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Impact of hydrolysates on monoclonal antibody productivity, purification and quality in Chinese hamster ovary cells

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Plant and yeast derived hydrolysates are economical and efficient alternative medium supplements to improve mammalian cell culture performance. We supplemented two commercial Chinese hamster ovary (CHO) culture media with hydrolysates from four different sources, yeast, soybean, Ex-Cell CD (a chemically defined hydrolysate replacement) and wheat to improve the productivity of two cell lines expressing different monoclonal antibodies (mAbs). Yeast, soybean and Ex-Cell CD improved the final mAb titer by increasing the specific productivity (qP) and/or extension of the culture period. Wheat hydrolysates increased peak viable cell density but did not improve productivity. IgG recovery from protein A purification was not compromised for all cultures by adding yeast, soybean and Ex-Cell CD hydrolysates except for one sample from soybean supplemented culture. Adding these three hydrolysates neither increased the amount of host cell protein, DNA or aggregate impurity amounts nor affect their clearance after purification. Profiling of the glycan types revealed that yeast and soybean hydrolysates could affect the distribution of galactosylated glycans. Ex-Cell CD performed the best at maintaining glycan profile compared to the non-supplemented cultures. Overall, yeast performed the best at improving CHO culture growth and productivity without being detrimental to downstream protein A processes but could affect mAb product glycan distribution while Ex-Cell CD yielded lower titers but has less effect on glycosylation. The hydrolysate to use would thus depend on the requirements of each process and our results would provide a good reference for improving culture performance with hydrolysates or related studies.

[**Key words:** Chinese hamster ovary; Monoclonal antibody; Hydrolysates; Productivity; Purification; Glycosylation; Aggregation; Host cell protein]

Culture media formulations which supports high cell growth and productivity without compromising product quality is required to meet the increasing demand for therapeutic monoclonal antibodies (mAb) produced using Chinese hamster ovary (CHO) cells in large scale suspension cultures (1,2). Although animal serum is a rich and complex source of nutrients, its use is limited due to safety concerns. Having a chemically defined (CD) serum free media formulation is ideal for consistency and safety but CD media can be highly cell line specific, costly and time consuming to develop and produce (3,4). Performance of many CD media is still unsatisfactory at high cell densities and high productivity (5). The basal media can also be supplemented with protein hydrolysates which are the products obtained after the hydrolysis of proteins using either enzymes, acid or alkali (6).

To maintain an animal component free media formulation, protein hydrolysates derived from enzymatic digests of non-animal sources are preferred for cell cultures producing therapeutic proteins. Hydrolysate feeds are an economical and easy method to boost performance of most cell culture processes (3,7) and are still potentially used in some large scale manufacturing processes (6–8). Yeast (9–11), soybean (9,12,13) and wheat (14) are three commonly used hydrolysate sources. The hydrolysates are a source of nutrients like amino acids, peptides, vitamins and minerals (3) and supplementing CHO cell cultures with these hydrolysates have been shown to improve culture performance and product yield (8,11,15). There are some concerns related to hydrolysate batch variations due to differences in raw materials and the manufacturing (3,12) but this variability can be reduced through ultrafiltration or screening for critical components using techniques like near magnetic resonance (3,7).

The use of any media supplements can affect both the upstream process as well as the downstream process. Hydrolysate supplements could possibly contribute to both product-related (e.g., aggregates and unwanted glycoforms) and process-related (e.g., host cell protein (HCP) and DNA) contaminants (16). These impurities need to be cleared during the downstream process. Most reports related to hydrolysate use in cell culture focus on the upstream part of the process, characterizing cell growth and product titer, and the downstream process is usually neglected. It has also been shown that animal tissue, soy, wheat and yeast hydrolysate addition can cause shifts in the glycan profiles of both γ- and β-interferon produced in CHO cell cultures (3,17). There are no reports of the effect of hydrolysates on cell lines which are producing mAbs. These glycan variant impurities are hard to remove during the downstream process and need to be controlled during the culture process. Any evaluation of media additions have to thus cover both upstream and downstream parts of the process.

