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Elucidating uptake and metabolic fate of dipeptides in CHO cell cultures using ^{13}C labeling experiments and kinetic modeling

Harnish Mukesh Naik [a,1], Xiangchen Cai [b,1], Pranay Ladiwala [a], Jayanth Venkatarama Reddy [a], Michael J. Betenbaugh [a], Maciek R. Antoniewicz [b,*]

[a] Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, 21218, USA
[b] Department of Chemical Engineering, University of Michigan, Ann Arbor, MI, 48109, USA

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ABSTRACT

The rapidly growing market of biologics including monoclonal antibodies has stimulated the need to improve biomanufacturing processes including mammalian host systems such as Chinese Hamster Ovary (CHO) cells. Cell culture media formulations continue to be enhanced to enable intensified cell culture processes and optimize cell culture performance. Amino acids, major components of cell culture media, are consumed in large amounts by CHO cells. Due to their low solubility and poor stability, certain amino acids including tyrosine, leucine, and phenylalanine can pose major challenges leading to suboptimal bioprocess performance. Dipeptides have the potential to replace amino acids in culture media. However, very little is known about the cleavage, uptake, and utilization kinetics of dipeptides in CHO cell cultures. In this study, replacing amino acids, including leucine and tyrosine by their respective dipeptides including but not limited to Ala-Leu and Gly-Tyr, supported similar cell growth, antibody production, and lactate profiles. Using ^{13}C labeling techniques and spent media studies, dipeptides were shown to undergo both intracellular and extracellular cleavage in cultures. Extracellular cleavage increased with the culture duration, indicating cleavage by host cell proteins that are likely secreted and accumulate in cell culture over time. A kinetic model was built and for the first time, integrated with ^{13}C labeling experiments to estimate dipeptide utilization rates, in CHO cell cultures. Dipeptides with alanine at the N-terminus had a higher utilization rate than dipeptides with alanine at the C-terminus and dipeptides with glycine instead of alanine at N-terminus. Simultaneous supplementation of more than one dipeptide in culture led to reduction in individual dipeptide utilization rates indicating that dipeptides compete for the same cleavage enzymes, transporters, or both. Dipeptide utilization rates in culture and cleavage rates in cell-free experiments appeared to follow Michaelis-Menten kinetics, reaching a maximum at higher dipeptide concentrations. Dipeptide utilization behavior was found to be similar in cell-free and cell culture environments, paving the way for future testing approaches for dipeptides in cell-free environments prior to use in large-scale bioreactors. Thus, this study provides a deeper understanding of the fate of dipeptides in CHO cell cultures through an integration of cell culture, ^{13}C labeling, and kinetic modeling approaches providing insights in how to best use dipeptides in media formulations for robust and optimal mammalian cell culture performance.

1. Introduction

Over the last four decades, recombinant protein therapeutics have been increasingly used for the treatment of diseases including cancer, autoimmune disorders, hematologic diseases, and various other medical conditions. More than 300 biotherapeutics have been approved by the U.S. FDA (Food and Drug Administration) since 2002, and this number continues to rise, with the market value of mAbs alone expected to surpass US$200 billion in 2023 (Grilo and Mantalaris, 2019). Of the multiple mammalian cell lines employed to produce biotherapeutics, Chinese Hamster Ovary (CHO) cells are the most widely utilized host for multiple reasons including, but not limited to, their ability to produce proteins with human-like glycosylation patterns, high productivity, robustness, and scalability in suspension cell culture bioreactors (Dhara

* Corresponding author. Department of Chemical Engineering, University of Michigan, 2800 Plymouth Road, Ann Arbor, MI, 48109, USA.
E-mail address: mranton@umich.edu (M.R. Antoniewicz).
[1] Equal contribution.

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et al., 2018; Kim et al., 2012). Multiple approaches are often employed to help improve productivity and product quality for CHO cells including optimization of basal medium and feed, cell engineering, altering processing such as intensified fed-batch and perfusion processes, and optimizing process parameters (e.g., feed times and nutrient levels, temperature, dissolved oxygen, pH etc.) (Hiller et al., 2017; Mohan et al., 2008; Naik et al., 2023; Templeton et al., 2013).

Mammalian cell culture media consists of numerous components including glucose, amino acids, trace metals, vitamins, lipids, buffers, etc. Amino acids, one of the principal components of cell culture media and feed, are consumed in large amounts by CHO cells. They act as critical nitrogen and carbon sources to the cells, contributing directly, and following metabolic conversion, to biomass components including native cellular proteins, DNA, and other macromolecules. In addition, amino acids can contribute to the generation of energy needed for cell growth and recombinant protein production (Carrillo-Cocom et al., 2015; Duarte et al., 2014). However, amino acids can offer major challenges in terms of solubility and stability in both basal and feed media formulations. For example, tyrosine has the lowest solubility of all essential amino acids, with a maximal concentration of about 0.5 g/L in water at physiological pH and room temperature. Hydrated salts of tyrosine are only slightly soluble at neutral pH. Characterization of feed media precipitate by Hoang et al. revealed that tyrosine made up the major portion of the precipitate (approximately 77 wt%) (Hoang et al., 2021). As a result, in cell culture processes using highly concentrated feeds, tyrosine is often fed at very high pH to increase its solubility in the final cell culture media. Such an approach leads to challenges in pH control, salt concentration, and precipitation in bioreactors. In the same study by Hoang et al., phenylalanine was found to be the second most abundant amino acid in feed media precipitate (Hoang et al., 2021). Furthermore, branched-chain amino acids including leucine, isoleucine, and valine present low intrinsic solubility in cell culture media as they are capable of forming mixed crystals with one another and with glutamine as well as undergoing complex formation with electrolytes, which subsequently alters their own hydration (Salazar et al., 2016). In total, the limited solubility and poor stability of these amino acids can lead to loss of media batches and suboptimal cell culture performance due to insufficient nutrient levels for cellular demands.

To overcome the solubility and stability challenges posed by amino acids, researchers have evaluated the application of both naturally occurring and synthetically prepared peptides in mammalian cell cultures. In early work, Franek et al. demonstrated that plant-based protein hydrolysates can serve both as a source of free amino acids and bioactive peptides acting on cell signaling functions to exert specific effects on cell growth and productivity (Franek et al., 2000). More recently, advances in serum-free chemically defined basal media and feeds have often included synthetically prepared peptides such as dipeptides and tripeptides as well as modified amino acids. Tyrosine-containing dipeptides are almost 250 times more soluble than free tyrosine, enabling higher concentrations of tyrosine residues in CHO cell cultures. In one study, Kang et al. observed that addition of Tyr-His and Tyr-Lys in place of free tyrosine led to a 67% increase in cell viability, a 37% increase in titer, and lower lactate and ammonium levels in batch cultures. Since these dipeptides are highly soluble, they only need to be fed once at high concentrations at the time of inoculation to provide sufficient tyrosine even for lengthy cell culture processes (Kang et al., 2012). Multiple other studies have shown that replacing free amino acids with dipeptides can help supply more nutrients to cell cultures and improve cell growth and protein production (Christie and Butler, 1994; Emmerson and Phang, 1993; Franek et al., 2000, 2003; Franek and Katinger, 2002; Sanchez-Kopper et al., 2016; Verhagen et al., 2020).

While several studies have demonstrated that dipeptides can be used to replace free amino acids to improve cell culture performance, there is still not a clear understanding of the quantitative fate and dispositions of these dipeptides including uptake kinetics, transport, and cleavage in CHO cell cultures. Franek et al. observed a decrease in concentration of oligopeptides and an increase in free amino acids in cell culture supernatants when they supplemented cultures with synthetic oligopeptides (Franek et al., 2003; Franek and Katinger, 2002). Multiple studies reported peptidase activity in spent media and cytosolic cell fractions, and researchers proposed that these peptidases are released into the media as the culture duration progresses, potentially leading to extracellular hydrolysis of dipeptides (Christie and Butler, 1994; Emmerson and Phang, 1993). Other studies observed a fast clearance of dipeptides from culture media and proposed rapid transport of dipeptides into the cells rather than extracellular hydrolysis (Kang et al., 2012; Vazquez et al., 1993). Using LC-Q-TOF and dipeptide standards, Sánchez-Kopper et al. concluded that dipeptides are imported into cells, especially during the initial phases of the culture, and decomposed inside the cells into free amino acids that are either used for biomass and protein synthesis or secreted back in the medium (Sanchez-Kopper et al., 2016).

