Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 87 (2004) 387–392
Food Chemistry
www.elsevier.com/locate/foodchem

Rapeseed protein hydrolysates: a source of HIV protease peptide inhibitors

Maria del Mar Yust, Justo Pedroche, Cristina Megías, Julio Girón-Calle, Manuel Alaiz, Francisco Millán, Javier Vioque *

Department Fisiol Tecnol Prod Vegetables, Instituto de la Grasa, Padre García Tejero 4, 41012 Sevilla, Spain

Received 6 March 2003; received in revised form 13 November 2003; accepted 14 November 2003

Abstract

Inhibitors of the human immunodeficiency virus (HIV) protease can be obtained from microbial and plant sources. The presence of inhibitors in protein hydrolysates obtained by treatment of rapeseed protein isolates with the food grade endoprotease alcalase was investigated. Inhibitory activity was determined by measuring growth of *E. coli* expressing the HIV protease. Hydrolysis of rapeseed protein with alcalase resulted in the generation of peptides that inhibited the HIV protease, which implies that those peptides can be absorbed from the medium by the cells in the inhibition assays. Two fractions rich in peptidic HIV protease inhibition were obtained by size exclusion chromatography. These results exemplify a new approach in the research of HIV protease inhibitors.

Keywords: Rapeseed; Protein hydrolysate; HIV protease; Peptide inhibitors

1. Introduction

The human immunodeficiency virus (HIV) is quickly becoming one of the world's deadliest viruses. It is estimated that five new victims are infected with the HIV every minute (Semple, 2000). Gene products required for viral replication are suitable targets for fighting the disease. Currently, the most successful drugs target reverse transcription, an essential process to HIV infection (Semple, 2000). Other drugs inhibit the production of HIV protease, an enzyme required to assemble new virus particles.

The HIV protease is a virally encoded protease that serves to cleave the "gag" and "gag-pol" polyprotein precursor into mature, functional proteins. The "gag" gene codes for structural proteins that form the shell around the viral RNA. The "pol" gene codes for enzymes such as reverse transcriptase, RNAase H or integrase (Semple, 2000).

Among the approaches to develop, anti-HIV drugs those directed against the HIV-protease seem to be the most promising. The HIV-protease inhibitors developed so far are substrate-based and function at the active site. Most of them are peptide mimetic compounds based on the substrate's primary sequence. Indinavir is among the most extended inhibitors of HIV-protease used nowadays in therapy against HIV infection. However several side effects, associated with this drug have been described, such as fat accumulation (Miller et al., 1998), hepatitis (Matsuda, Gohchi, & Yamanaka, 1997), kidney stone formation (Hug et al., 1999) or cutaneous side effects (Calista & Boschini, 2000). These negative effects, but especially the high rate of mutation of the virus, that generate new strains resistant to known inhibitors, have promoted the search for new HIV enzyme inhibitors. There are reports of HIV protease inhibitors from natural sources, such as α-microbial alkaline protease inhibitor (Stella et al., 1991) and lignin-like substances derived from an edible mushroom (Ichimura, Watanabe, & Maruyama, 1998).

Rapeseed is one of the most important oilseed crops in the world, ranking fourth with respect to oil

* Corresponding author. Tel.: +34-954-611-550; fax: +34-954-616-790.
E-mail address: jvioque@cica.es (J. Vioque).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2003.11.020

