ORIGINAL RESEARCH

Chickpea protein hydrolysate as a substitute for serum in cell culture

Julio Girón-Calle · Javier Vioque · Justo Pedroche · Manuel Alaiz · María M. Yust · Cristina Megías · Francisco Millán

Abstract The growth of mammalian cells in vitro requires the use of rich culture media that are prepared by combining serum with specific nutrient formulations. Serum, the most expensive component of culture media, provides a complex mixture of growth factors and nutrients. Protein hydrolysates that can support in vitro cell growth and eliminate or reduce the need to use serum have been obtained from different sources. Here we describe the use of two food grade proteases to produce a chickpea protein hydrolysate that has been added to cell culture medium in order to determine whether it can be used as a substitute for serum. Medium containing the hydrolysate has been tested using two human cells lines: the monocytic THP-1 cell line which grows in suspension, and the epithelial Caco-2 cell line which grows as a monolayer. The chickpea protein hydrolysate was a good substitute for serum in the first case, but did not allow growth of Caco-2 cells. Supplementation of culture media with this inexpensive and safe hydrolysate would greatly reduce the cost of cell culture.

J. Girón-Calle (✉) · J. Vioque · J. Pedroche ·
M. Alaiz · M. M. Yust · C. Megías · F. Millán
Instituto de la Grasa (Consejo Superior de Investigaciones
Científicas), Avenida Padre García Tejero, 4,
41012 Sevilla, Spain
e-mail: jgiron@cica.es

Introduction

Complex nutrient mixtures are needed in order to grow mammalian cells in vitro. Culture media are usually prepared by combining a mixture of nutrients including salts, amino acids, vitamins, glucose, and serum (Keenan et al. 2006). Serum, most frequently fetal bovine serum (FBS), is the most expensive component in cell culture media, and sustains growth by providing a mixture of growth factors and nutrients which are not always well characterized and/or too expensive in pure form. Other concerns with the use of serum are the changing composition of different serum batches, and the need to address special safety measures to avoid animal transmitted diseases. In order to address these concerns and limitations, serum-free and low-serum culture media have been developed. Some of these media include natural extracts that may be of animal origin, while others have a well defined composition (Ballez et al. 2004; Burteau et al. 2003; Schlaeger 1996; Taylor et al. 1972a; Taylor et al. 1972b). Arguably, media with a well defined composition and free of any serum or natural extract are the best choice for culturing cells, specially for research purposes, but these media are expensive and their use is not widespread.

Protein isolates are used in the elaboration of many food products in order to improve their functional and/ or nutritional properties. Protein hydrolysates have the additional advantage of having improved functional properties as compared to the original protein isolates from which they are prepared (Frokjaer 1994; Vioque et al. 2001). More recently, potential health-promoting properties of peptides in these hydrolysates have been discovered, including effects on cell growth (Kitts 1994; Yoshikawa et al. 2000). Chickpea seeds are an inexpensive source of high quality protein, and procedures have been developed for the production of protein isolates and hydrolysates from these seeds using food-grade microbial enzyme preparations (Sánchez-Vioque et al. 1999). The goal of this work was to investigate cell proliferation under exposure to chickpea protein hydrolysates in order to determine whether these hydrolysates can be used to sustain cell growth as a complete or partial replacement for serum. Chickpea hydrolysates were obtained by treating protein isolates with two food-grade microbial enzyme preparations, alcalase and flavourzyme. Caco-2 epithelial cells and THP-1 monocytic cells were used as cell models. Cell growth was correlated with the degree of hydrolysis of the chickpea hydrolysates, and long-term growth experiments using the most promising conditions were carried out.

