Peptides Modulate Growth and Productivity of Mammalian Cell Cultures and Suppress Apoptosis

Frantisek Franek

Production of therapeutic proteins in large-scale animal cell cultures continues to increase. The availability and cost of desired products such as interferons and therapeutic antibodies depend not only on sophisticated modifications of cell-line genomes, but also on the volumetric productivity achievable in bioreactor technology. The yield of product at harvest largely depends on the metabolism that controls the channeling of nutrients to synthesize both biomass and product.

Peptones, traditional components of microbial fermentation broth, were recognized as beneficial supplements to animal cell culture media more than 25 years ago (**1**). Peptones are mixtures of peptides obtained by partial hydrolysis of proteins. The prevalent opinion on their mode of action considers them to be a rich and inexpensive source of amino acids needed for cell mass synthesis and for the synthesis of secreted proteins (**2**). A variant view on the role of peptides emerged from our studies in which we fractionated soy and wheat protein enzymic hydrolysates by liquid chromatography. We found marked differences in the growth-promoting activities of various chromatography fractions. Very high activities were found with wheat gluten hydrolysate fractions, the amino acid composition of which was rather unbalanced, leaving essential amino acids insufficiently represented (**3**). Therefore, we started to evaluate the effects of a set of pure synthetic peptides, the composition and molecular mass of which were likely to be similar to those of the most active hydrolysate fractions (**4, 5**). We paid special attention to the antiapoptotic activity of tested peptides. These studies confirmed specific signal roles of various peptides comprising three to five amino acid residues.

PRODUCT FOCUS: THERAPEUTIC AND DIAGNOSTIC PROTEINS

PROCESS FOCUS: PRODUCTION

WHO SHOULD READ: PROJECT MANAGERS, PROCESS SCIENTISTS

KEYWORDS: PROCESS OPTIMIZATION, PEPTONES, APOPTOSIS, SYNTHETIC PEPTIDES

LEVEL: INTERMEDIATE

MATERIALS AND METHODS

Mouse hybridoma ME-750 was cultured in DMEM/F12/RPMI 1640 (3:1:1) media supplemented with BME amino acids; 2.0 mM glutamine; 0.4 mM each of alanine, serine, asparagine, and proline (**6**); 15 mM HEPES; 2.0 g L^{-1} sodium bicarbonate; and an iron-rich growth-promoting mixture containing 0.4 mM ferric citrate (**7**).

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All media components and additives were from Sigma-Aldrich (www.sigmaaldrich.com; St. Louis, MO). The cultures in 25-cm^2 T-flasks were kept at 37 °C in a humidified atmosphere with 5% CO_2. The culture volume was 6.0 mL. Monoclonal antibody concentrations were determined by immunoturbidimetry (**8**).

Synthetic peptides were products of Bachem (www.bachem.com; Bubendorf, Switzerland) and of

PolyPeptide Laboratories (www.polypeptide.com; Prague, Czech Republic). Chromatography fractions of wheat gluten enzymic hydrolysate were prepared as described earlier (3). Peptides were added to the cultures as concentrated solutions in saline. Viable cells and apoptotic cells were counted in a hemocytometer. The number of apoptotic cells was determined by microscopic counting of cells displaying apoptotic morphology (shrunken cells with ruffled membranes). Alternatively, apoptosis was quantified by fluorescence microscopy of cells stained with bisbenzimide H33342 (9).

Table 1: Alanine oligomers exert positive effects on growth, viability, and production of a hybridoma cell line.

	Parameters on Day Six of Batch Culture		
Peptide	Viable Cells $\times 10^{-3}$ mL^{-1}	Apoptotic Index %	Monoclonal Antibody mg L^{-1}
Control	1060	49	30
Ala	1030	46	31
Ala-Ala	1160	30	38
Ala-Ala-Ala	1450	22	43
Ala-Ala-Ala-Ala	1480	25	48
D-Ala-D-Ala-D-Ala	1510	23	44
ß-Ala-ß-Ala-ß-Ala	1390	28	38

Note: All tested peptides were present at 0.2% (w/v) concentrations. If not stated otherwise, alanines constituting the peptides were of L-configuration. Apoptotic index: (dead cells/total cells) × 100.

Table 2: Various peptides exert diverse effects on growth, viability, and production of a hybridoma.