production after soybean, palm, and cottonseed (FAO, 2002). Rapeseed is rich in protein and therefore defatted rapeseed meal may constitute a good source of proteins for humans (Finlayson, 1976). Protein isolates obtained from defatted rapeseed meal have been used for the fortification of foods (Dev & Mukherjee, 1986; El Nockrashy, Mukherjee, & Mangold, 1977; Gonçalves et al., 1997; Zhou, He, Yu, & Mukherjee, 1990). The preparation of rapeseed protein hydrolysates present certain advantages with respect to functional and nutritional properties (Vioque et al., 1999; Vioque, Sánchez-Vioque, Clemente, Pedroche, & Millán, 2000). Protein hydrolysates may be a source of bioactive peptides, which are short chain peptides with certain biological properties that include immunomodulatory, antimicrobial, antithrombotic and antihypertensive effects (Clare & Swaisgood, 2000; Smacchi & Gobbetti, 2000). In recent years, peptides derived from different proteins have been recognized as HIV protease inhibitors (Lee & Maruyama, 1998). But peptides are usually of restricted value for therapeutic purposes because of their limited ability to penetrate cell membranes. HIV protease is a toxic protein when expressed in *E. coli* since many cellular proteins are cleaved by this protease. In this paper we describe the generation of HIV protease peptide inhibitors by treatment of rapeseed protein with a food grade endoprotease. These peptides are detected by their ability to enter the cells of *E. coli* expressing the HIV protease, inhibiting the HIV protease, and improving cell growth.

2. Materials and methods

2.1. Materials

Rapeseed seeds were a gift from Koipesol Semillas S.A. (Sevilla, Spain). The seeds were grounded and extracted with hexane in a soxhlet extractor for 9 h in order to remove most of the fat content. Isopropyl-β-D-thiogalactoside (IPTG) was supplied by Boerhinger. Pepsin and trinitrobenzenesulphonic acid (TNBS) was from Sigma. Haemoglobin was from Merck. Alcalase 2.4 l was purchased from Novo Nordisk. All other reagents were of analytical grade.

2.2. Preparation of protein isolate

Rapeseed flour (20 g) was suspended in 200 ml of 0.2% NaOH solution pH 12, and extracted by stirring for 1 h at room temperature. After centrifugation at 8000*g* for 20 min, two additional extractions of the pellet resulting from the centrifugation were carried out with half of the volume of alkaline solution. The pH of the supernatant was adjusted to the isoelectric point (pH 4.3) and the precipitate formed was recovered by centrifugation as described above. The precipitate was washed with distilled water adjusted to pH 4.3 and freeze-dried until further use. Chemical composition of rapeseed protein isolate is shown in Table 1.

Table 1
Rapeseed protein isolate chemical composition

Component	Composition (%)
Protein	91.1
Ash	2.3
Moisture	2.1
Fiber	0.02
Glucosinolates	0.21
Phytic acid	0.23
Soluble sugars	1.4
Polyphenols	1.3
Fats	0.3

2.3. Hydrolysis

Protein isolates were hydrolyzed in batches with alcalase in a pH-stat. A hydrolysis curve was obtained by the pH-stat technique using the following hydrolysis parameters: substrate concentration, 10%; enzyme/substrate ratio, 0.3 AU/g; pH 8; temperature 50 °C. Hydrolysis was conducted in a 1000 ml reaction vessel equipped with a stirrer, thermometer and pH electrode. pH was maintained by adding 2 N NaOH. Hydrolysis was stopped after 50 min by acidification to pH 5 with 5 N HCl. Hydrolysates were clarified by ultrafiltration through 0.45 mm filters to remove insoluble substrate and residual enzyme. Filtrates were lyophilized for storage at −20 °C.

2.4. Degree of hydrolysis

The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (Adler-Nissen, 1979). The total number of amino groups was determined in a sample 100% hydrolyzed by incubation with 6 N HCl at 120 °C for 24 h.

2.5. Biogel P2 chromatography

Rapeseed protein hydrolysates (2–3 ml, 10 mg/ml) were injected into a Biogel P2 (BIORAD) gel filtration column (2 × 55 cm) at a flow rate of 10 ml/h 50 mM ammonium bicarbonate pH 8.3. Protein elution last 4 h. Fractions collected every 10 min (1.67 ml) from Biogel P2 chromatography were lyophilized to remove water and ammonium bicarbonate.