Materials and methods

Materials

Kabuli type chickpea (*Cicer arietinum*) was purchased in a local market. Alcalase 2.4 L FG and Flavourzyme were a gift of Novozymes (Bagsvaerd, Denmark). PD-10 Sephadex G25 columns were purchased from Amersham (Uppsala, Sweden). 2,4,6-Trinitrobencensulfonic acid, 2,5-diphenyltetrazolium bromide (MTT), neutral red, and Trypan blue were from Sigma (St. Louis, MO). Tissue culture media, serum and other reagents for cell culture were from GIBCO (Barcelona, Spain). Filters (glass fiber, 0.45 and 0.22 μm) were purchased from Millipore (Bedford, MA).

Preparation of protein isolates and hydrolysates

Chickpea protein isolates were prepared by extraction in water pH 10.5 (10% w/v), precipitation at the isoelectric point pH 4.3, and washing with water (Sánchez-Vioque et al. 1999). Additional washes (2 mL/g original flour) were carried out using acetone (one wash), diethyl ether (two washes), ethanol (five washes), and water (two washes) by vigorous shaking and centrifugation at 3,000g for 10 min. The resulting isolates were lyophilized.

Hydrolysis was carried out at 50 °C under vigorous stirring and a constant pH of 8.0 for hydrolysis with alcalase and pH 7.0 for hydrolysis with flavourzyme (Clemente et al. 1999b; Pedroche et al. 2002). Isolates were suspended in distilled water (2% w/v), and alcalase (0.03 Anson Units/g of isolate) and flavourzyme (5 Leucine Aminopeptidase Units/g of isolate) were added immediately and 75 min later, respectively. Alcalase and flavourzyme had been previously pre-cleaned using PD-10 Sephadex G25 columns in order to eliminate preservatives and other low molecular weight components that could be present in these commercial preparations. Temperature was kept at 50 °C throughout the whole process and pH was adjusted by addition of NaOH or HCl as necessary. Hydrolysis was stopped by inactivation of the proteases by heating at 95 °C for 20 min. The resulting suspensions were adjusted to pH 7 if needed and clarified by centrifugation at 3,000g for 10 min. The supernatants were filtered through glass fiber, 0.45 and 0.22 μm nitrocellulose filters before they were added to cell growth-media.

Characterization of isolates and hydrolysates

Protein content was calculated by multiplying nitrogen content, determined using an elemental analyzer, by 6.25. Minor components (moisture, ash, fiber, carbohydrates, polyphenols, and lipids) were determined as described (Sánchez-Vioque et al. 1999). The degree of hydrolysis (DH), defined as the percentage of covalent bonds that are broken during hydrolysis, was determined according to Adler-Nissen (1979) by reaction of free amino groups with 2,4,6-trinitrobenzensulfonic acid. Hydrolysis of covalent bonds in proteins or peptides leads to formation of free amino groups. The number of free amino groups was referred to the total number of amino groups as determined in samples subjected to complete chemical hydrolysis (HCl 6 N, 100 °C, 24 h). Amino acid analysis was carried as described (Alaiz et al. 1992).

Cell culture and treatment

Cells were kept at 5% (v/v) CO_2 in Dulbeco's Modified Eagle Medium (1,000 mg/L glucose, 110 mg/L pyruvate, and 580 mg/L glutamine) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) MEM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. For routine maintenance THP-1 cells were subcultured every 2–3 days by resuspension in fresh medium. Caco-2 cells were subcultured once a week by standard trypsinization, and medium was renewed once in between passages. Experiments were initiated by replacing the medium with fresh medium containing the hydrolysates to be tested and 0, 0.5 or 10% (v/v) FBS. Cells were not pre-conditioned or adapted for growth in the absence of serum.

Determination of cell proliferation and viability

2,5-Diphenyltetrazolium bromide (MTT) viability assay

Cells were grown and treated in 96-well plates. Cells were incubated for 1 h in the usual culture conditions after addition of the same volume of medium containing MTT (1 mg/mL). After this incubation, 100 µL HCl (0.1 N) in isopropanol were added to dissolve the blue formazan crystals formed by reduction of MTT (Kops et al. 1997). Absorbance at 570 nm using a background reference wavelength of 630 nm was measured using a dual-wavelength plate reader.