In this study, we aim to identify a simple, commercially available media formulation for culturing mAb producing CHO cell lines which does not interfere with product quality and purification using

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TABLE 1. Media osmolality, cell growth and productivity performance.

Cell line and medium	Hydrolysate	Osmolality (mOsm kg^{-1})	Growth rate (h^{-1})	Culture length (days)	Peak VCD (10^6 cells mL^{-1})	IVCD (10^7 cell mL^{-1} day^{-1})	End titer (mg L^{-1})	qP (pcd, pg cell^{-1} day^{-1})
Hum431-PC	None (Ctrl)	328 ± 10	0.0164 ± 0.0003	16 ± 0	10.0 ± 0.4	8.9 ± 0.0	475 ± 11	5.4 ± 0.1
(CHO DG44 producing	Yeast	366 ± 3	0.0139 ± 0.0004	21 ± 0	7.2 ± 1.3	9.0 ± 1.3	1130 ± 71	12.7 ± 2.7
Humira in PowerCHO2 media)	Soybean	364 ± 22	0.0155 ± 0.0008	21 ± 0	10.9 ± 1.8	12.1 ± 0.6	784 ± 72	6.6 ± 0.9
	Ex-Cell CD	380 ± 30	0.0131 ± 0.0000	21 ± 0	7.5 ± 0	8.9 ± 0.2	641 ± 37	7.2 ± 0.6
	Wheat	336 ± 8	0.0156 ± 0.0004	14 ± 0	12.1 ± 0.3	7.7 ± 0.1	379 ± 6	4.9 ± 0.1
Her293-PC	None (Ctrl)	328 ± 10	0.0173 ± 0.0016	16 ± 0	8.5 ± 0.6	8.6 ± 0.9	760 ± 23	8.9 ± 1.2
(CHO DG44 producing	Yeast	366 ± 3	0.0156 ± 0.0001	17 ± 0	8.8 ± 0.5	8.8 ± 0.4	1265 ± 35	14.5 ± 0.9
Herceptin in PowerCHO2 media)	Soybean	364 ± 22	0.0164 ± 0.0003	22 ± 0	9.4 ± 1.3	13.4 ± 3.4	1250 ± 0	9.7 ± 2.5
	Ex-Cell CD	380 ± 30	0.0141 ± 0.0006	16 ± 0	7.4 ± 0.3	6.1 ± 0.9	931 ± 47	15.3 ± 1.5
	Wheat	336 ± 8	0.0170 ± 0.0001	14 ± 0	9.9 ± 0.2	7.6 ± 0.1	709 ± 1	9.4 ± 0.1
Her293-FC	None (Ctrl)	272 ± 6	0.0169 ± 0.0005	14 ± 0	18.5 ± 1.6	10.1 ± 0.4	1135 ± 21	11.3 ± 0.6
(CHO DG44 producing	Yeast	302 ± 2	0.0127 ± 0.0005	18 ± 0	16.4 ± 0.1	11.6 ± 0.6	1715 ± 49	14.8 ± 0.4
Herceptin in FortiCHO media)	Soybean	286 ± 3	0.0162 ± 0.0010	16 ± 0	16.7 ± 0.8	11.0 ± 0.3	1340 ± 0	12.2 ± 0.4
	Ex-Cell CD	307 ± 4	0.0126 ± 0.0012	18 ± 0	13.6 ± 4.0	10.7 ± 0.1	1610 ± 42	15.0 ± 0.6
	Wheat	278 ± 1	0.0189 ± 0.0001	14 ± 0	22.3 ± 0.8	13.0 ± 0.3	1100 ± 0	8.5 ± 0.2

