Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth

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Abstract

The Baculovirus Expression Vector System has become widely used for the production of recombinant proteins for research and diagnostics. Serum-free culture media able to support high cell densities have been developed for the large scale culture of insect cells. While serum elimination aims at avoiding the risks associated with the introduction of an ill defined component of bovine origin, additives such as protein hydrolysates from animal sources are still used. An alternative could be the supplementation of culture media with protein hydrolysates derived from plants. In this study, we describe the replacement of lactalbumin hydrolysate with a laboratory produced hydrolysate of rapeseed proteins. Its effect on Sf9 cell growth kinetics, substrate consumption and by-product formation in low-serum or serum-free medium was evaluated. Cells were unable to grow in the presence of a rapeseed protein hydrolysate generated by PTN 3.0 Special® enzyme and containing only 24% of peptides under 1 kDa in size. On the other hand, serum-free medium supplementation with a rapeseed protein hydrolysate obtained with Orientase 90N® enzyme had a strong growth promoting effect, leading to a 60% increase in maximal cell density without affecting cell metabolism. This significant positive effect could be explained by the higher degree of hydrolysis of this digest, with 74% of peptides under 1 kDa in size.

Introduction

Insect cell culture technology, originally developed for the production of viral pesticides for agriculture, has become widely used for the production of recombinant proteins for research and diagnostics during the last 20 years. Indeed, the Baculovirus Expression Vector System (BEVS) allows the expression of large quantities of biologically active proteins (Altmann et al. 1999). For these reasons, performant culture media able to support high density suspension cultures of insect cells are required for the large-scale production of heterologous proteins. Medium development for the culture of lepidopteran cell lines started more than 40 years ago (Wyatt 1956). Hink (1970) replaced the

insect haemolymph previously used by fetal bovine serum, a cheaper source of growth factors, lipids and other components supporting cell growth. He also used lactalbumin hydrolysate and yeastolate as a source for amino acids and vitamins. Currently, conventional media formulations for the culture of lepidopteran cell lines, such as TC-100, TNM-FH or IPL-41, are usually supplemented with 10% of fetal bovine serum, at least at the laboratory scale.

For large-scale culture of insect cells, the use of serum-supplemented media presents several serious drawbacks including high cost, variation of quality from one lot to another, and potential introduction of pathogenic agents into the culture medium, e.g. viruses, mycoplasmas or prions. In the presence of

serum, the downstream processing of recombinant proteins is also made more difficult because of its high protein content. For these reasons, many studies have focused on the reduction or complete elimination of animal serum from insect cell culture media. Inlow et al. (1989) successfully developed a serum-free medium for the culture of Sf-9 cells, by addition of yeastolate and a complex lipid emulsion to IPL-41 basal medium. Further improvements led to the commercialisation of several serum-free media currently available for lepidopteran cell culture, such as ExCell401 (JRH Biosciences), Sf900II (Gibco) or EXTRA-SB5 (Eurobio).

Protein hydrolysates have been widely used for the replacement of fetal bovine serum in mammalian and insect cell cultures. They are generated by acid or enzymatic hydrolysis of animal tissues (tryptose broth, peptones), milk proteins (lactalbumin, casein), microbial or yeast biomass (bactopeptone, yeastolate), or, more recently, plant proteins (soy, rice, wheat). For example, Primatone RL, an enzymatic digest of meat, has been used as a low-cost supplement in serum-free media for mammalian cells (Schlaeger 1996) or insect cells (Schlaeger et al. 1993). These hydrolysates mainly provide stable amino acids in the form of oligopeptides, but also lipids, vitamins and other low molecular weight components to culture media. Although they have been used for several years, literature remains scarce on the kinetic behaviour of cells cultivated in the presence of such hydrolysates.

