REPORT

Protein hydrolysates from the alga Chlorella vulgaris *87/1 with potentialities in immunonutrition*

✉ Humberto J Morris[1], Olimpia V Carrillo[2], Ángel Almarales[3], Rosa C Bermúdez[1], María E Alonso[2], Leonardo Borges[4], María M Quintana[4], Roberto Fontaine[1], Gabriel Llauradó[1], Martha Hernández[5]

[1]Center for Studies on Industrial Biotechnology, Faculty of Natural Sciences, University of Oriente
Ave. Patricio Lumumba s/n, Santiago de Cuba 5, CP 90500, Cuba
[2]Faculty of Biology,University of Havana, UH
Ave. 25 / J and I, Vedado,CP 10400, Havana, Cuba
[3] Center of Technological Applications for Sustainable Development CATEDES
Guantánamo, Cuba
[4]Solar Energy Research Center, CIES
Ministry of Science Technology and Environment, Santiago de Cuba, Cuba
[5]Center of Bioplants, UNICA
University of Ciego de Ávila, Cuba
E-mail: hmorris@cebi.uo.edu.cu

ABSTRACT

Chlorella vulgaris (Chlorophyta, Chlorophyceae) has received a particular attention in the programmes of microalgae utilisation in biotechnology. Enzymatic hydrolysis of cell proteins represents a very promising method to increase protein digestibility and thus, for obtaining hydrolysates with improved nutritional and functional properties. However, this technology has been little approached and the biological evaluation of hydrolysates has had a strictly nutritional nature. The design of hydrolysis conditions that combined for the first time, the use of *C.vulgaris* 87/1 treated with ethanol and pancreatin at pH values of 7.5-8.0, led to a product with a degree of hydrolysis of 20-22% and yields of 50-55%, characterised by a high digestibility (97.2%) and nitrogen solubility over a wide pH range (2.0-10.0). Hydrolysis curves were fitted to an exponential model, common to many food proteins. The bulk of the product dry matter consists of soluble peptides and free amino acids (47.7%) with three main peptides of molecular masses between 2 and 5 kDa. The oral administration of *Chlorella* hydrolysate (500 mg/kg) to undernourished Balb/c mice provided benefits in terms of liver protein metabolism and the induction of anabolic processes in gut mucosa. The hydrolysate also enhanced the immunological recovery, as judged by the stimulation of haemopoiesis, monocyte-macrophage system activation, as well as humoral and cell mediated immune functions, like T-dependent antibody response and the reconstitution of delayed-type hypersensitivity (DTH) response. These results represent the first findings in the world concerning the immunomodulating effects of a microalgae protein hydrolysate.

Keywords: protein hydrolysates, Chlorella vulgaris, immunonutrition, alga

*I*ntroduction

In the last decade, the biotechnology of microalgae has been an attractive option for obtaining foods and biomolecules, comprising proteins, vitamins, pigments, polyunsaturated fatty acids and substances with antitumoral and immunomodulating activities [1].

The microalga *Chlorella vulgaris* (kingdom *Protoctista*, division *Chlorophyta*, order *Chlorococcales*, genus *Chlorella*, and specie *vulgaris*) has received a considerable attention in the programmes of microalgae utilisation in nutrition and biomedicine. Recent reports point out that the world production of *Chlorella* amounts to 2000 ton/year and the biomass is applied in the protein enrichment of conventional foods and in the formulation of functional foods and nutritional supplements [2].

In Cuba, the south region of oriental provinces differs climatologically from the rest of the country, due to the high annual values of radiation and temperature, favourable conditions for developing microalgal biotechnology, particularly with strains isolated from the zone. Different projects related to the mass culture and the obtainment of new nutritional and pharmaceutical products from the microalga *C. vulgaris* 87/1, isolated from Chalons dam in Santiago de Cuba, have been carried out in the Solar Energy Research Center (CIES) and in the Center for Studies on Industrial Biotechnology (CEBI).

One of the main research approaches has been the evaluation of *Chlorella* as a source of protein products. However, *Chlorella* has a strong cellulosic wall, and therefore the proteins offer poor results in humans, when using intact cells. Enzymatic hydrolysis has been suggested as a very promising method to increase protein digestibility and for obtaining hydrolysates with improved nutritional and functional properties [3].

