

Working Towards a Chemically-Defined Replacement for Hydrolysates

Z.W. Deeds, C.S. Updike, B.J. Cutak, and M.V. Caple
Sigma-Aldrich Biotechnology, 3050 Spruce Street, Saint Louis, MO 63103, USA

Abstract

Protein hydrolysates are commonly used in cell culture processes either as a component of a complete medium formulation or as part of a feeding supplement for a fed-batch bioreactor process. Due to the undefined nature of hydrolysates, there is a push to develop a chemically-defined substitute. It is well known that hydrolysates serve a nutritive function as a source of free amino acids, small peptides, carbohydrates, vitamins and many other potential contaminants. They also have been reported to contain bioactive peptides¹ that could potentially activate specific pathways within the cells. The data presented here are the initial results of studies performed to elucidate the essential components of commercially available wheat gluten and meat hydrolysates. Reverse-phase HPLC was used to initially separate the complex mixture into fractions containing either classes of components or individual components so they could be subsequently tested in a cell culture system utilizing Chinese Hamster Ovary cells.

Introduction

As an integral part of many cell culture media formulations and bioreactor feeding supplements protein hydrolysates have multiple advantageous functions. First and foremost, hydrolysates serve as a source of nutrition in the form of free amino acids and small peptides. Contaminants such as carbohydrates and vitamins are also often present and undoubtedly have a positive impact. However, there are other benefits that have been reported that cannot be attributed solely to nutritional enhancements. Schlaeger et al. report that a meat hydrolysate, Primatone RL, has an anti-apoptotic effect,² while Franek et al. suggest that peptides within the hydrolysates can mimic growth and survival factors.¹

Although protein hydrolysates can yield positive effects, by their nature they are an undefined component, which is not desirable for pharmaceutical production purposes. In many cases the methods by which the hydrolysates are created are not very well controlled. Other complications arise from the starting protein sources, which can be affected by such factors as storage temperature and the time of year it was harvested. All of these starting material and processing differences manifest themselves as lot-to-lot variability of the hydrolysates, which may lead to changes in production and product quality. For these reasons there is a demand for a product that mimics hydrolysates, but is chemically-defined and eliminates the lot-to-lot variability of hydrolysates. The data presented here represent the initial steps that have been taken to identify the active components in two commercially available hydrolysates.

Materials and Methods

Sigma-Aldrich Corporation (St. Louis, MO) supplied all chemicals, media and solutions unless otherwise stated.

Cell Lines

Cell lines CHO-IgG1 and CHO-IgG2 are proprietary clones expressing recombinant antibodies.

Culture Media

The media used in this study are CHO Kit 2 Base Formulation (Product Code C 4739) that is modified to contain no hydrolysates and 4.5 g/L Glucose and CHO CD-3 Medium (Product Code C 1490). The stocks were kept in modified CHO DHFR^r Medium (Product Code C 8862) without hydrolysates.

Cell Growth and Recombinant Protein Production Assays

The cells were routinely cultured in suspension in modified CHO DHFR^r Medium (Product Code C 8862) and were used to seed experiments conducted in 125 mL (50 mL working volume) disposable Erlenmeyer shake flasks (Corning) or 125 mL and 250 mL (100 mL and 150 mL liquid volume respectively) Techne spinner flasks. Initial cell density was 50,000 viable cells/mL. The cells were cultured in a ThermoForma incubator at 37 °C, 5% CO₂, and 130 rpm shaker speed/80 rpm stir speed.

The early experiments were counted using a Schärfe System Casy 1™ Model TTC and viability was assessed using the Trypan Blue Exclusion Method. Later assays were counted using a ViCell™ XR from Beckman Coulter. Spent medium samples were collected for the analysis of nutrients/metabolites and IgG concentration.

The shakers/spinners were fed on days 4 or 5, depending on the cell density (0.8 to 1.2x6 viable cells/mL). The feed included Glucose (2 g/L) and L-Glutamine (2 mM) to prevent carbon source limitation. Additionally, the Hydrolysate Base (1 or 2) was supplemented at this time.

Quantification of Recombinant IgG

The IgG secreted into the medium was measured by HPLC (Dionex DX500 and AD20, Sunnyvale, CA) using a protein-G affinity column (Applied Biosystems, Foster City, CA). The analytical method is based on affinity chromatography utilizing an analytical column packed with poly-flow through particles designed for very rapid mass transport. The protein-G has a high affinity for IgG under neutral pH. Neither media components nor other proteins, such as albumin, are retained by the column. The bound IgG is then eluted with an acidic solution. The IgG is detected and quantified by UV absorbance at 210 nm.