Given the limited observations present in previous studies, it is evident that the uptake, cleavage, and utilization kinetics of dipeptides have not been sufficiently described or understood. Therefore, in this study, we examined the fate of several dipeptides in CHO cell cultures by employing ^{13}C tracing techniques and kinetic modeling (Gonzalez and Antoniewicz, 2017). Specifically, CHO cells were cultured in media missing a single amino acid supplemented with a dipeptide and ^{13}C labeled tracer of the missing amino acid. Data from ^{13}C labeling experiments were integrated into a kinetic model to estimate dipeptide utilization rates and provide insights into where dipeptide cleavage occurs. We evaluated whether dipeptides are cleaved intracellularly, extracellularly, or both. Further, we determined how several factors including dipeptide concentration, dipeptide composition, orientation of amino acids in the dipeptide, and the number of dipeptides supplemented impact dipeptide utilization rates. Ours is the first study to employ ^{13}C labeling to elucidate the metabolic fate of dipeptides in mammalian cell culture. It will therefore serve as a useful and pioneering tool for others to utilize in the future as dipeptide usage becomes more common in cell culture bioprocessing. Moreover, better characterization of the ways in which dipeptides are processed in cell culture will further provide insights into how proper amounts and addition times of dipeptides can help design media formulations to facilitate the replacement of insoluble and unstable amino acids and ensure sustained availability of critical amino acids over extended cell culture runs. Ensuring nutrient availability and utilization will be critical for maximizing biomass synthesis and recombinant protein production and enabling robust biomanufacturing processes for the generation of valuable therapeutics in mammalian cell culture.

2. Results

2.1. Replacing amino acids with dipeptides does not impact CHO cell culture performance

In order to determine whether dipeptides can replace amino acids in CHO cell culture to support cell growth and protein production, CHO-GS cells were cultured in batch mode using media missing a single amino acid, supplemented with either the missing amino acid or a dipeptide containing the missing amino acid. For all dipeptides tested in this study, the growth behavior and protein production were not affected by this switch. As an example, Fig. 1 shows the growth data, glucose and lactate profiles, and titers for experiments performed with leucine-free media supplemented with either 4 mM leucine, 4 mM Ala-Leu, or 8 mM Ala-Leu (Fig. 1A and B); and tyrosine-free media supplemented with either 1.1 mM tyrosine, 1.1 mM Gly-Tyr, or 2.2 mM Gly-Tyr (Fig. 1C and D). No significant differences were observed in culture performance. These results demonstrate that dipeptides such as Ala-Leu and Gly-Tyr can successfully replace the essential amino acids leucine and tyrosine in cell culture media. Similar results were obtained for a CHO–K1 cell line; as an example, Supplemental Fig. S1 shows data for experiments where leucine-free medium was supplemented with either 4 mM leucine, 4 mM

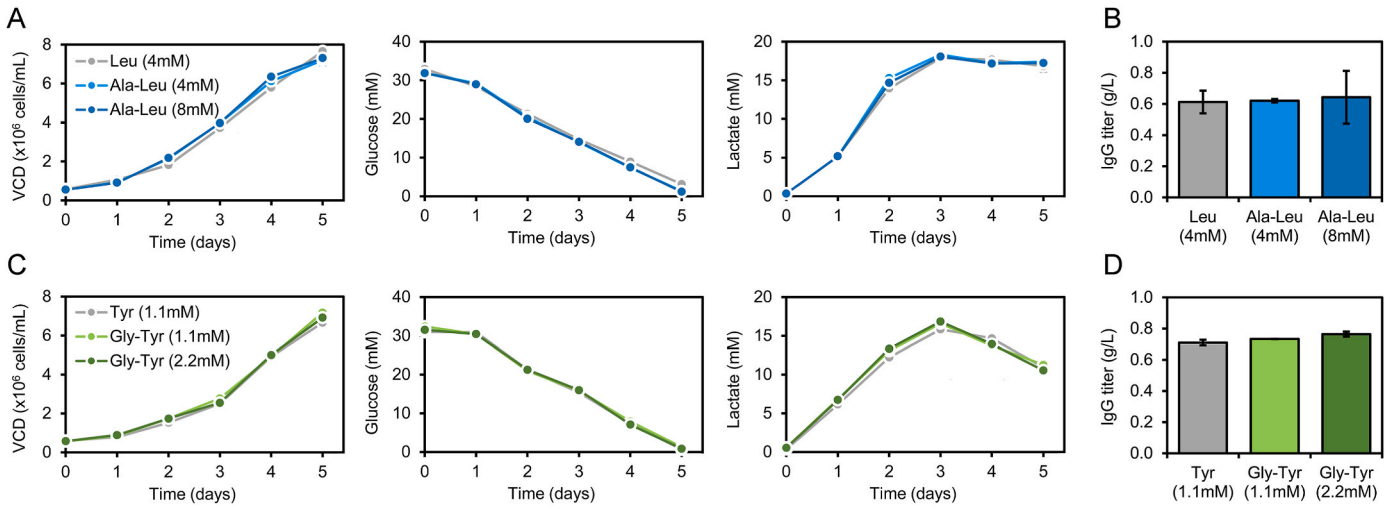

Fig. 1. Replacing leucine and tyrosine with dipeptides in cell culture media does not impact CHO cell culture performance. (**A**) CHO-GS cells were cultured in leucine-free medium supplemented with either 4 mM leucine, 4 mM Ala-Leu, or 8 mM Ala-Leu. Viable cell density (VCD), glucose and lactate profiles were not impacted. (**B**) IgG final titer was also not impacted. (**C**) CHO-GS cells were cultured in tyrosine-free medium supplemented with either 1.1 mM tyrosine, 1.1 mM Gly-Tyr, or 2.2 mM Gly-Tyr. VCD, glucose and lactate profiles were not impacted. (**D**) IgG final titer was also not impacted. Error bars represent standard deviations (n = 2, biological replicates).

Ala-Leu, or both 4 mM leucine and 4 mM Ala-Leu. Again, no differences in cell culture performance were observed.

2.2. ^{13}C labeling experiments confirm intracellular cleavage of dipeptides

To determine if dipeptides are cleaved inside CHO cells, ^{13}C isotope tracing experiments were employed. Specifically, CHO–K1 cells were first pre-cultured for several days in leucine-free medium supplemented with 4 mM fully labeled leucine, i.e. [U–^{13}C]leucine, to ensure that all leucine in the CHO cells was fully ^{13}C labeled, and then sub-cultured into leucine-free medium containing 4 mM [U–^{13}C]leucine and 4 mM Ala-Leu. For the next 6 days, supernatant samples and CHO cell pellets

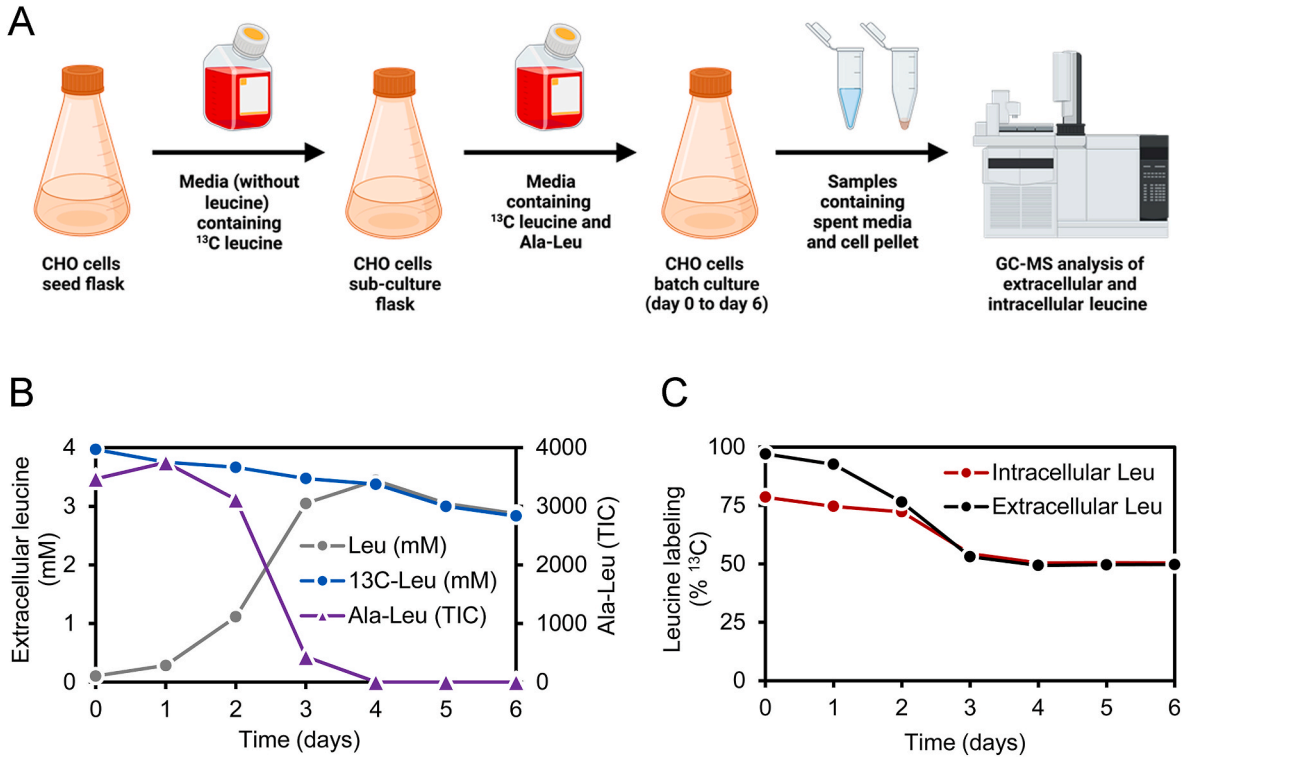

Fig. 2. ^{13}C labeling experiments confirm dipeptides cleavage inside CHO cells. (**A**) Schematic of experiment design and analysis. CHO–K1 cells were pre-cultured in leucine-free medium supplemented with 4 mM [U–^{13}C]leucine and then sub-cultured into leucine-free medium supplemented with 4 mM [U–^{13}C]leucine and 4 mM Ala-Leu. Created with BioRender.com. (**B**) Extracellular concentration profiles of unlabeled leucine and fully labeled leucine were measured. For Ala-Leu, the total ion counts (TIC) from GC-MS analysis was used as a surrogate for dipeptide concentration. (**C**) ^{13}C labeling of intracellular and extracellular leucine was also measured by GC-MS. During the first two days, intracellular leucine labeling was lower than extracellular leucine labeling, indicating intracellular dipeptide cleavage. The first sample was collected 10 min after sub-culturing the CHO cells from a medium containing [U–^{13}C]leucine into a medium containing [U–^{13}C] leucine and unlabeled Ala-Leu.