The applications of protein hydrolysates in the food industry comprise hypoallergenic products, formulations for athletes, surgical and geriatric patients and enteral diets. Moreover, several pharmacological actions have been reported for protein hydrolysates. The inhibition of angiotensin converting enzyme, the antioxidant ability and the hypocholesterolemic effects have been the most studied [4]. Additionally, different peptides exhibit an immunoenhancing activity, and therefore, they could be an approach to modulate the immune system through nutritional interventions, which is the main goal of *immunonutrition* [5].

✉ Corresponding author

1. Shimizu Y, Li B. Microalgae as a source of bioactive molecules: special problems and methodology. In: Proksch P, Mueller WEG, editors. Frontiers in marine biotechnology. Wymondham: Horizon Biosciences; 2006. p. 145-74.

2. Pulz O, Gross W. Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 2004;65:635-48.

3. Stoilov IL, Georgiev TD, Taskov MV, Koleva ID, inventors. Oral preparation for patients with chronic renal insufficiency and other protein metabolic diseases. WO patent 95/2952. 1995 Nov 2.

4. Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. Int Dairy J 2006;16:945-60.

5. Biziulevicius GA, Kislukhina OV, Kazlaukaitè J, Zukaitè V. Food-protein enzymatic hydrolysates possess both antimicrobial and immunostimulatory activities: a ´cause and effect´ theory of bifunctionality. FEMS Immunol Med Microbiol 2006;46:131-8.

From a practical point of view, the milk proteins have been the most commonly used to obtain hydrolysates for nutritional purposes. At present, the potential of plant proteins for producing hydrolysates and bioactive peptides offer new opportunities for microalgae research. This technology has been little approached and the reported processes, mainly in the genus *Scenedesmus* and *Spirulina*, are complex and difficult to scale up [3]. Moreover, the biological evaluations of microalgae hydrolysates have had a strictly nutritional nature, and information about the immunomodulating properties was not available

This communication reports the development of a new enzymatic procedure to obtain a protein hydrolysate from the microalga *C. vulgaris* 87/1 and the evaluation of the immunonutritional properties in a model of protein-energy malnutrition (PEM).

Results and discussion

The algal culture was carried out by autotrophic outdoor cultivation of *C. vulgaris* 87/1 in an open circulating cascade system of 500 m^2 (Solar Energy Research Centre, Santiago de Cuba). The spray dried biomass was partially extracted with ethanol to attenuate its intense green colour, which limits the acceptance for nutritional purposes.

The protein quality evaluation was the starting assay to obtain hydrolysates with immunonutritional properties. The protein content of *Chlorella vulgaris* 87/1 ethanol extracted biomass was higher than 40 and 30%, in terms of crude and true protein, respectively. The nutritional quality could be considered satisfactory, as judged by the *in vitro* protein digestibility (75.9%), supplementary value (15%) and the essential amino acid profile (45.3%), with the exception of sulphur amino acids, defined as the limiting ones. The nutritional value was enhanced by the low levels of nucleic acids and the presence of a significant fraction of fibber and minerals [6]. These results sustain the continuance of the research work initiated in Cuba, aimed at the use of Chlorella biomass in the formulation of nutritional supplements and to investigate the potential therapeutic properties of *Chlorella*-based products.

The experiences on microalgae protein hydrolysis have been conducted for nearly thirty years. The applied procedures have been characterised by the preferential use of *Scenedesmus* and *Spirulina* proteins, as well as alkaline proteases. None of the reported methods combine the ethanol treatment of biomass with the use of an active protease at a pH, closed to neutral value in the hydrolysis of *Chlorella* proteins [7]. Pancreatin, with an optimum activity at pH values of 7.5-8.0 was used in the method developed in this work. At this pH, pancreatin hydrolysed the algal protein in an efficient way, thus avoiding the difficulties related to the neutralization of the reaction medium and undesired changes in the amino acids. The use of pancreatic enzymes, alone or together with other proteases, has been much extended in hydrolysis protocols of many food proteins.

The results of our study, in terms of amino nitrogen levels, indicate that cells proteins are more readily hydrolysed after extraction with ethanol [7]. The ethanol extracts take up 12-15% of the algal biomass and contain a number of biologically active compounds as chlorophylls, carotenoids, sterols and polyunsaturated fatty acids.

Though kinetic models for food protein hydrolysis with animal or bacterial proteases have been formulated, our study is the first report in the modelling of microalgal protein hydrolysis. The hydrolysis curves fitted empirical expressions, solutions of an exponential type of differential equation, which describe the increase in the degree of hydrolysis upon the time. The model, common to other food proteins, could represent a useful tool for the design of enzymatic reactors.