Neutral red viability assay

Cells in 96-well plates were incubated in fresh culture medium containing the vital stain neutral red (50 µg/mL) for 30 min. Cells were then washed using phosphate buffer saline and the stain was dissolved using 75 µL acetic acid (1% v/v) in ethanol (50% v/v in water) before determination of absorbance at 540 nm using a plate reader (Karczewski et al. 1999).

Trypan blue staining

Trypan blue was added to cells in suspension (10% v/v of a stock consisting of 0.4% w/v Trypan blue in phosphate buffer saline), and a counting chamber was used for determination of viable cells at the microscope.

Results

Hydrolysis of chickpea proteins using alcalase and flavourzyme

Protein isolates were prepared by extraction of chickpea flour at alkaline pH and precipitation of protein at the isoelectric pH followed by washes with ethanol, diethyl ether and acetone. The resulting isolates had 98% protein (w/w), the remaining weight being moisture and fiber 1% (w/w) each. Carrying out hydrolysis with alcalase and flavourzyme results in degrees of hydrolysis that can not be reached by using any of these enzymes alone (Clemente et al. 1999b). Figure 1 shows the effect on the degree of hydrolysis of treatment with alcalase for 75 min followed by treatment with flavourzyme for 105 min. Subtilisin A, an endoproteinase from *Bacillus licheniformis*, is the main component of alcalase while flavourzyme is a mixture of fungal proteases and peptidases. Treatment with alcalase caused a rapid increase of the degree of hydrolysis, which reached a value of 15% after 75 min. Further treatment with flavourzyme approximately doubled the degree of hydrolysis in the same time. Hydrolysis with alcalase and flavourzyme did not significantly change the amino acid composition as compared with the original protein isolate and with other chickpea hydrolysates previously described (Clemente et al. 1999b).
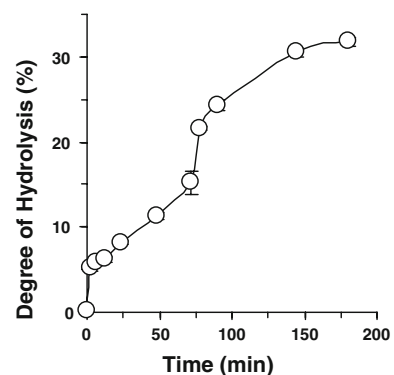

Fig. 1 Treatment of chickpea isolates with alcalase and flavourzyme. Alcalase was added at time 0 and flavourzyme 75 min later. The degree of hydrolysis was determined by reaction with 2,4,6-trinitrobenzensulfonic acid after heat-inactivation of the enzymes and removal of insoluble material by centrifugation. Data are averages ± standard deviation of three determinations

Effect of chickpea hydrolysates with increasing degree of hydrolysis on cell growth

The possibility that chickpea hydrolysates obtained by treatment with alcalase and flavourzyme could support cell growth in serum-free or low-serum media was explored using Caco-2 and THP-1 cells that were seeded in media containing 10% (v/v) hydrolysates of increasing degree of hydrolysis in addition to 0, 0.5 or 10% (v/v) FBS. The anchorage-dependent Caco-2 cells were grown for up to four days (Fig. 2), and the faster growing THP-1 cells, which grow in suspension, were cultured for up to three days (Fig. 3), which corresponds to the end of the exponential growth phase in standard conditions.

