Short communication

The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density perfusion cultures of mammalian cells

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Abstract

Protein hydrolysates as substitutes for serum have been employed by many in cell culture medium formulation, especially with the shift to low protein or protein-free media. More recently, vegetable hydrolysates have also been added as nutritional supplements to fortify the amino acid content in small peptide form for batch and fed-batch fermentations. Several of these new hydrolysates (peptones of soy, rice, wheat gluten etc.) were tested as protein-free medium supplements for the production of a recombinant therapeutic protein. Multiple peptone-supplemented, continuous perfusion bioreactor experiments were conducted, varying dilution rates and basal medium composition over the various runs. Cell specific rates and product quality studies were obtained for the various peptones and compared with peptone-free medium. The potential for peptones to decrease intrinsic and proteolytic degradation of the product was also investigated. It was found that peptones confer a nutritional benefit, especially at low dilution rates, for the recombinant BHK cell line used in this investigation. The specific productivity increased 20–30% compared to the peptone-free controls. However, this benefit was also fully delivered by using fortified medium in place of the peptone-enriched media. Therefore, while peptones may be considered as useful medium additives when development time is limited, their addition may be avoided by systematic medium development if permitted by the time line of the project.

Introduction

The optimization of culture medium is one of the key steps for the production of any therapeutic product in addition to optimization of physical fermentation parameters such as temperature, pH or DO. The goals of medium optimization include the improvement of productivity, quality and cost savings. In the past two decades, serum has been successfully eliminated for the production of mammalian cell derived proteins. However, these serum-free or even protein-free media often resulted in a decrease of specific productivity and sometimes changes in product quality. In addition, the removal of serum or other complex protein

supplements may also affect the culture from a nutritional standpoint (trace elements, growth factors, etc.). Since an important goal of process optimization is elimination of any animal or human derived proteins to avoid viral, mycoplasma or prion contamination, the supplementation of small peptides like peptones as a replacement of complex protein mixtures might be a viable option. For the case of the production of a highly glycosylated therapeutic protein that is highly susceptible to proteolytic and/or intrinsic degradation, the addition of peptones might even make the process more robust and decrease product degradation rates.

The use of protein hydrolysates as a substitute for serum has been attempted by many in cell culture (ori-

ginally as Primatone, a tryptic meat digest). More recent reports on this subject (Jan et al., 1994; Dyring et al., 1994; Zhang et al., 1994; Keen and Rapson, 1995; Xie et al., 1997; Nyberg et al., 1999) indicate that addition of peptones resulted in higher cell growth and increased productivity. Nevertheless, Gu et al. (1997) reported negative effects of Primatone, expressed in decreased terminal sialylation at each of the glycosylation sites of CHO-secreted rInterferon. Whereas early work often employed Primatone or peptones derived from bovine milk, currently, these are not options since the elimination of animal- and human-derived proteins is a primary goal for the production of protein therapeutics. More recently, vegetable hydrolysates (peptones of soy, rice, wheat gluten) have become commercially available and have been used to supplement basal medium in order to fortify the amino acid content in small peptide form for batch and fed-batch fermentations (Amatsuji et al., 1998; Green Cross, WO Patent).

Through metabolic flux analysis it was shown that the growth of hybridoma cells is more efficient when medium was enriched with peptone (Primatone RL) rather than with free amino acids (Bonarius et al., 1996). Both cell growth and antibody production were increased. In addition, 50% less lactate was produced and twice as much substrate was used in the tricarboxylic acid (TCA) cycle in comparison with the equivalent free amino acid mixture. One possible reason for their observation is that the amino acid balance of the peptone-enriched medium for this particular cell line allowed more efficient use. Furthermore, the authors believed that the peptide transport into the cell is more energy efficient than that for free amino acids.

