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Gluten of Spelt Wheat (*Triticum aestivum* Subspecies *spelta*) as a Source of Peptides Promoting Viability and Product Yield of Mouse Hybridoma Cell Cultures

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The enzymic hydrolysate of gluten from spelt wheat (*Triticum aestivum* subsp. *spelta*), an ancient protein-rich wheat subspecies, was subjected to repeated chromatography runs on the small pore size exclusion chromatography matrix, Biogel P-2. Two small peptide fractions were purified by rechromatography. The amino acid analyses carried out upon total hydrolysis of these fractions have shown a very high proportion of glutamic acid/glutamine, leucine, and methionine. The biological activity of the peptide fractions was tested on a model hybridoma at a concentration range from 0.02 to 0.2%. The most striking effect of peptide fractions, apparent even at the lowest concentrations tested, was a significantly higher persistence of viable cells on day 6, i.e., at the decline phase of the cultures. Culture viability values in the presence of peptide fractions were 64−74%, in comparison with 56% in the control culture. The results of this work are consistent with the concept that peptide molecules may act as antiapoptotic agents, survival factors, rather than serving as metabolic substrates.

KEYWORDS: Spelt wheat (*Triticum aestivum* subsp. *spelta*); gluten; peptides; apoptosis; hybridoma

INTRODUCTION

Monoclonal antibodies (mAbs) destined either for therapeutic or for diagnostic purposes become one of the major products in biopharmaceutics. The availability of these hybridoma cell products, in quantities of kilograms or even tons, is dependent on the volumetric productivity achievable in bioreactors. The productivity can be positively influenced either by genetic manipulations with the production cell line or by medium additives modulating the cell metabolism in favor of enhanced and continuing proteosynthesis.

The biological safety of mAb preparations can be guaranteed only when the media used for hybridoma cells culturing are protein-free and free of animal-derived components. However, hybridoma cultures carried out in protein-free media are more sensitive to various kinds of stress than the cultures conducted in classical serum-containing media. Under suboptimal culture conditions, hybridoma cells are prone to undergo the process of programmed cell death called apoptosis. Apoptosis is a specific mode of cell suicide that reduces viable cell population. Conditions in the cultures deviate from the physiological range, for example, when nutrients decrease and toxic metabolites increase (*1*−*5*).

We have found that the onset of apoptotic death may be prevented, or at least postponed, when the cultures are supplemented with certain pure amino acids (*6*, *7*). Other authors who added crude peptide mixtures (peptones) to the culture media considered peptones as a source of amino acids needed for cell mass synthesis and for the synthesis of the secreted protein (*8*−*11*). However, a different view on the role of peptides emerged from our study in which we fractionated soy and wheat protein enzymic hydrolysates by chromatography. The cultures were stimulated by the addition of wheat gluten hydrolysate fractions, which were limiting in essential amino acids (*12*). Our results didn't support the metabolic hypothesis of the effect of the hydrolysates and led us to conclude that the peptides act as survival factors, which suppress the apoptotic death rate without being metabolized. In the presence of peptides, cells survive and function even at suboptimal culture conditions and continue to synthesize the desired product.

The reported series of experiments focused on the capability of gluten-derived peptides to prolong the time period during which hybridoma cells remain viable and continue to synthesize mAbs. We followed our previous study of the action of peptide fractions obtained by enzymic hydrolysis of common wheat (*Triticum aestivum*) gluten (*12*). In this work, we prepared analogous peptide fractions from spelt (*T. aestivum* subsp. *spelta*), a protein-rich ancient wheat subspecies. Spelt grains contain 16−18% protein. The present study reports on the fine resolution of spelt wheat gluten hydrolysate by chromatography, on the amino acid composition of the most active peptide fractions, and on the positive effects of the fractions on culture viability and on the increase in mAb yield.

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MATERIALS AND METHODS

Materials. All chemicals used were of analytical grade. Ultrafiltration membranes were from Millipore Ltd. (16900 Prague 6, Czech Republic). Biogel P-2 was from BIO-RAD Ltd. (14700 Prague 4, Czech Republic). Cell culture media and Pronase were from Sigma-Aldrich (18621 Prague 8, Czech Republic). The spelt wheat flour was obtained from PRO-BIO Ltd. (78832 Stare Mesto pod Sneznikem, Czech Republic).

Cell Culture. 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 (*7*), 15 mM HEPES, 2.0 g L^{-1} sodium bicarbonate, and the iron-rich growth-promoting mixture containing 0.4 mM ferric citrate (*2*). 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. The cultures were inoculated at a density of $(250 \pm 50) \times 10^3$ cells mL^{-1} and incubated until the decline phase, i.e., for 6 days.

Gluten Hydrolysate. Spelt wheat flour was first defatted with acetone at −5 °C. The extracted flour was extensively washed with water to remove starch and water soluble proteins. The water insoluble gluten was minced, suspended in deionized water, and hydrolyzed by treating with Pronase (0.2% original flour mass) under stirring for 2 h at 37 °C. The insoluble portion was removed by centrifugation, and the supernatant was subjected to successive ultrafiltration runs using polythersulfone membranes Biomax 30 000 NMWL and 5000 NMWL. The final ultrafiltrate was freeze-dried.