The degree of hydrolysis went up to 20-22%, higher to those considered appropriate for applications in the field of specialized nutrition (> 10%). The amino nitrogen/ total nitrogen ratio of 26.4%, as well as the content of free amino acids and soluble peptides (47.7%) ensured the protein solubilization and assimilation. With respect to the essential amino acid to total amino acid ratio, the value of 44.7% is clearly above the third of the total amino acid content, which is an index of the nitrogenous equilibrium according to the nutritional recommendations [7].

The molecular weight pattern of an aqueous extract of *C. vulgaris*, determined by gel filtration chromatography in a Sephadex G-100 column showed the protein heterogeneity with molecular masses ranging from 120 to 12 kDa. A major chromatographic peak of molecular mass (lower than 10 kDa) appears as a result of the hydrolysis, which represents about 53% of the peptidic component. Three main peptides with molecular weights between 2 and 5 kDa were identified by gel filtration on Sephadex G-25 [7, 8]. Molecular mass profiles with a prevalence of peptides lower than 10 kDa have been associated with a decreased residual antigenicity. This was demonstrated in *Chlorella* hydrolysate through a biological assay in guinea pigs. The hydrolysis also modified the nitrogen solubility curve at different pH values; the improvement of this property is an important factor for the inclusion of the hydrolysate in foods.

As an alternative to pancreatin, different proteolytic enzymes (from animal, plant and microbial origin) were evaluated in *Chlorella* protein hydrolysis (Figure 1). The results showed that a wide spectrum of proteases (trypsin, papain, bromelain and *Bacillus subtilis* crude extracts) can be used for the production of protein hydrolysates from algae extracted with ethanol. The use of enzymatic combinations is suggested to obtain preparations in which a higher degree of hydrolysis could be required [9].

The balanced biochemical composition of *Chlorella* hydrolysate accounted for its potential biostimulating effect. The microbial growth promotion exerted by *Chlorella* hydrolysate was a useful model in the evaluation of the nutritional quality of this preparation [10, 11].

The studies of oral acute toxicity at repeated doses for 28 days (2000 mg/kg) did not show alterations in the clinical signs and the biochemical parameters of animals. Therefore, the product can be considered as safe.

From a clinical perspective, the new technologies for the production of protein hydrolysates from non-conventional protein sources could offer solutions to many aspects of the immunonutritional therapy. Thus,

6. Morris HJ, Carrillo O, Bermúdez RC. Enfoque integral en la utilización de los métodos químicos de evaluación de la calidad proteica. Revista Cubana de Salud Pública 2003;29(1):42-7.

7. Morris HJ, Almarales A, Carrillo O, Bermúdez RC. Use of *Chlorella vulgaris* cell biomass for the production of enzymatic protein hydrolysates. Bioresource Technol 2008;99(16):7723-9.

8. Morris HJ, Borges L, Martínez CE, Almarales A, Abdala RT. Composición bioquímica y propiedades bioestimulantes de un hidrolizado proteico de la microalga *Chlorella vulgaris* (Chlorophyta). Rev Cubana Quím 2001;13(3):28-36.

9. Morris HJ, Almarales A, Carrillo O. Utilización de combinaciones enzimáticas en la obtención de hidrolizados proteicos a partir de la microalga *Chlorella vulgaris*. Rev Cubana Aliment Nutr 2001; 15(2):85-9.

10. Morris HJ, Sanz A, Almarales A. Utilización de un hidrolizado enzimático de *Chlorella* vulgaris en la formulación de medios de cultivo de *Bacillus subtilis*. Tecnol Quim 1999;19(3):32-7.

11. Quintana MM, Gómez LM, Morris HJ. Bioproductos. In: Quintana MM, Alfaro OG, Morris HJ, Ramírez AM, Gómez LM, Armas E, Leal A. *Chlorella*: cultivos y aplicaciones algunas investigaciones cubanas. Santiago de Cuba: Editorial Oriente; 2007. p. 139-72.

the metabolic and immunological effects of the oral administration of *Chlorella* hydrolysate during the recovery of protein-energy malnutrition (PEM) were studied. The results provided the first evidences regarding the biochemical aspects of the *in vivo* mechanism.

