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A *Porphyra columbina* hydrolysate upregulates IL-10 production in rat macrophages and lymphocytes through an NF-κB, and p38 and JNK dependent mechanism

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ABSTRACT

The marine environment represents a relatively untapped source of functional ingredients. Here we characterise a hydrolysate obtained from *Phorphyra columbina* (PcRH) and its effects on primary splenocytes, macrophages and T lymphocytes *in vitro*. Our product had a high degree of hydrolysis, due to the use of a mixture of endo-peptidase and exo-peptidase, and was enriched in Asp, Ala and Glu. PcRH had mitogenic effects on rat splenic lymphocytes. IL-10 secretion was enhanced by PcRH in splenocytes (235%), macrophages (150%) and in lymphocytes (472%), while the production of TNFα and other proinflammatory cytokines by macrophages was inhibited (15–75%), especially under lipopolysaccharide stimulation. The effect of the hydrolysate on IL-10 was evoked by JNK, p38 MAPK and NF-κB dependent pathways in T lymphocytes. We conclude that PcRH has immunomodulatory effects on macrophages and lymphocytes, activating NF-κB and MAPK dependent pathways, and predominantly inducing IL-10 production.
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1. Introduction

The demand for functional foods and nutraceuticals has increased significantly in response to the increasing awareness of the influence of diet on health. The therapeutic potential of some food components has drawn the attention of clinicians, food manufacturers, researchers and consumers. The therapeutic potential of functional foods and nutraceuticals is attributable to the presence of specific compounds that are part of the diet and/or their molecular derivatives released during food metabolism (Agyei, Apostolopoulos, & Danquah, 2011). Dietary proteins, in addition to their ability to supply calories and amino acids, have also been known to offer health benefits *in vivo* and *in vitro* either in intact form or as hydrolysates. Peptides from food protein hydrolysates that induce beneficial biological functionalities are called bioactive peptides. Bioactive peptides may be produced either by microbial fermentation, by enzyme digestion *in vivo* or by means of proteolysis by enzymes *in vitro*, and can modulate the physiological activity of major body systems (Korhonen & Pihlanto, 2006). Such functionalities include antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects (Hartmann & Meisel, 2007; Requena et al., 2010; Yang et al., 2009).

It is well known that the immune function is of vital importance to prevent and control infection and neoplasia. However, there are very few strategies available to modulate the immune response effectively. Nutritional interventions that involve optimising the intake of essential nutrients and utilising promising functional foods have become a widely applied approach to promote immune-related health. Over the past few years, the potential of bioactive peptides derived from dietary proteins to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused a lot of scientific interest and attention (Ma, Bae, Lee, & Yang, 2006).

Seaweeds, with their diverse bioactive compounds, have opened up potential opportunities in the pharmaceutical and agro-food processing industries. The consumption of seaweeds as part of the diet has been claimed to be one of the prime reasons for low incidence of breast and prostate cancer in Japan and China, compared to North America and Europe. Seaweeds also contain

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other compounds of nutritional value, including proteins/aminoacids, polysaccharides (e.g., alginates, fucans and laminarans), oligoelements and polyphenols (Kumar et al., 2011).

Phorphyra columbina is a red seaweed native to Patagonia, Argentina. It has a high protein content (approximately 30% dry weight) and total dietary fibre (39–54% dry weight). Although other marine products have been the subject of intense study in the last decades, with the focus on antimicrobial, antitumour, antiviral and other pharmaceutical properties, seaweeds have received relatively little attention (Aneiros & Garateix, 2004). Moreover, the information about immunological properties from seaweeds' protein fractions is scarce. The aim of this study was to characterise a protein hydrolysate obtained from *P. columbina* and evaluate its immunomodulatory properties.

2. Materials and methods

2.1. Reagents

Flavourzyme (F) was obtained from Sigma Chemical Co. (St. Louis, MO) and fungal protease concentrate (FC) was provided by Genencor (Arroyito, Argentina). The other reagents were obtained from Sigma (Barcelona, Spain).

2.2. Preparation of hydrolysate from P. columbina

For obtaining the hydrolysate, the red marine alga *P. columbina* was dispersed at 50 g kg^{-1} in distilled water and then centrifuged at 3000*g* for 30 min. The pellet was subjected to various extraction steps with hot and cold water. This methodology is the traditional approach intended to obtain fico-colloids. Subsequently, the extraction residue was hydrolysed. A hydrolysate was obtained using 800-ml batch thermostated reactor. The reaction pH was continuously measured using a pH meter (IQ Scientific Instruments, Loveland, CO) and adjusted adding 2 N of NaOH or 2 N of HCl with a burette. Working conditions for the enzymes were T, 55 °C; pH, 4.3; E/S ratio; 50 g kg^{-1} and T, 55 °C; pH, 7.0; E/S ratio, 20 g kg^{-1} for FC and F, respectively. *P. columbina* residue hydrolysate (PcRH) was prepared using FC enzyme (3 h) and F enzyme (4 h) in a sequential way with a total reaction time of 7 h. Once the hydrolysis was complete, the enzymes were inactivated by thermal treatment, following the manufacturer's guidelines. The inactivated hydrolysate was centrifuged at 2000*g* for 30 min and the supernatant was frozen and lyophilised. Free amino groups were measured using OPA, according to Nielsen, Petersen, and Dambmann (2001), and the degree of hydrolysis (DH) was calculated as follows:

$$\mathrm{DH}(\%) = [(h - h_0)/h_{\mathrm{tot}}] \times 100$$

where h_{tot} is the total number of peptide bonds in the protein substrate calculated from amino acids profile (8.47 mEq g^{-1} protein), h is the number of peptide bonds cleaved during hydrolysis and h_0 is the content of free amino groups in the substrate.