In the past few years, the risks associated with the use of animal-derived materials in culture media have been increasingly revealed. Not only the supplementation with fetal bovine serum is questioned, but also the use of animal protein hydrolysates or any component of animal origin due to the possible introduction of pathogenic agents in biopharmaceutical processes. The current trend to develop media devoid of any animal material has increased the interest in plant protein hydrolysates which have been added to culture media for several years, sometimes in combination with other protein hydrolysates. Indeed, a variety of plant protein hydrolysates produced from rice, soy, wheat, potato or pea are now commercially available, but soy source remains the most often used. For example, Vaughn and Fan (1991) proposed an insect cell medium based on IPL-41 containing yeastolate, Primatone and soy protein hydrolysate. Donaldson and Shuler (1998) used a serum-free

medium for the BTI-Tn5B1-4 insect cell lines containing yeastolate and soy protein hydrolysate, which led to comparable cell growth and recombinant protein production to that obtained in the commercial Excell 405 serum-free medium. Keen and Rapson (1995) developed a serum-free medium including soy protein hydrolysate for the large-scale culture of CHO cells. More recently, Heidemann et al. (2000) demonstrated that several other plant peptones confer nutritional benefit for the culture of BHK cells and increase their productivity. Moreover, Price et al. (1999) indicated that some vegetable hydrolysates are able to support the growth of insect cells. A more precise approach was also proposed by Franek et al. (2000) who generated soy and wheat peptide fractions by size-exclusion chromatography with a reduced content of non-peptidic components. They observed that the different peptide fractions did not equally support mouse hybridoma growth, demonstrating that the role of protein hydrolysates is not only nutritional and that some of the peptides may exert other specific effects on cultured cells. The same authors also focused recently on the study of synthetic peptide effect on hybridoma cell culture (Franek and Katinger 2002).

Rapeseed proteins represent a new potential raw-material source for animal cell culture. Indeed, the culture of rape is widely developed for the production of oil. After oil extraction, defatted rapeseed cake-cakes containing 30–45% of proteins are left and could be used as low-cost raw-material for the production of rapeseed protein hydrolysates. Today, these proteins are mainly used as food adjuvants. Nevertheless, the well-balanced amino acid composition of rapeseed proteins (Godon 1996) compared to other oilseed crops like soybean makes them especially interesting to be tested in animal cell culture. The first step to use this new raw-material is the production of the extract of rapeseed proteins followed by a well-controlled enzymatic hydrolysis. Schweizer (2002) recently studied the fractionation and identification of peptides generated from rapeseed protein hydrolysis, but the use of rapeseed proteins in cell culture has not been reported.

Therefore, the aim of the present study was to assess the ability of rapeseed proteins and peptides to support insect cell growth in low-serum or serum-free media by comparison with the bovine lactalbumin hydrolysate usually added in insect cell media. Therefore, we first characterized some laboratory produced

rapeseed protein hydrolysates, and then performed shake flask suspension cultures by using various medium combinations so as to compare kinetics of cell growth, substrate consumption and by-product formation.

Materials and methods

Cell culture and media

Sf9 cells were obtained from Gérard Devauchelle (Station de Pathologie Comparée, INRA-CNRS, St-Christol-lez-Alès, France). The cell line, previously adapted to grow in EXTRA medium (Eurobio) supplemented with 5% fetal calf serum (FCS), was firstly adapted to grow in the same medium with only 2% FCS. This medium usually contains 3.3 g l^{-1} lactalbumin hydrolysate, but this component was excluded in the experiments described in this paper. Medium containing 2% serum was referred to as SCM. The Sf9 cell line was also adapted to grow in EXTRA-SB5 serum-free medium (Eurobio). This was designated SFM+LH for serum-free medium containing lactalbumin hydrolysate. In some experiments, rapeseed protein concentrate (RC) or rapeseed protein hydrolysate (RH) was added to the serum-free medium (SFM+RC or SFM+RH). The rapeseed protein stock solutions have been added to the media at 8% (v/v).

Cells were cultured at 27 °C, in T75-flasks or 250 ml shake Erlenmeyer flasks with 50 ml working volume. T-flasks were used for routine maintenance, adaptation to modified media, and to grow cells used to seed shake flasks. Cells were passaged every 3 days, and for each modified medium tested, cells were subcultured at least six times in their new medium before kinetic studies were performed in shake flasks. For suspension cultures, 0.1% Pluronic-F68 (Life Technologies) was added to the medium to protect cells from shear forces. Agitation speed in shake flasks was 100 rpm.