Liquid Chromatography. The spelt wheat gluten hydrolysates were fractionated by low-pressure chromatography on Biogel P-2 Fine equilibrated in 0.2% (v/v) formic acid. The column (11.5 cm × 50 cm) was loaded with 25 g of hydrolysate dissolved in 200 mL of 0.2% formic acid and run at the flow rate of 250 mL h^{-1}. The fractions were pooled according to the record of absorbance at 260 nm (Uvicord S, LKB, Sweden) and freeze-dried.

Determination of Amino Acid Composition. Enzymic hydrolysate fractions were subjected to total hydrolysis in 6 M hydrochloric acid for 20 h at 110 °C. For determination of tryptophan, hydrolysis in 6 M hydrochloric acid for 25 min at 160° was used. Aliquots for the determination of cystine content were oxidized by peracetic acid. Half-cystine was then determined as cysteic acid. The composition of total hydrolysates was determined on an automatic analyzer Biochrom 20 (Amersham Pharmacia Biotech, Freiburg, Germany). The experimental error involved in amino acid analyses was ±5%.

Assay of Hydrolysate Fractions Activity. The tested peptide fractions were added to the cultures as concentrated solutions in saline. Viable and apoptotic cells were counted in a hemocytometer. Apoptotic cells were identified by apoptotic morphology, i.e., shrunken cells with ruffled membranes, and were distinctive from the round-shaped live cells. No third type of cells, e.g., swollen necrotic cells, was seen in the static cultures used for the assays. A total of at least 500 cells were counted in each flask. The assays were conducted in triplicate. The experimental error involved in the estimation of cell density and viability was ±10%. The significance of differences from the control, at a 0.05 confidence level, was calculated using Student's *t*-test.

The concentration of mAb in the culture media was determined by immunoturbidimetry (*13*). Briefly, aliquots of media were diluted by 5% solution of poly(ethyleneglycol) and incubated for 1 h with porcine anti-mouse immunoglobulin affinity-purified antibody. The turbidity was measured at 340 nm. The mAb concentrations were determined using a calibration curve. The experimental error associated with the estimation of the mAb concentration was ±5%.

RESULTS

Fractionation of Gluten Enzymic Hydrolysate by Chromatography. The elution profile of the enzymic hydrolysate of spelt wheat (*T. aestivum* subsp. *spelta*) gluten proteins was characterized by multiple overlapping peaks, analogous to the earlier profile of common wheat (*T. aestivum*) gluten hydrolysate (*12*). A volume of the column effluent destined to further processing was selected on the basis of our previous experience with common wheat gluten hydrolysate. To obtain a21 (peptides formed by 2−10 amino acid residues), the most active fraction (*12*), the effluent was collected between 1.8 and 2.2 breakthrough volumes of the column. The collected volume was freeze-dried and rechromatographed. The elution curve of the a21 fraction displayed two main overlapping peaks. The preparation was subjected to repeated chromatography to obtain a211 and a212 subfractions (**Figure 1**). All hydrolysate fractions were stored as freeze-dried powders.

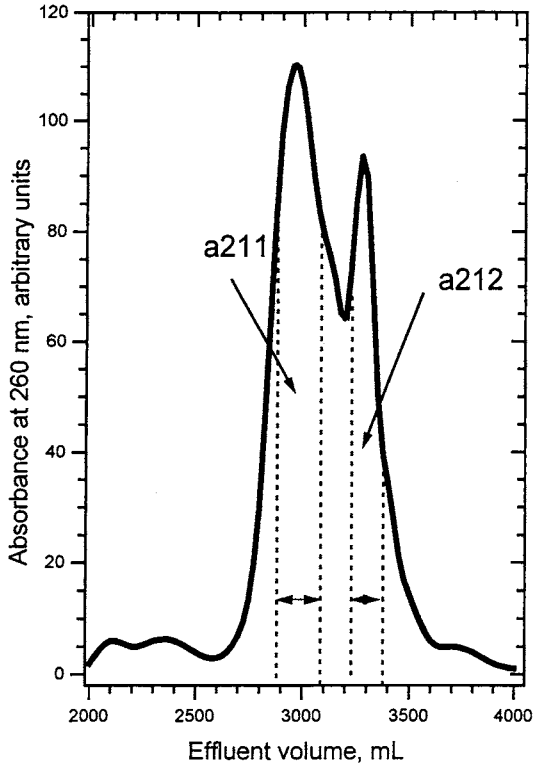

Figure 1. Rechromatography of the fraction a21 on Biogel P-2 fine. Column volume, 5200 mL; sample, 25 g. Collected subfractions a211 and a212 are marked by horizontal arrows.

