JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Immunomodulatory Properties of the Protein Fraction from *Phorphyra columbina*

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ABSTRACT: The phycobiliproteins from *Rhodophyta*, R-phycoerythrin (R-PE) and C-phycocyanin (C-PC), have been shown to exert immunomodulatory effects. This study evaluated the effects of a *Phorphyra columbina* protein fraction (PF) and R-PE and C-PC on rat primary splenocytes, macrophages, and T-lymphocytes in vitro. PF featured various protein species, including R-PE and C-PC. PF showed mitogenic effects on rat splenocytes and was nontoxic to cells except at 1 g L^{-1} protein. IL-10 secretion was enhanced by PF in rat splenocytes, macrophages, and especially T-lymphocytes, whereas it was markedly diminished by R-PE and C-PC. The production of pro-inflammatory cytokines by macrophages was inhibited. The effect of PF on IL-10 was evoked by JNK/p38 MAPK and NF-κB-dependent pathways in macrophages and T-lymphocytes. It was concluded that PF has immunomodulatory effects on macrophages and lymphocytes that appear to be predominantly anti-inflammatory via up-regulated IL-10 production and cannot be accounted for by R-PE and C-PC.

KEYWORDS: *immunomodulation, IL-10, phycobiliproteins, red seaweeds*

INTRODUCTION

The current application of chemical compounds isolated from diverse classes of algae is enormous. Since 1975, three areas of research in aquatic natural products have emerged: toxins, bioproducts, and chemical ecology. Over 15000 novel compounds have been chemically determined. Focusing on bioproducts, recent trends in drug research from natural sources suggest that algae are a promising group to furnish novel biochemically active substances.[1]

In many countries, the food industries consume a wide range of algae, which are well-known to have high contents of fiber, minerals, vitamins, and different antioxidants. In the past few decades, the emphasis has moved from wild harvests to farming and controlled cultivation to produce valuable new products on a large scale.

Seaweeds, with their diverse bioactive compounds, have opened up potential opportunities in pharmaceutical and agri-food processing industries. The consumption of seaweeds as part of the diet has been claimed to be one of the prime reasons for the low incidence of breast and prostate cancers in Japan and China compared to North America and Europe. Seaweeds also contain other compounds of nutritional value, including proteins/amino acids, polysaccharides (e.g., alginates, fucans, and laminarans), oligoelements, and polyphenols.[2]

Phorphyra columbina is a red seaweed native to the Patagonia, Argentina. It has high protein (approximately 30% dry weight) and total dietary fiber (39−54% dry weight) contents. It has characteristic long shaped blades with red edges and yellow areas inside.

The major constituent proteins of the red algae representing up to 50% of the total protein content of cells are the phycobiliproteins. They are a family of fluorescent proteins covalently linked to tetrapyrrole groups, namely, bilins (prosthetic group). The phycobiliproteins in intact cellular compartments (phycobilisomes) are weakly fluorescent. However, when the phycobiliproteins are released from the cells, they become highly fluorescent in a region of the spectrum that is well separated from the autofluorescence of other biological cell matter.[3]

Among the phycobiliproteins from *Rhodophyta*, R-phycoerythrin (R-PE) is one of the most important. It is an oligomeric water-soluble chromoprotein of 240 kDa characterized by an absorption spectrum with three peaks or shoulders at 499, 545, and 565 nm. This reddish-pink pigment is of great interest because it possesses original spectral properties. Purification of R-PE is performed by using different techniques such as ammonium sulfate precipitation and chromatographic techniques (ion exchange, gel filtration, etc.). R-Phycoerythrin is used commonly not only for applications in immunology, cell biology, and flow cytometry but also as a dye in the cosmetics industry and in natural foods.[4] Huang et al.[5] also reported that R-PE subunits isolated from red algae can be used as a photosensitizer in photodynamic therapy of carcinoma cells.

Received: March 2, 2012
Revised: July 31, 2012
Accepted: August 6, 2012
Published: August 6, 2012

dx.doi.org/10.1021/jf300928j | *J. Agric. Food Chem.* 2012, 60, 8146−8154

Recent studies have also shown their application in immunomodulatory and anticarcinogenic activities. In addition, they have potential as natural colorants for use in food and cosmetics, particularly as substitutes for synthetic dyes, which are generally toxic or otherwise unsafe.[3]

Another pigment present in the red algae, albeit in lower proportion, is phycocyanin. It is often used as a dietary nutritional supplement in many countries due to its therapeutic value, including hepatoprotective, antiaggregant, neuroprotective, and reactive oxygen species (ROS)-scavenging actions. In addition, the anti-inflammatory activity of C-phycocyanin (C-PC) has been demonstrated in various in vitro studies and in vivo experimental models such as mice with arthritis or sepsis.[6]

The aim of this study was to evaluate the immunomodulatory properties of *P. columbina* protein fraction (PF) and purified phycobiliproteins (R-PE and C-PC).

MATERIALS AND METHODS

Reagents. Except where indicated, all reagents were obtained from Sigma (Barcelona, Spain).

Preparation of Protein Fraction. To obtain the PF, the red marine alga *P. columbina* was dispersed in distilled water and centrifuged, and the supernatants were ultrafiltered using a 10 kDa cutoff Molecular/Por Cellulose-Ester membrane and Molecular/Por Stirred Cell S-43-70 system. The fraction with molecular weight >10 kDa (PF) was used for subsequent trials. The protein content of PF was determined with the bincinchoninic acid method using bovine serum albumin as standard. The TCA fraction was obtained by PF precipitation with trichloroacetic acid and centrifugation. The supernatant obtained (S_{TCA}) was then neutralized and used.

PF Characterization. *Spectroscopic Measurements.* PF, R-PE (Sigma *52412-F*), and C-PC (Sigma *52468-F*) were dispersed at 1 g L^{-1} in 0.1 M phosphate buffer (pH 6.8). Absorption spectra were recorded with a UV−visible spectrophotometer (Milton Roy Genesys 5) against a blank containing phosphate buffer. To estimate the R-PE and R-PC (mg g^{-1} protein) contents in PF, a calibration curve for the absorbance at 566 nm for R-PE and at 620 nm for R-PC as a function of protein concentration in 0.1 M phosphate buffer (pH 6.8) was plotted.