were collected daily for GC-MS analysis (Fig. 2A). For the supernatant samples, concentrations of unlabeled and labeled leucine were measured (Fig. 2B). For Ala-Leu, the total ion counts (TIC) from GC-MS analysis was used as a surrogate for dipeptide concentration (Fig. 2B). For the cell pellets, after washing them with a saline solution, labeling of intracellular leucine was quantified and compared to extracellular leucine labeling (Fig. 2C).

First, we verified that CHO cells became fully ^{13}C-labeled during the pre-culture. At the end of the pre-culture, free intracellular leucine labeling was measured to be more than 97%, and leucine in biomass proteins was more than 90% labeled. During the first two days of the culture with the dipeptide, intracellular leucine labeling was significantly lower compared to extracellular leucine labeling (Fig. 2C). This was true even for the first sample, which was collected only 10 min after sub-culturing the CHO cells from a medium containing [U–^{13}C]leucine into a medium containing [U–^{13}C]leucine and unlabeled Ala-Leu. For this first sample, intracellular leucine labeling was 78% compared to 98% for extracellular leucine. Since the only source of unlabeled leucine in this experiment was the dipeptide Ala-Leu, it is clear that the dipeptide must have been cleaved inside the CHO cells initially. As the culture progressed, however, the labeling of both intracellular leucine and extracellular leucine decreased, reaching a constant value of about 50% for both after day 3 (Fig. 2C), when the dipeptide was fully exhausted (Fig. 2B). The drop in leucine labeling was primarily due to the accumulation of unlabeled leucine in the medium, as shown in Fig. 2B. The fact that unlabeled leucine accumulated in the medium indicates that the dipeptide Ala-Leu was cleaved by the CHO cells at a rate that was significantly higher than net utilization of intracellular leucine for growth and protein production. In other words, excess cleavage of the dipeptide resulted in net production of unlabeled leucine. After day 3,

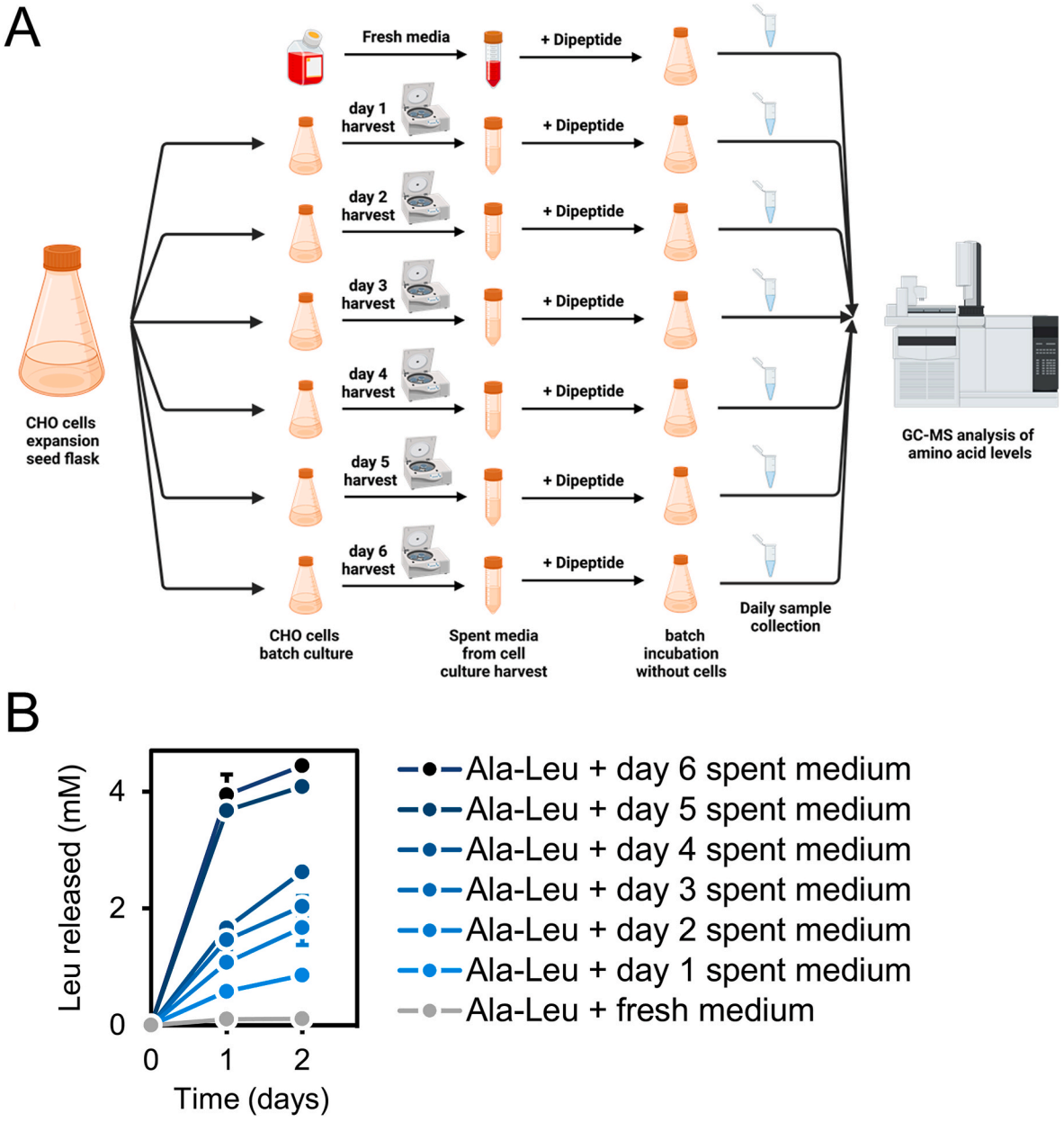

Fig. 3. Extracellular cleavage of dipeptides increases with age of cell culture. (**A**) Schematic of experiment design and analysis. CHO-GS cells were grown in batch culture for 6 days. Cell-free spent media were collected daily and supplemented with 4 mM Ala-Leu. As a control, Ala-Leu was also added to fresh medium. Created with BioRender.com. (**B**) Unlabeled leucine was released into spent media, indicating extracellular dipeptide cleavage. Dipeptide cleavage rate increased with cell culture age, while no dipeptide cleavage was observed in fresh medium. Error bars represent standard deviations (n = 2, biological replicates).

when the dipeptide was fully consumed, unlabeled and labeled leucine in the medium reached a similar level and were then consumed at the same rate by the CHO cells (Fig. 2B), indicating that there was no kinetic isotope effect. Similar results were obtained for tracer experiments performed with the CHO-GS cell line (Supplemental Fig. S2). Taken together, these results demonstrate that dipeptides are cleaved intracellularly by CHO cells in cell culture.

2.3. Extracellular cleavage of dipeptides increases with age of cell culture

Next, we evaluated if in addition to intracellular cleavage, extracellular cleavage of dipeptides may be taking place in CHO cell cultures. To evaluate this, CHO-GS cells were grown in batch culture for 6 days. Spent media were collected daily. After removing CHO cells from the collected spent media, 4 mM of the dipeptide Ala-Leu was added and the flasks were placed back in the incubator. Samples were collected daily for GC-MS analysis to monitor changes in leucine concentration (Fig. 3A). As a control, the dipeptide was also added to fresh medium and incubated under the same conditions. When the dipeptide was incubated with fresh medium, no cleavage of Ala-Leu was observed. This was true for all dipeptides tested in this study. However, when Ala-Leu was added to spent media, unlabeled leucine was released during the cell-free incubation. Interestingly, the rate of leucine release increased with the age of the cell culture (Fig. 3B). As an example, when Ala-Leu was incubated with spent medium collected on day 3 of the culture, it took 2 days for 2 mM of leucine to be released. However, when Ala-Leu was incubated with spent medium collected on day 5, about 4 mM of leucine was released within the first day, suggesting that nearly all of the dipeptide had been cleaved by the first sampling point. Similar results were obtained for experiments performed using spent media collected from CHO–K1 cell cultures (Supplemental Fig. S3). Taken together, these results suggest that in addition to intracellular dipeptide cleavage, extracellular dipeptide cleavage is taking place in CHO cell cultures, especially during later stages of CHO cell cultures.