	Parameters on Day Six of Batch Culture		
Peptide	Viable Cells $\times 10^{-3}$ mL^{-1}	Apoptotic Index %	Monoclonal Antibody mg L^{-1}
Control	1060	49	30
Some peptides enhance both the cell growth and the product yield:			
Ser-Ser-Ser	1470	31	41
Thr-Thr-Thr	1190	34	41
Val-Val-Val-Val	1200	28	39
Other peptides enhance selectively either the cell growth or the product yield:			
Gly-Gly-Gly	1660	25	32
Gly-Phe-Gly	1350	35	29
Gly-Glu-Gly	1270	19	30
Gly-Lys-Gly	930	38	49
Chromatography fraction of wheat gluten enzymic hydrolysate enhances both the cell growth and the product yield:			
Fraction a21	1750	36	63
Synthetic glutamine-containing peptide, derived from wheat gluten sequence, enhances mainly the product yield:			
Pro-Gly-Gln-Gly-Gln	1260	32	51

Note: All tested peptides were present at 0.2% (w/v) concentrations.
Apoptotic index: (dead cells/total cells) × 100.

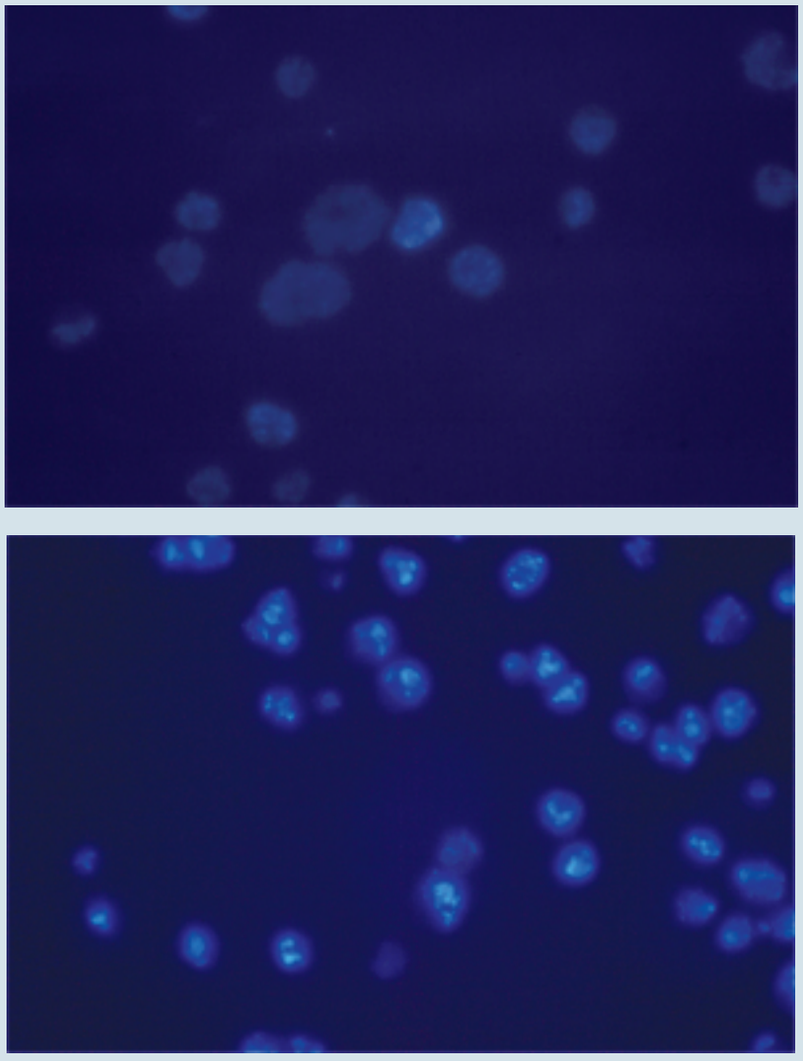
Results and Discussion

A Novel View — Intact Peptide Is the Real Agent: Testing the effects of alanine and alanine peptides provided the most convincing data on the specific roles of peptides. Supplementation of hybridoma cultures by monomeric alanine resulted in no significant improvement of the culture parameters. This was not surprising, because this nonessential amino acid was generally known to be produced rather than consumed by most cultured cell lines. Moreover, our protein-free hybridoma medium has already been enriched with this amino acid (see Methods). On the other hand, upon supplementation of the cultures with alanine oligomers, the viable cell density and the antibody yield increased, whereas the apoptotic index significantly decreased. Peptides formed by D-alanine and oligomers of beta-alanine were found to be active as well (Table 1).

The fate of the peptide in the culture was examined with tetraalanine. Amino acid and peptide analyses have shown that 70% of the starting amount of tetraalanine was still present as a peptide after four days of culture. In parallel, the concentration of monomeric alanine in the medium increased, so that the balance of peptide-bound and free alanine was virtually zero (5). These analyses have confirmed that the peptide molecules were largely stable during the course of the culture and that the amino acid liberated from the peptide was not significantly used in cell metabolism.