Polyacrylamide Gel Electrophoresis. The electrophoresis in polyacrylamide gel (SDS-PAGE) was performed according to the method of Laemmli[7] with 12% acrylamide gels. The polypeptides used as molecular mass markers were Precision Plus Protein Kaleidoscope Standards from Bio-Rad. The gel plates were fixed and stained with a solution containing 0.125% Coomassie Blue R-250, 50% methanol, and 10% acetic acid in water. This analysis was carried out on PF and R-PE.

Phenolic Compound Analysis. Phenolic compounds were extracted (four times) from 0.2 g of PF in 5 mL of HPLC grade methanol at room temperature. The extract was sonicated for 15 min and centrifuged, and the supernatant was concentrated under nitrogen flow at room temperature and finally filtered through a Millipore 0.45 μm pore size filter. An aliquot was separated for the analysis of free aglycones/unconjugated phenolic compounds. The remaining sample was subjected to acid hydrolysis (50 min at 90 °C) to release the aglycone portion from glycosylated phenolic compounds and hydrolyzing conjugated molecules. Analysis was carried out by HPLC.

Phenolic Compound Profile of PF. Compounds were separated on a 150 mm × 4.6 mm, 5 μm particle size, Supelcosil LC-ABZ column and an Alltech C18 5 μm guard column. The mobile phase was a gradient prepared from formic acid in water (2%, pH 3, solvent A) and formic acid in methanol (2%, pH 3, solvent B). The gradient program was (0.7 mL min^{-1}, room temperature) 0.01−8.00 min, 15% B isocratic; 8.01−25.00 min, 15−50% B; 25.01−40.0 min, 50% B isocratic; 40.01−50.0 min, 50−90% B; 50.01−55.00 min, 90−15% B. The detector was set at 280, 320, 360, and 520 nm for simultaneous monitoring of the different groups of phenolic compounds. Data were processed using Shimadzu LC solution software.

Spleen Mononuclear Cell Isolation. Primary mononucelar cells were obtained from female Wistar rats obtained from Janvier SAS (Le Genest Saint Isle, France) as described by López-Posadas et al.[8] Lymphocytes were obtained by negative magnetic immunolabeling (CD11b, CD161.a, and CD45RA) using monoclonal antibodies by BD Biosciences (Erembodegem, Belgium) and specific MACS microbeads (Miltenyi Biotec, Madrid, Spain). Monocytes were isolated similarly but with a negative selection using anti-CD161.a-biotin, CD45RA-PE, and anti-CD3-biotin antibodies. Separation protocols were set up and validated by flow cytometry. The cells were maintained at 37 °C in standard culture conditions.

Cell Proliferation Assay. Cell proliferation was measured by [^{3}H]-thymidine incorporation. Splenocytes (10^6 cells mL^{-1}) or macrophages (10^6 cells mL^{-1}) were cultured with [^{3}H]-thymidine (1 mCi/well, GE Healthcare, Bucks, UK) in the presence or absence of PF (1 or 0.1 g L^{-1} of proteins) for 1 h before the addition of bacterial lipopolysaccharide (LPS, 1 μg mL^{-1}) and were incubated for 24 h. After this period, the cells were fixed in ice-cold 70% trichloroacetic acid and solubilized 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 analyzer (Packard Instrument, Meriden, CT, USA) and expressed as disintegrations per minute [dpm; mean ± standard deviation (SD)]. All assays were performed in triplicate.

Cytokine Determination. For cytokine determinations the cell suspensions (10^6 cells mL^{-1} in RPMI or DMEM medium) were treated with PF, R-PE, or C-PC and stimulated with concanavalin A (ConA; 5 μg mL^{-1}) or LPS (1 μg mL^{-1}) depending on the cell type. Cell culture medium was collected after 24 h (splenocytes and macrophages + LPS) or 48 h (splenocytes and lymphocytes + ConA), 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, USA). Samples were run in triplicate, and results are expressed as cytokine concentration (pg mL^{-1}).

Lactate Dehydrogenase (LDH) Assay. Cellular toxicity was measured as the release of LDH. Cells were cultured in the conditions described above, and LDH activity in supernatants was measured spectrophotometrically using sodium pyruvate (25 mmol L^{-1}) as substrate in 50 mmol L^{-1} sodium phosphate buffer (pH 7.5).[8]

NF-κB and Mitogen-Activated Protein Kinase (MAPK) Inhibitors Assay. To explore signaling pathways, the kinase inhibitors [PD98059 for the MAPK ERK1/2, SB203580 for p38 MAPK, SP600125 for c-Jun N-terminal kinase (JNK), and Bay11-7082 for NF-κB] were added to the cell culture medium (10 μmol L^{-1} in all cases) 30 min before the addition of PF (0.1 g L^{-1} of protein).

Statistical Analysis. All analyses were performed in triplicate from each sample (n = 3 per experiment), and results are expressed as the mean ± SD. The data were analyzed by one-way analysis of variance, using the software Statgraphics Plus 3.0. Least significant difference tests were used to determine statistical differences between samples on preselected pairs. The significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

PF Characterization. *Spectra of Protein Fraction and Pigment Composition.* A PF was obtained from the water extract of *P. columbina* using ultrafiltration with a cutoff of 10 kDa. This was expected to contain hydrosoluble pigments characteristic of red algae, such as phycoerithryn and phycocyanin, which belong to the phycobilin family of fluorescent heteroproteins present in members of *Rhodophyta*.[3] Thus, a visible spectrum was obtained and compared with those corresponding to pure R-PE and C-PC. As shown in Figure 1, both proteins appear to be present in PF, based on the detection of peaks characteristic of R-PE (566, 545, and 495 nm) and C-PC (600−640 nm). These results are in agreement with those of Niu et al.[10] and Sampath-Wiley and Neefus[11]