Cell density was estimated with 10% accuracy using a haemocytometer and viability was measured by trypan blue exclusion. The maximal viable cell density (X_{max}) is the one observed in culture medium during the kinetic studies. The maximal specific growth rate (μ_{max}) is calculated from the slope of the logarithmic curve of the increasing viable cell density *versus* the culture time. Samples for substrate and by-product analysis were taken from the cultures

Table 1 Composition of the rapeseed cattle-cakes and protein concentrate (% w/w of total matter) (Schweizer 2002).

	Rapeseed cattle-cakes % (w/w)	Protein concentrate % (w/w)
Proteins	33	75
Fibres	40	8
Moisture	10	11
Lipids	7	4
Ash	7	1
Other	3	1

every 24 h, and supernatants were kept frozen until analysis.

Preparation of rapeseed protein concentrate and hydrolysate

Rapeseed cattle-cakes were obtained from Robbe-Novance (France). They were broken into meal (particles of diameter under 250 μm) with a grinder (Bioblock Retsch SM100). The preparation of rapeseed protein concentrate was then carried out in two steps. One-hundred g of defatted rapeseed meal were mixed with 1 l NaOH 0.2 M at pH 12.9 for 1.5 h with agitation. The supernatant was collected by centrifugation (3000 g, 10 min). Proteins in the supernatant were then precipitated by adjusting the pH to 4.5 with 6 N HCl. The precipitate was recovered by centrifugation (3000 g, 10 min), washed with deionised water and freeze-dried. The dried concentrate was stored at 4 °C. The stock solution used for media supplementation was obtained by dissolution of freeze-dried concentrate in deionised water to a concentration of 8 g l^{-1} followed by filter sterilization. The composition of the rapeseed cattle-cakes and protein concentrate was analysed by Schweizer (2002) and given in Table 1. The protein percentage (% w/w of total matter) is more than doubled in the concentrate.

The rapeseed protein hydrolysates were obtained by enzymatic hydrolysis of the protein concentrate. Two enzymes were used separately: Orientase 90N® (Quest Int.), produced by *Bacillus subtilis* and PTN 3.0 Special® (Novo Nordisk), extracted from pig pancreas. Hydrolysis was performed in a 2-l thermostated reactor containing rapeseed protein concentrate, enzyme, and deionised water to reach a final volume of 1 l. The temperature was controlled at 50 °C and pH was set at 8 for PTN 3.0 Special® enzyme and at 7 for Orientase 90N® by addition of 0.2 N NaOH. Agitation speed was 450 rpm. After 5 h,

Table 2 Hydrolysis degree, size distribution of peptides and free amino acids in rapeseed protein concentrate and hydrolysates obtained by action of Orientase 90N® or PTN 3.0 Special® (% w/w of soluble nitrogenous weight).

	Degree of hydrolysis	Free amino acids % (w/w)	Size <1 kDa % (w/w)	Size 1–10 kDa % (w/w)	Size >10 kDa % (w/w)
Rapeseed concentrate			9	17	74
Orientase 90N hydrolysate	32%	1.2	74	23	3
PTN 3.0 Special hydrolysate	13%	1.4	24	66	10

hydrolysis was stopped by heating the reaction mixture at 95 °C for 10 min. Rapeseed protein hydrolysates were stored at 4 °C after freeze-drying. Stock solutions used to supplement culture media were prepared by dissolution of 8 g l⁻¹ freeze-dried hydrolysates in deionised water and filter sterilization.

Analytical methods

The extent of proteolytic degradation was measured by means of the degree of hydrolysis (DH), i.e. the percentage of peptidic bonds cleaved during hydrolysis, according to the pH-stat method described by Adler-Nissen (1982). The volume of sodium hydroxide added during hydrolysis to maintain the pH at its set point is used to calculate the number of protons liberated during hydrolysis, which is linearly correlated to the number of peptidic bonds cleaved.

Size repartition of the peptides was evaluated by Size-Exclusion HPLC (BioCAD®700^E equipped with a U.V. detector, PerSeptive Biosystems), using a Superdex Peptide HR 10/30 column (Pharmacia Biotech). Elution was performed with acetonitrile/H₂O/trifluoroacetic acid (40/60/0.1, v/v/v). Peptides were detected at 214 nm.

