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Immunomodulating capacity of commercial fish protein hydrolysate for diet supplementation

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Abstract

Dietary proteins harbour bioactive peptides that can be released by a fermentation process. Fish proteins are a valuable and little-exploited source of potentially active biopeptides. The aim of this research was to evaluate the effects of a commercially available fermented fish protein concentrate (Seacure®) (FPC) derived from a fermentation process, on the mucosal immune response in a murine model. BALB/c mice received the FPC or the non-fermented powder at different concentrations (0.20, 0.25 or 0.30 mg/ml) for 2, 5 or 7 consecutive days. At the end of each feeding period, histological studies of the gut were carried out and the phagocytic activity of peritoneal macrophages, the number of IgA+ cells in the small intestine lamina propria and bronchial tissue and the number of IL-4+, IL-6+, IL-10+, IFNγ+ and TNFα+ cells in the small intestine lamina propria were determined. Different accumulative doses of FPC did not induce any inflammatory immune response and the normal morphology of the small intestine was not affected. Phagocytic activity of peritoneal macrophages was enhanced following FPC administration at 0.3 mg/ml for 7 consecutive days. The number of IgA+ cells increased in the small intestine lamina propria but not in the bronchial tissue. IL-4, IL-6 and IL-10 were all significantly increased in the lamina propria of the small intestine of animals that received FPC. At the same time, some pro-inflammatory cytokines such as IFNγ and TNFα also increased, but the intestinal homoeostasis was maintained and no tissue damage was observed. We conclude that FPC is an immunomodulating food with a demonstrated capacity to enhance non-specific host defense mechanisms.

Keywords: Fish proteins; Immunomodulation; Intestinal mucosa; Peptides

Introduction

The development of vaccines and antibiotics has contributed enormously to the control of various infectious diseases. However, in recent years, opportunistic or complicated infections, often attributed to stress-associated immunosupression, are difficult to treat with antibiotics alone and continue to create a challenge in the clinical field. Most immunomodulatory pharmaceuticals are also not suitable for chronic or ongoing preventive use. In this sense, there is an ongoing

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interest to identify new immunomodulators to enhance non-specific host defence mechanisms (Yang et al., 2001). Although treatment and prevention of infectious diseases are the most common reasons to use immunomodulators, other reasons, such as amelioration of stress-induced immunosupression, general well-being and as a way to reduce treatment costs, are also well suited to immunomodulation. Non-pharmaceutical immunomodulating substances identified to date include bacteria, bacterial products, complex carbohydrates, cytokines, fermented foods, nutraceuticals and traditional medicinal plants (Blecha, 2001).

Development of immunomodulators from natural sources for diet supplementation, both animal and human, is an active area of research. Dietary proteins harbour bioactive peptides that are inactive within the sequence of the parent proteins but that can be released during gastrointestinal digestion, food processing or by a fermentation process (Korhonen and Pihlanto, 2003). Although most examples come from animal protein sources, plants also contain bioactive sequences. For example, a cabbage fermentation extract and young barley leaf powder were seen to reinforce Ig productivity in both systemic and intestinal immune systems (Miyazaki et al., 2001). Koh et al. (2002) reported the immunomodulating capacity of a water-extract of rice bran previously fermented by *Saccharomyces cerevisiae*. Immunostimulants represent a modern and promising tool in aquaculture, enhancing the resistance of cultured fish to disease and stress (Bagni et al., 2000). In the market, there are already animal feed supplements with demonstrated immunostimulating capacities (Chambon-Savanovitch et al., 2001; Cook et al., 2003).

It is well demonstrated that the hydrolysis of milk proteins yields a large number of biologically active immunomodulating peptides (LeBlanc et al., 2002). Beneficial effects of bioactive peptides encrypted in milk proteins allowed these substances to be claimed as potential nutraceuticals for food and pharmaceutical applications (Biziulevicius et al., 2003).

While milk is perhaps the most exploited source of bioactive peptides, fish proteins are another valuable source of potentially active biopeptides (Galassi and Lerici, 1976; Lacera et al., 1985). The aim of this research was to evaluate the effects of a fermented fish powder on the mucosal immune response in an in vivo model in a broader effort to further assess the role of bioactive peptides derived from a variety of means of microbial proteolysis as potential immunomodulators. Seacure® is a commercially available human protein supplement derived from the fermentation of fish protein by a proprietary yeast strain. A murine model previously used for the study of the immunomodulating capacity of lactic acid bacteria (Perdigón et al., 2001), cheese (Medici et al., 2004), kefir (Vinderola et al., 2005) and other fermented milks (Matar et al., 2001) was now employed to evaluate the immunomodulating capacity of this fish powder concentrate intended for human diet supplementation. This research was particularly important, because relative to the bacterial fermentation of milk proteins, e.g., much less is known about bioactives from fish sources or from yeast-derived fermentations (Farnworth, 2003).