2.4. ^{13}C labeling experiments were integrated into a kinetic model to estimate dipeptide utilization rates

In order to quantitatively characterize dipeptide uptake and metabolic kinetics, we next constructed a kinetic model to estimate metabolic fluxes by fitting data from ^{13}C labeling experiments. Since ^{13}C labeled dipeptides are not easily available, we used ^{13}C labeled amino acids as tracers to assist in estimating dipeptide utilization rates. In cell culture experiments, CHO cells were grown in media missing a single amino acid supplemented with ^{13}C labeled tracer of the missing amino acid and a dipeptide containing the missing amino acid. For example, to quantify the utilization rate of Ala-Leu, leucine-free medium was supplemented with [U–^{13}C]leucine and Ala-Leu. For each experiment, cell growth, amino acid concentrations (both labeled and unlabeled), and ^{13}C labeling of intracellular amino acids were measured by GC-MS, similar to the data shown in Fig. 2. The data was then fit to a kinetic mathematical model consisting of four ordinary differential equations (ODEs) to estimate fluxes in the model. The mathematical details of the model are described in the Methods and Materials section. Fig. 4 shows a schematic representation of our model.

In all experiments performed in this study, we observed a rapid increase in the levels of the missing amino acid in the medium during the early stages of culture, typically during the first 2–3 days of the culture, indicating relatively fast cleavage of dipeptides. This was followed by a second phase, i.e., after the dipeptide was fully exhausted, where CHO

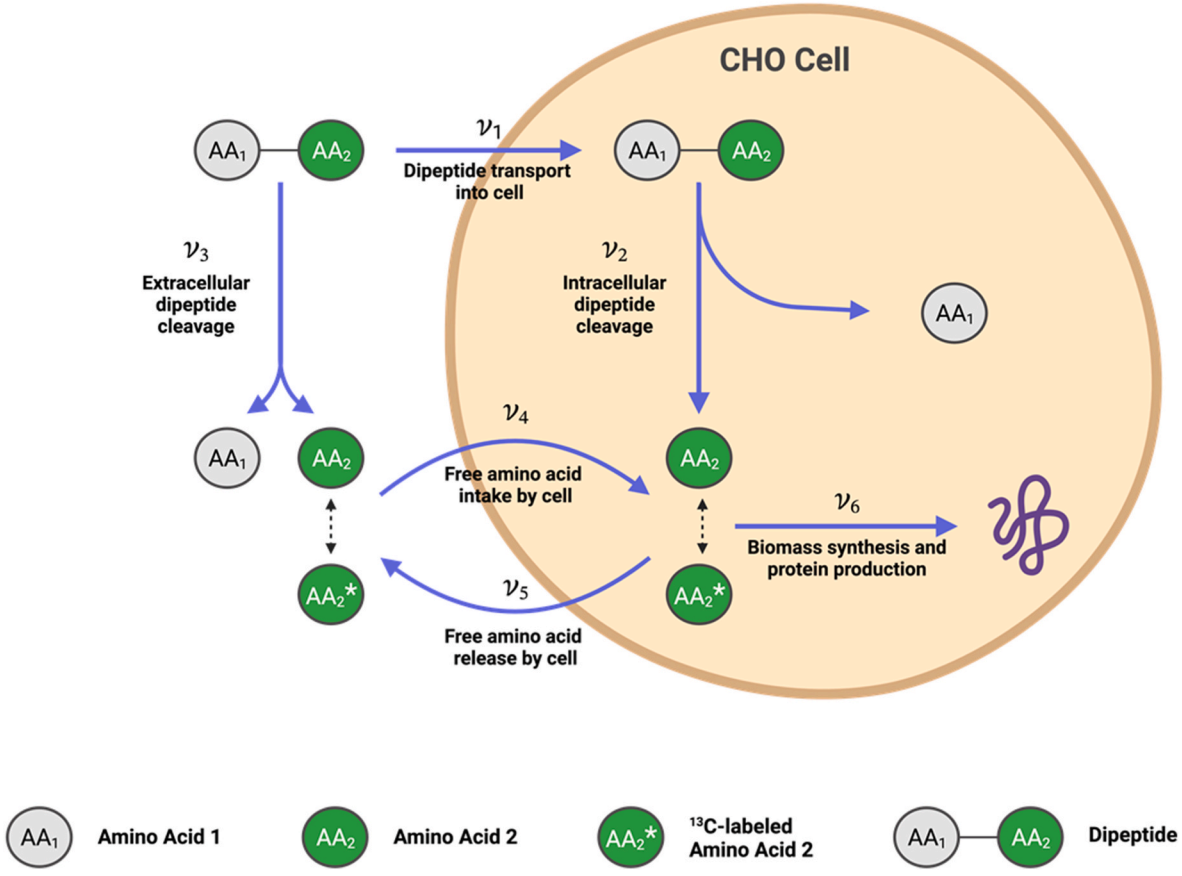

Fig. 4. Schematic of dipeptide metabolism in CHO cell cultures. ν1: transport of dipeptide into CHO cells; ν2: cleavage of dipeptide inside CHO cells; ν3: extracellular cleavage of dipeptide; ν4: import of free amino acid into CHO cells; ν5: export of free amino acid into medium; ν6: utilization of amino acid for biomass synthesis and protein production. In ^{13}C labeling experiments, fully labeled amino acid was added to the cell culture medium. Created with BioRender.com.

cells utilized both the labeled and unlabeled amino acids from the medium at rates proportional to their respective concentrations. To describe these observations mathematically, our dynamic model has two distinct phases: 1) the dipeptide utilization phase, where dipeptides are cleaved into their respective amino acids and any excess amino acids released accumulate in the medium; and 2) the post-dipeptide utilization phase, where amino acids from the medium are consumed to support cell growth and protein production. Data fitting was performed in MATLAB using least-squares regression tools. To our knowledge, ours is the first study that used ^{13}C tracing techniques incorporated into a mathematical framework to provide an in-depth characterization of dipeptide utilization kinetics in CHO cell cultures.

2.5. Orientation and type of amino acids in dipeptides impacts dipeptide utilization rates

By applying the mathematical model and ^{13}C labeling techniques described above, dipeptide utilization rates were determined for a range of commercially available dipeptides. First, we evaluated how the order in which amino acids are conjugated in the dipeptides, as well as the type of amino acids used to form the dipeptide, affected dipeptide utilization rates. Specifically, we evaluated six alanine-containing dipeptides, including three in which alanine occupied the N-terminus of the dipeptide (Ala-Leu, Ala-Tyr, and Ala-Phe), and three in which alanine occupied the C-terminus of the dipeptide (Leu-Ala, Tyr-Ala, and Phe-Ala). Dipeptide utilization rates were determined for two CHO cell lines, a CHO-GS cell line and a CHO–K1 cell line (Fig. 5).

We observed that when alanine was present on the N-terminus, dipeptide utilization rates were significantly higher (by 2- to 5-fold) compared to when alanine was present on the C-terminus (Fig. 5A and B). For example, for the CHO-GS cell line, Ala-Tyr utilization rate was 5-fold higher (38 nmol/10^6 cells/hr) than Tyr-Ala utilization rate (7 nmol/10^6 cells/hr); a similar 5-fold difference was observed for Ala-Phe vs. Phe-Ala (220 vs. 40 nmol/10^6 cells/hr), while a 2-fold difference was observed for Ala-Leu vs. Leu-Ala (187 vs. 100 nmol/10^6 cells/hr, Fig. 5A). No differences in growth rates were observed for any of these dipeptides, indicating that while some dipeptides were consumed more slowly than others, the metabolism of dipeptides was never rate limiting. Similar results were obtained for CHO–K1 cells (Fig. 5B). We again observed that when alanine was present on the N-terminus, the dipeptide utilization rates were significantly higher compared to when alanine was present on the C-terminus. Interestingly, there were some notable differences in the utilization rates between the two CHO cell lines. For example, leucine-containing dipeptides (Ala-Leu and Leu-Ala) were utilized about 3-fold faster by CHO-GS cells compared to CHO–K1 cells, while tyrosine-containing dipeptides (Ala-Tyr and Tyr-Ala) were utilized about 2-fold faster by CHO–K1 cells compared to CHO-GS cells (Fig. 5A and B).

We also compared alanine-containing dipeptides with glycine-containing dipeptides, including those with alanine or glycine on N- and C-terminus. Due to limited availability of dipeptides, some experiments were only performed using one of the CHO cell lines. Again, we did not observe any differences in growth behavior between alanine-containing and glycine-containing dipeptides. Overall, we found that alanine-containing dipeptides had a higher utilization rate than glycine containing dipeptides. As an example, the utilization rate of Ala-Tyr was 5-fold higher than the utilization rate of Gly-Tyr (38 vs. 7 nmol/10^6 cells/hr), while a relatively smaller difference was observed for Leu-Ala compared to Leu-Gly (100 vs. 76 nmol/10^6 cells/hr, Fig. 5C).