Physiologically, the uptake of peptones is linked to specialized transport systems, which are different from amino acid transporters. Inside the cells, the peptones are first clipped by proteases and the resulting free amino acids can then be used as nutrients via the TCA cycle or as precursors for other amino acids, nucleic acids or incorporated into proteins. In addition to the specialized peptide transport systems, it has been found that high and low affinity carriers with different uptake kinetics exist for the same dipeptide (Skopicki et al., 1991). Unfortunately, detailed information concerning peptide uptake in cultured cells is unavailable. Based on investigations made on the mechanistic transport of di- and tripeptides in kidney proximal tubular cells, it was found that the active transport of peptides in these cells is energized by an

H⁺ gradient rather than a Na⁺ gradient commonly seen in amino acid and glucose transport systems. Since peptide-H⁺ cotransport is electrogenic, membrane potential also contributes to the uphill transport of peptides. The H⁺ gradient required to drive renal peptide transport is generated in vivo by concerted action of the basolateral Na⁺-K⁺-ATPase and the brush-border Na⁺/H⁺ exchanger (Hopfer, 1997). The optimal pH for peptide uptake in kidney cells is in the range of 6.0–7.0 (Ramamoorthy et al., 1995), and some investigators reported even lower pH (6.0–5.5) for optimal uptake (Ganapathy and Leibach, 1983; Silbernagl et al., 1987). Thus, the uptake of peptides seems to be independent of free amino acid uptake and the transport can be affected by other peptides but not by free amino acids (Brandsch et al., 1995a, b).

We conducted 2- and 12-liter perfusion cultures in order to quantify the effects of various peptones on nutrition profiles as well as cell specific productivity. The product was purified from cell culture supernatant and fully characterized. Special degradation tests were implemented to evaluate the potential of decreasing proteolytic or intrinsic degradation.

Materials and methods

Cell line, basal medium, and cultivation system

A recombinant BHK-21 cell line producing a therapeutic glycoprotein was cultured in a protein-free medium (referred to as 'standard') based on a 1:1 mixture of DMEM and Ham's F12 (JRH, Lenexa, KS, U.S.A.) buffered by 2 g/l NaHCO₃ (Mallinkrodt, St. Louis, MO, U.S.A.). The experiments were conducted either in 2-L or 12-L stirred tank bioreactors (Applikon, Schiedam, The Netherlands) equipped with cell retention devices. DO was maintained at 50% air saturation by bubble free oxygenation via diffusion silicone tubing (Kuhlmann, 1988). Agitation speed was set to 80 rpm in the 15-L systems and to 100–120 rpm in the 2-L system. pH was controlled at 6.8 by automatic base addition, temperature was controlled at 34.5 °C (for details see Chuppa et al. (1997)). The cell density was controlled between 10 and 30 × 10⁶ cell/ml by purging cells from the reactor based on optical density probe readings (Aquasant, Bubendorf, Switzerland). Spinner flask and roller bottle experiments were used to screen peptones identified as potential medium additives for future bioreactor runs, and to investigate their dose effect.

Peptones

All peptones used in this study were obtained from Quest International (Naarden, The Netherlands). This paper describes the use of three peptones in detail, NZ-Soy Peptone[®] (hydrolyzed using porcine enzymes), wheat peptone HyPep 4601[®] and NZ-Soy BL-4[®], both complete vegetarian hydrolysates. Beside these three peptones two other complete vegetarian peptones were also tested; HyPep 5603[®], a rice peptone and HySoy[®], a soy peptone with a high carbohydrate content. Results of these two peptones are only discussed briefly in the results and discussion part. All these peptones represent a mixture of mostly di- and tripeptides with low free amino acid content (<6%). The aqueous solubility of the tested materials is approximately 200 g/l. The concentrations used in experiments ranged from 1–5 g/l. The addition of 5 g/l of peptone to commercially available cell culture medium results in an approximately 3.5 increase of total amino acid content (based on manufacturer specification). The peptones were sterile filtered together with the basal powder during medium batching since it had been shown that autoclaving stock solutions of peptones may be problematic and reduces the nutritional benefit (Schlaeger, 1996).

