Peptide Mapping of a Wheat Gluten Hydrolysate by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

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Abstract

Animal cell culture medium is a mixture of amino acids, vitamins and salts, which is traditionally supplemented with serum or other animal derived components. Protein hydrolysates prepared from a variety of sources including animal tissues, milk protein and plant tissue are capable of replacing or reducing the need for more expensive components such as serum. More recently, due to increasing concern about the potential contamination of animal derived components by adventitious agents, plant hydrolysates have been used to replace animal source medium components. In particular, we have found a wheat gluten hydrolysate, optimized for high levels of small peptides and rich in stable glutamine, to have particular value as a component in the formulation of serum free cell culture media. A decrease in productivity, with no corresponding decrease in cell growth, is associated with lot-to-lot variability of this hydrolysate. We have developed an RP-HPLC method with photodiode array detection for peptide mapping of wheat gluten hydrolysate in order to examine lot-to-lot variability. Significant differences noted in the profile are correlated to observed differences in the biological activity of cells grown in medium containing different lots of hydrolysate. The profiling method can be used to monitor changes in hydrolysate peptide composition due to variations in processing conditions. This presentation will demonstrate that a primary variable affecting the peptide map is the source of wheat gluten starting material.

Introduction

Efforts to replace costly, undefined serum products in cell culture media have led to the study of low-cost protein hydrolysates from animal tissues, milk products, micro-organisms and plant tissues[1]. Use of animal origin serum-replacement products has been re-evaluated due to increased concern about the potential for contamination from adventitious agents. Franek et al. reported that protein hydrolysates can either partially or fully replace serum as a concentrated balanced nutrient mixture[2]. The cell culture media developed by Sigma-Aldrich are frequently used in large-scale protein production by the biotechnology industry which is increasingly requiring serum-free media. In an effort to find a serum substitute for cell culture media, we have evaluated wheat gluten hydrolysates.

Wheat gluten hydrolysates are a known source of high levels of small peptides, larger oligopeptides, and are particularly rich in stable glutamine[3]. Sigma-Aldrich has observed the positive effect of wheat gluten hydrolysates on cell culture productivity, but the degree of productivity enhancement varies between batches of wheat gluten hydrolysate.

In general, the chemical composition of protein hydrolysates is somewhat ill-defined. In order to control the quality of cell culture media formulated with protein hydrolysates, one must be able to control the quality of the hydrolysates. We report here the use of peptide mapping by reversed-phase high performance liquid chromatography to screen wheat gluten hydrolystates as potential raw materials in serum-free cell culture media.

Materials and Methods

Chemicals and Equipment:

Sigma-Aldrich Corporation supplied all chemicals used, except the wheat gluten hydrolysate, which was obtained from Quest International. Deionized water was purified using an ELGA water purification system. The HPLC system consisted of a Waters 2690 Alliance separation module equipped with a column heater coupled to a Waters 996-photodiode array detector. A Supelcosil C18 column (25 cm x 4.6 mm; 5 mm) was used for the separation of the hydrolysates. Data collection and processing was performed using Waters Millennium software 3.05.01.

Chromatography:

Mobile phase A was prepared by adding 1.0 mL trifluoroacetic acid (TFA) to 1000 mL HPLC grade water. Mobile phase B was prepared by adding 1.0 mL TFA to 1000 mL acetonitrile. Separation of hydrolysate components was performed using a 90 minute linear gradient from 0 to 30% B. The column was washed for 5 minutes with 100% B and re-equilibrated for 15 minutes between injections. The flow rate was 0.7 mL per minute and the column temperature was maintained at 25 ˚C. UV data were recorded from 210 nm to 285 nm. The injection volume was 100 mL.

Sample preparation:

The wheat gluten hydrolysate was prepared at 5 g/L in hot (56 ˚C– 60 ˚C) HPLC grade water, filtered through a 0.2 mm filter, and diluted to 2.5 g/L with mobile phase A for injection.

