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Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* on undernourished mice

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Abstract

This study examined the effects of oral administration of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* (Cv-PH) on the recovery of both innate and specific immune responses of undernourished Balb/c mice after a 3-day fasting period. Cv-PH was prepared by hydrolysis of ethanol-extracted cell biomass with pancreatin (20 AU/g) at pH 7.5 and 45 °C for 4 h. The treatment with Cv-PH (500 mg/kg) for 8 days provided benefits in terms of haemopoiesis, as judged by the recovery of bone marrow cellularity and the leukocyte counts in peripheral blood, particularly the lymphocyte pool, which increased up to 128% compared to control animals. Starved mice treated with Cv-PH showed a higher number of peritoneal exudate cells and the macrophage activation was demonstrated by the enhancement in glucose consumption and acid phosphatase activities relative to non-supplemented mice. The increased carbon clearance in peripheral blood suggested the stimulation of mononuclear phagocytic system. Cv-PH also stimulated both humoral and cell mediated immune functions positively, such as T-dependent antibody response and the reconstitution of delayed-type hypersensitivity response (DTH). These findings indicate that *Chlorella* protein hydrolysate can be used for developing physiologically functional foods with immunopotentiating activity.

Keywords: Enzymatic hydrolysis; Protein hydrolysate; Immunostimulant activity; Microalga; *Chlorella*; Functional foods

1. Introduction

Enzymatic protein hydrolysates have been reported as suitable sources of protein for human nutrition because of their gastrointestinal absorption, which seems to be more effective than both intact protein and free amino acids [1]. Therefore, protein hydrolysates have been widely used in specific formulations, in order to improve nutritional and functional properties [2]. These uses include clinical applications, such as hypoallergenic infant formula, biostimulating preparations, special foods, geriatric products, therapeutic diets and sport drinks [3–5].

The protein sources most commonly used in nutritional products are casein and whey proteins. However, plant proteins are finding commercial application as an alternative to proteins from animal sources [6]. There is also a persistent demand for innovative proteinaceous food materials and single cell protein (SCP) production is a major step in this direction [7]. In this context, microalgae provide through photosynthesis an efficient mean of converting solar energy into biomass [8] and in recent years, microalgal biotechnology has gained importance due to the development of new production technologies [9,10].

Green microalgae (*Chlorophyta*, *Chlorophyceae*) are suitable for protein products sold as health foods and food supplements [11–13]. The enzymatic hydrolysis of cell proteins of unicellular green algae has been described as a very promising method for improving protein digestibility and to obtain a balanced protein product for human nutrition [14]. However, little data were available about the use of *Chlorella* protein hydrolysates in clinical nutrition. The present study was designed to assess the effects of oral administration of *Chlorella* hydrolysate on the recovery of both innate and specific immune responses of undernourished mice. Since strong links exist between nutritional and immunological status, an extended knowledge of the immunostimulating activity of algal hydrolysates would be useful in understanding their role in the field of pharmacological nutrition.

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2. Materials and methods

2.1. Microorganism, cultivation conditions and biomass processing

Algae samples were obtained by autotrophic outdoor cultivation of *Chlorella vulgaris* 87/1 in open circulating cascade systems of 500 m^2. This strain was isolated from Chalons dam in Santiago de Cuba in 1987 and is deposited at the Culture Collection of the Solar Energy Research Center (CIES). The growth medium contained (g/l): NH_4NO_3 (1.2), $MgSO_4{\cdot}7H_2O$ (1.0) and a food grade NPK (8:12:12) fertilizer formula (0.9). The algal suspension was bubbled with 1% CO_2. The algae were harvested by continuous-flow centrifugation (separator Alfa Laval, Sweden) up to 10% dry matter in the slurry. The dark-green algae slurry was spray dried in a Niro Atomizer drier (input 200–210 °C, output 80–90 °C). The powder thus obtained (moisture content 7%) was preserved in plastic boxes for further use. Dry algae samples of 500 g were extracted with ethanol (2 l) at 45 °C for 3 h via gentle agitation.