The undernourished Balb/c mice treated with the hydrolysate for eight days (500 mg/kg) showed biostimulating effects in hepatic recovery: 1) an increasing in the total protein content and in cholinesterase enzymatic activity (marker of protein synthesis) and 2) a lower arginase enzyme activity (marker of urea synthesis). Moreover, the algal hydrolysate promoted trophic effects in jejunal mucosa, presumably mediated by the peptides contained in the product, responsible for the increase in gut DNA and protein synthesis (including sucrase and maltase enzymes) [12].

Protein hydrolysates modulate immunological responses; however, the identity of target cells and their specific functions are little known. The oral treatment with *Chlorella* protein hydrolysate provided benefits in terms of haemopoiesis, as judged by the recovery of bone marrow cellularity and leukocyte count in peripheral blood, particularly in the lymphocyte pool [13].

The algal hydrolysate stimulated peritoneal and splenic macrophages, as well as Kupffer cells. The macrophage activation in response to immunomodulating diet peptides is possible, as a result of signals derived from the mucosa associated lymphoid tissue (MALT) in the gut [14].

Our results point out the activation of the immunological response in undernourished mice treated with *Chlorella* hydrolysate as a supplement, distinguished by an appropriate Th2 response (antibody titres to sheep red blood cells) and the reconstitution of delayed-type hypersensitivity (DTH) response [14]. These findings could be related to the presence in the peptides identified in the hydrolysate of sequences derived from *Chlorella* proteins with antitumour or antiviral properties, associated with the immune system, or sequences coming from other proteins not studied yet from the structural and pharmacological points of view [15].

Given the analyzed markers, the *Chlorella* protein hydrolysate could be considered as an immunomodulating preparation (Figure 2). These immunoenhancing effects in a microalgal hydrolysate are reported for the first time.

The present knowledge of the role of *C. vulgaris* biomass and derived products, for instance protein hydrolysates, in the prophylaxis and therapy of several diseases is still at the empirical level. This work is a contribution to the study of the protein quality, biochemical composition and biostimulating properties of *Chlorella* protein hydrolysates. Our results accounted for its possible use in immunonutrition, for developing physiologically functional foods and/or for specific formulations with clinical applications.

Scientific novelty

The design of hydrolysis conditions, that combined for the first time the use of ethanol-treated *C. vulgaris* 87/1 biomass and pancreatin at a pH closed to neutral values, allowed the developing of a new, simple and efficient method for obtaining a protein hydrolysate with improved nutritional and functional properties. The kinetic modelling of the enzymatic hydrolysis of algal proteins was made for the first time, and it demonstrated that the process could be considered as a particular case of food protein hydrolysis. A microalgal protein hydrolysate with potentialities in the immunonutrition was obtained and our results represent the first report of the immunoenhancing effects in animal model

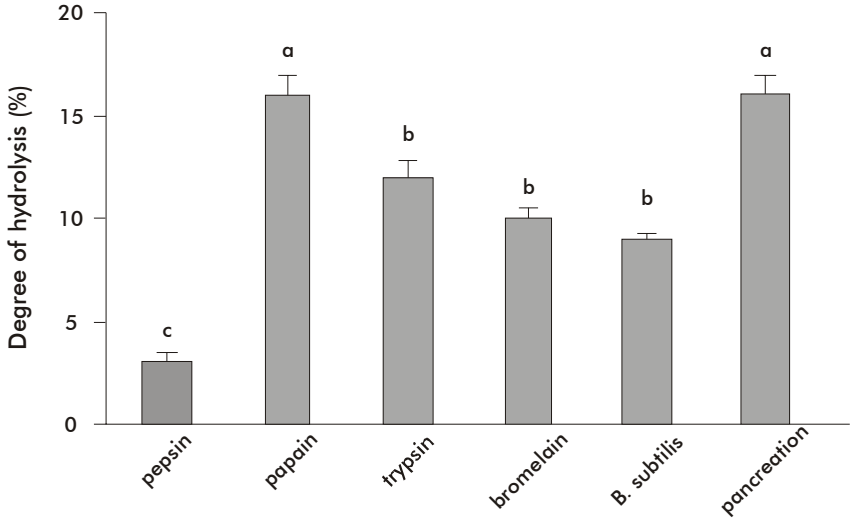

Figure 1. Degree of hydrolysis of cell proteins in ethanol extracted *Chlorella* biomass with different roteases. A 10% suspension in water of extracted biomass was hydrolysed (20 U/g) at 37 °C for 4 h, accompanied by continuous stirring. The pH was adjusted to the optimum for each enzymatic preparation. Each value is the mean of three experiments. Means without the same letter above bars are significantly different at the 5% level according to the Student-Newman-Keuls Test.