Cell culture:

Chinese Hamster Ovary (CHO) cell line expressing a proprietary recombinant antibody was cultured in

CHO Protein-Free Animal Component-Free medium (Sigma product C5467) supplemented with 2.5 g/L of wheat gluten hydrolysate. The cultures, in 100 mL Techne spinner flasks, were kept at 37 ˚C in a humidified atmosphere containing 5% CO2. Quantitation of the recombinant antibody was performed by affinity HPLC using a Protein G column from Applied Biosystems.

Results and Discussion:

Wheat gluten hydrolysate is an enzymatic digest of wheat gluten that provides a high quality source of peptides which is particularly rich in stable glutamine[3]. During the course of serum-free medium development for CHO cells, the incorporation of this wheat gluten hydrolysate in cell culture media was found to have a positive effect on protein expression versus no supplementation with the wheat gluten hydrolysate (Figure 1A). This effect was reproduced in various other CHO cell culture experiments in which supplementation with 2.5 g/L wheat gluten hydrolysate was found to be optimal. Several lots of this hydrolysate were analyzed in experiments designed to further demonstrate a correlation between cell culture productivity and the presence of this hydrolysate. These experiments showed that different lots of wheat gluten hydrolysate produced differing degrees of cell culture productivity (Figure 1B).

Figure 1A: Effect of the Presence of Hydrolysates on Cell Culture Productivity

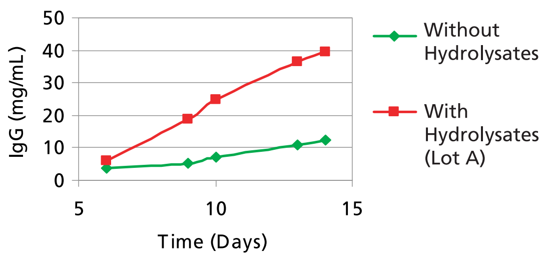
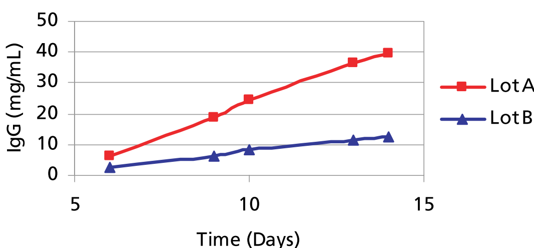
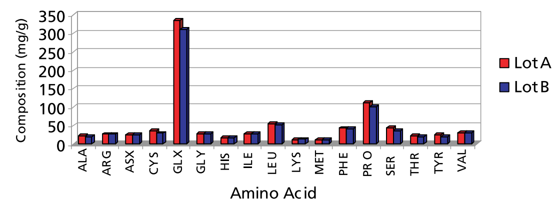


Figure 1B: Variation in Cell Culture Productivity With Respect to Lot of Hydrolysate



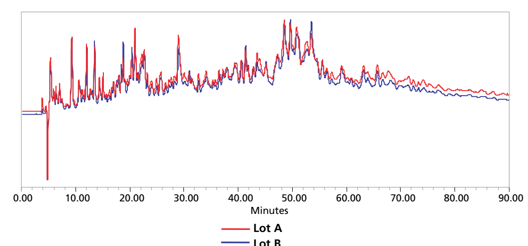
Because the raw material was a hydrolysate, the investigation began with basic analytical testing. The initial testing included amino acid composition analysis and elemental analysis by ICP atomic emmision spectroscopy. Figure 2 shows the results of amino acid composition analysis for two lots of wheat gluten hydrolysate that resulted in different cell culture productivity enhancements. No significant differences were observed in the amino acid composition of the two lots and no significant differences were observed in the elemental composition of the two lots. This seems to indicate that both lots were produced from similar wheat gluten preparations and that the productivity differences observed for the wheat glutens were related to the peptide composition. It was suspected that differences in final peptide composition might arise from the duration of hydrolysis, temperature applied during hydrolysis, or pH of the hydrolysis reaction.