2.6. HPLC C_{18} chromatography

Fractions 7 and 11 collected from Biogel P2 chromatography were redissolved in water and injected in a HPLC reverse-phase column (C_{18} Hi-Pore RP-318,

250 × 10 mm BIO-RAD column). Elution was achieved by a linear gradient of acetonitrile (0–30% for 60 min) containing 0.1% trifluoroacetic acid at a flow rate of 4 ml/min at 30 °C. Elution was monitored at 215 nm.

2.7. Pepsin assay

Pepsin was assayed in a total volume of 250 μl containing 25 μg of enzyme, 400 μg of substrate (haemoglobin) and 800 μg of hydrolysate. The buffer was NaAcetate 50 mM, 0.1 M NaCl, pH 3.5. A positive control was made with all the components except for protein hydrolysates. The incubation was developed for 20 min at 37 °C and stopped by addition of 350 μl trichloroacetic acid 5% (w/v). The mixture was centrifuged at 13600*g* for 5 min and the absorbance at 280 nm was measured in the supernatant. This value is indicative of pepsin activity, since peptides generated by the enzyme remain soluble after addition of trichloroacetic acid.

2.8. Cell assay

Cell assay for inhibition of the HIV protease was developed according to Büttner, Dornmair, & Schramm (1997). *E. coli* BL21 containing the plasmid PT_5 which includes cDNA coding for the HIV-1 protease was used. 50 μl of overnight culture were diluted into 5 ml fresh LB-medium containing 50 μg/ml ampicillin and with or without 20 mM IPTG. IPTG induced the expression of HIV protease. To determine the inhibitory activity on HIV protease, different amounts (1.25, 2.5, 5 and 12.5 mg) of rapeseed protein hydrolysates were added. Samples were shaken at 37 °C for several hours. Cell growth was determined by measuring the turbidity at 490 nm. Generation times were calculated from the optical densities readings fitted to the equation $y = B\,e^{At}$ (y: absorption at 490 nm, t: time in minutes) and following the equation $T = \ln(2)/A$. The ratio of the generation times of cells grown with or without HIV protease peptide inhibitors gives the "relative change in growth".

Net growing decrease (NGD) was calculated from the growth curve slopes in the exponential phase as follows:

$$\mathrm{NGD} = \mathrm{slope}_{(-\mathrm{IPTG})} - \mathrm{slope}_{(+\mathrm{IPTG})}/\mathrm{slope}_{(+\mathrm{IPTG})}.$$

If there is growth improvement then the NGD of the experimental assay is smaller than the NGD from the control (cultures without addition of rapeseed peptides), since $\mathrm{slope}_{(+\mathrm{IPTG})}$ with peptide inhibitors is higher than the $\mathrm{slope}_{(+\mathrm{IPTG})}$ of control.

3. Results and discussion

A rapeseed protein hydrolysate was obtained by hydrolysis with the alkaline protease alcalase. This is a microbial protease that is used in the food industry for the preparation of hydrolysates with improved functional or nutritional properties as compared to the original protein preparations. Fig. 1 shows the kinetic of hydrolysis of the rapeseed protein isolate with this protease. After 10 min, hydrolysis of the substrate is almost complete. Aliquots taken at different times of the hydrolysis were assayed for inhibition of the protease pepsin, which is, as the HIV protease, an aspartic endopeptidase. Both proteases share the signature sequence Asp–Thr–Gly, an overall similarity of primary structure, inhibition by pepstatin and are inactivated by mutation of the putative active-site aspartates (Wlodawer & Vondrasek, 1998). Therefore pepsin may be representative for the HIV proteases in the aspartic group and this assay was used as a prescreening for aspartic protease inhibitors. Fig. 1 shows that the highest degree of inhibition of pepsin is observed in the hydrolysate obtained by treatment with alcalase for 10 min. Thus, this hydrolysate was further used for determination of HIV protease inhibitory activity. Inhibition at 0 min hydrolysis time may be due to peptides naturally present in the starting material or to other secondary metabolites. In this regard, this point could be considered as "background inhibition".