As expected, the growth of cells was influenced by the concentration of FBS in the medium. Thus, Caco-2 cells hardly grew in the absence of serum, and a normal growth was only sustained by medium containing 10% (v/v) FBS as routinely used for cell culture. The growth of cells cultured in the presence of 0.5% (v/v) FBS was limited but better than in the absence of serum (Fig. 4, see cell growth for 0% (v/v) hydrolysate and 0, 0.5 and 10% (v/v) FBS). Addition of hydrolysate enhanced or inhibited cell proliferation depending primarily on the degree of hydrolysis, but also on FBS concentration and the duration of culture (Fig. 2). Most prominently, supplementation with the 15% DH hydrolysate together with 0.5% (v/v) FBS increased proliferation of Caco-2 cells grown for 2 days to about twice the control cell number corresponding to cells which were supplemented with the non-hydrolyzed isolate, although this effect dissipated at longer culture times. Thus, supplementation with hydrolysates of any degree of hydrolysis in addition to 0.5% (v/v) FBS inhibited the growth of cells cultured for 3 or 4 days. Supplementation with the 32% DH hydrolysate and 10% (v/v) FBS inhibited proliferation of Caco-2 cells grown for 2 to 4 days.

As expected, the proliferation of THP-1 cells was also limited by serum concentration (Fig. 5, see cell growth for 0% (v/v) hydrolysate and 0, 0.5, and 10% (v/v) FBS). Addition of chickpea hydrolysates had effects similar to those observed in Caco-2 cells. Thus, certain hydrolysates inhibited growth in some cases, while media supplemented using hydrolysates with higher degree of hydrolysis (20% and higher) were able to sustain growth at higher rates than media alone at least at some time points (Fig. 3). In general,
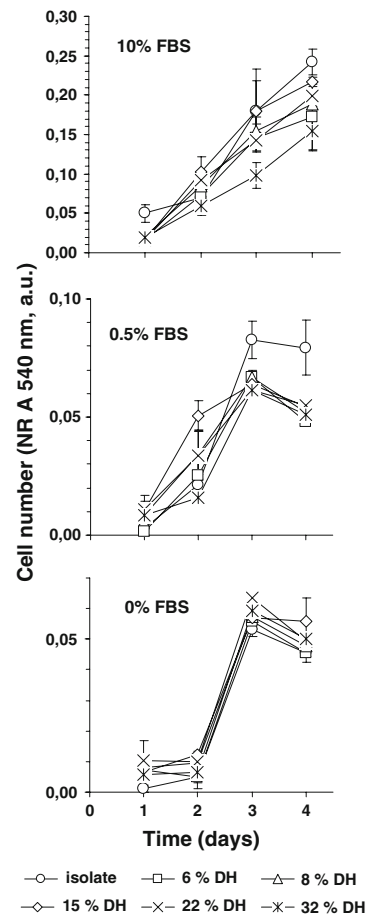

Fig. 2 Proliferation of Caco-2 cells cultured in the presence of chickpea non-hydrolyzed isolate (0% degree of hydrolysis) and hydrolysates of increasing degree of hydrolysis (6–32%). Cells (5×10^3 cells/well) were cultured in 96-well plates in culture medium containing 0, 0.5 or 10% (v/v) FBS, and 10% (v/v) hydrolysate. Cell number was estimated after incubating for 1–4 days by measuring uptake of the vital stain neutral red by the cells. Data represent average ± standard deviation of three incubations

the effect of chickpea hydrolysates on growth was more apparent in cells grown in media containing 0 or 0.5% (v/v) FBS than in cells grown in media containing 10% (v/v) FBS.