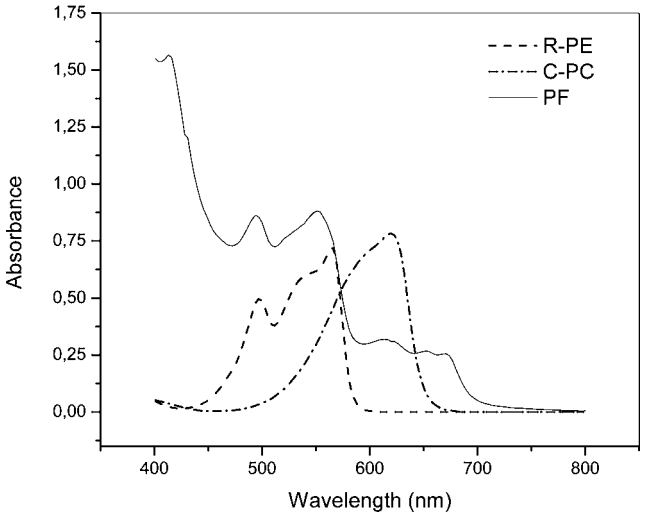

Figure 1. Spectra of protein fraction (PF) from *Porphyra columbina* and R-phycoerythrin (R-PE) in 0.1 M phosphate buffer (pH 6.8).

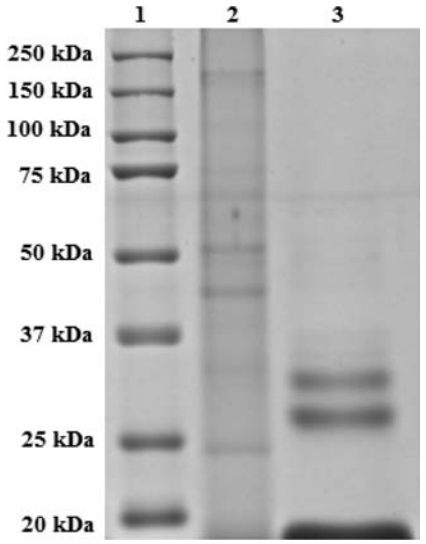

Figure 2. SDS-PAGE analysis of the protein fraction. Lanes: 1, molecular weight markers; 2, protein fraction (PF) from *Porphyra columbina* and 3, R-phycoerythrin (R-PE).

Table 1. Phenolic Compounds of PF

phenolic compound	concentration[a] (mg kg^{-1} dw)
gallic acid	2.47 ± 0.02
cathechin	0.21 ± 0.01
epicatechin	5.20 ±0.02
ferulic acid	nd
coumaric acid	0.23 ± 0.01
rutin	nd
cinnamic acid	nd
ellagic acid	nd
quercetin	0.17 ± 0.01
kaempferol	0.02 ± 0.00

[a]Data are presented as the mean ± SD; dw, dry weight; nd, not detected.

obtained with a phosphate buffer extract (0.1 M, pH 6.8) for *Porphyra* sp. and *Porphyra purpurea*, respectively. However, other peaks are also present, suggesting the presence of other pigments in the sample.

On the other hand, the absorbance at 280 nm was considerably higher than that at 566 and 620 nm (3.75), suggesting that PF contains other proteins in addition to R-PE and C-PC, consistent with the denaturing SDS-PAGE profile (Figure 2). An idea of the purity of R-PE and C-PC may be reached on the basis of the A_{566}/A_{280} and A_{620}/A_{280} ratios, respectively.[9] Thus, a value of 4.5 is indicative of a high-purity extract for R-PE, and this index decreases as the presence of contaminants rises.[12] The A_{566}/A_{280} and A_{620}/A_{280} of PF were 0.194 and 0.08, consistent with the complexity of the extract and indicating that it contains more R-PE than C-PC. This was confirmed by direct quantitation of the proteins using specific calibration curves: PF contains 37.6 and 19.9 mg of R-PE and C-PC per gram of proteins, respectively.

SDS-PAGE of Protein Fraction. As shown in Figure 2, R-PE featured three components: two near 30 kDa and one prominent band that runs between 14.4 and 20.1 kDa. The former matches the γ subunit molecular weight (30−33 kDa),[13] whereas the strong band probably corresponds to a superposition of the α and β subunits (molecular weights of 17 and 19 kDa, respectively).[10] Additional bands are also detected with apparent molecular weights between 37 and 250 kDa, representing other components in PF apart from the phycobiliproteins.

Phenolic Compound of Protein Fraction. PF showed a low content of phenolic compounds (Table 1). The sum of gallic acid, cathechin, epicatechin, coumaric acid, quercetin, and kaempferol was 8.3 mg 100 g^{-1}. Epicatechin and gallic acid were the most abundant phenolic compounds, whereas ferulic acid, rutin, cinnamic acid, and ellagic acid were not detected. Hence, phenolic compounds are unlikely to account for PF effects.

Effect of PF on Splenocyte and Macrophage Cell Viability and Proliferation ([^{3}H]-Thymidine Incorporation Assay). As shown in Table 2, PF exerted a mitogenic effect in splenocytes at the concentration of 0.1 g L^{-1}. This is in

Table 2. Effect of PF on Splenocyte and Macrophage Proliferation, Assessed by the [^{3}H]-Thymidine Uptake Assay (1 mCi/Well)

		uptake of [^{3}H]-thymidine[a] (dpm)	
PF (g L^{-1} of protein)	LPS (μg mL^{-1})	in splenocytes	in macrophages
0	0	427.1 ± 11.9 a	243.6 ± 17.3 a
0.1	0	691.8 ± 6.8 b	513.0 ± 14.5 b
1	0	379.9 ± 17.4 a	220.2 ± 26.9 a
0	1	1036.1 ± 82.7 d	
0.1	1	872.3 ± 48.1 c	

[a]Data are presented as the mean ± SD. Different letters indicate significant differences between samples ($p < 0.05$).