On-line measurements and off-line analyses

pH and DO were monitored by Ingold electrodes (Mettler Toledo, Wilmington, MA, U.S.A.). The accuracy of these on-line measurements were confirmed off-line by a NOVA blood gas analyzer (NOVA Biomedical Corp., MA, U.S.A.). The same instrument was also used to measure dissolved CO₂ concentrations. The cell concentration was determined using a hemacytometer and cell viability was estimated by Trypan blue exclusion. Cell size was determined by an electronic particle counter (CASY1, Schärfe Systems, Reutlingen, Germany). Glucose and lactate concentrations were measured off-line using a YSI Model 2700 analyser (Yellow Springs Instruments, OH, U.S.A.). A modification of the same instrument, equipped with appropriate enzymatic membranes and software, was used for glutamine and glutamate measurement. Ammonia and LDH concentrations were measured using an Ektachem DT60 analyzer (Eastman Kodak, NY, U.S.A.). Free amino acids were measured via HPLC (Dynamax-System, Varian, Walnut Creek, CA, U.S.A.) using the OPA method (similar to Bunte-meyer et al., 1991). Samples were filtered through

a 10 kd MW cut-off membrane (Millipore, Bedford, MA, U.S.A.) to remove proteins and cell debris.

Product concentration was determined by a specialized assay. Culture harvest were collected for further downstream processing to compare product quality for different medium conditions. The recombinant protein was purified by a series of chromatographic steps following cell removal from the harvest. Purity and quality were evaluated either by Coomassie blue or silver-stained SDS-PAGE or western blot analysis, respectively. Oligosaccharide mapping was used to analyze the glycosylation patterns of the recombinant protein. Briefly, glycans from dialyzed, purified fermenter samples were released by using the N+O mode of an automated hydrazinolysis instrument (GlycoPrep 1000, Oxford GlycoSystems, Abington, UK). The released pool of glycans were analyzed by HPAE-PAD (Hardy and Townsend, 1988; Hughes and Johnson, 1981) (Dionex LC, Sunnyvale, CA, U.S.A.) using a CarboPac PA1 column and a linear gradient of 20–200 mM Sodium Acetate in 100 mM sodium hydroxide and a flow rate of 0.8 ml/min. Quantization of the major glycans were carried out by peak area integration and expressed as relative percent peak areas.

Results and discussion

Small scale cultures (batch mode)

Small-scale experiments in spinner and roller-bottle cultures (batch mode) were used to evaluate various peptone concentrations. Addition of 1–5 g/l gave the best results in terms of final titer (not shown); higher concentrations led to growth inhibition due to toxic ammonia levels at the end of the batch cultures. Almost all peptone-enriched cultures showed benefits compared to standard medium, with the average final product titer at the end of the batch being approx. 30% higher with peptone formulations. However, the cell specific rates (q_{Lac} , q_{Glc} , q_{Gln}) during exponential growth showed no significant differences.

Bioreactor runs (perfusion mode)

Peptones were tested in either 2-L or 15-L perfusion fermentors. Altogether nine long-term perfusion cultivations were conducted. Most of these experiments were performed keeping a constant cell density of 20 million cells/ml at a dilution rate of 10 volumes/day

Glycomap produced under Standard Conditions

Glycomap produced under Peptone Conditions (NZ-Soy)

Figure 1. Glycosylation profile of the purified product produced under standard- and peptone (NZ-Soy) conditions.

(cell specific perfusion rate (cspr) of 0.5 nl/cell/d) throughout the production phase of the fermentation.