Figure 2: Amino Acid Results for Two Lots of Wheat GlutenHydrolysate



An HPLC method was developed to qualitatively evaluate differences in the peptide composition of wheat gluten hydrolysates. The instrument method employed a shallow gradient because of the high concentration of small peptides in the hydrolysates. Although it is not conventional to initiate a gradient separation with a 100% aqueous mobile phase, it was necessary to achieve retention of several hydrophilic components. Initially the absorbance at 214 nm was monitored because it is generally accepted as the standard peptide-mapping wavelength[4]. Figure 3A shows the chromatographic traces at 214 nm obtained for two lots of wheat gluten hydrolysate that resulted in different cell culture productivity enhancements. It is apparent that both hydrolysates contain a considerable number of distinct peptide components. The nearly identical overlay of the two lots of hydrolysate at 214 nm does not provide any significant differentiation between the samples (Figure 3A).

Figure 3A: Overlaid Chromatograms at 214 nm of Two Lots of Wheat Gluten Hydrolysate



Because the spectral range from 210 nm to 285 nm was collected throughout the separation using a photodiode array detector, chromatographic traces at wavelengths other than 214 nm could be extracted

and compared in search of differences between the hydrolysate samples. The UV spectra of proteins and peptides are greatly dependent on the spectra of the amino acids of which they are composed[5]. The absorbance at 280 nm is often used for protein and peptide detection based on the aromatic amino acid residues. Figure 3B shows a portion of the 280 nm chromatograms extracted from the same data set as Figure 3A. The selected region contains peak area differences between the hydrolysate samples that can be seen in Figure 3B. The two peaks demonstrating the greatest difference between the samples are arbitrarily labeled as Peak 1 and Peak 2 as a designation for further studies. Comparison of the areas of Peak 1 and Peak 2, as illustrated in Figure 4, shows a clear difference between the two lots. The area of Peak 1 seems to provide a parameter that is correlated to the biological activity of the hydrolysate, however, the area of Peak 2 appears to be inversely related to biological activity (refer to Figure 1B and Figure 4). In short, more Component 1 and less Component 2 seems to increase productivity while the reverse, less Component 1 and more Component 2, appears to decrease productivity.

Figure 3B: Overlaid Chromatograms at 280 nm of Two Lots of Wheat Gluten Hydrolysate

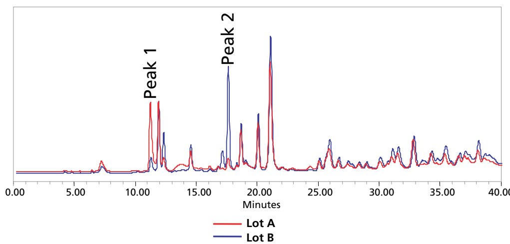
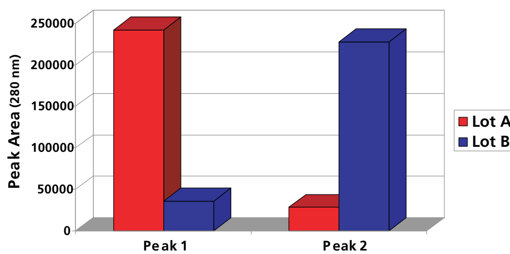
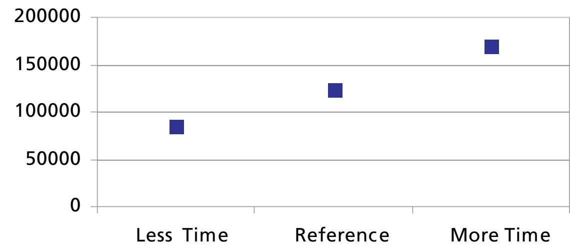