2.3. PcRH characterisation

2.3.1. Amino acid analysis

Samples (2 mg) were hydrolysed with 4 ml of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatisation with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC), according to the method of Alaiz, Navarro, Giron, and Vioque (1992), using D,L-α-aminobutyric acid as internal standard. The HPLC system consisted of a Model 600E multi-system with a 484 UV–vis detector (Waters, Milford, MA) equipped with a 300 × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 m; Waters). A binary gradient was used for elution with a flow of 0.9 ml/min. The solvents used were (A) sodium acetate (25 mM) containing sodium azide (0.02% w/v) pH 6.0 and (B) acetonitrile. Elution was as follows: time 0.0–3.0 min, linear gradient from 91% A to 86% A; 3.0–13.0 min, elution with 86% A; 13.0–30.0 min, linear gradient from 86% to 69% A; 30.0–35.0 min, elution with 69% A. Eluted amino acids were detected at 280 nm. The column was maintained at 18 °C. Tryptophan was determined by HPLC-RP chromatography, after basic hydrolysis according to Yust et al. (2004).

2.3.2. Fast protein liquid chromatography (FPLC)

Gel filtration chromatography was carried out in an AKTA purifier system equipped with a Superdex peptide column (GE Life Sciences, Piscataway, NJ). Injection volume was 200 µl (10 mg protein PcRH ml^{-1}) and elution was carried out using 0.75 M ammonium bicarbonate at 1 ml min^{-1}. Elution was monitored at 214 nm and molecular mass was estimated using molecular weight (MW) standards from Pharmacia: blue dextran (2,000,000 Da), cytochrome C (12,500 Da), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da).

2.4. Spleen mononuclear cell isolation

Female Wistar rats were obtained from Janvier S.A.S. (Le Genest Saint Isle, France), sacrificed by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in medium. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lyses buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA{\cdot}2H_2O$, pH 7.3) for 30 min on ice. For splenocyte experiments, mononuclear cells were washed and suspended in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 0.1 g l^{-1} streptomycin, 2.5 g l^{-1} amphotericin B and 0.05 mM β-mercaptoethanol. To purify lymphocytes, mononuclear cells were resuspended in rinse buffer (phosphate buffer saline pH 7.2, 0.5% BSA, 2 mM EDTA) and single-cell suspensions were obtained using a 70-µm cell strainer (BD Falcon™, Madrid, Spain). For lymphocyte isolation, non-target cells were magnetically labelled and retained in an MACS® separator. Target cells passed through the column were collected, rinsed and suspended in RPMI medium supplemented as above. Magnetic labelling was indirect, using biotin- or phycoeritrin-labelled antibodies against CD11b, CD161a and CD45RA (BD Biosciences, Erembodegem, Belgium) and specific MACS® microbeads (Miltenyi Biotec, Madrid, Spain).

To isolate macrophages, we used the same protocol as for lymphocytes with the following changes. Target cells were suspended in DMEM medium supplemented with 10% FBS, penicillin 100 U ml^{-1}, streptomycin 0.1 g l^{-1}, amphotericin B 2.5 g l^{-1}; and the labelling antibodies were anti-CD161a-biotin, CD45RA-PE and anti-CD3-biotin. Separation protocols were set up and validated by flow cytometry. The cells were maintained at 37 °C in standard culture conditions. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was approved by the Animal Welfare Committee of the University of Granada (Granada, Spain).

2.5. Protein determination

For the following experiments protein content of PcRH was determined by bincinchoninic acid method using bovine serum albumin as standard (Smith et al., 1985).

2.6. Cell proliferation assay

Cell proliferation was measured by [^{3}H]-thymidine incorporation. Splenocytes and lymphocytes (10^6 cells ml^{-1}) were cultured with [^{3}H]-thymidine (1 mCi/well, GE Healthcare, Little Chalfont, UK), in the presence or absence of PcRH (1 or 0.1 g l^{-1} of proteins) for 1 h before the addition of concanavalin A (ConA, 5 μg ml^{-1}) and were incubated for 48 h. After this period the cells were fixed in ice-cold 70% trichloroacetic acid and solubilised in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted with a liquid scintillation counter (Beckman Coulter, Madrid, Spain). [^{3}H]-Thymidine uptake was measured with a Tri-Carb liquid scintillation analyser (Packard Instrument, Meriden, CT) and expressed as c.p.m. (mean ± SD). All assays were performed in triplicate.

2.7. Cytokine determination

For cytokine determinations the cells suspensions (10^6 cells ml^{-1} in RPMI or DMEM medium) were cultured in the presence or absence of PcRH (1, 0.1 and 0.01 g l^{-1} of protein) and stimulated with concanavalin A (ConA, 5 μg ml^{-1}) or lipopolysaccharides (LPS; 1 μg ml^{-1}) depending on cell type. Cell culture medium was collected after 24 or 48 h, cleared by centrifugation (3000*g*, 5 min, 4 °C) and frozen at −80 °C until assayed for cytokine content by commercial ELISAs (Biosource Europe, Nivelles, Belgium, and Becton Dickinson, Franklin Lakes, NJ). In all the experiments, samples were run in triplicate and results are expressed as cytokine concentration (pg mL^{-1}).

2.8. Lactate dehydrogenase assay

Cellular toxicity was measured as the release of lactate dehydrogenase. Cells were cultured under the conditions described above and lactate dehydrogenase activity in supernatants was measured spectrophotometrically using sodium pyruvate (25 mM) as substrate in 50 mM sodium phosphate buffer (pH 7.5) (Halprin & Ohkawara, 1966).