Theoretical and practical importance of the result

The theoretical importance of the result lies on the contribution to the knowledge of the biological me-

12. Morris HJ, Borges L, Martínez CE, Carrillo O. Aspectos bioquímicos de la intervención con un hidrolizado enzimático de *Chlorella vulgaris* durante la recuperación de la malnutrición proteico energética en ratones Balb/c. Rev Cubana Aliment Nutr 2002;16(1):5-12.

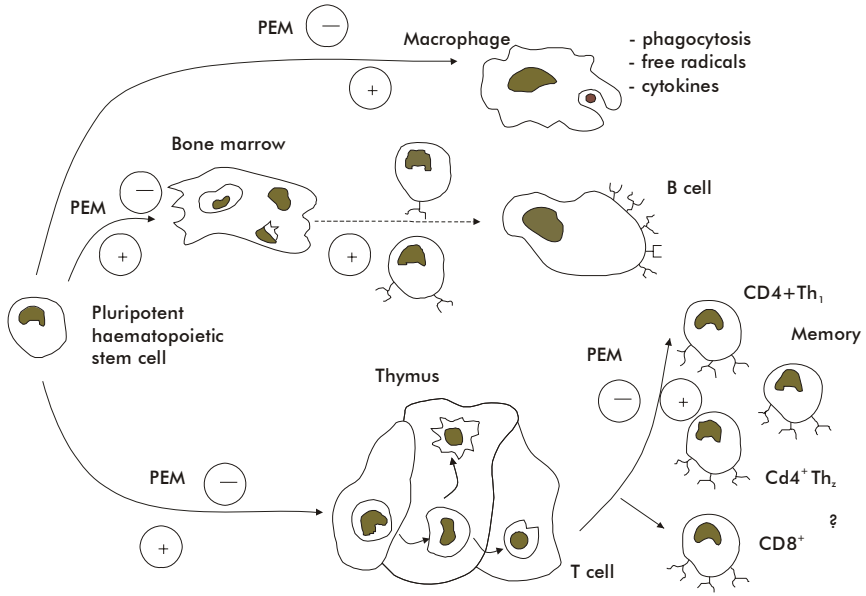

Figure 2. Alterations in the immunological response during protein-energy malnutrition (PEM) and the immunoenhancing effects of *Chlorella vulgaris* protein hydrolysate in Balb/c mice. The sign – indicates an inhibitory effect of PEM in the immune function. The sign + refers to the immunomodulating action of *Chlorella* protein hydrolysate in undernourished mice.

chanism of microalgal protein hydrolysates, specially the *in vivo* immunomodulating effects. The practical importance is justified, taking into account that the production of protein hydrolysates from *Chlorella vulgaris* 87/1 could be conceived as part of a whole strategy to value the algal biomass. The hydrolysates represent an innovative proteinaceous bioresource with a wide profile of applications in food and pharmaceutical industries. The extension of the results related to the immunomodulating activity to other experimental systems, could lead to the formulation of new national pharmaceutical products.

Acknowledgments

The authors sincerely acknowledge the collaboration of Yamila Lebeque, Yaixa Beltrán, Onel Fong, Claudina Zaldívar, Joaquín Díaz, Jane Marcos, Orlando Alfaro and Amado Ramírez in the development of this work.

13. Morris HJ, Borges L, Martínez CE, Carrillo O. Restauración de la inmunocompetencia en ratones Balb/c malnutridos con la administración de un hidrolizado enzimático de *Chlorella vulgaris*. Reviata Cubana de Farmacia [on line] 2003; 37(3). Disponible en: URL: htpp://scielo. sld.cu/scielo.php?script

14. Morris HJ, Carrillo O, Bermúdez RC, Lebeque Y, Fontaine R, Llauradó G, *et al*. Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* on undernourished mice. Enzyme Microbial Tech 2007;40(3):456-60.

15. Morris HJ, Carrillo O, Alonso ME, Bermúdez RC. Are the peptide sequences encrypted in food *Chlorella* protein a possible explanation for the immunostimulatory effects of microalgal supplements? Med Hypotheses 2008;70(4):896.