Table 3. Level of Lactate Dehydrogenase[a] (mU μL^{-1}) in the Culture Medium from Splenocytes with or without PF

PF (g L^{-1} of protein)	basal	LPS	ConA
0	20.3 ± 2.7 a	20.1 ± 2.0 a	22.5 ± 0.4 a
0.1	21.1 ± 1.3 a	19.7 ± 1.1 a	21.8 ± 1.0 a
1	113.8 ± 2.0 b	138.0 ± 4.1 b	139.6 ± 0.5 b

[a]Data are presented as the mean ± SD. Different letters indicate significant differences between samples ($p < 0.05$).

dx.doi.org/10.1021/jf300928j | J. Agric. Food Chem. 2012, 60, 8146−8154

Table 4. Level of Lactate Dehydrogenase[a] (mU μL^{-1}) in the Culture Medium from Macrophages and Lymphocytes with or without PF

	macrophages		lymphocytes	
PF (g L^{-1} of protein)	basal	LPS	basal	ConA
0	24.7 ± 1.3 a	24.7 ± 1.3 a	24.3 ± 2.0 a	24.3 ± 2.0 a
0.1	23.8 ± 2.9 a	24.6 ± 1.0 a	24.1 ± 2.4 a	25.5 ± 2.2 a
1	100.3 ± 2.7 b	104.5 ± 1.4 b	108.3 ± 2.4 a	163.8 ± 28.9 b

[a]Data are presented as the mean ± SD. Different letters indicate significant differences between samples ($p < 0.05$).

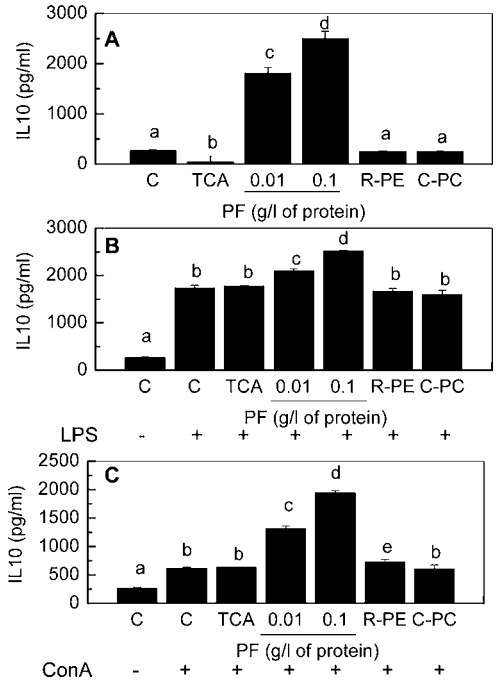

Figure 3. Effect of protein fraction (PF), C-phycocyanin (C-PC), and R-phycoerythrin (R-PE) on the production of interleukin 10 (IL-10) by splenocytes. Splenocytes were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF (0.01 and 0.1 g L^{-1} protein), C-PC (0.01 g L^{-1} protein), R-PE (0.01 g L^{-1} protein) and TCA supernatant from PF (S_{TCA}) in the absence (A) or presence of bacterial lipopolysaccharide (LPS, 1 μg mL^{-1}) (B) or the presence of concanavalin A (ConA, 5 μg mL^{-1}) (C). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as the mean ± SD; letters with $p < 0.05$ indicate significant differences.

accordance with the mitogenic effect observed with other protein extracts of natural origin, for instance, the fungal protein Fip-*vvo* isolated from *Volvariella volvácea*, which augments [^{3}H]-thymidine uptake with a maximum at 5 mg L^{-1}.[14] Similar results were obtained with a protein obtained from the red seaweed *Tichocarpus crinitus*, with an optimum concentration of 0.78 mg L^{-1}.[15] Thus, PF probably contains proteins that stimulate lymphocyte proliferation within the splenocyte mixed cell population, possibly of the lectin type, but at any rate capable of binding surface receptors and inducing the activation and mitogenic response.[14] In addition, as shown in Table 2, PF also exerted a mitogenic effect at the concentration of 0.1 g L^{-1} on macrophages. However, the effect was lower than that obtained for splenocytes, consistent with the absence of lymphocyte collaboration. The effect on both cell types was concentration dependent, as it was completely lost at 1 g L^{-1}. This was probably due to a cytotoxic action, because LDH activity is significantly increased in the cell medium in these conditions (Tables 3 and 4). This is typically the consequence of enzyme release secondary to cell lysis or membrane leakage. Of note, this effect was observed also in the presence of cell stimuli (LPS, ConA). However, there was no toxicity with the concentration of 0.1 g L^{-1}, which was used for subsequent experiments.

The mitogenic effects of LPS and PF were not additive, so that cell proliferation was comparable in the LPS and LPS+PF groups. This is not surprising because LPS was used in submaximal concentration in this experiment. In turn, PF mitogenic activity was about half as efficacious as mitogenic as LPS, although obviously quite less potent.

Effect of PF on IL-10 Secretion in Splenocytes, Macrophages, and Lymphocytes. Interleukin-10 (IL-10), a pleiotropic cytokine that inhibits inflammatory and cell-mediated immune responses, is produced by a wide variety of cell types including T and B cells and monocytes/macrophages.[16] Figure 3 illustrates the IL-10-enhancing effect of PF in rat splenocytes in basal conditions and under LPS or ConA stimulation. Optimal concentration was again 0.1 g L^{-1}, with the higher concentration of 1 g L^{-1} being toxic for the cells, as mentioned above, resulting in marked ablation of cytokine production (data not shown). A lower concentration (0.01 g L^{-1}) was completely nontoxic as well (data not shown), but also much less efficient on IL-10 release.

Becaue LPS and ConA act as macrophage/T cell stimuli, respectively, our results suggest that both cell types may be

Table 5. Level of Lactate Dehydrogenase[a] (mU μL^{-1}) in the Culture Medium from Splenocyte with or without R-PE and C-PC

phycobiliprotein	proteins (g L^{-1})	basal	LPS	ConA
	0	24.2 ± 0.6 a	24.9 ± 2.0 a	25.5 ± 0.2 a
R-PE	0.01	23.6 ± 1.0 a	22.2 ± 2.2 a	23.9 ± 1.6 a
	0.1	30.6 ± 0.9 b	43.3 ± 0.2 b	43.5 ± 4.1 b
C-PC	0.01	25.7 ± 1.7 a	22.7 ± 1.9 a	23.6 ± 2.2 a
	0.1	41.6 ± 2.1 c	53.5 ± 1.9 c	51.5 ± 2.1 c

[a]Data are presented as the mean ± SD. Different letters indicate significant differences between samples ($p < 0.05$).