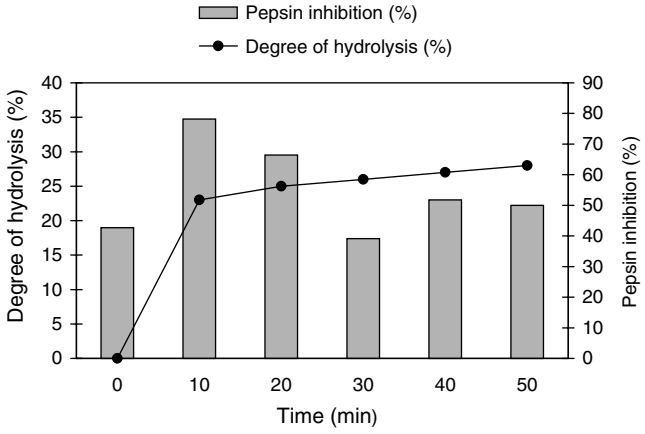

Fig. 1. Time course of the hydrolysis of rapeseed protein by alcalase and pepsin inhibition by the hydrolysates obtained at different times of hydrolysis. Data correspond to the average of two independent experiments.

HIV protease inhibition was assayed using an *E. coli* cell assay. In this assay the improvement in *E. coli* growth expressing the HIV protease is determined. Fig. 2 shows the growth improvement in *E. coli* cultures after addition of different amounts of rapeseed protein hydrolysate. 5 mg of protein hydrolysate allowed for the highest growth rates, with an improvement of 18% in the generation time with respect to the negative control, which consisted of cells grown in the presence of IPTG and therefore expressing the HIV protease and absence

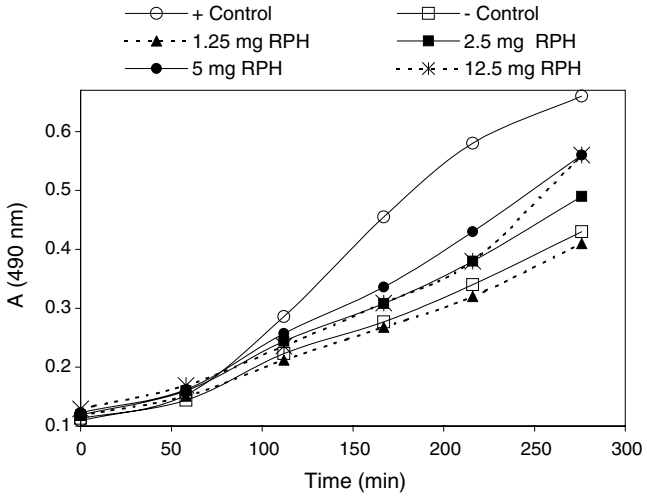

Fig. 2. Effect of a rapeseed protein hydrolysate (10 min treatment with alcalase) on *E. coli* growth. Positive control: *E. coli* in the absence of IPTG or peptide inhibitors. Negative control: *E. coli* in the presence of IPTG, which allows for expression of the HIV protease. Others: *E. coli* in the presence of IPTG and different amounts of 10 min rapeseed protein hydrolysate (RPH).

of hydrolysate extract. Interestingly, higher amounts of protein hydrolysate (12.5 mg) did not have a larger effect on *E. coli* growth, maybe because of the presence of antimicrobial peptides that inhibit cell growth at higher protein concentrations.

The same type of hydrolysates, obtained by alcalase treatment for 10 min, was subjected to size exclusion chromatography using a Biogel P2 gel filtration column (Fig. 3). Several fractions, from 4 to 16, were assayed for HIV protease inhibitory activity.