2.9. NF-kB and MAPK inhibitors assay

In order to explore signalling pathways, the kinase inhibitors [PD98059 for the mitogen-activated protein kinase MAPK ERK1/2, SB203580 for p38 MAPK, SP600125 for c-Jun N-terminal kinase (JNK) and Bay11–7082 for NF-kB] were added to the cell culture medium (10 mM in all cases) 30 min before the addition of PcRH (1 g l^{-1} of protein). The cells were then incubated for 24 or 48 h and the supernatants were used to determine cytokine concentrations as described above.

2.10. Antioxidant capacity

2.10.1. ABTS$^{\cdot+}$ radical cation decolorisation assay

To estimate the antioxidant capacity, the ABTS$^{\cdot+}$ radical cation decolorisation assay according to Cian, Lugren, and Drago (2011) was used. The absorbance reading was taken at 6 min after initial mixing. ABTS$^{\cdot+}$ inhibition with PcRH was determined at 1.8 g l^{-1} of protein. To determine the concentration causing an inhibition of 50% (IC_{50}) serial dilutions of PcRH from 0 to 2.37 g l^{-1} protein were made.

2.10.2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

DPPH radical-scavenging activity assay was measured according to the method of Sánchez-Moreno, Larrauri, and Saura-Calixto (1998). An aliquot of 10 μl of PcRH at 0.9 g l^{-1} protein was mixed with 190 μl of methanol solution containing 0.08 mM DPPH radical. The mixture was allowed to stand for 60 min in the dark, and the absorbance was monitored at 517 nm. The difference between the blank and the sample was used for calculating the scavenging activity as percentage inhibition. Serial dilutions of PcRH from 0 to 0.9 g l^{-1} protein were made to determine the concentration causing an inhibition of 50% (IC_{50}).

2.10.3. Copper-chelating activity by assay of β-carotene oxidation

Copper-chelating activity was determined by the assay of β-carotene oxidation according to Megías et al. (2008) with modifications. A solution of β-carotene 10 g l^{-1} in chloroform was prepared. After addition of 1 ml Tween 20, chloroform was evaporated under nitrogen and then the solution was dissolved in 0.1 N pH 7.0 phosphate buffers. A solution 50 μM $CuSO_4$ was prepared and 10 μl were added to each well. The assay mixture was: 200 μl β-carotene, 10 μl $CuSO_4$, 200 μl PcRH. The concentration of PcRH was 1 g l^{-1} of protein. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm. Positive control (+): β-carotene 200 μl + $CuSO_4$ 10 μl, and negative control: β-carotene 200 μl. The difference between the negative control and the sample was used for calculating the copper-chelating activity as percent inhibition.

2.11. Statistical analysis

All results are expressed as mean ± SD. The data were analysed by one-way analysis of variance, using the software Statgraphics Plus 3.0. Least significant difference test was used to determine statistical differences between samples. Significance was established at $p < 0.05$.

3. Results

3.1. Hydrolysis reactions and PcRH characterisation

Fig. 1A shows the hydrolysis curve of PcRH. The hydrolytic curve showed a high rate of hydrolysis in the first hour, after which the proteolysis rate kept increasing gradually with time, albeit more slowly. Addition of the F enzyme after 3 h of reaction increased DH again, augmenting the efficiency of the reaction (Fig. 1A). The final DH obtained with the enzymatic system

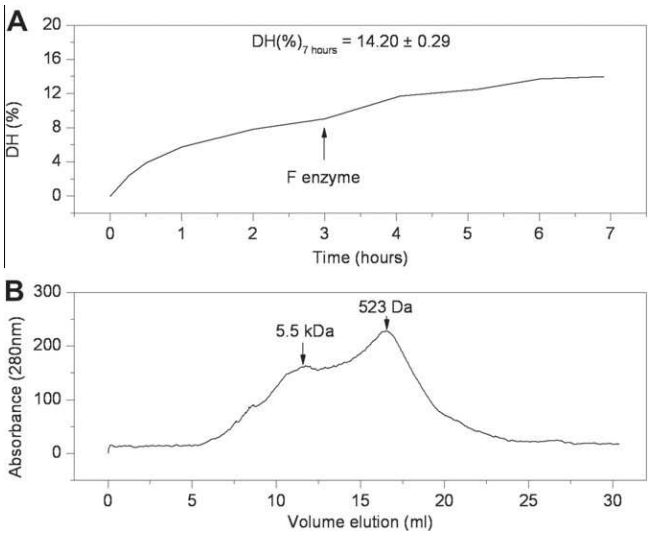

Fig. 1. Enzymatic hydrolysis of *Porphyra columbina* residue, with FC + F proteases (A) and FPLC gel filtration profile of PcRH (B). The profile shown is representative of several that were carried out. All samples were analysed in triplicate.