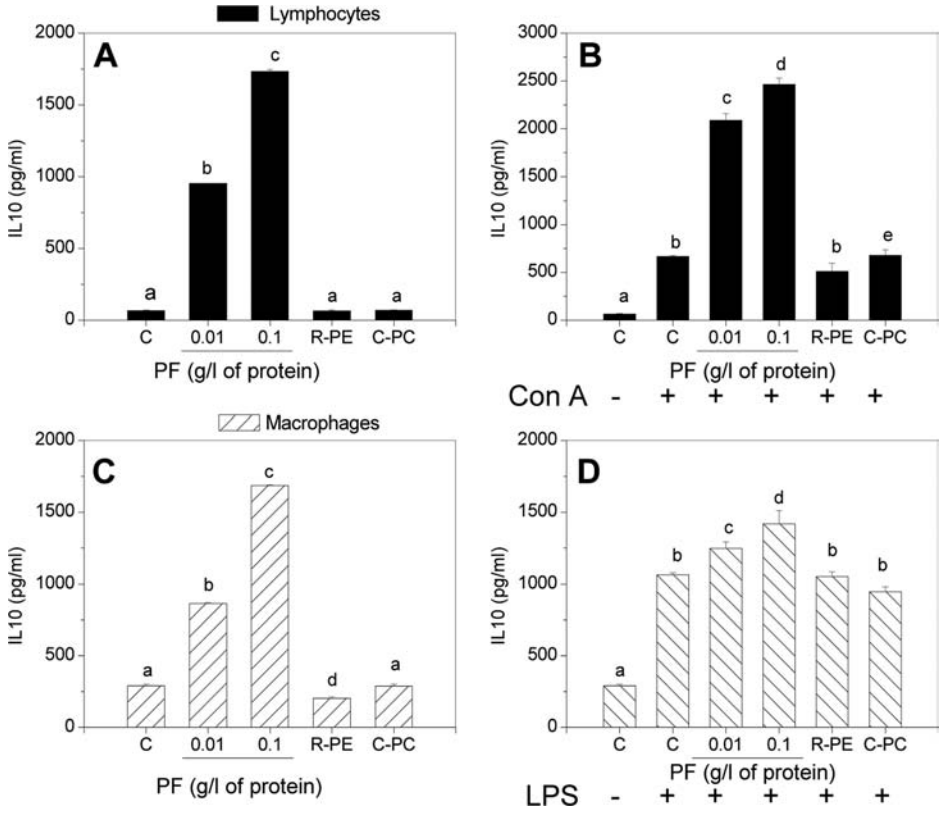

Figure 4. Effect of protein fraction (PF), C-phycocyanin (C-PC), and R-phycoerythrin (R-PE) on the production of interleukin 10 (IL-10) by lymphocytes and macrophages. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF (0.01 and 0.1 g L^{-1} protein), C-PC (0.01 g L^{-1} protein), and R-PE (0.01 g L^{-1} protein) in the absence (A, C) or presence of concanavalin A (ConA, 5 μg mL^{-1}) (B) or in the presence of bacterial lipopolysaccharide (LPS, 1 μg mL^{-1}) (D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as the mean ± SD; letters with $p < 0.05$ indicate significant differences.

Table 6. Level of Lactate Dehydrogenase (mU μL^{-1}) in the Culture Medium from Macrophages and Lymphocytes with or without R-PE and C-PC[a]

		macrophages		lymphocytes	
phycobiliprotein	protein (g L^{-1})	basal	LPS	basal	ConA
	0	24.2 ± 2.0 a	23.1 ± 2.5 a	24.2 ± 2.0 a	25.5 ± 0.2 a
R-PE	0.01	22.4 ± 0.4 a	24.3 ± 0.7 a	23.8 ± 1.5 a	21.8 ± 1.0 a
	0.1	51.1 ± 1.4 b	71.5 ± 2.1 b	50.4 ± 1.6 a	49.9 ± 3.9 b
C-PC	0.01	24.2 ± 2.8 a	26.9 ± 1.2 a	22.4 ± 0.6 a	23.7 ± 1.7 a
	0.1	63.3 ± 4.4 c	105.8 ± 7.1 c	60.1 ± 1.0 a	52.0 ± 1.9 b

[a]Data are presented as the mean ± SD. Different letters indicate significant differences between samples ($p < 0.05$).

modulated by PF, giving rise to the IL-10 increase. To rule out the presence of nonproteic compounds in PF that could account for the observed actions, proteins were precipitated with trichloroacetic acid (TCA) and bioactivity was evaluated in the supernatant (S_{TCA}). This was completely devoid of enhancing activity and actually inhibited splenocyte basal IL-10 secretion, as shown in Figure 3A. Thus, the effects on splenocytes are ascribed to the proteins present in the extract. Interestingly, when pure R-PE and C-PC were tested at the concentration of 0.01 g L^{-1} (which was not toxic to the cells, as opposed to the concentration of 0.1 g L^{-1}; Table 5), the effects on IL-10 production were almost completely lost compared to that of PF, indicating that these are unlikely to account for the immunomodulatory effects.

The effect of PF on macrophages and T lymphocytes was confirmed using primary cultures of both cell types isolated in vitro (Figure 4). Thus, IL-10 was up-regulated in basal conditions and under stimulation with LPS or ConA, respectively. Of note, the effect was more pronounced in T cells than in macrophages, suggesting that the former are the main target of PF. As with splenocytes, the concentration of 1 g L^{-1} was toxic for both macrophages and lymphocytes (Table 4). Consistent with the results obtained with splenocytes, R-PE and C-PC had little effect on either T lymphocytes or monocytes. It is appropriate to note that the concentration of 0.01 g L^{-1} was not toxic to the cells (T lymphocytes or monocytes), as opposed to the concentration of 0.1 g L^{-1} (Table 6).