Hydrolysate Fractionation and Testing

The HPLC system consisted of a Shimadzu LC-6A separation module equipped with a binary gradient with high pressure mixing and a Shimadzu SPD-6AV UV detector. A preparative C18 column (2.5 cm x 22.5 cm) was used for the separation of the hydrolysates. Data collection and processing were performed using the Class-VP Chromatography Data System.

The primary chromatography method (TFA Separation) consisted of mobile phase A (0.1% trifluoroacetic acid in HPLC grade water) and mobile phase B (0.1% TFA in acetonitrile). Separation of the hydrolysate components was performed using a 300 minute linear gradient from 0 to 30% B. The flow rate was 5 mL/min and the column temperature was maintained at 25 °C. The injection volume was 5 mL of a 200 g/L solution of the representative hydrolysate.

The hydrolysate fractions were collected at one-minute intervals with a fraction collector and then pooled in five-minute intervals to simplify testing (66 fractions compared to 330). The samples were frozen in a -70 °C freezer and then lyophilized to remove the solvents. After lyophilization the fractions were resuspended in 5 mL water so they would be compatible with cell culture testing and mass spectrometry. Five milliliters of water were used since the initial hydrolysate injection volume was 5 mL.

The fractions were cell culture tested as outlined above (feeding on Day 4 or 5). They were fed at an amount that would be equivalent to the representative quantity in 2 g/L of hydrolysates.

Results

Hydrolysate Base Feeding Results

Base 2 into Modified C 4739 with CHO-IgG2 cells:
- Total Productivity: 40.3% of Hydrolysate Control
- Specific Productivity: 55.9% of Hydrolysate Control
Base 2 into CHO CD-3 (Product Code C 1490) with CHO-IgG1 cells:
- Total Productivity: 45.3% of Hydrolysate Control
- Specific Productivity: 54.3% of Hydrolysate Control

Table 1: Comparison of Base 2 performance to Hydrolysates. Hydrolysate Control is the Wheat Gluten Hydrolysate at 2 g/L. All conditions were fed on day 4 or 5 with Base 2 and 2 g/L Glucose and 2 mM L-Glutamine.

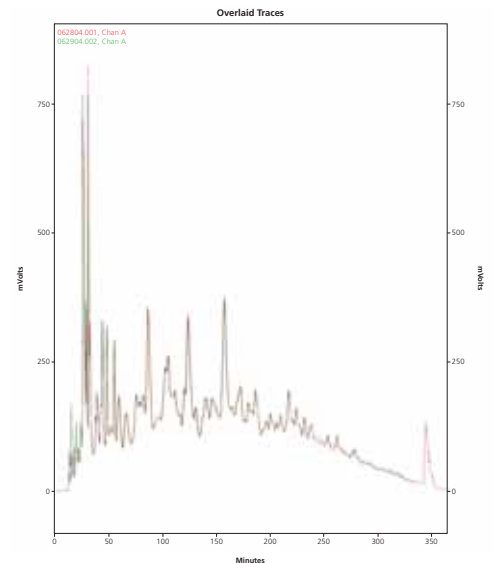


Figure 1: RP-HPLC Chromatogram. Two 280 nm traces for the wheat gluten hydrolysate fractionation are shown.

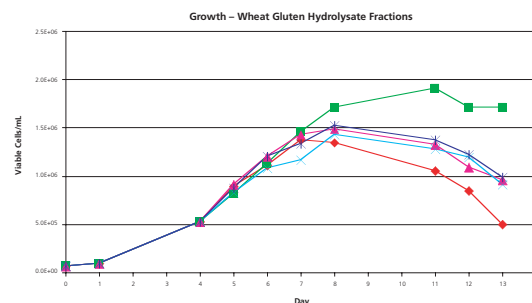


Figure 2: Growth Results for Wheat Gluten Hydrolysate Fraction Screening with CHO-IgG2. Performed in 125 mL shakers (50 mL working volume) inoculated at 50,000 viable cells/mL. None of the Wheat Gluten fractions showed significant improvements in growth.