We also tested if extracellular dipeptide cleavage rates were similarly affected by the composition of the dipeptide and orientation of the amino acids in the dipeptide. For this, we quantified dipeptide cleavage rates in cell-free spent media supplemented with one of the following dipeptides: Ala-Leu, Leu-Ala, Ala-Tyr, Tyr-Ala or Gly-Tyr. We indeed found that dipeptides with alanine at the N-terminus had a higher utilization rate than dipeptides with alanine at the C-terminus and dipeptides with glycine instead of alanine at N-terminus, as we observed in cell culture experiments (Supplemental Fig. S4). Taken together, these experiments demonstrate that both the type of amino acids present in dipeptides as well as the orientation of the amino acids can impact dipeptide utilization rates in cell culture and cell-free experiments.

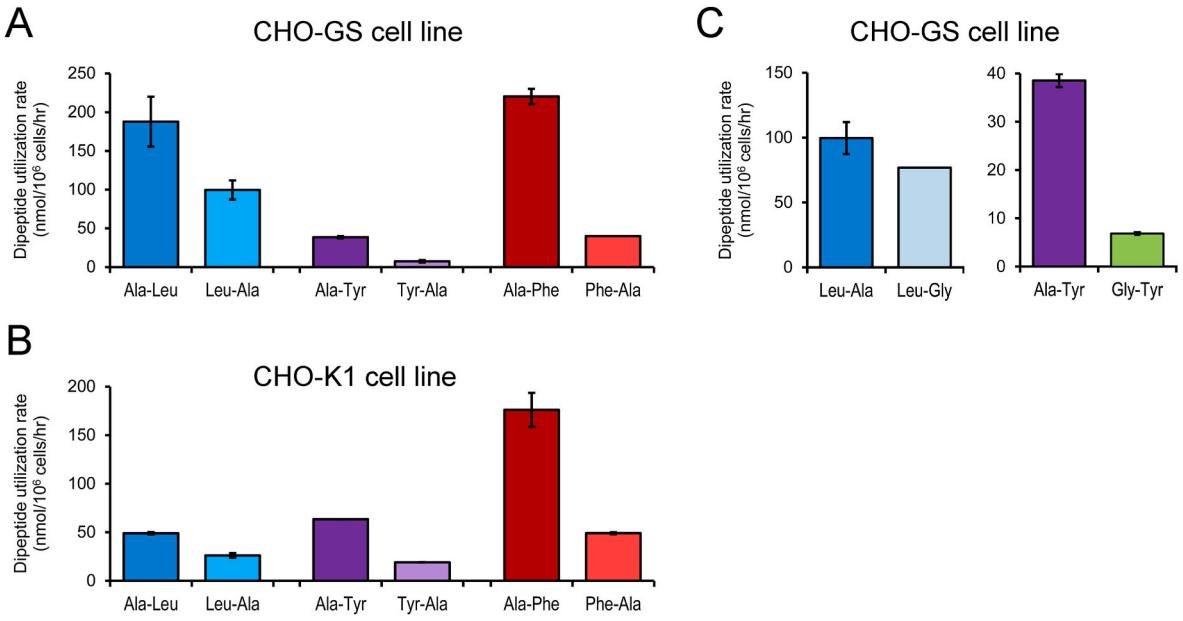

Fig. 5. Orientation and type of amino acids in dipeptides impacts dipeptide utilization rates. Dipeptides with alanine at N-terminus (4 mM Ala-Leu, 1.1 mM Ala-Tyr, 8 mM Ala-Phe) are consumed faster than dipeptides with alanine at C-terminus (4 mM Leu-Ala, 1.1 mM Tyr-Ala, 8 mM Phe-Ala) by CHO-GS cells (**A**), and by CHO–K1 cells (**B**) in cell culture. Alanine-containing dipeptides (1.1 mM Ala-Tyr, 4 mM Leu-Ala) are consumed faster than glycine-containing dipeptides (1.1 mM Gly-Tyr, 4 mM Leu-Gly) by CHO-GS cells in cell culture (**C**). In plots **(A)** and **(C)**, error bars represent standard deviation from biological replicates ($n = 2$). In plot **(B)**, error bars represent standard deviation from technical replicates.

2.6. Dipeptide cleavage rates depend on dipeptide concentration and follow Michaelis-Menten kinetics

Next, we evaluated whether dipeptide cleavage rates were affected by dipeptide concentration. First, to study the effect of dipeptide concentration on intracellular cleavage rates, CHO cells were cultured for 3 days in leucine-free medium supplemented with Ala-Leu at different concentrations (1, 2, 3, 4, and 6 mM). No notable differences were observed in cell growth (Fig. 6A). However, we did observe that higher concentrations of Ala-Leu (up to 4 mM) resulted in increased rates of unlabeled leucine release from dipeptide cleavage (Fig. 6A). We determined that dipeptide utilization rates followed saturation kinetics (i.e. Michaelis-Menten kinetics), with an approximate first order concentration-dependence at lower dipeptide concentrations and a zero order, or near concentration-independent behavior, at higher concentrations. Michaelis-Menten kinetics were also observed for another dipeptide that was tested, Gly-Tyr (Supplemental Fig. S5A).

To determine if extracellular cleavage of dipeptides also followed Michaelis-Menten kinetics, CHO cells were grown for 5 days in batch culture and spent medium was collected. After removing CHO cells, Ala-Leu was added to aliquots of spent medium at different concentrations (1, 2, 4, 6, and 8 mM), and the increase in leucine concentration was monitored for 2 days by GC-MS (Fig. 6B). Higher Ala-Leu concentrations indeed resulted in faster release of leucine from dipeptide cleavage. The calculated dipeptide cleavage rates were again described well by classical Michaelis-Menten kinetics (Fig. 6B). Michaelis-Menten kinetics were also observed for cleavage of Gly-Tyr dipeptide in cell-free experiments (Supplemental Fig. S5B). Taken together, our results indicate that both intracellular and extracellular dipeptide cleavage rates depend on dipeptide concentration and can be described accurately by the classical Michaelis-Menten model with K_m values typically in low millimolar range (~2–8 mM).

2.7. Dipeptide cleavage rates are reduced when multiple dipeptides are present

Finally, we evaluated if the presence of multiple dipeptides in cell culture can affect dipeptide utilization rates. For this study, CHO cell culture experiments were performed using leucine-free media supplemented with [U–^{13}C]leucine and either Ala-Leu alone, or Ala-Leu together with another dipeptide (Ala-Phe, Phe-Ala, or Ala-Tyr). The utilization rates of Ala-Leu were then determined using the ^{13}C tracer methods and kinetic model described above. In all cases, no differences in growth rates were observed. However, the presence of the second dipeptide in cell culture reduced the utilization rate of Ala-Leu by about 40–50%. Lower dipeptide utilization rates were observed for both CHO cell lines used in this study (Fig. 7A and B). We also tested if extracellular dipeptide cleavage rates are similarly affected by the presence of additional dipeptides. For this, we quantified dipeptide cleavage rates in cell-free spent media supplemented either with Ala-Leu alone, or Ala-Leu together with another dipeptide (Ala-Phe, Phe-Ala, or Ala-Tyr). We indeed found similar reductions in the cleavage rates of Ala-Leu in cell-free experiments (Fig. 7C and D) as we observed in cell culture experiments (Fig. 7A and B). Taken together, these data suggest that the dipeptide substrates are likely competing with each other for the same cleavage enzymes. In theory, this could pose challenges when supplementing multiple dipeptides in culture. However, while supplementation of multiple dipeptides can lead to competition between dipeptides for their utilization, our data suggest that the net utilization rates of dipeptides, despite getting reduced due to multiple dipeptide supplementation, are still significantly higher than what is required by cells for cell growth and protein production.

2.8. High-throughput analysis of dipeptide kinetics and interactions in 96-well plates

The advantages of performing cell-free experiments described above are that they do not require special media or expensive tracers and can be performed in small scale. This is also desirable, for example, when