Net growing decrease was calculated for these fractions as described in Section 2. Only two fractions, 7 and 11, (marked with an arrow in Fig. 3) showed a NGD below the control (Fig. 4) indicating the presence in

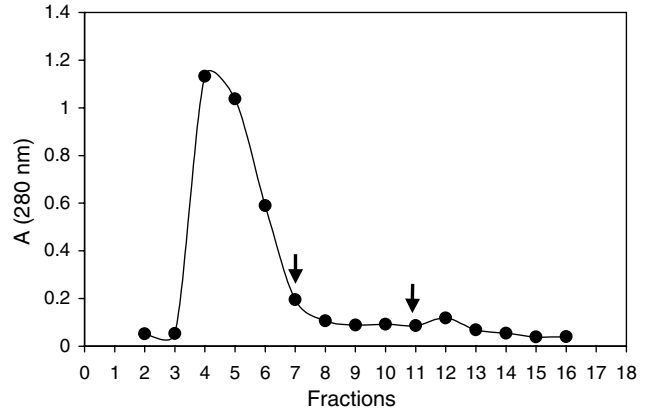

Fig. 3. Biogel P2 gel filtration chromatography of a rapeseed protein hydrolysate obtained by treatment with alcalase for 10 min. Arrows indicate fractions with HIV protease inhibitory activity.

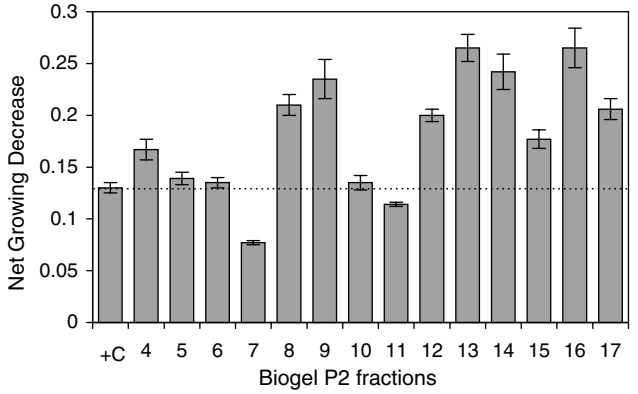

Fig. 4. Net growing decrease of fractions collected from Biogel P2 gel filtration chromatography. Data correspond to two independent experiments and the corresponding averages.

these fractions of HIV protease peptide inhibitors. These two fractions produced an improvement in the generation time of *E. coli* cultures of 22.5% and 20% respectively with respect to cell cultures grown with IPTG and absence of peptide inhibitors.

The stimulatory effect of these fractions on *E. coli* growth, that is, the inhibition of HIV protease, is clearly observed in Fig. 5 where the growth curve of the positive control (*E. coli* not expressing HIV protease), negative control (*E. coli* expressing HIV protease) and cells expressing HIV protease treated with inhibitory peptides fractions 7 and 11 are compared.

Fig. 6 shows the peptide profile by reverse phase HPLC of fractions 7 and 11 purified by Biogel P2 gel filtration chromatography. Both fractions are made up of a complex mixture of peptides that may exert their inhibitory activity on the HIV protease. In spite of the complexity of the profile several peaks are present in both.

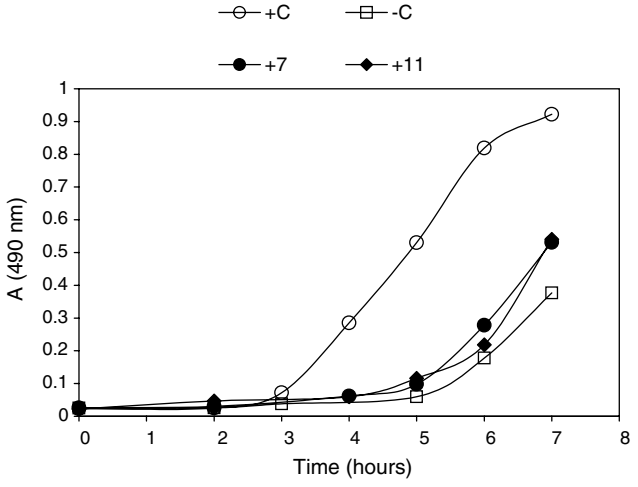

Fig. 5. Effect of fractions 7 and 11 from Biogel P2 chromatography on the growth of *E. coli* expressing HIV protease. Controls and treatments as in Fig. 2.