Effect of different concentrations of hydrolysate on cell proliferation

In order to further characterize the positive effects on growth, cells were exposed to different concentrations of a hydrolysate with a 22% degree of hydrolysis, which is within the range that stimulated
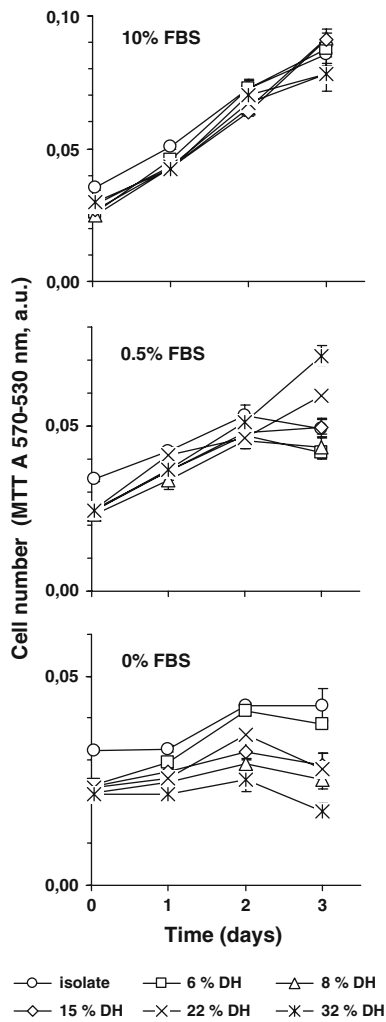
Fig. 3 Proliferation of THP-1 cells cultured in the presence of chickpea non-hydrolyzed isolate (0% degree of hydrolysis) and hydrolysates of increasing degree of hydrolysis. Cells (5×10^3 cells/well) were cultured in 96-well plates in culture medium containing 0, 0.5 or 10% (v/v) FBS, and 10% (v/v) hydrolysate. Cell number was estimated after incubating for 1, 24, 48, and 72 h by measuring reduction of MTT. Data represent average ± standard deviation of three incubations

Fig. 4 Proliferation of Caco-2 cells cultured in the presence of increasing concentrations of a hydrolysate with a 22% degree of hydrolysis. Cells (5×10^3 cells/well) were cultured in 96-well plates in culture medium containing 0, 0.5 or 10% (v/v) FBS, and 0–10% (v/v) hydrolysate. Cell number was estimated after incubating for 1, 2, 3, and 9 days by measuring uptake of the vital stain neutral red. Data represent average ± standard deviation of three incubations

growth under some circumstances in both cell lines. Caco-2 and THP-1 cells were cultured for up to 9 and 7 days, respectively. The results of these studies are shown in Figs. 4 and 5.