Enhancement of cell growth and product yield was observed also in cultures supplemented with some small peptides composed of serine, threonine, or valine. However, the effect of some other peptides was found to be rather selective. These tripeptides enhanced either cell growth or product yield (Table 2.) Examination of the action of the tripeptide Gly-Lys-Gly has lent support to our view that the effect of a peptide is different from the effect of free amino acids constituting the respective peptide. Similar to several other lysine-containing peptides, and in contrast to the above reported alanine peptides, the tripeptide Gly-Lys-Gly

Figure 1: Hybridoma cells in the exponential phase (top) and in the decline phase (bottom), chromatin-stained with bisbenzimide H33342



suppressed the maximum viable cell density to 70% of the control, but at the same time enhanced the antibody yield to 160% of the control. In the presence of a mixture of free glycine and lysine, the maximum viable cell density was not suppressed, and the antibody yield increased only to 117% of the control (**10**).

The growth-stimulating and the production-enhancing effects of peptides that have been used in our studies require peptide concentrations in the millimolar range, at least. This minimum concentration requirement is characteristic for the reported phenomena, and it contrasts with the range of active concentrations of most peptide hormones that may perform their physiological functions when present at levels several orders of magnitude lower. Another characteristic feature of the action of our peptides is the relatively low selectivity: The effects are not associated with the presence of any specific amino acid residue. Both neutral and charged, and both hydrophilic and hydrophobic residues may participate in the composition of active peptides.

Peptides Act As Survival Signals and Suppress Apoptotic Death

Whenever the culture conditions in a bioreactor start to deviate significantly from the physiological range, cultured animal cells tend to reduce their viable population size by setting into action the process of programmed cell death called *apoptosis*. The potential of cultured cell lines to reduce their population size by apoptotic death prevents the collapse of the culture that would inevitably follow a total exhaustion of nutrients and enables cells to survive as a smaller population under conditions of starvation. This trend is particularly pronounced in lymphocyte hybridomas because in these cell lines the properties of a transformed tumor cell and a highly differentiated lymphocyte are combined. Necrotic death occurs only exceptionally, e.g., upon mechanical insult or addition of a toxic agent (**7**, **11–14**).

Apoptotic suicide starts to reduce the population long before cell nutrients are exhausted. This feature of apoptotic death allows for application of signals that persuade the starving cells to postpone their suicide. Survival-signal molecules do not primarily promote the rate of cell growth, but they suppress the rate of apoptotic death, even if the levels of nutrients are critically low.

The idea of survival-signal action of peptides stems from our earlier studies on hybridoma cultures exposed deliberately to starvation in media diluted to 40% or to 20% by saline. We have found that apoptosis can be significantly suppressed by supplementing the diluted media with alanine, glycine, serine, asparagine, or proline (**6**). Because those amino acids are not essential nutrients, their beneficial effect may be tentatively ascribed to their survival-signal character.

Examination of the effects of a series of tri- and tetrapeptides (composed of glycine, alanine, serine, threonine, lysine, or phenylalanine) has shown that the common feature of the activity of these peptides is the suppression of apoptosis. Their effects on the viable cell density and on the final product yield are diverse. In addition to peptides that improve all culture parameters, one group promotes solely the growth of the culture; other peptides suppress growth and enhance the product yield (Table 1).

Further selection of the most active peptides and the understanding of their mode of action promise to be PROFITABLE to the efforts of intensifying biotechnological processes that provide therapeutic recombinant proteins or recombinant viruses.

The group enhancing both the viable cell density and the product yield includes natural and synthetic peptides derived from wheat gluten (**15**). However, the beneficial effects are not confined to peptides composed of natural L-isomers of amino acids. Examples presented in Table 1 document that a tripeptide formed by D-isomers and a peptide composed of beta amino acids are active in improving the culture parameters in a degree comparable to the effects of L-amino acid peptides.

A New Cell Culture Tool

Q: Can peptides accomplish whatever we want to do with animal cell culture? **A: An Optimist´s View:** Yes. It is only a matter of patience to select the correct peptide.

Our results yield clear evidence that peptides smaller than any known hormone represent a new class of agents enabling a broad spectrum of intervention in the cell processes. The number of sequence variants of tri- or tetrapeptides is huge. Only a very small fraction of this peptide population has been tested. Further selection of the most active peptides and understanding their mode of action promise to be profitable to the efforts of intensifying biotechnological processes that provide therapeutic recombinant proteins or recombinant viruses.

An argument may arise that synthetic peptides are too expensive for large-scale cultures. However, such a view takes into account the catalog prices of milligram or gram quantities of peptides. The cost of a selected tri- or tetrapeptide synthesized in quantities of hundreds of grams or kilograms will go down 20 to 50 times in comparison with current catalog prices.

The availability of pure growth- and/or production-promoting synthetic peptides would represent a challenge for novel media formulations, meeting the most severe criteria of biological safety. The presentation of a survival-signal to cultured cells will open the way to complex feeding strategies exploiting the extended lifespan of the cultures for continuing product synthesis.

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