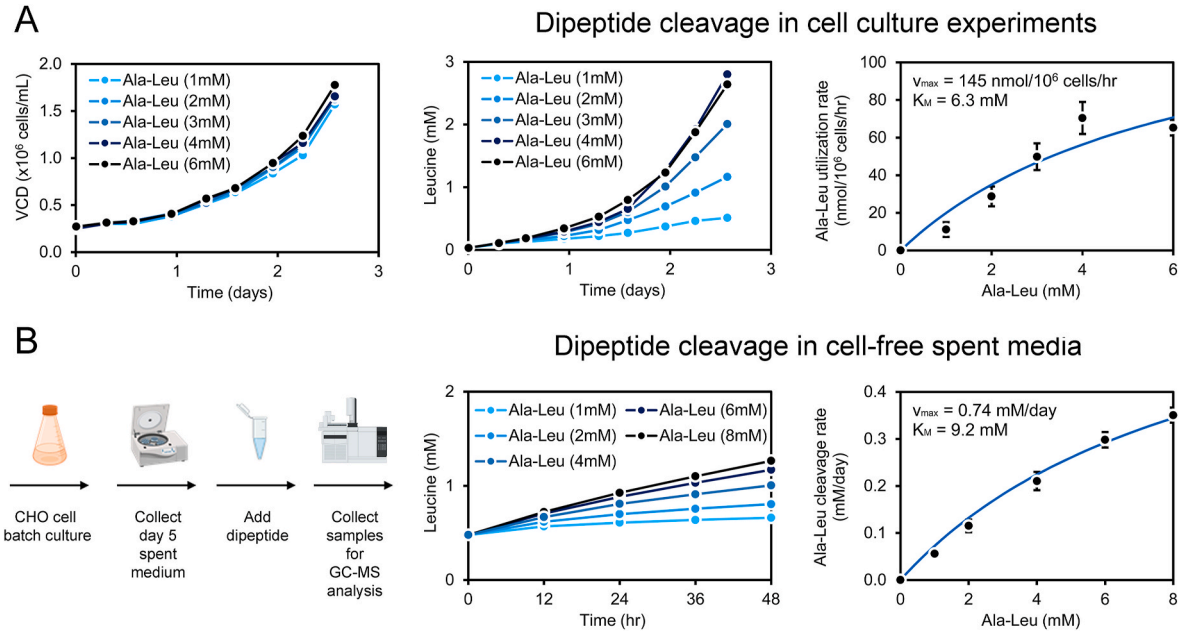

Fig. 6. Dipeptide cleavage rates depend on dipeptide concentration and follow Michaelis-Menten kinetics. (**A**) CHO–K1 cells were grown in batch culture in leucine-free medium supplemented with Ala-Leu at different concentrations, 1, 2, 3, 4, and 6 mM. Dipeptide utilization rates increased with increasing dipeptide concentration following Michaelis-Menten kinetics. (**B**) Dipeptide cleavage was monitored in cell-free spent media collected from day 5 of CHO–K1 cell culture. Dipeptide cleavage rates increased with increasing dipeptide concentration (Ala-Leu at 1, 2, 4, 6, 8 mM) according to Michaelis-Menten kinetics. Error bars for the estimated dipeptide utilization rates represent standard deviations calculated using Monte Carlo simulations. Created with BioRender.com.

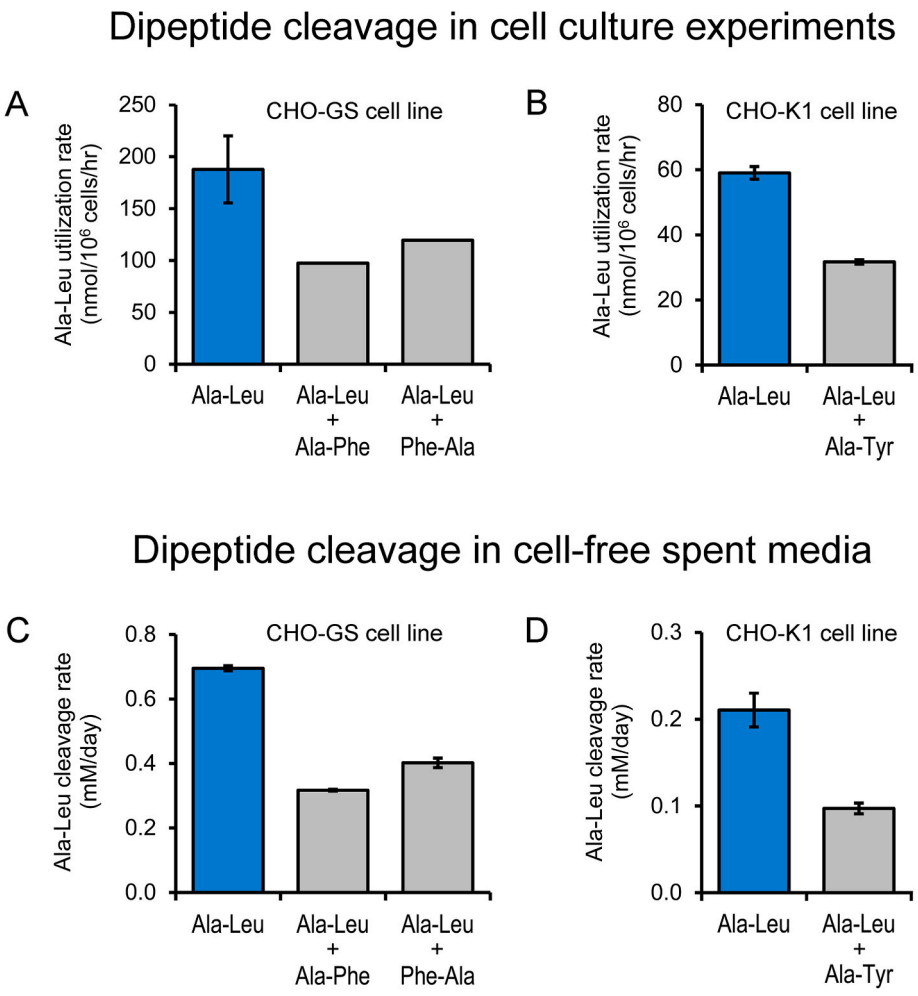

Fig. 7. Dipeptide cleavage rates are reduced when multiple dipeptides are present. (**A, B**) Utilization rates of Ala-Leu were quantified is CHO cell culture experiments with and without other dipeptides. For the CHO-GS cell line, the following conditions were evaluated: 4 mM Ala-Leu, 4 mM Ala-Leu +4 mM Ala-Phe, 4 mM Ala-Leu +4 mM Phe-Ala (**A**). For the CHO–K1 cell line, the following conditions were evaluated: 4 mM Ala-Leu, 4 mM Ala-Leu +4 mM Ala-Tyr (**B**). Cleavage rates of Ala-Leu were also determined in cell-free spent media from CHO cell cultures for the same dipeptide conditions (**C, D**). Error bars represent standard deviations ($n = 2$, biological replicates).

dipeptide cost or availability is a concern. To demonstrate this, we performed a series of cell-free experiments using 96-well plates and two additional peptides: Ala-Gln and Tyr-Tyr-Tyr. For the dipeptides Ala-Gln and Ala-Tyr, cleavage rates were determined at 1, 2, 4, 6, and 8 mM. For the tripeptide Tyr-Tyr-Tyr, cleavage rates were determined at 1, 2, 3, and 4 mM. Additionally, we determined to what extent Ala-Gln, Ala-Tyr, Tyr-Tyr-Tyr and Ala-Leu cleavage rates were affected when the different peptides were incubated simultaneously at 4 mM concentration. The results from these experiments are shown in Fig. 8.

The cleavage rates of all three peptides, i.e. Ala-Gln, Ala-Tyr and Tyr-Tyr-Tyr, followed Michaelis-Menten kinetics with K_m values in low millimolar range (1–9 mM, Fig. 8A–C). When Ala-Leu and Ala-Gln were incubated together, the cleavage rates of both dipeptides were same as their respective individual cleavage rates (Fig. 8D and G), suggesting that Ala-Leu and Ala-Gln were cleaved by different enzymes. In contrast, Ala-Tyr and Tyr-Tyr-Tyr significantly reduced the cleavage rate of Ala-Leu (Fig. 8H and I); however, Ala-Leu did not significantly reduce the cleavage rates of Ala-Tyr and Tyr-Tyr-Tyr (Fig. 8E and F). These data suggest that the peptides were likely cleaved by the same enzyme or enzymes, and that the enzymes had higher affinity towards Ala-Tyr and Tyr-Tyr-Tyr than Ala-Leu, consistent with the Michaelis-Menten kinetics results shown in Fig. 8B, C and 6B.

Taken together, these experiments demonstrate that small-scale cell-free experiments can provide valuable information about dipeptide cleavage kinetics and interactions that will be critical for media optimization and process control.

3. Discussion

In this study, we performed detailed characterization of dipeptide utilization rates and cleavage kinetics in CHO cell cultures and cell-free experiments to gain more fundamental insights into the factors that affect dipeptide metabolism. All of these studies were aided by novel ^{13}C isotope tracing techniques and models that were developed for this specific purpose. Overall, we show that multiple factors including dipeptide composition, orientation of amino acids in dipeptides, dipeptide concentrations, and the presence of multiple dipeptides all have a significant impact on dipeptide kinetics. Furthermore, we observe that dipeptides are cleaved intracellularly as well as extracellularly. Interestingly, we found that all dipeptides used in this study could successfully replace the respective amino acids in cell culture media without affecting growth characteristics and productivity of CHO cells. This suggests that in all cases the rates of dipeptide metabolism were high enough such as to not become rate limiting.