Table 1
Amino acids profile of PcRH.[*,a]

Amino acids	Total amino acids[b] (g/100 g ± SD)	Free amino acids[c] (g/100 g ± SD)	Peptide amino acids[d] (g/100 g ± SD)
Asp	12.33 ± 0.09	0.31 ± 0.02	12.04 ± 0.09
Glu	10.13 ± 0.01	0.02 ± 0.01	10.11 ± 0.01
Ser	6.36 ± 0.10		6.36 ± 0.10
His	1.04 ± 0.02		1.04 ± 0.02
Gly	9.63 ± 0.18		9.63 ± 0.18
Thr	6.42 ± 0.01		6.42 ± 0.01
Arg	6.40 ± 0.07		6.40 ± 0.07
Ala	12.09 ± 0.10	0.13 ± 0.01	11.96 ± 0.10
Pro	3.54 ± 0.21		3.54 ± 0.21
Tyr	2.31 ± 0.01		2.31 ± 0.01
Val	6.80 ± 0.01		6.80 ± 0.01
Met	1.51 ± 0.05		1.51 ± 0.05
Cys	0.89 ± 0.03		0.89 ± 0.03
Iso	2.88 ± 0.06	0.02 ± 0.01	2.87 ± 0.06
Trp	0.64 ± 0.09		0.64 ± 0.09
Leu	8.03 ± 0.01	0.23 ± 0.01	7.82 ± 0.01
Phe	3.61 ± 0.05	0.09 ± 0.01	3.52 ± 0.05
Lys	5.38 ± 0.02	0.19 ± 0.01	5.19 ± 0.02

[*] Data presented as mean ± SD. All samples were analysed in triplicate.
[a] PcRH: *Phorphyra columbina* residue hydrolysate.
[b] Total amino acids: amino acids profile of PcRH.
[c] Free amino acids: free amino acids profile of PcRH.
[d] Peptide amino acids: total amino acids profile – free amino acids profile.

Table 2
Level of lactate dehydrogenase (m U μ^{-1}) in the culture medium from splenocytes and lymphocytes with or without PcRH (1 or 0.1 g l^{-1} of protein).[A]

	Splenocytes		Lymphocytes	
PcRH (g l^{-1} of protein)	Basal	ConA	Basal	ConA
0	24.2 ± 0.6[a]	25.5 ± 0.2[a]	24.3 ± 2.0[a]	25.5 ± 2.0[a]
0.1	23.8 ± 2.1[a]	23.8 ± 1.5[a]	23.7 ± 1.7[a]	21.6 ± 1.6[a]
1.0	23.8 ± 0.9[a]	22.5 ± 1.6[a]	21.8 ± 1.0[a]	23.8 ± 1.5[a]

[A] Data presented as mean ± SD. All samples were analysed in triplicate. Different letters mean significant differences between samples ($p < 0.05$).

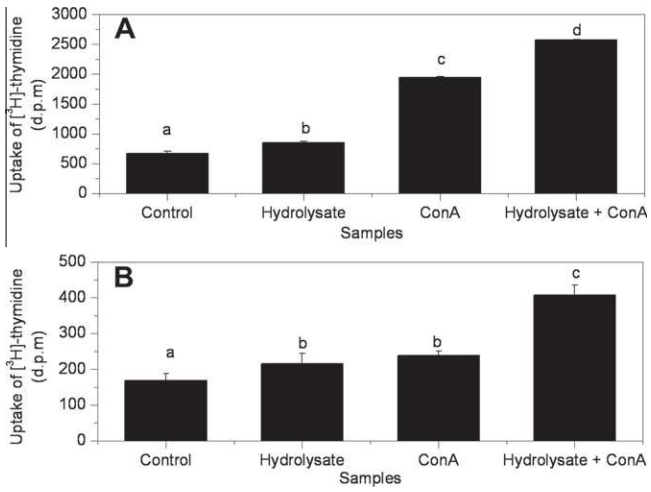

Fig. 2. Effect of PcRH on splenocyte (A) and lymphocytes (B) proliferation, assessed by the thymidine uptake assay. Cells were incubated with hydrolysate 1 g l^{-1} of protein with or without ConA (5 μg/mL). [^{3}H]-Thymidine (1 mCi/well) was added at the same time and uptake was measured 48 h after. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

(FC + F) was 14.20 ± 0.28%. Fig. 1B shows the FPLC gel filtration profile of PcRH, showing two main peaks. The first peak corresponds to peptide fractions with MW around 5.5 kDa and the second one to peptides of MW 523 Da. It should be noted that these are peptide

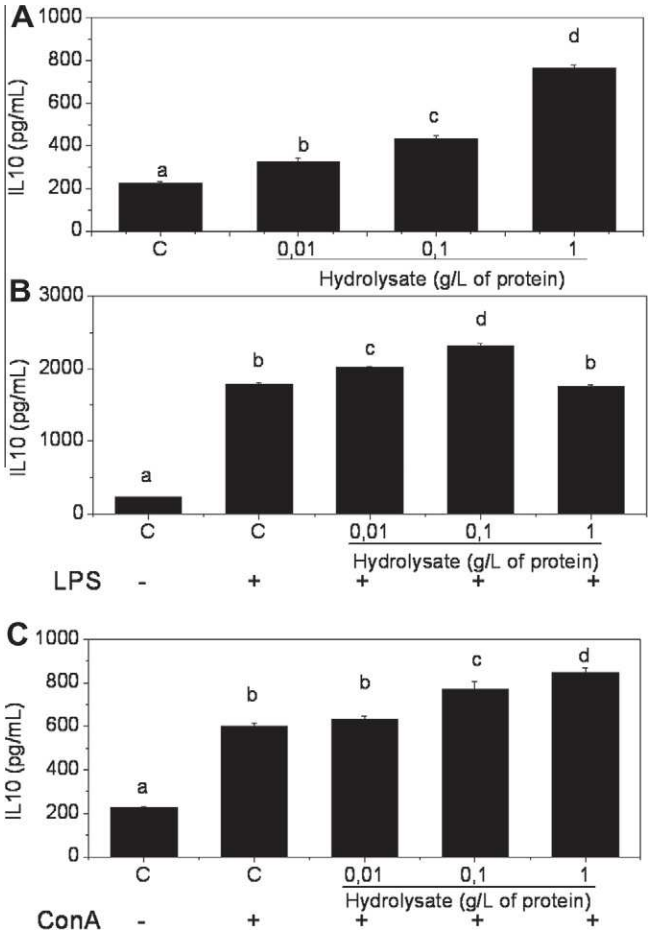

Fig. 3. Effect of PcRH on the production of IL-10 by splenocytes. Splenocytes were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PcRH (0.01, 0.1 and 1 g l^{-1} of protein) in absence (A), or presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (B), or presence of Concanavalin A (ConA, 5 μg/mL) (C). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

weight estimations. Considering 120 Da the average MW of amino acids, this fraction could be formed by tetrapeptides. The PcRH also have free amino acids, Asp, Ala and Glu being the most abundant (Table 1).