The concentration of free amino acids in protein hydrolysates was measured by reverse phase HPLC using a HP 1090 Liquid Chromatograph (Hewlett Packard) equipped with a Hypersil column (H5C18 20R, Interchim). Amino acids were quantified as their ortho-phthalaldehyde (OPA) derivatives by automatic precolumn derivatization (Godel et al. 1992). Detection was performed with a U.V. detector at 338 nm. A commercial solution of amino acids (Sigma, AA-S-18) was used as a standard.

Glutamine was converted into glutamate with asparaginase (Roche, 102903) and glutamate was measured by means of an enzymatic method (Diffchamb, 139092). Lactate was also analysed with an enzymatic method (Sigma 735-10), and ammonium ions by

means of a selective electrode (Orion, Espoo, Finland).

Glucose, fructose, and sucrose were measured by ion-exchange HPLC with Maxima 825 system (Waters) equipped with a Sugar Pak column (Waters) and a refractive index detector, using water as the mobile phase. Trehalose, which had the same retention time than sucrose on the Sugar Pak Column, was therefore analysed separately by HPLC with a Chroma system (Biosystem) equipped with an ion-exchange/size exclusion Polypore H column (Brownlee Labs) and a refractometer detector, using 0.04 N sulphuric acid as the mobile phase.

Results

Composition of rapeseed protein hydrolysates

The two enzymes used for the hydrolysis of rapeseed proteins display different specificities. PTN 3.0 Special® is known to hydrolyse proteins at the residues of alkaline amino acids, such as arginine or lysine, whereas Orientase 90N® cuts proteins in a non-specific way. As the size and nature of the peptides generated by enzymatic hydrolysis may play an important role in their ability to support cell growth, it was important to compare the size profiles of the peptides obtained after digestion by the two enzymes. The intact rapeseed protein concentrate was also analysed to allow comparison with digested proteins. The size profiles of rapeseed protein concentrate and hydrolysates obtained by size-exclusion HPLC are given in Table 2 as percentage of the total soluble nitrogenous material.

The rapeseed protein concentrate contains 74% of soluble nitrogenous compounds larger than 10 kDa. This result was expected, as the concentrate has not been treated by the proteases. Some smaller peptides can be found as well, as 9% of the peptides are below

Figure 1. Growth kinetics of Sf9 cells cultured in 250 ml Erlenmeyer flasks in 2% serum-containing medium without lactalbumin hydrolysate (SCM) and in the same medium supplemented with 8% Orientase 90N® rapeseed protein hydrolysate (SCM+RH). (A) Viable cell density in SCM+RH (◆) or SCM (◇). (B) Viability in SCM+RH (—) or SCM (---).

Table 3 Maximal viable cell densities ($X_{\max}$) and maximal specific cell growth rate ($\mu_{\max}$) of suspension cultures of Sf9 cells in the different culture media.

	SCM	SCM+RH	SFM	SFM+LH	SFM+RC	SFM+RH
$X_{\max}$ (10^6 cells ml^{-1})	2.3	2.6	2.5	2.5	3.0	4.0
$\mu_{\max}$ (h^{-1})	0.023	0.032	0.018	0.025	0.013	0.023

Figure 2. Metabolism of Sf9 cells grown in 250 ml Erlenmeyer flasks in SCM+RH. (A) Glucose (◆), fructose (▼), sucrose (□), lactate (◇). (B) Glutamine (●).

1 kDa in size. These oligopeptides of less than 10 amino acids could result from the process of protein extraction from the defatted rapeseed meal. The hydrolysate obtained after treatment by the PTN 3.0 Special® enzyme contains a majority of peptides between 1 and 10 kDa in size, with only 24% of peptides under 1 kDa. Polypeptides over 10 kDa can still be found in this hydrolysate. The degree of hydrolysis is only 13% after a 5-h hydrolysis. The specificity of PTN 3.0 Special® is probably the explanation for this rather poor digestion. The highest degree of hydrolysis was obtained with Orientase 90N®. The major portion of the soluble peptides has

sizes under 1 kDa, corresponding to peptides of less than 10 amino acids. Both hydrolysates contain less than 1.5% free amino acids and present 80% solubility of nitrogenous material.