2.2. Enzymatic hydrolysis of cell biomass

Pancreatin (Merck), having a specific proteolytic activity of 0.47 AU/mg of protein, was used for cell protein hydrolysis of the extracted algal biomass. One proteolytic unit was expressed as the amount of enzyme necessary to catalyze at an initial rate the release of 1 μmol tyrosine from a 2% denatured casein solution at pH 7.5 and 37 °C within 1 min.

A 10% suspension in water of the ethanol-extracted alga was hydrolysed at an enzyme/substrate ratio of 20 AU/g, pH 7.5 and 45 °C for 4 h in a 1000 ml reaction vessel, equipped with a stirrer, thermometer and pH electrode. The enzyme reaction was stopped by heat treatment at 85 °C for 15 min. The slurry thus obtained was centrifuged and the resultant solutions were spray-dried.

The yields of the technology were of 40–45 g of hydrolysate/100 g of cell biomass. The bulk of the product dry matter consists of soluble hydrolysed protein and free amino acids, accounted for 47.7%.

Three main peptides with molecular weights lower than 5000 Da were identified by size exclusion chromatography. The amino nitrogen/total nitrogen ratio of 26.4% was considered appropriate for protein assimilation. The amino acid pattern was comparable with that of FAO reference protein, except for the low content of sulphur amino acids [15].

2.3. Animals and diets

Female Balb/c mice (8 weeks) were purchased from LABEX® (Santiago de Cuba, Cuba) and housed individually. A total of 30 mice that were starved for 3 days with free access to salted water were studied. After this time, blood was collected from the orbital vein of 10 mice and then, the animals were killed (M group). The others were refed *ad libitum* for 8 days with commercial pelleted diet (M-DC group) or the commercial diet and the *Chlorella* protein hydrolysate administered orally at a dose of 500 mg/kg of body weight per day (M-CvPH group). A control group of 10 mice (C) was fed with the commercial diet throughout the study. In the experiments concerning the humoral immunity, the refeeding period was extended to 14 days.

2.4. Haematological methods

The blood specimens were analysed for white blood cell counts. Femoral bone marrow cells were withdrawn with Hanks' solution and counted with a Neubauer chamber.

2.5. In vivo peritoneal macrophage stimulation activity

2.5.1. Number of peritoneal exudate cells

Peritoneal exudate cells were collected from the peritoneal cavity of mice by washing with Hanks' solution. Murine peritoneal cells, suspended in RPMI 1640 medium (Gibco-BRL, USA) containing 5% fetal bovine serum, were cultured at 37 °C for 2 h in a CO_2 atmosphere and non-adherent cells were removed by washing with RPMI 1640 medium.

2.5.2. Assay for glucose consumption

Macrophages (adherent cells) were plated into a 96-well culture plate (Nunc Inc., Denmark) at a density of 1×10^5 cells/well (50 μl of a 2×10^6 cells/ml suspension). The plate was incubated at 37 °C in a CO_2 atmosphere and after incubation for 72 h, the glucose remaining in the culture supernatants was measured with Rapi-Gluco-Test assay kit (EPB Carlos J. Finlay, Cuba) based on the glucose oxidase method. The results are expressed as the glucose consumption, after subtracting the glucose content in the culture supernatant from that in the culture medium.

2.5.3. Assay for acid lysosomal phosphatase activity

Macrophages were cultured as described above. Macrophages monolayers in microplates (1×10^5/well) were solubilised by the addition of 0.1 ml of 0.1% Triton X-100 and the cell suspensions were collected in test tubes. Then, 0.5 ml of *p*-nitrophenyl phosphate was added as a substrate, and 0.4 ml of 0.2 mol/l acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 1 h and 0.5 ml of 0.2 mol/l borate buffer (pH 9.8) was added, then the optical density at 405 nm was measured. The acid phosphatase activity was expressed as the amount of *p*-nitrophenol released from *p*-nitrophenyl phosphate in cell lysates [16].