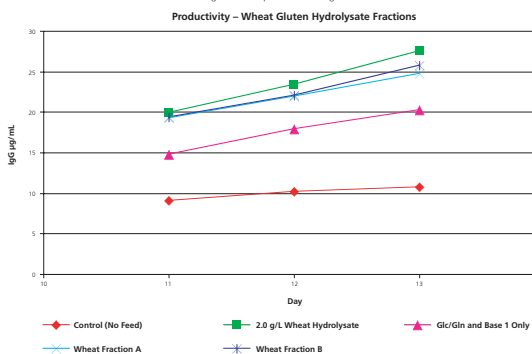


Figure 3: Productivity Results for Wheat Gluten Hydrolysate Fraction Screening with CHO-IgG2. Performed in 125 mL shakers (50 mL working volume) inoculated at 50,000 viable cells/mL. Fractions A and B yielded significant improvements in production.

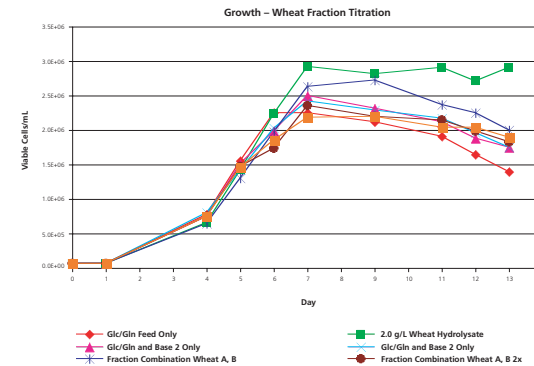


Figure 4: Growth Results for Wheat Gluten Hydrolysate Fraction Titration with CHO-IgG2. Performed in 125 mL spinners (100 mL working volume) inoculated at 50,000 viable cells/mL. When combined at 1x concentrations, Fractions A and B showed a moderate growth improvement. This was not seen at 2x and 4x concentrations.

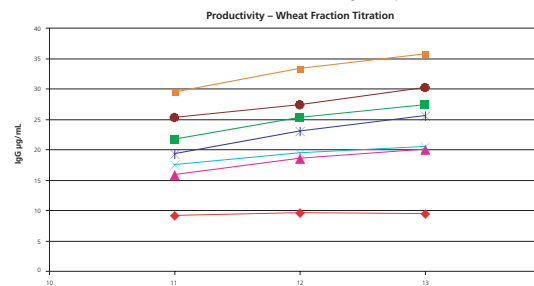


Figure 5: Productivity Results for Wheat Gluten Hydrolysate Fraction Titration with CHO-IgG2. Performed in 125 mL spinners (100 mL working volume) inoculated at 50,000 viable cells/mL. When combined, Fractions A and B gave a positive concentration dependent response.

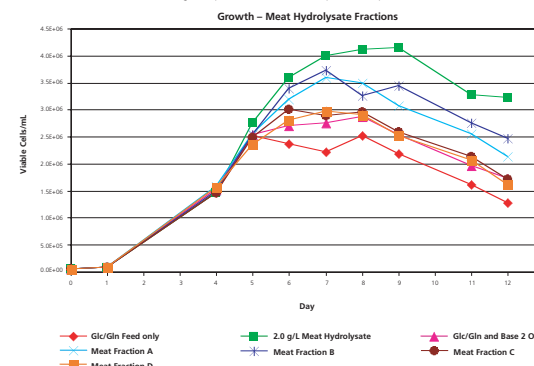


Figure 6: Growth Results for Meat Hydrolysate Fraction Screening with CHO-IgG2. Performed in 125 mL spinners (100 mL working volume) inoculated at 50,000 viable cells/mL. Fractions A and B gave a positive impact on growth.

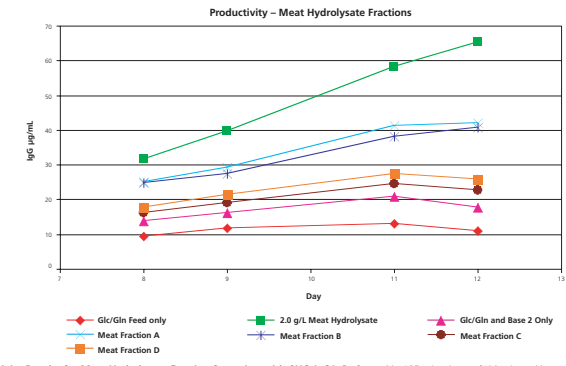


Figure 7: Productivity Results for Meat Hydrolysate Fraction Screening with CHO-IgG2. Performed in 125 mL spinners (100 mL working volume) inoculated at 50,000 viable cells/mL. Fractions A and B had a very significant positive effect on productivity, while Fractions C and D had a minor positive impact.

Discussion

In order to define the critical components in protein hydrolysates, the many functions that they serve must be addressed. The first of which is a nutrient source and was accounted for by creating a "Hydrolysate Base."