Effect of rapeseed protein hydrolysate in serum-containing medium

The effect of rapeseed protein hydrolysate produced by enzymatic digestion with Orientase 90N® was studied in 2% FCS-containing medium (SCM) in 250 ml shake flasks. The addition of rapeseed protein hydrolysate only slightly enhances Sf9 cell growth in

Figure 3. Growth kinetics of Sf9 cells cultured in 250 ml Erlenmeyer flasks in serum-free media. (A) Viable cell density in serum-free medium with (SFM+LH, ●) or without (SFM, ▽) 3.3 g/l lactalbumin hydrolysate. (B) Viable cell density in SFM (▽), in SFM supplemented with rapeseed protein hydrolysate (SFM+RH, ◆) or with rapeseed concentrate (SFM+RC, ○). (C) Viability in SFM (---) and SFM+LH (—). (D) Viability in SFM (---), SFM+RH (—) and SFM+RC (···).

serum-containing medium (Figure 1A). The maximal viable cell density is 2.6×10^6 cells ml^{-1} instead of 2.3×10^6 cells ml^{-1} in the control. Nevertheless, in medium supplemented with rapeseed protein hydrolysate, viable cells reach their maximal density 50 h before the control culture, showing a higher maximal specific growth rate in the presence of this hydrolysate (Table 3). However, the viability of the culture in SCM+RH decreases before the viability of the culture control, probably because of faster depletion of nutrients (Figure 1B).

Carbohydrate and glutamine consumption, as well as lactate and ammonia production are similar in the two cultures. For this reason, only the kinetic profiles corresponding to the culture in SCM+RH are presented in Figure 2. Glucose, with an initial concentration of 1.2 g l^{-1} , is exhausted from the culture medium after 100 h. When glucose is becoming limiting, cells start to use fructose and sucrose as carbon and energy sources. Fructose is depleted quickly after glucose. Sucrose concentration slowly decreases from its initial level of 2.5 to 1.8 g l^{-1} at the end of the experiment. No accumulation of lactate can be observed. Glutamine is not exhausted until the end of the culture, but like sucrose, its consumption rate is steady even during the decline phase. Ammonium

ions are not shown in this graph, as their concentration stays close to zero during the whole experiment.

Effect of rapeseed proteins in serum-free cultures

The effect of rapeseed proteins is expected to be more pronounced in the absence of FCS. Therefore, we studied the growth-promoting properties of rapeseed protein concentrate and hydrolysate in serum-free medium, where the bovine lactalbumin hydrolysate was used as a control.

The serum-free medium used in this study is usually supplemented with 3.3 g l^{-1} lactalbumin hydrolysate from bovine milk. Our first objective was to examine the effect of this supplementation on the cell growth. After the cell adaptation, as described in materials and methods part, we compared Sf9 cell growth in suspension cultures performed in 250 ml shake flasks with and without lactalbumin hydrolysate. As shown in Figure 3A, the addition of lactalbumin hydrolysate does not strongly affect Sf9 cell growth in serum-free medium. The maximal viable cell density is very similar in SFM and SFM+LH medium. Nevertheless, elimination of lactalbumin hydrolysate seems to decrease the cell growth rate

Figure 4. Metabolism of Sf9 cells grown in 250 ml Erlenmeyer flasks in serum-free media. (A) Carbohydrate and lactate evolution in SFM+RH: glucose (●), sucrose (□), fructose (△), trehalose (▼) and lactate (◆). (B) Glutamine consumption in SFM (□), SFM+RH (●) and SFM+RC (▼).

(Table 3). Indeed, maximal cell density is reached almost 100 h later in the absence of lactalbumin hydrolysate. Moreover, while the maximal viable cell density is maintained for more than 200 h in SFM+LH, viable cells remain at their higher level for a shorter period of time without lactalbumin hydrolysate. The decrease in viability is simultaneous in the two cultures (Figure 3C).