2.6. Carbon clearance from peripheral blood

The mice were injected via the tail vein with 0.2 ml of a colloidal carbon suspension, consisting of 3 ml of Pelycan drawing ink 17 black (Pelycan AG, Germany), 4 ml of saline and 4 ml of 3% gelatine solution. A 50 μl aliquot of the blood was taken every 5 min from the orbital plexus with a heparinised haematocrit tube after the injection of the carbon suspension, then immediately mixed with 4 ml of 0.1% (w/v) Na_2CO_3. The concentration of the colloidal carbon was estimated by absorbance at 675 nm. The clearance rate of carbon is expressed as the half-life of carbon in the blood ($t_{1/2}$, min), calculated by means of the following equation: $t_{1/2} = [\ln 2(t_2 - t_1)]/(\ln A_1 - \ln A_2)$, where A_1 and A_2 are the optical densities at times t_1 and t_2, respectively.

2.7. In vivo antibody response

Humoral immune response was evaluated through an immunization protocol with sheep red blood cells (SRBC) as antigen. Three groups, comprised of five mice, were designed: M-DC, M-CvPH and control as described above. After the starvation (day 0) mice were injected intraperitoneally (i.p.) with 0.2 ml of a 25% SRBC saline solution. After 7 days from the first injection, blood samples of 50 μl were drawn from the orbital plexus to measure antibody titres by a haemagglutination (HA) reaction. The reciprocal serum dilution, which just gave agglutination was considered to be the titre. At this time, mice received the second immunization and on day 14, antibody titres were determined.

2.8. Delayed-type hypersensitivity (DTH) response

After the starvation (day 0), five animals in M-DC and M-CvPH groups were immunized by an intradermal (i.d.) injection of 50 μl of 5 mg/ml bovine serum albumin (BSA) emulsified in Complete Freund Adjuvant (CFA) (Sigma, St. Louis, MO) at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20 μl of 5 mg/ml BSA into one rear foot pad, while the other rear foot pad received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of micrometer (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS injected foot pads. A similar immunization protocol was applied to control animals [17].

2.9. Statistical analysis

Data are expressed as mean ± S.E. One-way analysis of variance and *post hoc* Duncan tests were used to determine mean differences among the groups for all of the parameters studied.

Table 1
Effect of starvation and refeeding with commercial diet supplemented or not with *Chlorella vulgaris* protein hydrolysate (Cv-PH) on haemopoiesis of Balb/c mice

	Control	M	M-DC	M-CvPH
Bone marrow cellularity ($\times 10^6$/femur)***	12.5 ± 1.4 a	7.14 ± 0.82 b	8.42 ± 1.07 b	13.16 ± 1.64 a
White blood cell count (10^9 L^{-1})**	7.34 ± 0.28 a	3.51 ± 0.55 c	6.36 ± 0.18 b	8.98 ± 0.76 a
Granulocytes (10^9 L^{-1})**	2.42 ± 0.37 a	1.17 ± 0.64 b	1.43 ± 0.32 b	2.69 ± 1.08 a
Lymphocytes (10^9 L^{-1})**	4.84 ± 0.37 b	2.26 ± 0.64 c	4.86 ± 0.39 b	6.20 ± 0.99 a

Data are expressed as mean ± S.E. of 10 animals per experimental group. Different letters indicate significant differences among the groups in Duncan test: ($^{**}p < 0.01$, $^{***}p < 0.001$).

Table 2
Effect of starvation and refeeding with commercial diet supplemented or not with *Chlorella vulgaris* protein hydrolysate (Cv-PH) on the number of peritoneal exudate cells (PECs) and macrophage functional activities of Balb/c mice

Assay	C	M	M-DC	M-CvPH
No. of PECs ($\times 10^6$/mouse)***	2.4 ± 0.4 b	1.1 ± 0.4 c	3.0 ± 0.6 b	4.2 ± 0.6 a
Macrophage glucose consumption (μg/well)*	129 ± 10 a	84 ± 4 b	83 ± 13 b	114 ± 7 a
Macrophage acid phosphatase activity (nmol *p*-nitrophenol/ 60 min/1 × 10^5 cells)***	28 ± 5 b	17 ± 3 c	35 ± 4 b	47 ± 6 a

Peritoneal exudate cells (PECs) were isolated from the peritoneal cavity by lavage with Hanks' solution. PECs were cultured at 37 °C for 1 h in a CO_2 incubator. The macrophages (1 × 10^5 cells) were cultured in 96-well plates for 72 h and glucose remaining in the supernatants was measured by the Rapi-Gluco-Test, enzymatic method. The production of acid phosphatase by macrophages cultured for 72 h was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in cell lysates. All values are expressed as the arithmetic mean ± S.E. of 10 mice. Different letters indicate significant differences among the groups in Duncan test ($^{*}p < 0.05$ and $^{***}p < 0.001$).