3.2. Effect of hydrolysate on splenocyte and lymphocyte cell viability and proliferation

As a routine procedure, the possible effect of PcRH on splenocyte cell viability was assessed. The level of lactate dehydrogenase activity in the culture medium was similar in the presence and absence of PcRH, indicating that it was not toxic to the cells (Table 2). Of note, PcRH was also non-toxic to macrophages (data not shown). ConA, a well-known T cell mitogen, stimulated the proliferation of murine splenocytes ([^{3}H]-thymidine incorporation), as expected (Fig. 2A). The effects of PcRH on splenocyte (and lymphocyte) proliferation were evaluated in both unstimulated (without mitogen) and stimulated cells (with mitogen, Con A) as shown in Fig. 2A and B. PcRH had a small but significant enhancing effect on the proliferation of splenocytes, which was observed with and was actually more pronounced without ConA. The results were very similar when primary lymphocytes were studied instead of the mixed splenocyte population (Fig. 2B).

3.3. Effect of PcRH on IL-10 secretion in splenocytes, macrophages and lymphocytes

Fig. 3 shows the effect of PcRH on the production of IL-10 by splenocytes. The hydrolysate exhibited a concentration-dependent stimulatory effect on IL-10 secretion by splenocytes under basal conditions (Fig. 3A). When the cells were treated with LPS or ConA, which predominantly affect monocytes/macrophages and T lymphocytes, respectively, a large surge in IL-10 release was obtained (Fig. 3B and C). This was further increased by addition of PcRH in a concentration-dependent fashion, although the effects were not entirely alike. Thus the effect of the hydrolysate on LPS-evoked IL-10 secretion at 1 g l^{-1}, while ConA response was enhanced at 0.1 and 1 g l^{-1} but not at 0.01 g l^{-1}.

In order to confirm the cell target of these actions, PcRH was assayed in purified primary spleen macrophages and T-lymphocytes (Fig. 4). As in splenocytes, the hydrolysate induced IL-10 in a concentration-dependent way under basal conditions in both cell types (Fig. 4A), the response being more robust (and perhaps more potent) in lymphocytes. Macrophages appeared to develop a higher secretory response than lymphocytes under stimulation. Again, PcRH was able to enhance IL-10 under these conditions. This effect was more powerful in T cells than in macrophages or mixed splenocytes. Of note, the hydrolysate was ineffective at the concentration of 1 g l^{-1} in macrophages, just like in splenocytes, while the response did not reach a plateau in lymphocytes.

3.4. Signal transduction pathways

The stimulation of the MAPK signal transduction pathways by PcRH was studied in lymphocytes using inhibitors for ERK1/2 (PD98059), p38 MAPK (SB203580) and JNK (SP600125). As shown in Table 3, the addition of either SB203580 or SP600125 to the cell culture medium prevented the increase in IL-10 secretion by lymphocytes induced by PcRH, while PD98059 had no inhibitory effect.

The role of the NF-κB signalling pathway in the stimulation of lymphocytes by PcRH was also studied. Total inhibition of IL-10 secretion was observed when cells were cultured in the presence of Bay11–7082, indicating that this pathway also plays a pivotal role in the response to hydrolysate (Table 3).

3.5. Effect of PcRH on pro-inflammatory cytokine secretion in splenocytes

We examined the possible effect of PcRH on proinflammatory cytokines by measuring TNFα secretion in quiescent as well as LPS-stimulated splenocytes (Fig. 5A and B, respectively). As in the case of IL-10, PcRH augmented basal TNFα release, at concentrations