Proof of product quality

Based on the work by Gu et al. (1997), where rInterferon produced using peptone-containing medium was shown to exhibit poor glycosylation, the first goal was to test the glycosylation and biological activity of the product. To this end, the target protein was purified and fully characterized during distinct phases in which the fermentor was alternately fed with NZ-Soy medium at 5 g/l or standard medium. SDS PAGE and western blot analyses showed that the molecule

remained structurally intact during all periods of the cultures (results not shown). Oligosaccharide mapping of the product from the peptone-containing medium and peptone-free phases showed no significant difference (Figure 1). The elution profiles for neutral sugars, mono- di- tri- and tetra-sialylation structures were similar if peptone (here NZ-Soy) supplemented medium was used. The differences seen here reflect the normal micro heterogeneity always present during production. Pharmacokinetic tests and profiles in animals were also similar, further indicating that the molecules produced were comparatively intact and active (data not shown here).

Figure 2. Bench scale cultivation for the evaluation of HyPep 4601 wheat peptone: During this run, standard, fortified and peptone containing medium was used. The cell specific perfusion rate (cspr) varied between 0.35 and 5 nl/cell/day as the medium throughput was kept constant.

Comparison of cell specific rates

Cell specific rates were then determined for the perfusion culture for different perfusion mode operating parameters. These included various dilution rates, cell densities, and medium formulations. Both peptone-supplemented and fortified basal medium formulations (additional amino acids) were tested in these cultures.

Amino acid analyses of previous cultures indicated that asparagine, serine, and glutamine are depleted in standard medium at a cell specific perfusion rate (cspr) less than 0.35 nl/(cell d). This led to two modifications of the previously used basal medium. Fortified medium I contained double the glutamine, asparagine and serine; fortified medium II contained double the glutamine and asparagine and quadruple the serine concentration. These two modified formulations were evaluated in comparison to peptone-containing and the standard medium. Figure 2 shows the time course of a 75-day 2-L fermentation. The bioreactor was inoculated with cells into standard medium and then switched to HyPep 4601 Wheat peptone medium (both periods with 5 g/l) followed by periods of a amino

acid-fortified medium II, standard medium and finally back into the peptone-containing medium. The cspr was altered from 0.36 to 0.48 nl/(cell d) over the course of this run (between days 40–55 the cspr was changed stepwise). The specific productivity was related to cspr with a higher specific production rate obtained at a higher cspr. The medium throughput (10.3 l/d) and the residence time (4.44 hr) were kept constant during the run and changes in cell density led to changes in cspr. Figure 3 shows the average specific rates for the low and the high cspr period. At the lower cspr the metabolic profiles for glucose, lactate and the lactate yield showed no significant changes using the different media. The ammonia production rate using peptone media was slightly higher. A slightly higher glutamine uptake in the fortified medium was notified caused by the increased amount of glutamine in this medium. At high cspr the specific lactate production rate and the lactate yield were slightly lower in peptone media. Overall, in contrast to the observations of Bonarius et al. (1996), peptones did not significantly change the nutrient profile of this BHK cell line. At a given cspr the metabolic rates for the main energy sources were similar regardless of the me-

Figure 3. Cell specific rates during the bench scale cultivation as shown in Figure 2 using HyPep 4601 wheat peptone medium: The rates are given as average values; the error bars represent the standard deviation.

dium used. Nevertheless amino acid analysis clearly demonstrated a significant uptake of peptones over the free amino acids at low as well as at high cspr, since the residuals free amino acid concentration increased significantly when peptone medium was used (see below and Figure 6). At a cspr of 0.36 nl/(cell d) the peptone-containing and the fortified medium resulted in slightly better production compared to standard medium, most likely due to an amino acid limitation as the dilution rates is decreased. Fortified medium, however, eliminates this limitation.

At a cspr of 0.48 nl/(cell d) the specific productivity is slightly higher than at the lower cspr period. The same trend was seen with the apparent specific growth rate; slightly higher values at 0.48 nl/(cell d) period. The performance of the standard medium is slightly better compared to the wheat peptone period. One speculation is that this might indicate inhibition by amino acids due to higher residual levels at

this dilution rate. The overall better performance at higher cspr, for both media indicated that at lower cspr, nutrient limitation, growth inhibition, and/or enhanced proteolytic degradation are still present even with fortified media.