protein A chromatography. We evaluated four different hydrolysates: a soy hydrolysate, a yeast hydrolysate, a CD supplement based on hydrolysate components and a wheat hydrolysate. The hydrolysates were tested in two different mAb producing CHO cell lines and in two different commercially available basal and feed media optimized for CHO cultures. The effect of hydrolysate addition was evaluated based on cell growth and productivity. Most studies available do not proceed to perform evaluation of the effect of hydrolysate supplementation on the downstream process. We performed further studies on potential effects hydrolysate addition could have on protein A purification and product quality attributes like aggregation and glycosylation. These results would be beneficial to anyone looking for a simple culture and supplementing strategy using hydrolysates for high mAb expression using CHO cells.

MATERIALS AND METHODS

Cell culture and media Two cell lines producing IgG mAb were generated using CHO DG44 cells (Thermo Fisher Scientific, Grand Island, NY, USA), one cell line was expressing a Humira biosimilar, Hum431, and the other cell line was expressing a Herceptin biosimilar, Her293. Cells were passaged every 3–4 days by diluting the cultures to 3×10^5 cells mL^{-1} in fresh media. Cell viability and density were determined by trypan blue exclusion method using a Vi-Cell XR cell viability analyzer (Beckman Coulter, Indianapolis, IN, USA). Two different commercial basal media were used. One basal media was PowerCHO2 (PC, Lonza, Cologne, Germany) supplemented with 6 mM L-glutamine (Sigma Aldrich, St. Louis, MO, USA). CHO Xtreme feed from Lonza was subsequently added to the cultures in PC media at 20% of the starting culture volume on days 1 and 3. Another basal media used was CD FortiCHO (FC, Thermo Fisher Scientific) supplemented with 6 mM L-glutamine and 0.3% anti clumping agent (Thermo Fisher Scientific). Cultures in FC media were supplemented with CHO CD Efficient Feed A and B (Thermo Fisher Scientific) at 20% of the starting culture volume for each feed in a single bolus feed at the start of the culture.

Hum431 was adapted to PowerCHO2 (Hum431-PC) and Her293 was adapted to both PowerCHO2 and CD FortiCHO (Her293-PC and Her293-FC) for our evaluation.

Three different hydrolysates and a hydrolysate based supplement were evaluated in the study: Ultra-filtered yeast hydrolysate (catalog number: 96,863, Irvine Scientific, Santa Ana, CA, USA), peptone from glycine max soybean (catalog number: P0521, Sigma Aldrich), Ex-Cell CD hydrolysate fusion (catalog number: 24700C, Sigma Aldrich) and HyPep 4601 protein hydrolysate from wheat gluten (catalog number: H6784, Sigma Aldrich). All hydrolysates were in powdered formats and 5 g of each hydrolysate was measured and added separately to 1 L of basal media (both PowerCHO2 and CD FortiCHO). The media was mixed by stirring, followed by sterile filtration using 0.22 μm bottle top filters (Corning, NY, USA) to eventually obtain one control basal media without hydrolysates and 4 hydrolysate supplemented media for both PC and FC. Media osmolality was checked before and after hydrolysate addition using a VAPRO vapour pressure osmometer (ELITech Group, Puteaux, France).

Effect of the hydrolysates was compared using duplicate 250 mL shake flasks (Corning) with a final working volume of 50 mL of culture. All cultures were seeded at an initial density of 3×10^5 cells mL^{-1} in 35 mL of fresh media to reach a final working volume of around 50 mL after feeding. Supernatant and cultures were sampled at regular intervals to obtain cell density and viability using Vi-Cell XR automated cell counter, and measured titer using Immage 800 immunochemistry nephelometer (Beckman Coulter). Exponential cell growth rate, total integral viable cell density (IVCD) and specific antibody productivity (qP) were determined as previously reported (18) and listed in Table 1. Supernatant was harvested when viability dropped below 50% for purification and product quality analysis.