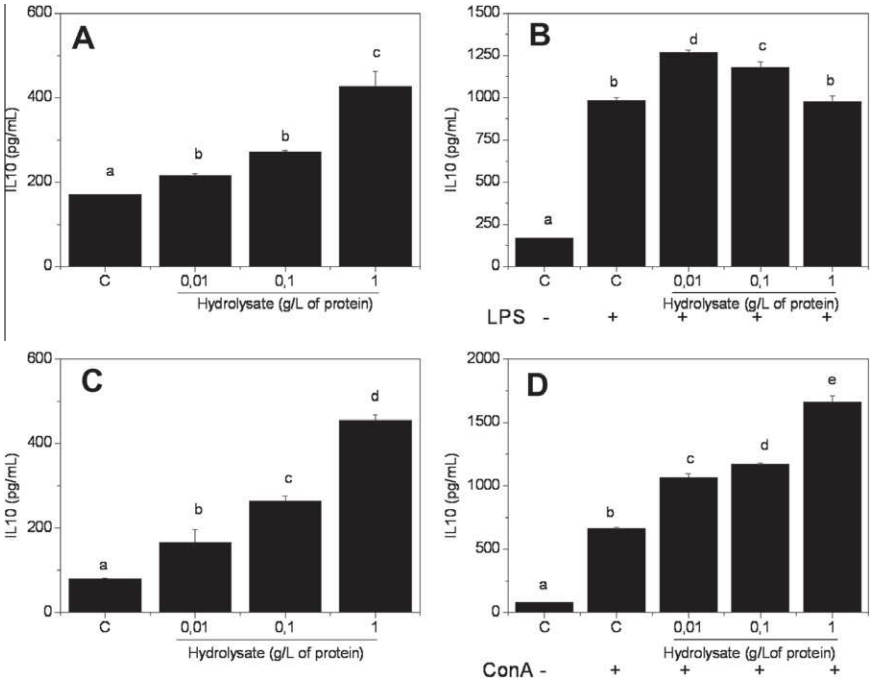

Fig. 4. Effect of PcRH on the production of IL-10 by lymphocytes and macrophages. Cells were plated in 24-well plates (0.5 × 10^6 cells/well) and cultured with PcRH (0.01, 0.1 and 1 g l^{-1} of protein) in absence (A and C), or presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (B), or presence of Concanavalin A (ConA, 5 μg/mL) (D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

of 0.1 g l^{-1} or higher. However, the hydrolysate had the opposite effect when cells were treated with LPS, i.e. a concentration-dependent inhibition of secretion. This effect was marked, with ~90% of cytokine release being blocked by PcRH at 1 g l^{-1}. Because of the magnitude of this effect, we decided to extend our study to other proinflammatory cytokines like IFN-γ.

As shown in Fig. 5C and D, the addition of PcRH to splenocytes decreased IFN-γ secretion in the cell culture medium in a concentration-dependent fashion. This effect was modest in quiescent cells, while a much more pronounced effect was obtained under ConA stimulation, with a maximal inhibition of approximately 50% at 1 g l^{-1}.

3.6. Effect of PcRH on pro-inflammatory cytokine secretion in macrophages and lymphocytes

As TNFα, IL-1β and IL-6 are produced mainly by macrophages we decided to evaluate the effect of PcRH on these pro-inflammatory cytokines on this cell type. Fig. 6A and B shows the results for TNFα. Interestingly, TNFα output was reduced significantly, albeit slightly, by PcRH in macrophages under basal conditions, an effect opposite to that obtained with splenocytes. The results obtained with LPS were very similar to those in splenocytes. Furthermore, the hydrolysate also inhibited basal and LPS evoked IL-1β secretion, although less markedly than in the case of TNFα (data not shown). IL-6 production was unaffected by PcRH alone, but LPS-induced secretion was totally prevented by the hydrolysate (Fig. 6C and D).

Contrary to what was observed for splenocytes, T lymphocytes were unaffected in basal conditions and responded to PcRH with a significant increase of IFN-γ secretion (~20%) with ConA (Fig. 7A and B).

Table 3
Effect of MAP kinase inhibitors (PD98059: ERK1/2 inhibitor, SB203580: p38 MAPK inhibitor and SP600125: JNK inhibitor) and NF-kB inhibitors (Bay 11–7082) on interleukin 10 (IL-10) by lymphocytes stimulated with hydrolysate.[A]

Samples	IL10 (pg ml^{-1})
Control	112 ± 0.51[a]
ConA	937 ± 51.5[c]
Hydrolysate	433 ± 3.03[b]
BAY11–7082 + hydrolysate	137 ± 0.51[a]
SB203580 + hydrolysate	121 ± 0.30[a]
PD98059 + hydrolysate	327 ± 10.10[b]
SP600125 + hydrolysate	130 ± 1.01[a]

[A] Data presented as mean ± SD. All samples were analysed in triplicate. Different letters mean significant differences between samples ($p < 0.05$).

3.7. Antioxidant capacity of PcRH

PcRH showed a good *in vitro* antioxidant capacity: 61.4 ± 0.9% and 81.1 ± 1.6% for $ABTS^{+\cdot}$ and DPPH inhibition, respectively. The 50% inhibition concentration values (IC_{50}) for $ABTS^{+\cdot}$ and DPPH assay for PcRH were 1.0 ± 0.02 g l^{-1} and 0.7 ± 0.03 g l^{-1} of protein, respectively. On the other hand, PcRH showed higher inhibition of β-carotene oxidation in the presence of copper (97.5 ± 0.6%).

4. Discussion

Here we present data on a hydrolysate obtained from *P. columbina* under controlled conditions *in vitro*. These were selected in order to obtain a high DH (the most widely used indicator for comparison among different proteolysis processes (Kong, Guo, Hua, Cao, & Zhang, 2008)), namely by way of the addition of F enzyme, a mixture of endopeptidase and exopeptidase. This combination has been shown to be more efficient than an endopeptidase alone (In, Chae, & Oh, 2002), probably due to the fact that it tends to reduce the effect of the competition between unhydrolysed protein and the peptides being constantly formed during hydrolysis