Since rapeseed protein concentrate contains about 30% of non-protein components (detailed in Table 1), it was important to first determine if it could exert toxic effects on Sf9 cultures as a result of the presence of some non-peptide substances. After cell adaptation, parallel culture experiments were performed in 250 ml shake flasks with SFM and SFM+RC. Kinetics of viable cell density and viability are reported in Figure 3B and D. Maximal cell density is higher in SFM supplemented with the rapeseed protein concentrate than in the reference medium, with 3.0×10^6 cells ml⁻¹ instead of 2.5×10^6 cells ml⁻¹ in the control. The positive effect of rapeseed protein concentrate on maximal cell density appears to be stronger than that of lactalbumin hydrolysate (Figure 3A). On the other hand, the maximal specific rate of cell growth is lower in SFM+RC medium where the maximal cell density is reached after 300 h only (Table 3). These observations clearly suggest that, even if there may be substances present to inhibit cell growth rate, rapeseed protein concentrate is essentially non-cytotoxic. However, the increased maximal cell density points out the fact that the concentrate might provide the cells with additional substrates that could be limiting in the reference medium.

Based on these encouraging results, the next experiments have been performed with the protein hy-

drolysates. As previously, the first step concerned the Sf9 cell adaptation to grow in serum-free medium (SFM) supplemented with 8% (v/v) rapeseed hydrolysates produced by action of Orientase 90N® or PTN 3N Special®. While cells adapted easily to the medium containing the protein hydrolysate produced with Orientase 90N®, they were unable to adapt to the medium supplemented with PTN 3N Special® hydrolysate, which rapidly led to cell death. This observation was unexpected, as Sf9 cells were able to adapt to the medium supplemented with rapeseed protein concentrate, which suggested the absence of toxic components in the raw-material. The cell death may have been caused by one or more specific peptides generated during hydrolysis which have cytotoxic effects, or by certain compounds present in the PTN 3N Special® enzyme mixture. Rapeseed protein hydrolysate produced with PTN 3N Special® enzyme was therefore not tested as a medium supplement in suspension cultures.

Cell growth and viability in SFM and SFM supplemented with Orientase 90N® hydrolysate (SFM+RH) were then compared in suspension cultures of Sf9 cells in 250 ml shake flasks (Figure 3B and D). The supplementation with rapeseed protein hydrolysate obtained by digestion with Orientase 90N® shows a striking effect on cell growth. Maximal cell density is increased by 60% in comparison with the reference medium, reaching 4.0×10^6 cells ml⁻¹ after 200 h. This effect is far greater than that of lactalbumin hydrolysate (Figure 3A) and rapeseed protein concentrate (Figure 3B). Moreover, the maximal specific rate of cell growth is higher in SFM+RH (Table 3), which results in a more rapid decrease in viability due perhaps to the faster nutrient depletion.

The evolution with time of carbohydrate, glutamine, lactate and ammonia concentrations was followed during the growth of Sf9 cells in serum-free medium supplemented with or without rapeseed protein concentrate or hydrolysate. No difference in carbohydrate and lactate metabolism could be detected between the cultures in SFM, SFM+RC or SFM+RH media. For this reason, the evolution of sugars and lactate has been represented only for the culture in SFM+RH (Figure 4A). The serum-free medium was found to initially contain 1.2 g l^{-1} trehalose in addition to glucose, fructose and sucrose. This sugar is present in large quantity in lepidopteran hemolymph and then can be metabolised by insect cells. As expected, Sf9 cells quickly consume trehalose, and its depletion occurs at 200 h. Glucose consumption is also fast, but due to its higher initial concentration of 3 g l^{-1} , its exhaustion occurs later, after 250 h. At this time, viable cell concentration has already started to decrease for 50 h (Figure 3B). After glucose depletion, a slight consumption of sucrose is observed from 2.5 to 2 g l^{-1} at the end of the culture, whereas fructose is completely consumed in the next 50 h. Lactate concentration remains very weak all along cell growth and death.