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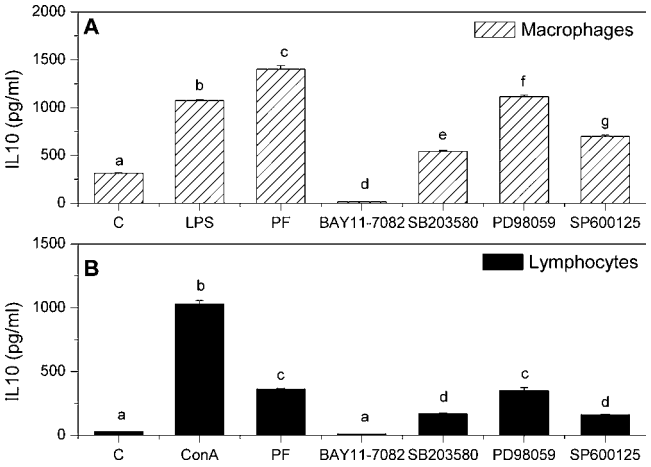

Figure 5. Effect of MAP kinase and NF-κB inhibitors on interleukin 10 (IL-10) by macrophages (A) and lymphocytes (B) stimulated with protein fraction (PF). Cytokine concentrations were determined by ELISA in the supernatants of cells preincubated for 1 h with the signal transduction inhibitors [MAP kinase inhibitors PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), or the NF-κB inhibitor Bay 11-7082] followed by a 24 h incubation with PF. Data are expressed as the mean ± SD; letters with $p < 0.05$ indicate significant differences.

Signal Transduction Pathways. Both the MAPK and the NF-κB signaling pathways have been shown to be implicated in the production of IL-10 in monocytes/macrophages.[16,17] The stimulation of the MAPK signal transduction pathways by PF was studied in macrophages and T lymphocytes using inhibitors for ERK1/2 (PD98059), p38 MAPK (SB203580), and JNK (SP600125). The addition of either SB203580 or SP600125 to the cell culture medium prevented the increase in the expression of IL-10 in both cellular populations, whereas PD98059 had almost no inhibitory effect (Figure 5), indicating that the effect of PF was p38/JNK dependent. The role of the NF-κB signaling pathway in the stimulation of macrophages and lymphocytes by PF was also studied. One of the ways to activate NF-κB by extracellular stimuli involves the rapid degradation of IκB-α following phosphorylation of Ser32 of IκB-a by IκB kinase (IKK, the so-called canonical pathway). Bay11-7082 inhibits this phosphorylation step and therefore blocks the NF-κB canonical activation pathway. Complete inhibition of the secretion of IL-10 was observed when cells were cultured in the presence of Bay11-7082, indicating that this pathway plays a pivotal role in the response to PF (Figure 5).

The dual involvement of MAPK and NF-κB is not unexpected because they are both activated by the IKK complex in macrophages, for instance, in response to LPS.[18] Our data show that the NF-κB pathway was absolutely required for the induction of IL-10, whereas p38 MAPK and JNK from MAPK pathways appeared to play a secondary role, possibly enhancing the effect of NF-κB.

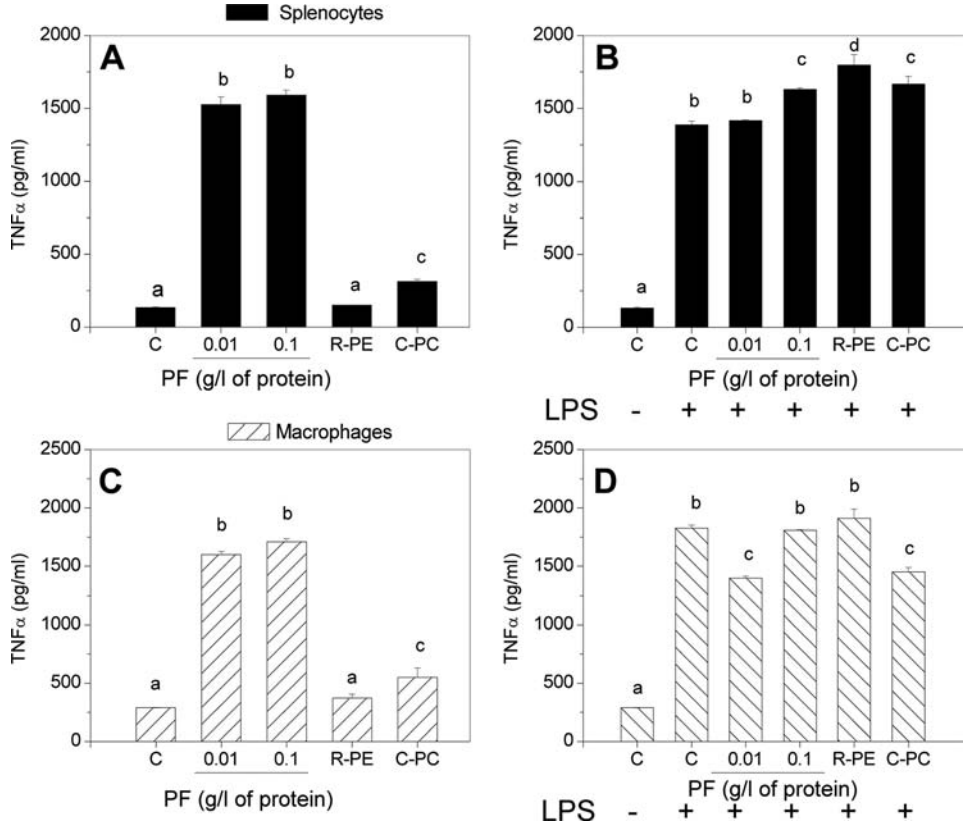

Figure 6. Effect of protein fraction (PF), C-phycocyanin (C-PC), and R-phycoerythrin (R-PE) on the production of tumor necrosis factor (TNFα) by splenocytes and macrophages. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF (0.01 and 0.1 g L^{-1} protein), C-PC (0.01 g L^{-1} protein), and R-PE (0.01 g L^{-1} protein) in the absence (A, C) or presence of bacterial lipopolysaccharide (LPS, 1 μg mL^{-1}) (B, D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as the mean ± SD; letters with $p < 0.05$ indicate significant differences.