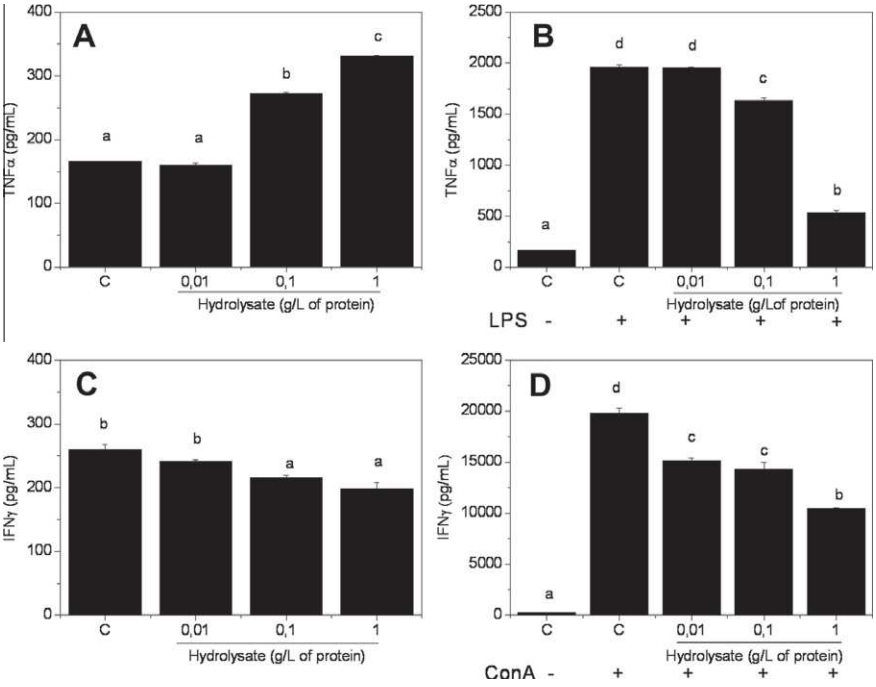

Fig. 5. Effect of PcRH on the production of TNFα and IFNγ by splenocytes. Splenocytes were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PcRH (0.01, 0.1 and 1 g l^{-1} of protein) in absence (A and C), or presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (B), or presence of Concanavalin A (ConA, 5 μg/mL) (D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

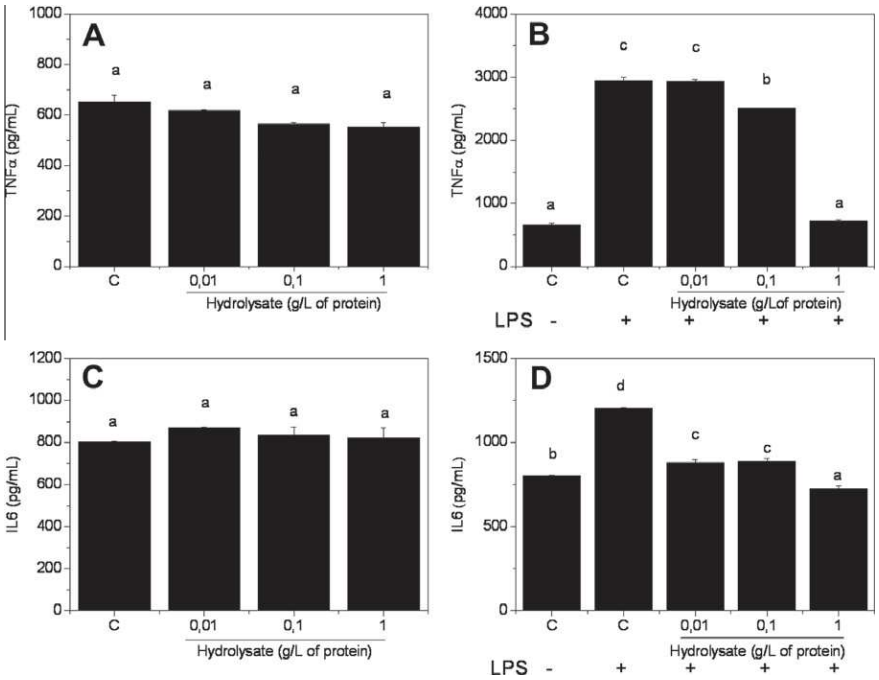

Fig. 6. Effect of PcRH on the production of TNFα and IL6 by macrophages. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PcRH (0.01, 0.1 and 1 g l^{-1} of protein) in absence (A and C), or presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (B and D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

(Adler-Nissen, 1986). The production of extensive protein hydrolysates by the sequential action of endopeptidases and exoproteases is considered the most effective way to obtain protein hydrolysates with defined characteristics (Kong et al., 2008).

The hydrolysate thus obtained, PcRH, has remarkable immunomodulatory properties, based on our *in vitro* experiments. These can be summarised as follows (Fig. 8): (1) mitogenic effects on murine splenic lymphocytes and splenocytes; (2) stimulation of IL-10 secretion in macrophages and specially T lymphocytes; (3) inhibition of the production of TNFα and other proinflammatory cytokines by macrophages, especially under LPS stimulation; (4) inhibition of the release of IFN-γ by splenocytes. Mechanistically, PcRH induces IL-10 by JNK, p38 MAPK and NF-κB dependent pathways in T lymphocytes, based on pharmacological evidence.

The mechanism of the effect on TNFα/IL-6/IL-1β in macrophages was not characterised in the present study, but is presumably due to actions that differ markedly from those on lymphocytes. One possibility is that IL-10 is induced as the predominant effect in these cells, producing an inhibition of proinflammatory cytokines in an autocrine/paracrine fashion. IL-10 has potent inhibitory effects on a number of immune cells including macrophages. For instance, IL-10 has been shown to stimulate monocyte expression of soluble TNF receptors, which act as natural inhibitors of TNFα, and to enhance the production of IL-1R antagonist (IL-1ra), which competitively inhibits IL-1β by binding to the membrane receptor (Foey et al., 1998). Whatever the exact mechanism, it should be noted that IL-10 is normally induced in inflammatory responses alongside proinflammatory cytokines and other mediators, but these typically dominate the inflammatory response. Thus the effect of PcRH is remarkable.