3. Results and discussion

The haematological parameters assayed in all groups are shown in Table 1. The oral treatment with *Chlorella* protein hydrolysate during refeeding provided benefits in terms of haemopoiesis, as judged by the recovery of bone marrow cellularity and the leukocyte count in peripheral blood, significantly higher compared with those of M-DC group and similar to control values. Particularly, the lymphocyte pool increased up to 128% relative to control group ($p < 0.01$). The granulocyte pool was also significantly higher after refeeding with Cv-PH than M-DC group. These parameters were decreased in fasted mice (group M). A significant contribution to the effect of Cv-PH on haemopoiesis, may be a routing of differentiation of bone marrow haemopoietic cells toward the granulocyte and lymphocyte pools and a more rapid recovery of these series of haemopoietic cells. It may involve both a non-specific effect and immunomodulating effects described in aqueous extracts of *Chlorella vulgaris* [18,19].

Since macrophages have been suggested to play important roles in immunological surveillance, we studied the influence of the oral administration of Cv-PH in the number of peritoneal exudate cells and the functional activities of macrophages (Table 2). Cv-PH at 500 mg/kg during refeeding remarkably increased the number of peritoneal exudate cells relative to the other groups ($p < 0.001$). The functional activities of macrophages were greatly enhanced by Cv-PH, as judged by the glucose consumption and the acid phosphatase levels. Macrophages from Cv-PH group exhibited higher values of glucose consumption than did group M-DC ($p < 0.05$) and reached control levels. A large quantity of glucose is known to be consumed by macrophages to produce the ATP and NADPH (via the hexose monophosphate shunt) required for phagocytosis and also to produce reactive oxygen intermediates [20]. Cv-PH group showed the highest values of macrophage lysosomal acid phosphatase activity ($p < 0.001$). Macrophages scavenger functions, including lysosomal enzymes, are responsible for killing microorganisms and cancer cells *in vivo* [16].

We evaluated the effects of Cv-PH administration on the *in vivo* phagocytic activity of undernourished mice (Fig. 1). This evaluation was performed by measuring the carbon clearance in peripheral blood as an index of the phagocytic activity of the liver and spleen, because a colloidal carbon suspension injected into mice via the tail vein is mainly phagocytized in these organs. Group Cv-PH reached control values of the half-life time of carbon in the blood at the end of the refeeding period. This indicated that Cv-PH possesses potentiating activity for the mononuclear phagocytic system. We measured the liver and spleen weights to elucidate whether the enhanced carbon clearance activity was caused by an increase in the number of total phagocytes or by

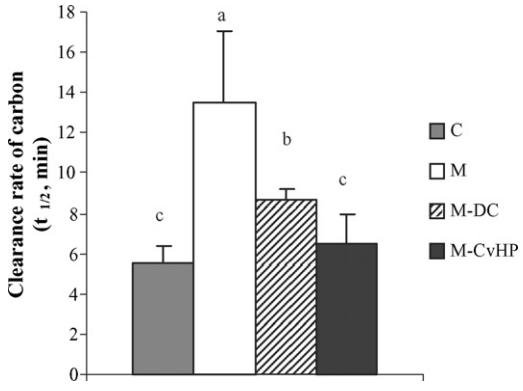

Fig. 1. Effect of starvation and refeeding with commercial diet supplemented or not with *Chlorella vulgaris* protein hydrolysate (Cv-PH) on clearance rate of carbon particles from blood circulation in Balb/c mice. All values are expressed as the arithmetic mean ± S.E. of 10 mice. Different letters indicate significant differences among the groups in Duncan test ($p < 0.01$).