dx.doi.org/10.1021/jf300928j | J. Agric. Food Chem. 2012, 60, 8146−8154

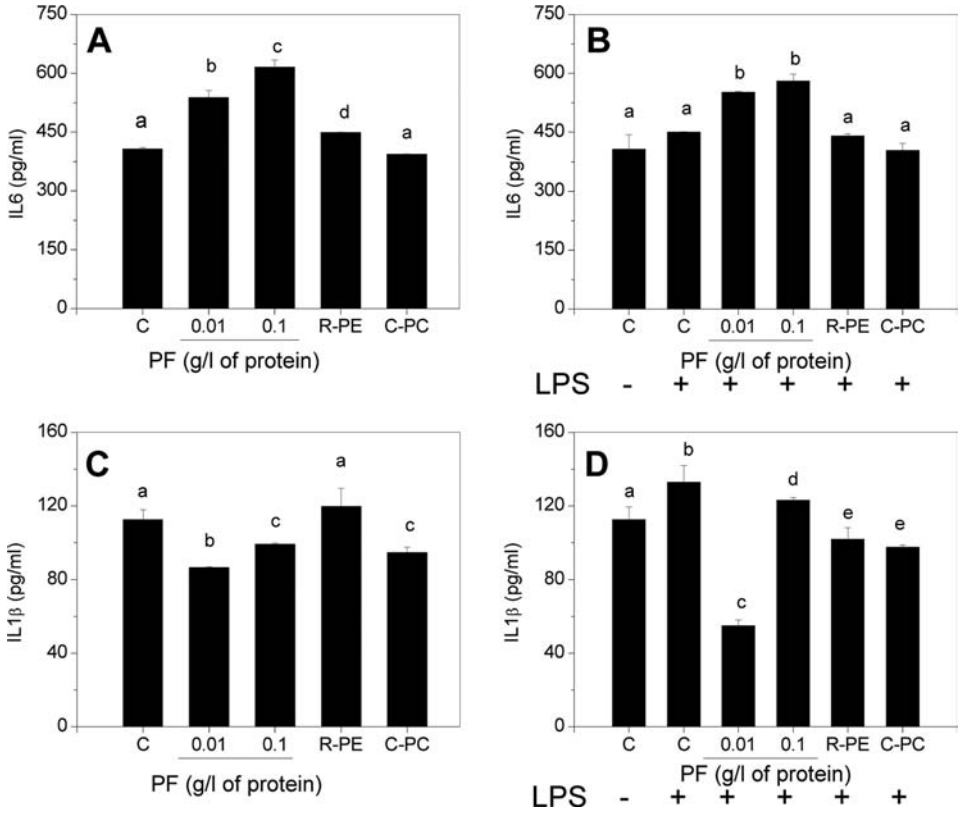

Figure 7. Effect of protein fraction (PF), C-phycocyanin (C-PC), and R-phycoerythrin (R-PE) on the production of interleukin 6 (IL6) and interleukin 1β (IL1β) by macrophages. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF (0.01 and 0.1 g L^{-1} protein), C-PC (0.01 g L^{-1} protein), and R-PE (0.01 g L^{-1} protein) in the absence (A, C) or presence of bacterial lipopolysaccharide (LPS, 1 μg mL^{-1}) (B, D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as the mean ± SD; letters with $p < 0.05$ indciate significant differences.

Effect of PF on TNFα, IL-6, IL-1β, and IFN-γ Secretion in Splenocytes, Macrophages, and Lymphocytes. TNFα is produced mainly by macrophages in response to a diverse array of stimuli, including bacterial, viral, and fungal or parasite invasion. TNFα evokes the recruitment of neutrophils and macrophages to the inflammatory site and enhances leucocyte function. Thus, this cytokine is a pivotal mediator of the acute response to infections but also a causative factor of many systemic complications in severe infections.[19] As shown in Figure 6A,C, PF (0.01 and 0.1 g L^{-1}) evoked TNFα secretion in quiescent splenocytes and macrophages, an effect that was essentially not reproduced by R-PE or C-PC. Interestingly, this effect was largely lost in LPS-treated rat splenocytes, and there was no effect at all in macrophages (Figure 6B,D). Both R-PE and C-PC exhibited the same marginal effects as PF on splenocyte but not macrophage TNFα secretion under LPS stimulation.

Conversely, PF was a weak inducer of IL-6 in macrophages in either basal or LPS stimulatory conditions, whereas it inhibited IL-1β release (Figure 7). These effects were again not reproduced globally by either R-PE or P-CP, indicating that these components are not chiefly responsible for PF effects. Thus, PF exerts an atypical modulatory effect dominated by induction of TNFα and IL-10 with little or no effect on IL-6 and IL-1β and extra up-regulation of IL-10 in the presence of LPS. This may in turn influence the changes in pro-inflammatory cytokines, because IL-10 has been shown to modulate the production of TNFα, IL-6, and IL-1β induced by LPS, IFN-γ, or both.[20]

We also looked at IFN-γ secretion by T lymphocytes, isolated or within the mixed splenocyte population. PF produced a mild inhibitory effect of basal cytokine release and a more robust one (~50%) in ConA-treated splenocytes (Figure 8A,B). However, the direct effect of PF on T lymphocytes was stimulatory rather than inhibitory (Figure 8D). The effect of R-PE and C-PC had a different profile, exhibiting a marked inhibition, which was especially prominent in ConA-stimulated splenocytes or T cells.