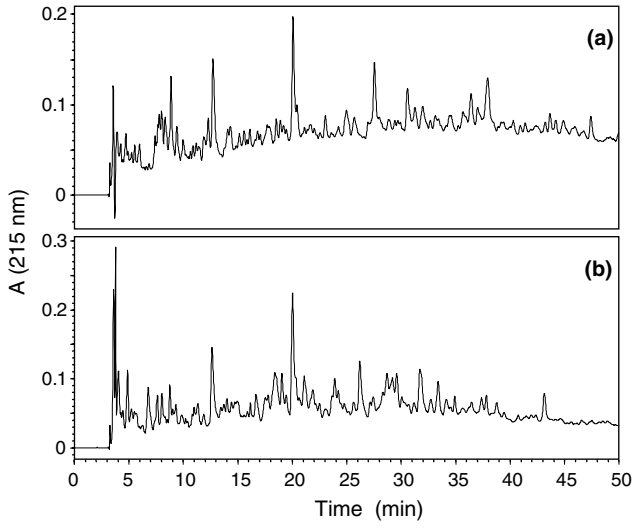

Fig. 6. HPLC C_{18} reverse phase chromatography of rapeseed protein hydrolysate fractions 7 (a) and 11 (b) from Biogel P2 chromatography.

The *E. coli* assay method used in this work to detect peptide inhibitors of the HIV protease has the advantage of not only identifying inhibitory peptides but also providing information about their possible absorption by cells. This is so because peptides need to enter the *E. coli* cells to exert their inhibitory activity. A question is if there is splicing of the peptide resulting in other peptides responsible of the inhibitory activity. This is an interesting point that could be addressed in future research to ascertain if peptides reach intact the protease.

Nevertheless, it should be kept in mind that the cell entry mechanism in *E. coli* may differ from the uptake mechanisms of peptides by human lymphocytes. It has been reported recently that the HIV protease is toxic not only to *E. coli*, but also to eucaryotic cells such as *S. cerevisiae* and mammalian COS7 cells (Blanco, Carrasco, & Ventoso, 2003). Thus, it could be possible in the near future to use other cell types for screening for HIV protease inhibition. This could help in the identification of HIV protease inhibitors in plant protein hydrolysates that could be useful as in vivo therapeutic agents by blocking HIV processing and infection. In addition to the design of synthetic HIV protease inhibitors, the identification of inhibitory peptides derived from natural proteins is a novel approach to the search for therapeutic drugs that is world while promising. From the point of view of Food Scientists, using food by-products such as oilseed protein isolates have the additional advantage of representing a value-added outlet for inexpensive by-products.

Acknowledgements

This work was supported by grant AGL 2001-0526 (F.M.) and AGL 2002-02836 (70% FEDER funds) (J.G.C.), and a Ramón y Cajal Contract (J.G.C.). The authors are very grateful to Professor Moshe Kotler from the Department of Molecular Genetics, The Hebrew University-Hadassah Medical School, Ein Karen, Jerusalem, Israel, for generously making available the HIV protease clone.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*, 1256–1262.

Blanco, R., Carrasco, L., & Ventoso, I. (2003). Cell killing by HIV-1 protease. *Journal of Biological Chemistry, 278*, 1086–1093.

Büttner, J., Dornmair, K., & Schramm, H. J. (1997). Screening of inhibitors of HIV-1 protease using an Escherichia coli cell assay. *Biochemical and Biophysical Research Communications, 233*, 36–38.