The growth of Caco-2 cells was stimulated by exposure to the hydrolysate at many time points and concentrations of hydrolysate, especially in cultures containing 0.5% (v/v) FBS (Fig. 4). Cells hardly grew in serum-free medium, and although cells exposed to the hydrolysate grew faster than controls
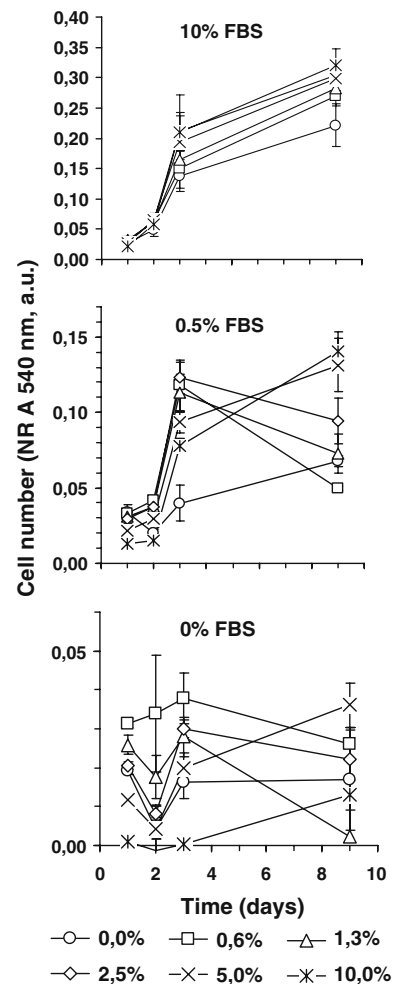
at some time points, differences were not significant. Addition of different concentrations of hydrolysate to 0.5% (v/v) FBS medium enhanced the growth of cells cultured for 2 to 9 days up to three fold as compared to control cells cultured in the same 0.5% (v/v) FBS medium without hydrolysate. The effect was highest even at low concentration after 2 or 3 days of culture, and became concentration-dependent in 9-days old cultures. The effect on cells cultured in medium containing 10% FBS (v/v) was much lower, and although a trend similar to that observed in cells
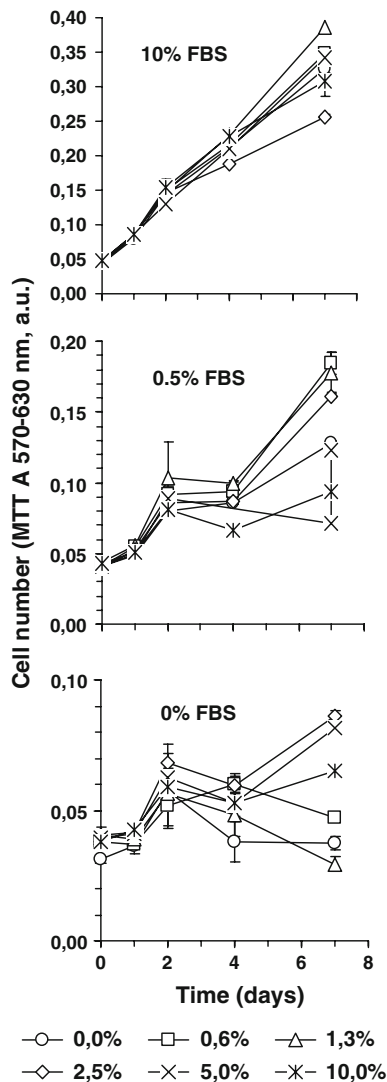
Fig. 5 Proliferation of THP-1 cells cultured in the presence of increasing concentrations of a hydrolysate with a 22% degree of hydrolysis. Cells (5×10^3 cells/well) were cultured in 96-well plates in culture medium containing 0, 0.5 or 10% (v/v) FBS, and 0–10% (v/v) hydrolysate. Cell number was estimated after incubating for 1 h, and 1, 2, 4 and 7 days by measuring reduction of MTT. Data represent average ± standard deviation of three incubations

grown at 0.5% (v/v) FBS was observed, differences with controls were only significant after 9 days in culture.

The effect of the hydrolysate on the growth of THP-1 cells was lower than the effect observed in Caco-2 cells, and only after 7 days in culture some significant differences can be observed (Fig. 5). Thus, growth was enhanced up to two-fold in THP-1 cells

cultured for 7 days in 2.5–10% (v/v) hydrolysate and 0% (v/v) FBS, and up to 40% in cells grown in 0.6–2.5% (v/v) hydrolysate and 0.5% (v/v) FBS, as compared to a growth enhancement of up to three-fold in Caco-2 cells exposed to hydrolysate in 0.5% (v/v) FBS medium.

Long-term culture

The long-term growth of THP-1 and Caco-2 cells was studied in order to find out whether the positive effect that chickpea hydrolysates had on cell growth at certain time points and concentrations is enough to allow for normal growth in serum-free or low serum medium. THP-1 cells were grown for several passages in medium containing 2.5 or 10% (v/v) hydrolysate (22% degree of hydrolysis) and 0, 0.5 or 10% (v/v) FBS, and Caco-2 cells were grown in medium containing 10% hydrolysate (22% degree of hydrolysis) and 0 or 10% (v/v) FBS. As controls, cells were also cultured in the same media containing no hydrolysate. Cumulative cell numbers were the result of multiplying cell numbers by splitting factors, which were adjusted as needed during culture in order to keep recommended cell concentrations.

In the absence of hydrolysate and the presence of 10% (v/v) FBS, THP-1 cells grew as expected (Fig. 6 upper panel). An FBS concentration of 0.5% (v/v) supported growth at a much lower rate, and finally, cells grew slowly and eventually died at 0% (v/v) FBS. Addition of 10% hydrolysate did not interfere with growth at 10% (v/v) FBS, slightly improved growth at 0.5% (v/v) FBS, and most interestingly, supported a good rate of growth at 0% (v/v) FBS (Fig. 6 lower panel). Caco-2 cells were not able to grow at 0% (v/v) FBS in the presence or absence of 10% (v/v) hydrolysate (data not shown).