Protein A purification Cell culture clarification was performed by centrifugation at 4000 ×*g* for 20 min at room temperature, followed by filtration through 0.22 μm membrane (Nalgene Rapid-Flow Filters, Thermo Fisher Scientific). The cell culture supernatant was then stored at 2–8°C for short-term usage or −20°C for long-term storage. Protein A affinity chromatography was performed with 3 mL of Toyopearl AF-rProtein A-650F (Tosoh Bioscience, Tokyo, Japan) media packed in a Tricorn 10/50 column (3.8 cm bed, GE Healthcare, Uppsala, Sweden), run at linear flow rate of 150 cm/h (volumetric flow rate 2 mL/min). The column was equilibrated with 5 column volumes (CV) of 50 mM HEPES, 150 mM NaCl, pH 7.0. 10 mL of cell culture supernatant was loaded and the column washed with 15 CV of equilibration buffer. Antibody was eluted with 10 CV of 100 mM acetic acid, pH 3.5. Protein was collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it descended below that value. The pH of the eluted mAb was neutralized by addition of 1 M Tris. The column was further cleaned with 20 CV of 0.1 M NaOH, followed by 5 CV of equilibration buffer and then stored in 20% ethanol (V/V). Chromatography experiments were conducted on an ÄKTA Explorer 100 or Avant 25 (GE Healthcare).

Size exclusion chromatography for analysis and quantification of purified mAb Aggregate content was measured by analytical size exclusion chromatography (SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex UltiMate 3000 UHPLC system (Thermo Scientific) operated at a flow rate of 0.6 mL/min and room temperature, using a buffer formulation of 50 mM MES, 20 mM EDTA, 200 mM arginine, pH 6.0. Sample injection volume was 100 μL.

mAb concentration was also monitored by SEC, comparing experimental results with a calibration curve prepared from known quantities of injected purified mAb. This approach avoids the overestimation error by affinity based methods that capture aggregates in addition to non-aggregated mAb.

Host cell protein and DNA quantification Host cell protein (HCP) content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC, USA) according to manufacturer's recommendations. DNA content was measured using a QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA) designed for absolute quantitation of DNA copy number. Samples were prepared according to manufacturer's recommendations. In brief, they were digested by proteinase K (Roche, Indianapolis, IN, USA), added at 10% v/v of 2 mg/mL proteinase K in 5% SDS to sample, for 16 h at 50°C, followed by DNA extraction using either a DNA extractor kit (Wako Pure Chemicals, Osaka, Japan) or QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). TaqMan PCR reaction mixture was assembled from a 2 × ddPCR Mastermix, 10 × primer and probes using a resDNASEQ Quantitative CHO DNA Kit (Applied Biosystems, Foster City, CA, USA) and DNA sample in a final volume of 20 μL. Each reaction mixture was loaded into a sample well of an eight channel disposable droplet generator cartridge, then 70 μL of droplet generation oil. Generated droplets were transferred to a 96-well PCR plate, heat-sealed, then placed on a thermal cycler and amplified to end-point by denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s then 60°C for 1 min. Analysis was performed with QuantaSoft analysis software (Bio-Rad Laboratories). Correlation between DNA copy number and DNA concentration was based on CHO host cell DNA standards from Applied Biosystems (resDNASEQ Quantitative CHO DNA Kit).

Glycan analysis IgG samples were first desalted using a PD 10 column (GE Healthcare) following manufacturer's protocol. Glycans were released from 100 μg IgG by incubating with 500 U of the PNGase F (New England Biolabs, Ipswich, MA, USA) in the reaction buffer at 37°C for 1 h, then purified by HyperCarb porous graphitized carbon cartridge (Thermo Fisher Scientific). The purified *N*-glycans were labelled with 2-aminobenzamide (2-AB) and excess 2-AB was removed by passing the labelling mixture through a MiniTrap G-10 desalting column (GE Healthcare). The purified, 2-AB-labelled glycans were then dried under vacuum. Before the

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