Table 1. Amino Acid Composition of Hydrolysate Fractions[a]

amino acid	a21	a211	a212	amino acid	a21	a211	a212
Asp + Asn	3.8	3.4	4.7	Ile	6.3	6.5	3.8
Met	3.4	1.9	13.5	Leu	23.5	24.5	15.0
Thr	3.4	3.7	2.9	Tyr	1.1	1.0	2.5
Ser	7.9	7.8	8.0	Phe	1.1	1.0	2.8
Glu + Gln	21.4	21.2	26.9	His	1.6	2.0	0.6
Gly	5.5	5.8	4.4	Lys	0.6	0.6	0.4
Ala	5.8	6.0	2.8	Arg	3.3	3.4	2.3
Val	6.6	6.7	3.8	Pro	4.4	4.3	5.2
1/2 Cys	0.3	0.2	0.4	Trp	ND[b]	ND[b]	ND[b]

[a] Relative quantities (molar percent) obtained upon total hydrolysis of the fractions are given. [b] Not detectable.

Amino Acid Composition of Hydrolysate Fractions. The amino acid compositions of hydrolysate fractions were found to be distinctly unbalanced. In a21 and in a211, the values of glutamine/glutamic acid and of leucine exceeded markedly the values of other amino acids. In a212, a minor subfraction of a21, the dominating amino acids were glutamine/glutamic acid, leucine, and methionine (**Table 1**).

Effects of Hydrolysate Fractions on Cell Growth and mAb Production. Under standard culture conditions, the maximum viable cell density of control batch cultures, reached on day 3, was on average 1630×10^3 cell mL^{-1}. The average cell viability

Table 2. Parameters of Hybridoma Cultures in the Presence of Gluten Hydrolysate Fractions[a]

additive	viable cells ($\times 10^{-3}$ cell mL^{-1})	total cells ($\times 10^{-3}$ cell mL^{-1})	viability (%)	mAb (mg L^{-1})
none (control)	1180	2110	56	22
a21				
0.02%	1290	2020	64	28
0.05%	1480	2080	71	31
0.10%	1680	2330	72	35
0.20%	1810	2590	70	36
a211				
0.02%	1330	2050	65	31
0.05%	1580	2230	71	33
0.10%	1750	2500	70	35
0.20%	1840	2490	74	38
a212				
0.02%	1370	2010	68	29
0.05%	1510	2190	69	30
0.10%	1520	2050	74	32
0.20%	1420	1950	73	32

[a] Values on day 6 of the cultures are given.

was 92%. The changes of the culture parameters, caused by the addition of hydrolysate fractions, were insignificant when monitored on day 3. Marked differences were found on day 6, at the decline phase.

The fractions obtained by chromatography were tested at a final concentration range from 0.02 to 0.2% (w/v) (**Table 2**). The addition of the hydrolysate fractions resulted in an increase of cell density at the highest concentrations of the fractions and in an increase of mAb yield. However, the most striking effect of the peptide fractions addition was the increase in the viability values of the cultures, i.e., 64−74%, as compared to 56% in the control culture. The viability was significantly higher than the control, even at the lowest concentrations of the additives (Student's *t*-test, $P = 0.05$). The total cell density was found to be significantly higher only at 0.2 and 0.1% concentrations of a21 and a211. The highest cell density and mAb yield occurred at 0.2% a21 and a211 and at 0.1% a212.

DISCUSSION

Spelt wheat was selected as the source of gluten for the present work because its proteins content is nearly twice that in common wheat flour. The fraction of small peptides from the spelt wheat gluten hydrolysate was subjected to fine resolution and rechromatography on a size exclusion chromatography matrix. The effluent volumes of the subfractions separated on the Biogel P-2 column (see **Figure 1**) indicate that the molecules of the a212 subfraction are smaller than those in a211. In each subfraction, the sum of the dominant amino acids (glutamic acid/glutamine, leucine, and methionine) represented about 50 molar percent. The high content of glutamine residues in the peptide fractions evidently reflects the occurrence of glutamine-rich repetitive sequences that comprise the central part of the high molecular mass subunit of glutenin (*14*). Nevertheless, the rechromatographed subfractions a211 and a212 are not pure substances. High-performance liquid chromatography displays at least 10 components in each subfraction (data not shown).

If we assume that the molecular masses of peptides in the tested fractions are in the range of 500−1000 Da, the molar concentrations of the peptides at the lowest 0.02% concentration applied in this work would be lower than the concentration of free glutamine in the media. This approximate calculation lends more support to the view that intact peptide molecules are responsible for the antiapoptotic effect, rather than amino acids liberated from the peptides.

The conclusion on the survival−signal activity of intact peptides has also been supported by the results of our testing the activities of some synthetic oligopeptides. Tetramers of the nonessential amino acid alanine significantly enhanced hybridoma growth and mAb production, while monomeric alanine was without effect (*15*). Using a model of a synthetic peptide, we demonstrated that the effect of the peptide was associated with the presence of the intact peptide molecule in the culture media. The addition of a mixture of free amino acids, constituting the respective peptide, did not exert a comparable effect (*16*).

The results of our present study add weight to the concept that the primary positive effect of the peptides is the prolongation of the productive lifespan of the cultures through suppressing the suicidal tendencies inherent in hybridoma cell lines (*1*−*5*). The application of natural (*12*, this work) or synthetic (*15*, *16*) peptides is expected to facilitate the development of media formulations and feeding strategies resulting in a substantial increase in the mAb production.

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