Discussion

The addition of a variety of protein hydrolysates to culture media in order to promote cell growth has been proposed. These proposals include protein hydrolysates from meat (Gu et al. 1997; Jan et al. 1994; Schlaeger 1996; Taylor et al. 1972a; Taylor et al. 1974; Taylor et al. 1972b), lactalbumin (Young 1976), soy, wheat and rice (Heidemann et al. 2000), and rapeseed (Deparis et al. 2003). Nevertheless,
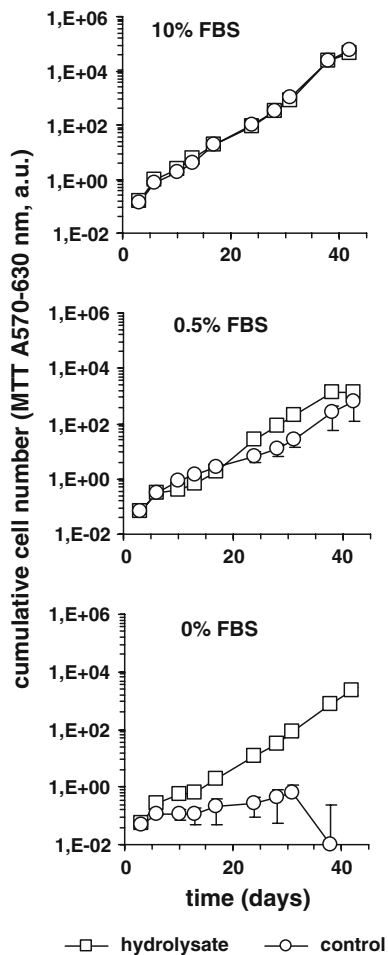
Fig. 6 Long-term culture of THP-1 cells in the presence of 10% (v/v) hydrolysate (22% degree of hydrolysis) and 0, 0.5 or 10% (v/v) FBS. Cumulative cell numbers were calculated by multiplying cell numbers by splitting factors, which were adjusted during culture in order to keep a cell concentration between 0.5×10^5 and 8×10^5 cells/mL

others have reported negative or negligible effects on growth (Franek et al. 2003; Franek and Katinger 2002; Giovannini et al. 2000), or even positive effects at lower concentrations and negative effects at higher concentrations (Gu et al. 1997). Using hydrolysates facilitates the production of serum-free and protein free media, although those hydrolysates of animal origin still have the potential of carrying adventitious infectious agents. Alternatives to using serum are of special interest for the production of biopharmaceuticals using intensive cell culture because large-scale production benefits most from costs reductions.

A variety of peptides having an effect on cell proliferation in vitro have been purified from this type of hydrolysates. While cell proliferation is promoted by peptides from fish (Ravallec Ple et al. 2001; Ravallec Ple et al. 2000), soybean (Franek et al. 2000), wheat (Franek et al. 2000), and milk (Kayser and Meisel 1996), cell growth inhibition by peptides derived from soybean (Kops et al. 1997) and milk (Ganjam et al. 1997) has been described. Peptides derived from wheat have been found to inhibit (Giovannini et al. 2000; Silano and De Vincenzi 1999) and promote (Rivabene et al. 1999; Rocca et al. 1983) cell death. Short-chain peptides with diverse biological properties, including cell growth regulation, can be released from food proteins during digestion and in vitro hydrolysis (Meisel and Bockelmann 1999; Vioque et al. 2001). These are the so called bioactive peptides, which are defined as peptides with biological effects beyond the nutritional value derived from their amino acid composition (Vioque et al. 1999). Our previous studies on the production and biological properties of chickpea hydrolysates (Clemente et al. 1999a; Girón-Calle et al. 2004; Pedroche et al. 2002; Yust et al. 2003) suggested the possibility that this material might represent an inexpensive and safe alternative to serum for cell culture. As described above, the idea of using protein hydrolysates in cell culture is not new, but the use of inexpensive and safe materials such as a grain legume and two commercially available food grade proteases would support the use of chickpea hydrolysates.