Figure 4: Comparison of Peak Areas Between Two Lots of Wheat Gluten Hydrolysate



The wheat gluten hydrolysates evaluated in this study were prepared by the addition of a proprietary mixture of enzymes to purified wheat gluten for a specific amount of time, at a specific temperature, and at a specific pH. A single lot of this hydrolysate, Lot A, was used to study these three processing conditions in samples prepared and supplied by the Quest International. Figure 5 shows the areas of Peak 1 as affected by different degrees of hydrolysis in relation to a reference point that is the normal hydrolysis time. The area of Peak 1 appears to be influenced by the length of time the wheat gluten is exposed to the enzyme. It was concluded that a greater amount of exposure to the enzyme did increase the size of Peak 1. The processing conditions of temperature and pH failed to affect the area of Peak 1 or Peak 2 (data not shown).

Figure 5: Effect of Hydrolysis Time on the Occurrence of Component 1 in Wheat Gluten Hydrolysate



Other lots were obtained and mapped with the profiling method described above. Figure 6 focuses on the area of Peak 1 for several different lots of hydrolysate. Differences in the areas of Peak 1 are significantly different from lot to lot. With the information supplied by the hydrolysate supplier, each lot in Figure 6 was connected to one of three different wheat gluten vendors used by the hydrolysate supplier. The three wheat gluten vendors are arbitrarily labeled as X, Y, and Z in Figure 6. It seems clear that the source of the wheat gluten has a more significant effect on the area of Peak 1 than any of the processing variables that were studied (see Figure 5). Significant differences in the area of Peak 2 were also observed between hydrolysates prepared from the three wheat gluten suppliers (data not shown). In an effort to more thoroughly monitor the profile of wheat gluten hydrolysates, we now routinely monitor 8 peaks in the same region of the 280 nm chromatogram, as seen in Figure 7.

Figure 6: Overlaid Chromatograms at 280 nm of Two Lots of Wheat Gluten Hydrolysate

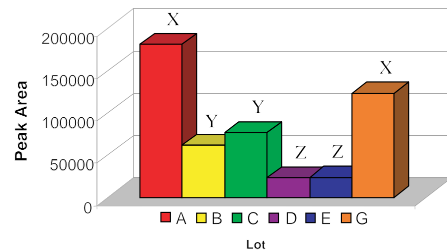
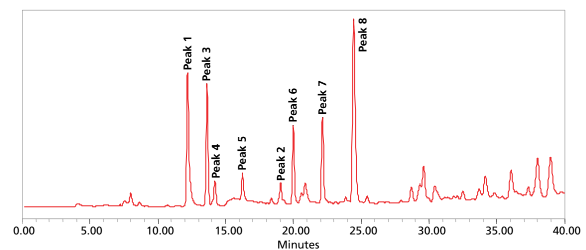


Figure 7: Comparison of Peak Areas Between Two Lots of Wheat Gluten Hydrolysate



Conclusion

The peptide map at 280 nm, generated using reversed-phase HPLC method, provides a fingerprint of 8 peaks for profiling wheat gluten hydrolysates. The variable that appears to influence the fingerprint more than any other variable studied was the supplier of the starting material, the wheat gluten. Differences in the chromatographic profile seem to be correlated of the lot-to-lot differences observed in cell culture productivity studies. Using this profiling method, in conjunction with collaboration from the hydrolysate vendor, allows for the selection of hydrolysate manufactured from wheat gluten obtained from a specific supplier. Further study is required to unambiguously identify the components that are responsible for the differences observed in the chromatographic profile and to understand the variables that influence the final concentration of these components in a wheat gluten hydrolysate preparation.

Acknowledgements:

We would like to acknowledge Quest International for its collaborative efforts in this project. We would also like to acknowledge J. Scott Ross and Matt Koch of Sigma-Aldrich Corporation for their assistance with this project.

References:

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