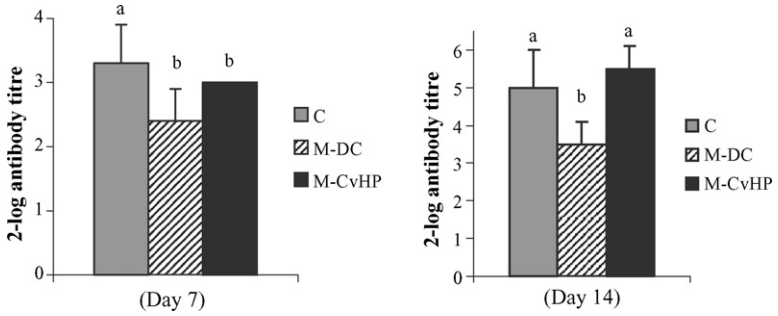

Fig. 2. Humoral immune response against a T-dependent antigen (sheep red blood cells, SRBC) at 7 and 14 days of inoculation. After the starvation period (day 0), five animals in each group were injected i.p. with 0.2 ml of a 25% of SRBC saline solution. Seven days from the first injection the antibody titres were measured by a haemagglutination reaction. The reciprocal serum dilution which just gave agglutination was considered to be the titre. At this time, mice received the second immunization and on day 14, antibody titres were determined as described. Different letters indicate significant differences among the groups in Duncan test, $p < 0.05$.

activated phagocytes. The results suggested that the increased phagocytic activity by Cv-PH was due to the activation of phagocytes and not to an increase in the number of total phagocytes (data not shown).

Another effect of Cv-PH on the immune system was a potentiation of the humoral response, which was determined by measuring antibody titres to SRBC, a T-dependent antigen (Fig. 2). There were no major differences in haemagglutination titres between the mice refed with the commercial diet supplemented or not with Cv-PH after a week of immunization. However, the secondary response (day 14) in the Cv-PH group evoked antibody titres that reached control values ($p < 0.05$). These findings suggested the stimulation of the functional abilities of T_h (T-helper cells) and/or expanded pools of memory B cells by Cv-PH.

Cell mediated immunity is severely affected in protein-energy malnutrition [21]. The effect of Cv-PH on cell-mediated immunity was determined by the DTH response. As shown in Table 3, control mice mounted a brisk DTH response to a rechallenge of BSA. After starvation, the oral administration of Cv-PH in addition to commercial diet promoted an increase of foot pad swelling compared to the commercial diet alone. The DTH response in M-CvPH group measured at 48 and 72 h after challenge was similar that of control mice ($p < 0.05$). The reconstitution of DTH response by Cv-PH reflected the induction of $CD4^+$ Th1 cells and the activation of macrophages by cytokines: tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) [17].

Malnutrition induced by dietary restriction produces a series of metabolic changes that lead to depression of immunocompetence, and several studies have assessed the effects of nutritional support on immunity [21]. Although the properties and composition of algae protein hydrolysates are certain to lead to preparations with biological activities [14] which have been administered to patients with different protein metabolic diseases [22], no information was available about the immunomodulating properties of green algae enzymatic protein hydrolysates.

In sum, the results of this study suggest that enzymatic protein hydrolysates from *Chlorella vulgaris* could enhance host-defense activity *in vivo* by stimulating mechanisms involved in both innate and specific immune responses of undernourished mice. Thus, Cv-PH can be used for developing physiologically functional foods with immunopotentiating activity for humans.

Table 3
Effect of starvation and refeeding with commercial diet supplemented or not with *Chlorella vulgaris* protein hydrolysate (Cv-PH) on the delayed-type hypersensitivity (DTH) response toward BSA antigen of Balb/c mice

	Foot pad thickness (mm)		
	24 h	48 h	72 h
Control	0.52 ± 0.07 a	0.46 ± 0.05 a	0.38 ± 0.06 a
M-DC	0.31 ± 0.09 c	0.30 ± 0.09 b	0.25 ± 0.08 b
M-CvPH	0.48 ± 0.09 b	0.46 ± 0.06 a	0.38 ± 0.05 a

Mice were immunized by an intradermal (i.d.) injection of 50 μl of 5 mg/ml BSA emulsified in CFA at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20 μl of 5 mg/ml BSA into one rear foot pad, while the other received a comparable volume of PBS. Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge. The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS injected foot pads. All values are expressed as the arithmetic mean ± S.E. of five mice. Different letters indicate significant differences among the groups in Duncan test ($p < 0.05$).

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