The time course of a 15-L perfusion culture is shown in Figure 4. The reactor was inoculated into standard medium at a cspr of 0.5 nl/(cell d). The fermentor performance was characterized using NZ-Soy BL-4 peptone medium (5 g/l), the two fortified media (I and II) and the standard medium at three different cspr ranging between 0.26–0.45 nl/(cell d). We felt that it would be easier experimentally to observe the beneficial effect of peptones at a lower cspr since nutrient depletion (including vitamins, trace elements, etc. in addition to amino acids) may occur (compared to higher perfusion rates). Therefore, the fermentor was first run at a cspr of 0.26 nl/(cell d). Supplementation of NZ-Soy BL-4 peptone (5 g/l) resulted in a 30%

15 L Bioreactor Run Comparison of Standard Medium, Fortified Standard Medium and NZ-Soy BL-4 Peptone

Figure 4. 15 l long term cultivation for the evaluation of NZ-Soy BL-4 peptone: Similar to the bench scale run the cspr was altered between 0.35 and 5 nl/cell/day and 0.45 nl/cell/day either by changing the medium throughput or changes in cell concentration.

Figure 5. Cell specific rates during the 15 l cultivation shown in Figure 4: Cell specific growth rates and cell specific productivity including standard deviation are shown for all media and cspr periods used during this culture.

higher specific productivity compared to standard medium (Figure 5). Fortified medium I also gave similar results as the NZ-Soy BL-4 medium. One interesting phenomenon was that an increase in titer of about 40% was seen within 8 hr of switching from standard medium to NZ-Soy BL-4 medium. Similarly, a titer decrease was also observed when the fermentor was switched from NZ-Soy BL-4 medium to standard medium (this suggests that the benefits of the addition of NZ-Soy BL-4 do not involve genetic regulation). The fortified medium gave similar results to the NZ-Soy

BL-4 medium, suggesting that the effect of NZ-Soy BL-4 was simply nutritional. Figure 5 shows the average specific productivity during the periods of running four media (NZ-Soy BL-4, fortified medium I and II, and standard medium) at a less-limiting cspr of 0.45. Once again the NZ-Soy BL-4 and medium I outperformed the standard medium by 30% in terms of specific productivity. Additionally, the specific productivities at a cspr of 0.45 were almost 100% higher than those at cspr of 0.26 for all three media tested.

The fermentor was then run at a cspr of 0.36

Figure 6. Residuals of free amino acids as a percentage of fresh medium during the 15 l perfusion cultivation shown in Figure 4. Values for three different media at low cell specific perfusion rate (0.26 nl/(cell d)) and high cell specific perfusion rate (0.45 nl/(cell d)) are given.

to confirm that NZ-Soy BL-4 and fortified medium gave higher specific productivity at various cspr. All three media (fortified medium II was only tested at high cspr) resulted in similar trends when comparing the relationship of cspr with specific productivity and growth rate. A higher cspr resulted in increased specific growth rates and productivity indicating that there may be nutrient limitations or growth inhibitors present at the lower perfusion rates. HPLC amino acid analysis showed that the supplementation of NZ-Soy BL-4 peptone has a dramatic effect on the uptake of several amino acids including aspartic acid, glycine, isoleucine, leucine and tyrosine at both low and high cspr (Figure 6). These amino acids are significantly

replaced by NZ-Soy BL-4 peptone, whereas glutamine or serine is still used as free amino acids. On the other hand, there was a significant amount of residual free amino acids in the supernatant, suggesting that there should not have been an amino acid limitation when NZ-Soy BL-4 was added to the standard medium since the total amino acid content became 3.5 times higher. The Monod constants for essential amino acids measured for hybridoma and CHO cells are extremely low in the range of 1 to 10 μM (Heidemann et al., 1995, 1998) and no reduction of growth rate should be expected. Hence, certain limitations may have occurred for other medium components (lipids, vitamins, trace elements) or the culture may have been negatively