PF therefore exerts complex mitogenic and immunomodulatory actions, which cannot be ascribed simply to its content in R-PE and C-PC, although these have significant effects on their own. Considered globally, our data indicate an atypical profile of cytokine modulation for PF. In particular, it is remarkable for the predominance of IL-10 secretion in different cell types and conditions. A picture thus emerges in which IL-10 (both macrophage- and T cell-derived) exerts immunomodulatory effects on T lymphocytes, and probably on macrophages themselves, limiting pro-inflammatory actions linked to MAPK and NF-κB activation.[21,22] This is consistent with the acknowledged mechanism of inhibitory effects on T lymphocytes by IL-10 mainly through inhibition of antigen-presenting cells.[23]

How IL-10 production is favored by PF over that of TNFα and especially IL-6 or IL-1β is unknown. These four cytokines are generally coregulated along with many other genes (iNOS, COX2, integrins, etc.) by pro-inflammatory stimuli such as LPS and other TLR and related ligands in various cell types, operating via the NF-κB pathway, with the overall result of cell

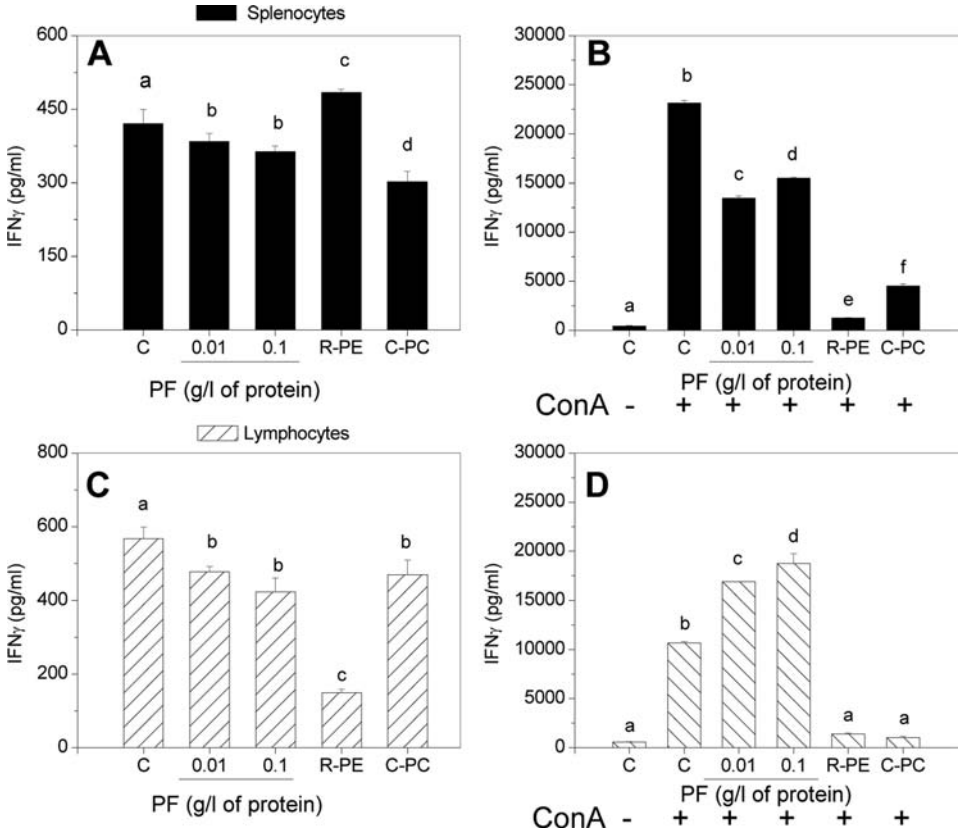

Figure 8. Effect of protein fraction (PF), C-phycocyanin (C-PC), and R-phycoerythrin (R-PE) on the production of interferon gamma (IFNγ) by splenocytes and lymphocytes. Cells were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF (0.01 and 0.1 g L^{-1} protein), C-PC (0.01 g L^{-1} protein), and R-PE (0.01 g L^{-1} protein) in the absence (A, C) or presence of concanavalin A (ConA, 5 μg mL^{-1}) (B, D). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as the mean ± SD; letters with $p < 0.05$ indicate significant differences.

activation and tissue inflammation. The signaling pathways, however, differ depending on the cell type, so that NF-κB and the proto-oncogene c-Maf are involved in antigen presenting cells, whereas Ikaros, GATA3, and AP-1 are implicated in Th2 cells and the transcription factor NF-Y is activated in B cells.[23] There is also a role for a variety of epigenetic mechanisms.

IL-10 may serve a counter-regulatory function as part of this stereotyped pro-inflammatory response. Conversely, IL-10 plays a determinant immunosuppresive/anti-inflammatory role when secreted by Treg lymphocytes or other cells. For instance, lack of IL-10 produces colonic inflammation in knockout mice, a well-known model of inflammatory bowel disease,[24] and IL-10 administration has proven anti-inflammatory effects in vivo.[25] The fact that IL-10 is consistently increased in basal or LPS/ConA stimulatory conditions and the magnitude of the response compared with that of other cytokines suggest that the overall effect of PF may be anti-inflammatory. However, care should be taken not to extrapolate this to the in vivo area, because there are many variables that may alter this balance in the living organism. The elucidation of the mechanism responsible for the relatively selective induction of IL-10 will require further experimentation.

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Author Contributions

R.E.C., R.L.-P., and S.R.D. conducted research. S.R.D., F.S.M., and O.M.-A. designed research. R.E.C., F.S.M., and O.M.-A. wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

Funding

This study was supported by the Ministery of Science and Innovation (SAF2008-01432, AGL2008-04332), by funds from Junta de Andalucía (CTS-6736 and CTS164), and by the Fundación Ramón Areces. R.E.C. was supported by Erasmus Mundus External Cooperation Window. R.L.P. was supported by a fellowship of the Ministery of Education and Science of Spain. We thank Project CAI + D 2009-PI-54-258 of the Universidad Nacional del Litoral for partial support of this research in Argentina and EADIC-LOTE 16. CIBERehd is funded by the Instituto de Salud Carlos III.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are thankful to Dr. Mercedes González and Franco Van de Velde for technical assistance.

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