Calista, D., & Boschini, A. (2000). Cutaneous side effects induced by indinavir. *European Journal of Dermatology, 10*, 292–296.

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: a prospectus. *Journal of Dairy Science, 83*, 1187–1195.

Dev, D. K., & Mukherjee, K. D. (1986). Functional properties of rapeseed protein products with varying phytic acid contents. *Journal of Agricultural and Food Chemistry, 34*, 775–780.

El Nockrashy, A. S., Mukherjee, K. D., & Mangold, H. K. (1977). Rapeseed protein isolates by countercurrent extraction and isoelectric precipitation. *Journal of Agricultural and Food Chemistry, 25*, 193–197.

FAO/WHO/UNU (2002). *Agricultural bulletin board on data collection, disemination and quality of statistics*, Geneva, World Health Organization.

Finlayson, A. J. (1976). The seed protein contents of some Cruciferae. In J. G. Vaughan, A. J. McLeod, & B. M. G. Jones (Eds.), *The biology and chemistry of the cruciferae*. London: Academic Press.

Gonçalves, N., Vioque, J., Clemente, A., Sánchez-Vioque, R., Bautista, J., & Millán, F. (1997). Obtention and characterization of rapeseed protein isolates. *Grasas y Aceites, 48*, 282–289.

Hug, B., Naef, M., Bucher, H. C., Sponagel, L., Lehmann, K., & Battegay, M. (1999). Treatment for human immunodeficiency virus with indinavir may cause relevant urological side-effects, effectively treatable by rehydration. *British Journal of Urology International, 84*, 610–614.

Ichimura, T., Watanabe, O., & Maruyama, S. (1998). Inhibition of HIV-1 protease by water-soluble lignin-like substance from an edible mushroom, *Fuscoporia obliqua*. *Bioscience, Biotechnology and Biochemistry, 62*, 575–577.

Lee, T.-G., & Maruyama, S. (1998). Isolation of HIV-1 protease-inhibiting peptides from-thermolysin hydrolysate of oyster proteins. *Biochemistry Biophysics and Research Communication, 253*, 604–608.

Matsuda, J., Gohchi, K., & Yamanaka, M. (1997). Severe hepatitis in patients with AIDS and haemophilia B treated with indinavir. *Lancet, 350*, 364.

Miller, K. D., Jones, E., Yanouski, J. A., Shankar, R., Feverstein, I., & Falloon, J. (1998). Visceral abdominal-fat accumulation associated with use of indinavir. *Lancet, 351*, 871–875.

Semple, J. W. (2000). A critical examination of current HIV therapies. *Biotechnology Advances, 18*, 635–652.

Smacchi, E., & Gobbetti, M. (2000). Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiology, 17*, 129–141.

Stella, S., Saddler, G., Sarubbi, E., Colombo, L., Stefanelli, S., Denaro, M., & Selva, E. (1991). Isolation of alpha-MAPI from

fermentation broths during a screening program for HIV-1 protease inhibitors. *Journal of Antibiotics, 44*, 1019–1022.

Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., & Millán, F. (1999). Production and characterisation of an extensive rapeseed protein hydrolysate. *Journal of the American Oil Chemical Society, 76*, 819–823.

Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., & Millán, F. (2000). Partially hydrolyzed rapeseed protein isolates with improved functional properties. *Journal of the American Oil Chemical Society, 77*, 447–450.

Wlodawer, J., & Vondrasek, J. (1998). Inhibitors of HIV-1 protease: a major success of structure-assisted drug design. *Annual Reviews in Biophysics and Biomolecular Structures, 27*, 249–284.

Zhou, B., He, Z.-Q., Yu, H.-M., & Mukherjee, K. D. (1990). Proteins from double-zero rapeseed. *Journal of Agricultural and Food Chemistry, 38*, 690–694.