Figure 7. Degradation profile at 34.5 °C, the supernatant was taken from the 15 l fermentor run (see Figure 4): Solid lines represent samples in the absence of additional salt addition, dotted lines represent samples incubated in the presence of 20 mM salt.

affected by an inhibitor. The presence of inhibitory substances especially at low dilution have been reported in literature (Kempken et al., 1991; Büntemeyer et al., 1992; Riese et al., 1994; Rønning et al., 1991; Siwiora et al., 1994), resulting in a decrease in specific growth rate and a drop in specific productivity. The presence of inhibitory substances is also supported by the fact that the specific growth rates were similar for three media at the same cspr (Figure 5).

In any case, the specific productivity was consistently higher in NZ-Soy BL-4 and fortified medium compared to the standard medium. This culture also demonstrated that the overall performance of NZ-Soy BL-4 peptone was significantly better compared to other peptones like HyPep 4601 wheat (Figure 3), HySoy or HyPep 5603 rice (data not shown) which gave no or only slight improvement compared to standard medium, especially at high cspr. This increase in specific productivity under NZ-Soy BL-4 and fortified medium could come from (1) a reduced degradation rate, or (2) an increased secretion rate or both. In order to further probe the mechanisms of NZ-Soy BL-4 and fortified medium we carried out degradation studies.

Degradation profiles

Previously purified product was spiked back into cell-free culture supernatant and incubated over a time period of 66 hours. Protein activity was measured throughout. Three different culture supernatants were used in this study: (1) standard fermentor supernatant; (2) standard fermentor supernatant with an additional 5 g/l NZ-Soy BL-4 added before spiking back the product; and (3) NZ-Soy BL-4 (5 g/l) harvest. Figure 7 shows the activity time profiles for these three supernatants with and without the addition of 20 mM salt, a potential stabilizer of the molecule. There was no significant difference in the degradation rate (based on protein activity) for the three fermentor supernatants tested. The addition of 20 mM salt, however, did slow down the degradation rate for all three. Additionally, other peptones at different concentrations were tested regarding their potential to inhibit or lower proteolytic degradation as described above. There was no apparent stabilization of the product in the medium by any of the peptones tested (results not shown). These results suggest that the effect of NZ-Soy BL-4 can be considered nutritional and not due to the protection of the glycoprotein from proteolytic or intrinsic degrad-

ation. Therefore, it is possible to avoid using NZ-Soy BL-4 by adding additional amino acids to the medium.

Summary

Comparable product quality and glycosylation patterns were obtained using peptone-supplemented media compared to standard medium. While at a given cspr the specific rates for the main energy sources were similar regardless of the medium used, the addition of peptones had a nutritional benefit at lower cspr. Under these conditions, the specific productivity increased 20–30%. During a 15-L fermentor run peptone even showed positive results at higher cspr (>0.45 nl/(cell day)) compared to standard medium. However, this benefit could also be delivered using media fortified with additional free amino acids. Best results were obtained using soy peptones (NZ-Soy or NZ-Soy BL-4) in contrast to peptones made of rice or wheat. Various degradation studies showed no potential for peptones as protease inhibitors to eliminate or slow down intrinsic instability in cell culture supernatant.

These results lead to the following main conclusions:

- (1) Peptones do not protect the molecule from proteolytic degradation;
- (2) The positive effect of peptones on product secretion is nutritional; and,
- (3) The same effect may be achieved by systematic medium design.

Consequently, this investigation shows that systematic medium development and, in particular, amino acid concentration optimization can successfully replace peptones. However, peptones can be a useful medium additive when time is limiting, and systematic media development is not feasible.

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