing cysteine and cystine	No ACCA was added
2	Complete basal medium containing cysteine and cystine	No ACCA was added	Modified feed medium lacking cysteine and cystine	1.76 mM ACCA
3	Complete basal medium containing cysteine and cystine	– 0.44 mM ACCA (for CHO-GS) – 0.88 mM ACCA (for CHO–K1)	Modified feed medium lacking cysteine and cystine	No ACCA was added
4	Complete basal medium containing cysteine and cystine	– 1 mM ACCA (for CHO-GS) – 2 mM ACCA (for CHO–K1)	Modified feed medium lacking cysteine and cystine	No ACCA was added

4.8. ^{13}C-metabolic flux analysis

Metabolic fluxes were determined by fitting extracellular uptake and secretion rates and the measured mass isotopomer distributions of intracellular metabolites to a compartmentalized metabolic network model using the Metran software (Yoo et al., 2008). The model contains three distinct metabolic compartments: extracellular, cytosol, and mitochondrion. The metabolites pyruvate, acetyl-CoA, citrate, α-ketoglutarate, malate, fumarate, oxaloacetate, alanine, glutamate, and aspartate are metabolically active in both the cytosol and mitochondrion. During the extraction process, intracellular pools of metabolites are homogenized. As such, the measured isotopic labeling of these metabolites reflects the mixture of distinct metabolic pools. In the ^{13}C-MFA model, we included mixing reactions to account for mixing of mitochondrial and cytosolic metabolite pools during extraction (Oates and Antoniewicz, 2022). Our model also accounts for dilution of intracellular metabolites due to incorporation of unlabeled CO_2 and influx of unlabeled metabolites from the medium such as aspartate. For ^{13}C-MFA, labeling data from two parallel labeling experiments for each condition, i.e. with either [1,2–^{13}C]glucose or [U–^{13}C]glutamine as the tracer, were fitted simultaneously to the network model to estimate intracellular fluxes. All data used for flux analysis are provided in the Supplementary Materials. To ensure that the global best solution was identified, flux estimation was repeated at least 20 times starting with random initial values. At convergence, a chi-square test was applied to test the goodness-of-fit, and accurate 95% confidence intervals were calculated by determining the sensitivity of the sum of squared residuals to flux parameter variations (Antoniewicz et al., 2006). The complete flux results, including upper and lower bounds of 95% confidence intervals for all fluxes, are reported in Supplementary Materials.

4.9. Protein purification

Cell culture harvests were purified using affinity chromatography and gravity flow as previously described by Wang et al. (2019). Protein A agarose bead slurry (Vector Lab, Burlingame, CA) was added to cell culture supernatants and these mixtures were incubated overnight at 4 °C on a rotator. Next day, the mixtures were loaded onto a 15-mL polypropylene column (QIAGEN) to retain the agarose beads followed by three washes with phosphate buffered saline (PBS) buffer. The bound IgG was eluted using 0.1 M glycine, pH 2.7 elution buffer. 1 M Tris-HCl, pH 9.0 neutralization buffer was immediately added to neutralize the acidic pH of the eluate. The eluates were subsequently dialyzed several times against PBS using Amicon Ultra Centrifugal Filters (MilliporeSigma) at 5000×*g* for 20 min at 10 °C using a filter with 10 kDa molecular weight cut-off. The final concentration of purified IgG was measured by a NanoDrop™ 2000/2000c Spectrophotometer and purity

was assessed on 10% SDS-PAGE gels (Bio-Rad) followed by Coomassie blue staining. Gels were de-stained in water overnight and clear bands for heavy chain and light chain of IgG were visible on the next day.

4.10. Charge variants profiling

Antibody charge variants were resolved utilizing a pH gradient cation exchange chromatography method on an Agilent 1260 infinity quaternary HPLC. This instrument includes a quaternary gradient pump, autosampler, thermostat column compartment, and a multiple wavelength detector. The column used was an Agilent Bio Mab NP5, 2.1 × 250 mm. The operating flow rate was 1 mL/min, and the column compartment was set to 30 °C. Detection was performed at 280 nm. For each chromatographic injection, 100 μg of protein was loaded onto the column. Mobile phase compositions include tris, piperazine, and an imidazole buffer system established from with modifications made for this study (Zhang et al., 2013). Mobile phases prepared for this study contained 4 mM tris-base, 4 mM piperazine, 4 mM imidazole at pH 7.65 (mobile phase A) and 4 mM tris-base, 4 mM piperazine, 4 mM imidazole, 16 mM NaCl at pH 10.8 (mobile phase B). The pH of the mobile phases was adjusted with concentrated sodium hydroxide or hydrochloric acid. All buffers were prepared using aqua solutions ultra-pure water and filtered through a 0.2 μm PES membrane before use. The pH gradient elution was established using a linear gradient from 44% A and 56% B to 29% A and 71% B in over 40 min. Data acquisition and data analysis were performed with Agilent OpenLab chromatography data software.

CRediT authorship contribution statement

Pranay Ladiwala: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Xiangchen Cai:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Harnish Mukesh Naik:** Writing – original draft, Investigation, Formal analysis, Data curation. **Lateef Aliyu:** Formal analysis, Data curation. **Martin Schilling:** Resources, Project administration, Conceptualization. **Maciek R. Antoniewicz:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Michael J. Betenbaugh:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was funded and supported by the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC) through the Industry-University Cooperative Research Center Program under U.S. National Science Foundation grant number 1624684.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2024.07.008.

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