Interestingly, the direct effect of the hydrolysate on these cells was stimulation of IFN-γ secretion, while the opposite was observed in splenocytes, a mixed population that features mostly lymphocytes and, secondarily, macrophages. Thus PcRH appears to downregulate IFN-γ expression indirectly, via actions on macrophages. Because TNFα/IL-6/IL-1β are generated through signalling

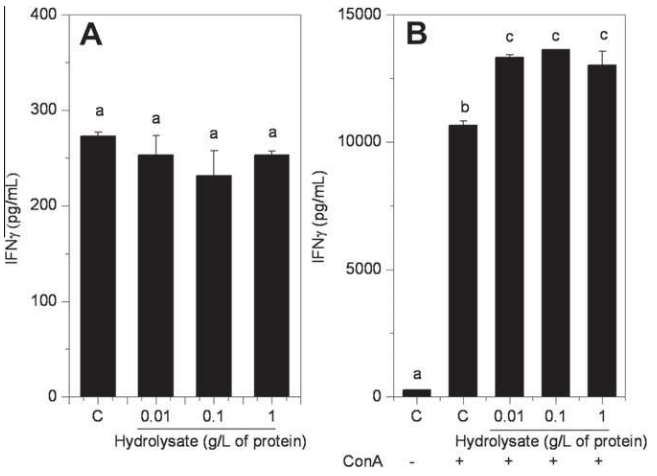

Fig. 7. Effect of PcRH on the production of IFNγ by lymphocytes. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PcRH (0.01, 0.1 and 1 g l^{-1} of protein) in absence (A), or presence of Concanavalin A (ConA, 5 μg/mL) (B). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD. All samples were analysed in triplicate; letters with $p < 0.05$ means significant differences.

pathways that involve NF-κB and MAPKs, it is unclear how PcRh can exert such different effects on IL-10 and proinflammatory cytokines. One possibility is that it acts as a mitogen and polarising agent in T lymphocytes, favouring the differentiation to Th2 or Treg cells, which are efficient IL-10 producers but poor IFN-γ secretors. Other protein hydrolysates have been shown to display mitogen properties. Thus Kong et al. (2008) found similar results with soy protein hydrolysates prepared with different enzymatic systems: alcalase, alcalase + flavourzyme, and papain. Likewise, Suetsuna, Chen, and Yamauchi (1991) reported that the oligopeptides derived from sardine muscle had mitogenic activity *in vitro*. Yang

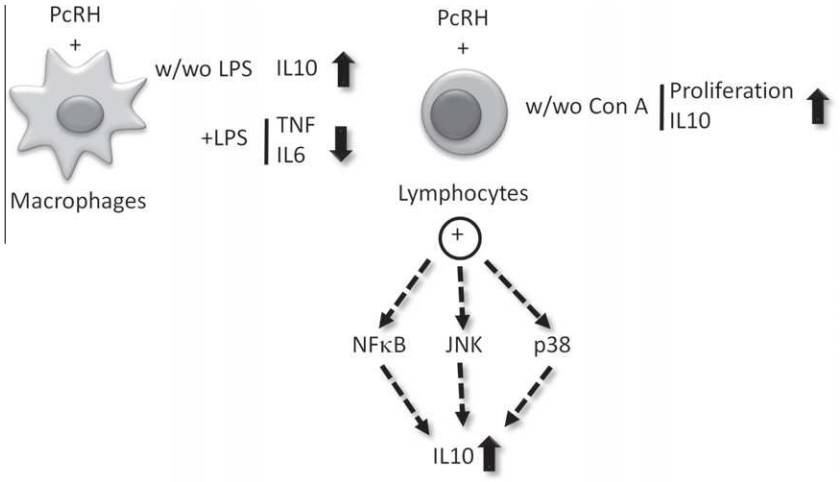

Fig. 8. Schematic diagram of the immunomodulatory mechanism proposed for PcRH. The hydrolysate induces the production of IL-10 by rat macrophages and T lymphocytes and inhibits the production of proinflammatory cytokines, especially after LPS stimulation. IL-10 response is evoked by JNK, p38 MAPK and NF-κB dependent pathways in lymphocytes.

et al. (2009) reported that a marine oligopeptide enriched in Glu > Asp > Lys > Leu > Arg > Gly with molecular weight between 100 and 860 Da caused a significant increase of about 120% in the ConA-stimulated proliferation of splenic lymphocytes compared with the control. Therefore, we may speculate that the PcRH peptides rich in Asp, Ala and Glu of around 523 Da may be responsible for the mitogenic response.

At present, the mechanism underlying the immunomodulatory effect of bioactive peptides remains uncertain. However, the striking effects of PcRH described here are consistent with a significant antiinflammatory/immunosuppressive action, provided of course that the bioactive peptides can gain access to their cell targets. In order to assess this possibility, peptides must be characterised further and their bioavailability evaluated.

On the other hand, the good antioxidant capacity exhibited for PcRH could be due to the liberation of low molecular weight peptides (around 523 Da) during the proteolysis process. Several studies have shown that low molecular weight hydrolysates generally possess higher radical-scavenging capacity than high molecular weight hydrolysates (Wang et al., 2010). In this regard, Torres-Fuentes, Alaiz, and Vioque (2011) reported that a combination of high His contents, around 20–30%, and small peptide size, provide the best chelating activities. However, the content of His in PcRH is relatively low, although the content of other chelating amino acids such as Asp and Glu (Saiga, Tanabe, & Nishimura, 2003) is high. Thus, these residues should be involved in the formation of complexes with Cu^{2+} and thus suppress β-carotene oxidation.

In conclusion, we have characterised PcRH, a protein hydrolysate obtained from *P. columbina*, which has substantial immunomodulatory properties *in vitro* on both macrophages and T lymphocytes, especially induction of IL-10 in both cell types, and antioxidative properties. PcRH may be useful in the management of inflammatory conditions and oxidative stress conditions. However, *in vivo* studies must be done to confirm these properties, followed by the isolation and sequencing of the peptides involved in these activities.

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