Hydrolysate Base 1 was originally developed to mimic the levels of amino acids, vitamins and metals present in a commercially available hydrolysate. Further optimization with matrix-style experiments utilizing Design-Expert™ software led to the development of Hydrolysate Base 2. As shown in Table 1 when Base 2 is fed into the modified C 4739 the total productivity with the CHO-IgG2 cell line increased to 40.3% of the 2.0 g/L wheat gluten hydrolysates control and the specific productivity increased to 55.9% of the control. Similar results were obtained using a much more rich formulation, CHO CD-3 (Product Code C 1490), with CHO-IgG1 cells. The data shows that with nutrients alone, the Hydrolysate Base 2 was able to recover some of the positive effects of hydrolysates.

The next step is to identify the other active components within the hydrolysates. Reverse-phase HPLC fractionation was used to aid with the separation of these compounds. In Figure 1 two chromatograms (at 280 nm) were overlaid to show the reproducibility of the TFA separation.

The complete hydrolysate fractionations were subsequently analyzed in experiments designed to demonstrate a correlation between cell culture productivity and the presence of bioactive peptides. These experiments showed that different fractions of commercially available hydrolysates enhanced antibody production. Figure 2 is a growth curve for the two positive wheat hydrolysates fractions. While fractions A and B did not improve cell growth like the hydrolysate control, they did have a positive impact on productivity (as seen in Figure 3). The IgG produced in the wheat gluten hydrolysate conditions was ~27 µg/mL while each of the fractions achieved ~25 µg/mL.

The positive fractions A and B were combined to determine if there was an increased growth and productivity response compared to the individual fractions. Fractions A and B were also tested at 2x and 4x the original concentration. Figure 4 shows the viable cell growth curve for this experiment. The 1x level showed an increase in growth; however the higher concentrations did not show an increase in growth, which potentially indicates an inhibitory response. Figure 5 shows the productivity values associated with the hydrolysate fraction titration. When combined, Fractions A and B gave a positive concentration dependent response. Fractions A and B when tested at the 2x and 4x levels surpassed the productivity level achieved by the wheat hydrolysate control which would indicate that the levels of the components in the positive fractions are not at the optimal level within the original hydrolysate.

A commercially available meat hydrolysate was also fractionated and analyzed in experiments as performed above. Four fractions were found to enhance cell proliferation and/or antibody production. Figure 6 represents a viable cell growth curve for the four positive meat hydrolysate fractions. Only two of the fractions, A and B, yielded enhanced cell growth when compared to the Base 2 control. Additionally, fractions A and B had a significant positive impact on production (see Figure 7). Fractions C and D had a minor impact on productivity, but are still worth investigating.

With the isolation of six hydrolysate fractions displaying positive biological effects further analytical experiments can now be performed to identify the active component(s) contained in the fractions.

Conclusions

- By analyzing the amino acid, vitamin, and metal composition of several protein hydrolysates, and with subsequent optimization, a Hydrolysate Base was designed to encompass some of the nutritive functions of hydrolysates
- Reverse-phase HPLC fractionation of commercially available wheat gluten and meat hydrolysates yielded six fractions that had a positive impact on productivity when tested with CHO cells
- Fractions A and B from the wheat gluten hydrolysate exhibited a concentration dependent response, indicating that the active components in these fractions are not at the optimal levels in the hydrolysate itself
- Current work is focused on identification of the component(s) of these fractions

Acknowledgements

We would like to thank all who contributed to this project at the Sigma-Aldrich Life Science and High Technology Center.

References

1. Franek F. et al., Plant Protein Hydrolysates: Preparation of Defined Peptide Fractions Promoting Growth and Production in Animal Cells Cultures. *Biotechnol. Prog.* **16**, 688-692 (2000).
2. Schlaeger, E. J. et al., The Protein Hydrolysate, Primatone RL, is a Cost-Effective Multiple Growth Promoter of Mammalian Cell Culture in Serum-Containing and Serum-Free Media and Displays Anti-Apoptotic Properties. *J. Immunol. Methods.* **194**, 191-199 (1996).
3. Schwartz, H. et al., Quality Control of a Wheat Gluten Hydrolysate for use as a Raw Material in Cell Culture Media. Poster presented at PITTCON 2002 (New Orleans, LA). Access at <http://www.sigmaaldrich.com/img/assets/4222/X56202-POSTER.OXD.pdf>

*Design-Expert® is a registered trademark of Stat-Ease, Inc.