Glutamine concentration evolution has been represented for the cultures performed in SFM, SFM+RC and SFM+RH (Figure 4B). Initial concentration of glutamine is slightly higher in SFM+RC, showing that the protein concentrate could provide free glutamine to the culture medium. The same phenomenon is not observed with the protein hydrolysate, but free glutamine, which is known to be unstable, is probably degraded at the end of the hydrolysis procedure when the reaction mixture is heated at 95°C for 10 min to stop the reaction. No limitation in glutamine is observed in either culture, as shown by its final concentration. But, as already observed in serum-containing media, its consumption is maintained at a significant rate even when the cell density becomes very low. Ammonia was not represented as its concentration stayed close to zero in all three cultures.

Discussion

The main objective of this work was to test the ability of rapeseed raw-material to be used as protein supplement in insect cell cultures. We performed kinetic

studies with suspension cultures of cells in serum-containing and serum-free media supplemented with either rapeseed protein concentrate or protein hydrolysates, and compared the cell growth performances with those obtained with bovine lactalbumin hydrolysate as supplement.

A rapeseed concentrate containing more than 70% proteins was hydrolysed using two enzymes with different specificities, from bacteria or pig pancreas origins. The size profiles of the peptides in the preparations indicated a better hydrolysis degree when the digestion was performed with Orientase 90N®. In this case, 74% soluble peptides of less than 10 amino-acids could be obtained. This demonstrates that the choice of the enzyme used for the protein degradation is of great importance because different proteases can not only generate different hydrolysis degrees but also different peptide structures.

The prerequisite for this new plant material to be considered for addition in animal cell culture media is the absence of any cytotoxic effect, which could mainly result from non-proteic substances. A very low cell growth rate was observed when cells were not previously adapted to the rapeseed concentrate (data not shown), but a short adaptation of five subcultures allowed the cells to grow very well in presence of the concentrate even in absence of fetal calf serum, demonstrating the absence of toxic effects. Moreover, the concentrate was shown to improve the maximal cell density, although if a slight reduction of cell specific growth rate suggested the presence of low inhibitory substances.

However, cells were unable to adapt to grow in the presence of PTN 3.0 Special® digested rapeseed proteins. This effect could not be attributable to toxic effects of non-protein components of the raw material since they would have been observed with the protein concentrate. For the same reasons, this effect was probably not due to the poor digestion of the protein. It is likely induced by the size profile of obtained peptides. In addition, some cytotoxins may be induced by non-identified components provided by the enzyme solution initially from pig pancreas. Due to the animal origin of this enzyme it is perhaps not suited to use it during preparation of plant protein hydrolysates. An important variability of growth-promoting properties of peptones from different origins or with different peptide composition has been reported in several studies (Jan et al. 1994; Franek et al. 2000, Franek and Katinger 2002). Unfortunately, the en-

zymes used for protein hydrolysis are scarcely specified while this information could be of great interest. Franek and Katinger (2002) studied the effect of synthetic oligopeptides on cultured hybridoma, and showed that oligoglycine promotes cell growth whereas other peptides suppress growth and enhance antibody production. This study clearly demonstrates that some specific peptides can be inhibitory for cell growth, but no lethal effects have been reported to our knowledge.

A significant promoting effect on the cell growth was clearly observed when the Orientase 90N® rapeseed protein hydrolysate was added to serum-free medium. In this case, maximal cell density and cell specific growth rate were increased by a factor of 1.6 and 1.3, respectively. Moreover, the effect on maximal cell density was much more pronounced than that observed with supplementation by lactalbumin hydrolysate, which did not increase the maximal cell density but only allowed a longer plateau at the highest cell concentration. These results pointed out the interest of this new plant peptide source, which could advantageously replace the bovine supplements such as fetal calf serum or bovine lactalbumin hydrolysate, and then reduce the risk of pathogenic agents from animal origin. However, when used in 2% fetal calf serum supplemented medium, the Orientase 90N® hydrolysate showed a much less significant effect. Apparently the fetal calf serum was more efficient as a growth-promoter than rapeseed protein hydrolysate in this case, but with the objective to minimize animal origin components in animal cell culture, the efforts have clearly to be focused on serum-free media.