The cell culture experiments performed in this study required special cell culture media (i.e. media missing specific amino acids) and expensive ^{13}C tracers. Fortunately, we found that cell-free experiments, which do not require special media or expensive tracers and can be performed

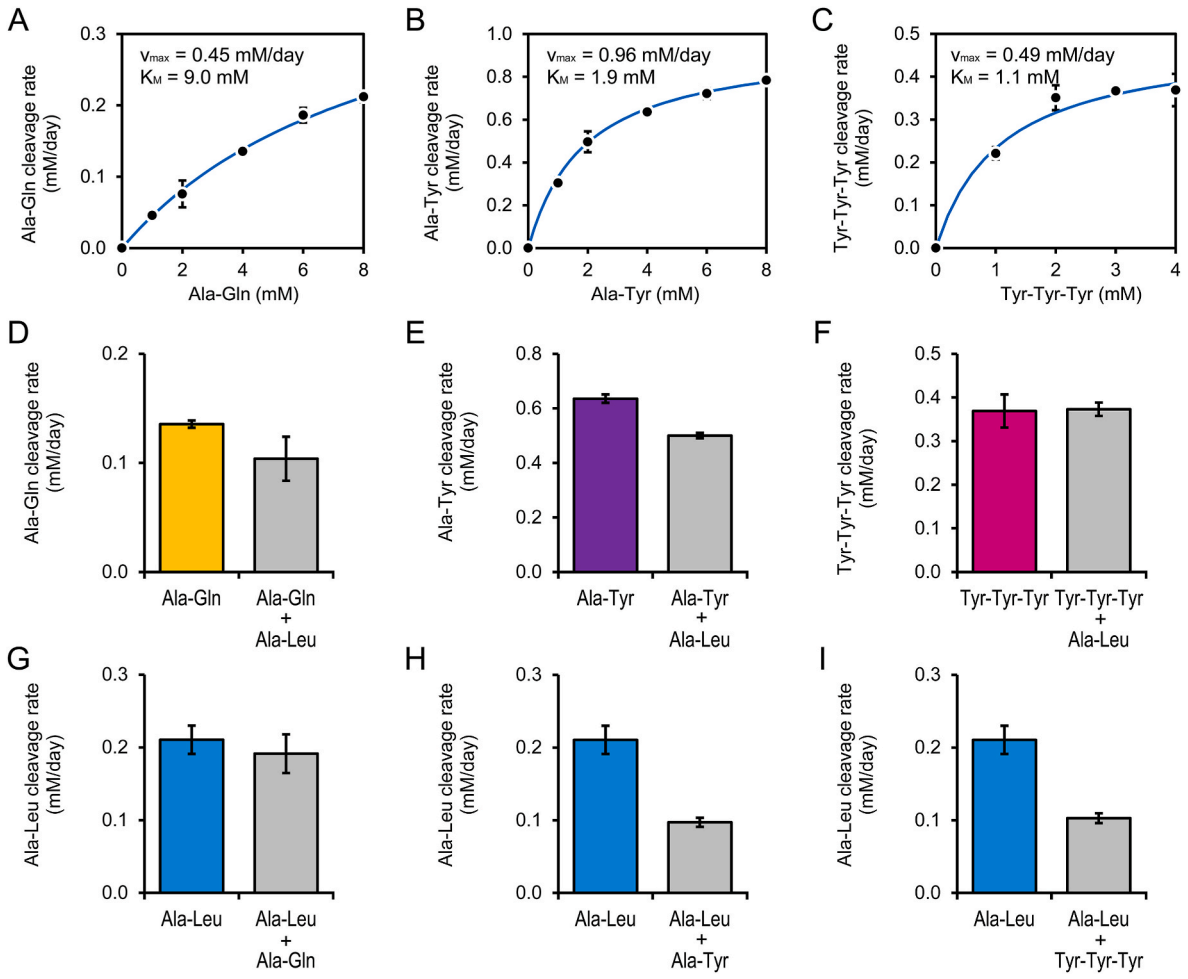

Fig. 8. High-throughput analysis of dipeptide kinetics and interactions. Cleavage rates of peptides were quantified in cell-free experiments in 96-well plates. Here, spent medium from a CHO–K1 batch culture collected on day 5 was used. (**A, B, C**) Individual peptides at different concentrations were added to the spent medium to determine Michaelis-Menten kinetic parameters. (**D, E, F, G, H, I**) Cleavage rates of peptides were compared when incubated individually at 4 mM, and together with another peptide (both at 4 mM). Error bars represent standard deviations ($n = 2$, biological replicates).

in small scale (e.g. 96-well plates), produced consistent results. Moreover, cell-free studies enabled a more direct estimation of Michaelis-Menten kinetics and permitted testing for a larger number of input parameters than was possible in cell culture experiments. We anticipate that these types of cell-free experiments could become a valuable tool in the future to systematically evaluate dipeptides and other cell culture media components for process optimization.

We did not attempt to identify specific dipeptide transporters or peptidases responsible for metabolism of dipeptides, as this was beyond the scope of this project. However, recent CHO genome sequencing and annotation studies did identify two potential dipeptide importers, PepT1 (SLC15A1) and PepT2 (SLC15A2), in a CHO–K1 cell line (Hammond et al., 2012; Hefzi et al., 2016). PepT1 and PepT2 were found to differ in selectivity and uptake kinetics with PepT1 reported to be a low-affinity and high-capacity transporter for peptides while PepT2 is a high-affinity and low-capacity transporter (Rubio-Aliaga and Daniel, 2008). Covitz et al. observed higher dipeptide uptake rates when PepT1 was overexpressed in CHO cells compared to CHO cells with native PepT1 (Covitz et al., 1996). Ito et al. demonstrated that a bulky hydrophobic side chain increases affinity towards PepT1 (Ito et al., 2013). We observed a faster utilization rate for Ala-AA than for AA-Ala dipeptides. Dipeptides with alanine on the N-terminus (Ala-AA) have a bulkier, more hydrophobic C-terminus when compared to dipeptides with alanine on the C-terminus (AA-Ala) that have a lighter, less hydrophobic C-terminus. This suggests that a more hydrophobic C-terminus may facilitate faster dipeptide utilization rate. In cases where dipeptides have the same structural groups at the C-terminus, it is possible that the hydrophobicity of the N-terminus may be responsible for the faster uptake of a dipeptide. This could explain why we observed a faster uptake rate for Ala-AA compared to Gly-AA.

Previous studies from our group and others have reported the release of enzymes and host cell proteins into the medium, which may be responsible for the extracellular cleavage of peptides. We previously identified multiple dipeptidyl peptidases (DPP), including DPP3 and DPP9 and the cytosolic non-specific dipeptidase (CNDP2), in CHO supernatants (superome; CHO–SO) even with a 96% cell culture viability (Kumar et al., 2015). Two other CHO secretomics studies, Slade et al. and Kol et al., also reported the presence of dipeptidyl peptidase 2 (DPP2) in spent media samples taken when cell culture viabilities were greater than 98% and during late exponential phase respectively (Kol et al., 2020; Slade et al., 2012). Consistent with these findings Emmerson et al. and Christie et al. reported peptidase activity both in spent media and cytosolic cell fractions in CHO cells and murine hybridoma cells (Christie and Butler, 1994; Emmerson and Phang, 1993). Consequently, dipeptides were likely subjected to some extracellular cleavage by these or other specific and non-specific dipeptidase enzymes released by cells. While previous studies (Christie and Butler, 1994; Emmerson and Phang, 1993; Franek et al., 2003; Franek and Katinger, 2002; Kang et al., 2012; Sanchez-Kopper et al., 2016; Vazquez et al., 1993) indicate either intracellular cleavage or extracellular cleavage of dipeptides, our

results indicate that dipeptides are simultaneously cleaved both intracellularly and extracellularly by CHO cells.

In some cases, especially during the later stages of cell culture when host cell proteins accumulate in the culture at high levels, dipeptides may be extracellularly cleaved into free amino acids rapidly. This may lead to accumulation of amino acids in cell culture media beyond their solubility levels and eventually cause precipitation, especially if the dipeptides were supplemented at high concentrations. However, the understanding of dipeptide utilization rates and behaviors gained from this study could guide the controlled release of amino acids into the media, thereby making precipitation a low-risk phenomena and a rare occurrence, especially for intensified fed-batch processes where individual amino acid solubility poses a big challenge in feed media design. Specifically, the dipeptides could be supplemented at lower concentrations in the basal medium to meet the amino acid requirements of the cells and then at high concentrations in feed media. Amino acids are consumed at fairly high rates in CHO cell fed-batch cultures which support high viable cell densities. Therefore, the addition of dipeptides at high VCDs would lead to their faster consumption without causing precipitation.

Another alternative to limiting the precipitation of free amino acids is to mitigate the extracellular cleavage of dipeptides by host cell proteins by reducing the accumulation of host cell protein in CHO cell cultures. Indeed, previous studies have employed CRISPR-Cas9 and TALEN technologies to eliminate host cell proteins from CHO cell cultures (Chiu et al., 2017; Kol et al., 2020) as well as the supplementation of protease inhibitor cocktail to inhibit the degradation activity of host cell proteins in cell culture (Chakrabarti et al., 2016).