Short-term incubations of Caco-2 and THP-1 cells in media containing chickpea hydrolysates showed some effects on growth, specially when hydrolysates were added to low serum and serum-free media, but these effects were either positive or negative depending on the degree of hydrolysis and/or incubation time (Figs. 2 and 3). These data most likely reflect the formation of both growth-promoting and growth-inhibiting peptides, resulting in hydrolysates with changing properties as the degree of hydrolysis increases. In addition, the formation of pro- or anti-apoptotic peptides could also complicate the situation. Data on the effect of hydrolysate concentration (Figs. 4 and 5) were again a little puzzling because both positive and negative concentration-dependent effects were found, which is consistent with previous reports on the diverse effects of protein hydrolysates

on a variety of cell types as described above. Long-term incubations of Caco-2 and THP-1 cells showed that the chickpea hydrolysate supports growth of THP-1 but not Caco-2 cells in the absence of serum. Thus, THP-1 cells kept growing under these conditions for as long as the culture was kept (19 passages, 45 days) (Fig. 6), while Caco-2 cells stopped growing only three passages into the culture.

The failure to support Caco-2 cells growth is consistent with the fact that cells that grow in suspension, like THP-1 and many other cell lines used for production of biopharmaceuticals, are more easily grown in serum-free medium than anchorage-dependent cells such as the Caco-2 cell line (Keenan et al. 2006). Alternatively, it would also be conceivable that this failure might be due to the presence of residual amounts of non-peptidic bioactive components that were previously found to inhibit Caco-2 growth (Girón-Calle et al. 2004). Nevertheless, this is unlikely because the protein isolates that were used for production of hydrolysates for the present study were extensively washed with water, ethanol, acetone, and diethyl ether in order to eliminate these bioactive components. In addition, growth of Caco-2 cells was not hampered by the hydrolysate in the presence of serum, so that the presence of residual growth-inhibiting components in the hydrolysates is unlikely.

In addition to effects on growth, protein hydrolysates may have other desirable or undesirable effects as components of culture media. Thus, decreased apoptosis (Schlaeger 1996), decreased sialylation of interferon (Gu et al. 1997), and increased antibody production (Jan et al. 1994) have been described in media supplemented with meat hydrolysates. Induction of apoptosis by wheat gliadin peptides (Giovannini et al. 2000), increased interferon-gamma secretion by rice and wheat hydrolysates (Burteau et al. 2003), and protection of recombinant interferon from hydrolysis by plant hydrolysates (Mols et al. 2005) have also been reported. According to studies in our laboratory, chickpea hydrolysates produced using alcalase have antioxidant properties as measured by inhibition of β-carotene oxidation in vitro (unpublished), inhibit the angiotensin converting enzyme (Pedroche et al. 2002; Yust et al. 2003), and might have other peptidase and protease-inhibiting activities as well. These properties would be particularly beneficial for intensive cell culture aimed at production of biopharmaceuticals because oxidation and

proteolysis frequently lower yields of production. The possibility in the near future of in vitro meat production (Pincock 2007) adds interest to the idea of large-scale serum-free tissue culture in order to meet both safety and economical requirements. The present study proves that chickpea hydrolysates obtained by treatment of protein isolates with the proteases alcalase and flavourzyme are one more potential alternative to serum that present some additional advantages such as being very inexpensive, safe, and having antioxidant properties. Like other alternatives to serum, the effects of these hydrolysates are cell-dependent, and preliminary assays with the particular cells of interest would be needed.

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