These good preliminary results on insect cell growth with the hydrolysate obtained from Orientase 90N® action could be attributed to its high content in small size peptides, as 74% of the peptides have molecular sizes under 1 kDa. Indeed the degree of digestion of the proteins and the size of the generated peptides probably play a crucial role in the growth-promoting properties of protein hydrolysates. The ability of cultured animal cells to metabolise dipeptides like glycyl-L-glutamine and L-alanyl-L-glutamine has been reported by Minamoto et al. (1991). Grimble et al. (1987) showed that intestinal cells more easily use di- or tri-peptides than larger peptides. Moreover, Franek and Katinger (2002) showed that tri-, tetra- and penta synthetic peptides have greater effects on hybridoma growth than larger

peptides. A more detailed peptide composition of our rapeseed protein hydrolysate will enable us to better understand the nature of the components supporting cell growth, using fractionation.

On the other hand, we observed that the supplementation of culture medium with rapeseed protein hydrolysate or concentrate did not induce any significant changes in cell metabolism. Carbohydrate consumption and lactate concentration were identical in the different media tested and no more ammonium ions appeared when protein hydrolysate was added. Analyses of the free amino-acids at the end of culture showed that most amino acids were not exhausted from serum-free media with or without rapeseed hydrolysate. Thus it could be concluded that the very low level of free amino acids contained in the protein hydrolysate were probably not responsible for the 60% increase in maximal cell density. It is likely that the growth promoting effect of rapeseed protein hydrolysate is mainly due to its peptides. This suggests that the cells have a preference for the peptides to free amino acids. Bonarius et al. (1996) noticed that medium supplementation with Primatone RL was more efficient than free amino-acid addition to support hybridoma cell growth. While this observation could be linked to an amino acid balance in Primatone RL more adapted for this specific cell line, the authors also thought that peptide transport into the cell is more energy efficient than free amino-acid transport.

Moreover, it has recently became clear that the role of peptones is not only nutritional. Schlaeger (1996) indicated that Primatone RL displays anti-apoptotic properties in mammalian cell cultures, outlining a non-nutritional property of this protein hydrolysate. In another study, Franek et al. (2000) tested different peptide fractions of soy protein and wheat gluten hydrolysates obtained by size-exclusion chromatography. Some fractions were found to be more efficient than the whole hydrolysate to improve hybridoma cell growth and immunoglobulin production, and the peptide fractions tested varied significantly in their growth-promoting effects. The authors concluded that some peptides exert very specific effects and might mimic growth-promoting or survival factors. By studying the effect of synthetic peptides on cultured hybridomas the same authors suggested that peptides of different length and amino acid composition can either enhance cell growth and increase viability or suppress cell growth.

In conclusion, our kinetic results demonstrate the

ability of a rapeseed protein hydrolysate produced by Orientase 90N® digestion to enhance Sf9 cell growth in serum-free medium more effectively than bovine lactalbumin hydrolysate without affecting the general metabolism of the cells. It is the first time that this plant source is reported as a potential non-animal supplement in animal cell culture. This preliminary study opens a number of perspectives for future studies. A comparison with other plant peptones commercially available will be performed and the rapeseed protein hydrolysate will be tested with other animal cell lines including mammalian cells. Further studies will be needed to better understand the nature of the growth-promoting activity of rapeseed protein hydrolysates as it is not clear at the moment if the positive effect on cell growth is a result of peptide composition, peptide length, or general amino acid balance of rapeseed proteins. We will focus on the separation and identification of peptides in different fractions to more accurately evaluate the peptides implicated in growth enhancement. The peptide transport and metabolism in cultured cells still remains questionable. Described in the literature mainly for intestinal cells, how *in vitro* cultivated cells use peptides as nutrients, anti-apoptotic factors or other property effectors remains to be better understood. To definitely validate this new plant source, it will also be necessary to better characterize cell markers after long-term cultivation in presence of rapeseed peptides. Some preliminary results have already demonstrated that the addition of the rapeseed peptides does not compromise the infection of Sf9 cells by a baculovirus neither the production of a recombinant protein (data not shown). Additional studies are still required to check the biological quality of the produced recombinant proteins.

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