4. Conclusions

Taken together, in this study we have rigorously elucidated dipeptide uptake and cleavage kinetics in CHO cell cultures by combining data generated from ^{13}C labeling experiments with a kinetic model of dipeptide cleavage, transport, and utilization. We believe this represents the first ever study to employ ^{13}C isotopic labeling techniques to determine dipeptide utilization rates. With this approach, we demonstrated that different dipeptides can support cell growth and protein production in the absence of free amino acids. Our results indicate the co-existence of intracellular and extracellular dipeptide cleavage in CHO cell cultures. Furthermore, dipeptide utilization rate depends on several factors, including order and type of amino acids conjugated to form the dipeptides, concentration at which dipeptides are supplemented in cell culture, and presence of multiple dipeptides simultaneously. We observed that alanine-containing dipeptides are consumed faster than glycine-containing dipeptides and dipeptides with alanine at N-terminus are consumed faster than dipeptides with alanine at C-terminus. When multiple dipeptides are fed at the same time, they potentially compete for the same cleavage enzymes or transporters or both, thereby, reducing the consumption rates of each other. By testing dipeptides at different concentrations in CHO cell cultures, our results indicate that dipeptides follow classical Michaelis-Menten kinetics. Furthermore, cell-free studies suggest that all of these behaviors are relevant to the cleavage dynamics in extracellular environments. Indeed, these cell-free studies, which are easily performed at small scales, could be used as a prototype to gain an initial understanding of dipeptide metabolism before testing them in large-scale CHO cell cultures. Understanding dipeptide metabolism in CHO cell cultures could better facilitate the selection of appropriate dipeptides. Estimating the utilization rates of dipeptides and using them in combination could guide media and feed design for controlled release of amino acids into the culture. As bioprocesses have transitioned from traditional fed-batch to intensified fed-batch processes and continuous modes of manufacturing, a higher demand for critical amino acids will be required. Indeed, dipeptides can serve as alternatives to sparingly soluble and unstable amino acids in cell culture basal media and feed in future media designs. Gaining a deeper understanding of dipeptide metabolism could help deliver these nutrients efficiently and improve bioprocess performance.

5. Materials and methods

5.1. Materials

Dipeptides Ala-Leu (Cat. No. A1878), Leu-Ala (Cat.No. L9250), Leu-Gly (Cat. No. L9625), Ala-Tyr (Cat. No. A4003), Tyr-Ala (Cat. No. T5129), Ala-Phe (Cat. No. A3128), Phe-Ala (Cat. No. P3251), Ala-Gln (Cat. No. A0550), and Tyr-Tyr-Tyr (Cat. No. T2007) were purchased from MilliporeSigma (Saint Louis, USA). The dipeptide Gly-Tyr was supplied by Evonik Nutrition and Care GmbH (Darmstadt, Germany). Amino acid tracers [U–^{13}C]leucine, [U–^{13}C]tyrosine, [U–^{13}C]phenylalanine, [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 0.1N 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 culture media were purchased from MilliporeSigma (Saint Louis, USA): Immediate Advantage medium (Cat. No. 87093C) and custom media missing specific amino acids. All media were filter-sterilized prior to use in cell culture experiments using a 0.22 or 0.4 μm pore-size membrane filter.

5.2. Cell culture

Two CHO cell lines were used in this study: a CHO-GS cell line (CHOZN® GS−/− ZFN-modified CHO cell line) producing IgG, and a CHO–K1 cell line (DHFR expression system) producing IgG. CHO cells were grown in suspension batch cultures in a humidified incubator operating at 37 °C, 125 RPM and 5% CO_2. For the CHO–K1 cell line, 6 mM glutamine and 0.4 vol% anti-clumping agent (Gibco, Canada) were added to the medium. Tracers and dipeptides were added as described in the text. Batch CHO cell cultures were seeded at ~0.3×10^6 cells/mL in 25–30 mL medium and CHO cells were grown in 125 mL shaker flasks (Corning Erlenmeyer cell culture flasks, Sigma-Aldrich, Cat No. CLS431143). 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.

5.3. Analytical methods

Viable cell density (VCD) and cell viability (%) were determined from cell counts based on the trypan blue dye exclusion method using a hemocytometer for CHO-GS cells, or an automated cell counter (Corning Cytosmart) for CHO–K1 cells. 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 or YSI 2500 biochemical analyzer (Yellow Spring Instrument). 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).

5.4. Cell-free experiments

To quantify dipeptide cleavage in cell-free experiments, dipeptides were added directly to filtered spent media. Flasks containing spent media with dipeptides were placed in a humidified shaking incubator operating at 37 °C, 125 RPM and 5% CO_2. Alternatively, dipeptides were added to small aliquots of spent media (200 μL) and pipetted into 96-well plates that were then placed in a humidified shaking incubator operating at 37 °C, 125 RPM and 5% CO_2. For these experiments, spent medium from a CHO–K1 batch culture was collected on day 5, centrifuged and filtered to remove CHO cells. Next, 1% PenStrep was added to the spent medium, to prevent microbial contaminations during the incubation, and 100 μL was aliquoted into each well of a 96-well plate. One or more dipeptides were then added to each well. Samples were collected at regular intervals for subsequent GC-MS analysis to quantify amino acid concentrations using a [U–^{13}C]algal amino acid or [U–^{13}C] glutamine solution as internal standard.

5.5. 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 (Oates and Antoniewicz, 2022). For dipeptides, the total ion counts (TIC) from GC-MS analysis was used as a surrogate for dipeptide concentration. The following TBDMS-derivatized dipeptides were monitored: Ala-Leu (*m/z* 373, 24.13 min), Leu-Ala (*m/z* 373, 24.34 min), Ala-Tyr (*m/z* 537, 35.42 min), Tyr-Ala (*m/z* 537, 35.67 min), Ala-Phe (*m/z* 407, 28.75 min), Phe-Ala (*m/z* 407, 28.85 min), Ala-Gln (*m/z* 502, 32.00 min), and Gly-Tyr (*m/z* 523, 36.48 min). 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 70 eV. 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 4ms 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).

5.6. Kinetic model

We constructed a kinetic model to quantify dipeptide utilization fluxes in CHO cell cultures based on ^{13}C labeling experiments. The model is shown schematically in Fig. 4. The model captures the fact that dipeptides are cleaved both intracellularly and extracellularly in CHO cell cultures. As such, the dipeptide concentration in the culture medium is impacted by two fluxes, ν_1 and ν_3. Intracellular cleavage of dipeptides is described by flux ν_2. The concentrations of free amino acids in the medium are governed by the inward and outward amino acid fluxes ν_4 and ν_5, respectively. Finally, amino acids inside CHO cells are directed toward biomass synthesis and protein production via flux ν_6. Our kinetic model assumes that CHO cells are grown in batch culture, and that the medium contains a ^{13}C labeled amino acid, such as [U–^{13}C]leucine, [U–^{13}C]tyrosine or [U–^{13}C]phenylalanine, and a dipeptide containing the same essential amino acid, e.g. Ala-Leu. Because we used custom culture media lacking specific amino acids in this study, extracellular amino acid ^{13}C labeling was 100% at the beginning of tracer experiments.

Mathematically, the kinetic model is composed of four coupled ordinary differential equations (ODEs) that describe the changes in biomass concentration (X), dipeptide concentration in the medium (DP), the concentration of labeled amino acid in the medium (AA_{13C}), and the concentration of unlabeled amino acid in the medium (AA_{12C}) during a batch culture:

$$\frac{dX}{dt} = \mu X \tag{1}$$

$$\frac{dDP}{dt} = -(v_1 + v_3)X \tag{2}$$

$$\frac{dAA_{13c}}{dt} = (v_5 f_i - v_4 f_e)X \tag{3}$$

$$\frac{dAA_{12c}}{dt} = (v_3 + v_5(1 - f_i) - v_4(1 - f_e))X \tag{4}$$

In equation (1), μ represents the growth rate of CHO cells. In equations (3) and (4), f_i and f_e represent the percent ^{13}C labeling of intracellular and extracellular amino acid, respectively, which were calculated as follows:

$$f_e = AA_{13c} / (AA_{13c} + AA_{12c}) \tag{5}$$

$$f_i = f_e v_4 / (v_5 + v_6) \tag{6}$$

The model assumes that there is no accumulation of amino acids or dipeptide inside CHO cells, i.e. pseudo steady-state assumption (Chen et al., 2019), which results in the following two flux constrains:

$$v_1 = v_2 \tag{7}$$

$$v_2 + v_4 = v_5 + v_6 \tag{8}$$

The kinetic model has two distinct metabolic phases: 1) the dipeptide utilization phase, where all fluxes in the model are allowed to have positive non-zero values; and 2) the post-dipeptide utilization phase, starting when the dipeptide is fully exhausted, where the fluxes v_1, v_2, and v_3 are set to zero. For a given set of fluxes and initial values for X, DP, AA_{13C} and AA_{12C} at t = 0, the ODEs were solved numerically using MATLAB. For simplicity, we assumed that the growth rate of CHO cells (μ) and the biomass-specific flux towards cell growth and protein production (v_6) were constant for the two metabolic phases.

To estimate fluxes in the model, data fitting was performed in MATLAB using least-squares regression tools, such as *fmincon* and the "GlobalSearch" optimization toolbox. For a given ^{13}C labeling experiment, the following time course data was fitted: i) viable cell density; ii) extracellular concentration of labeled amino acid; iii) extracellular concentration of unlabeled amino acid; iv) extracellular amino acid ^{13}C labeling; and v) intracellular amino acid ^{13}C labeling. Thus, by measuring cell concentrations, amino acid concentrations, and percent labeling over time, we were able to estimate fluxes in the model to quantify the dipeptide utilization rate (ν_1 plus ν_3).

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2024.03.002.

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