Materials and methods

Samples

The fish protein concentrate (FPC) was prepared from pacific whiting (*Merluccius productus*) and donated by Proper Nutrition, Inc., Reading, PA, USA. Briefly, the raw fish was cleaned, filleted, minced and fermented for 0–24 h. The fermentation process required 10% sucrose (w/w), 20% demineralized water and 0.2% proprietary yeast culture. The ferment was then pasteurized at 80 °C for 20 min and spray dried into a powder. Samples were taken at various time points (0, 10, 15 and 24 h) throughout production to characterize the kinetics of proteolysis. A 24 h sample was considered "fermented," while a 0 h sample was considered a "non-fermented" control for additional comparisons. All samples were pasteurized and spray dried as described above. The 24 h fermented product is available commercially as the human protein supplement Seacure®.

Proteolysis

The extent of proteolysis in FPC was evaluated using the *o*-phthaldialdehyde (OPA) method developed by Church et al. (1983). Concentration of samples was determined in reference to a standard curve of L-leucine ranging from 0 to 3 mM.

Size exclusion high-performance liquid chromatography (HPLC)

High-performance size-exclusion chromatography of protein/peptide samples was achieved using a HP1100 HPLC system (Agilent Technologies, Canada). Samples (100 mg/ml PBS) were prepared prior to HPLC injection by centrifugation at 6000*g* for 20 min at 4 °C (Micromax RF, IEC, USA). The supernatant was filtered using a 0.22 μm Millex-GP syringe filter (Millipore, Canada) and maintained at 4 °C until injection. Sample (50 μl) was loaded on a LKB TSK-G2000SW gel filtration column (600 × 7.5 mm, TosoHaas, USA) using 5 mM ammonium acetate pH 6.5 (Fisher Scientific, Canada) as the elution buffer prepared as described previously (Lemieux et al., 1991). Protein/peptides were eluted with a flow rate of 0.7 ml/min and monitored at 214, 220, 224

and 280 nm using an HP1100 Diode Array Detector (LeBlanc et al., 2002). Peptidic fractions in non-fermented and fermented products were selected based on elution time (32–62 min). Fractions were collected with a Gilson FC104 Fraction Collector, then pooled and concentrated using an Automatic Environmental SpeedVac® System (savant, USA) and stored at 4 °C for later use.

Reverse-phase HPLC (RP-HPLC)

To compare peptide elution patterns, RP-HPLC was performed as described previously (Matar et al., 1996) using a 3.5 µm Zorbaz SB-C18 column (4.6 × 150 mm, Agilent Technologies). Briefly, the column was equilibrated with solvent A (0.115% trifluoroacetic acid; TFA) at a flow rate of 1 ml/min and peptides were eluted with a solvent B (60% acetonitrile in 0.1% TFA) as follows; 0–30 min: 0–60% B; 30–35 min: 60–100% B; 35–42 min: 100–0% B. Eluted peptides were monitored at 214, 220, 224 and 280 nm.

Animals and feeding procedures

BALB/c female mice 6–8 weeks old weighing 20–25 g were obtained from Charles River (Montreal, Canada). A total of 19 experimental groups were set up (control mice and mice fed for 2, 5 or 7 days with three different concentrations of fermented or non-fermented FPC suspensions administered in the drinking water). Each experimental group (sample) consisted in five mice housed together in plastic cages kept in a controlled atmosphere (temperature 22 ± 2 °C; humidity 55 ± 2%) with a 12 h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care.

Animals were offered an aqueous solution of FPC or the non-fermented product (0.20, 0.25 or 0.30 mg/ml) for 2, 5 or 7 consecutive days (in replacement of water). FPC solutions were done in phosphate buffered saline (PBS) solution (Sigma-Aldrich, St. Louis, MO, USA). The daily water intake was 3.1 ± 0.3 ml/day/mouse. All groups of mice received simultaneously a conventional balanced diet ad libitum. The control group received the same conventional balanced diet, but with water instead of FPC solution.

At the end of each feeding period, animals were anesthetized (single peritoneal dose of ketamine/xylazine solution) and sacrificed by cervical dislocation to obtain the different tissues for the immunological studies.

Ex vivo phagocytosis assay in peritoneal macrophages

The assay was performed according to Perdigón et al. (1986). Briefly, peritoneal macrophages were harvested (in sterile conditions) by washing the peritoneal cavity with 5 ml of PBS containing 10 U/ml of Heparin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Bovine Serum Albumin (Jackson ImmunoResearch, West Grove, PA, USA). The macrophage suspension was washed twice with the same buffer, and it was adjusted to a concentration of 10^6 cell/ml. A heat-killed (100 °C, 15 min) *Candida albicans* suspension (10^7 cell/ml) was opsonized with mouse autologus serum (10%) for 15 min at 37 °C. Opsonized yeast (0.2 ml) were added to 0.2 ml of each macrophage suspension. The mixture was incubated for 30 min at 37 °C. The phagocytosis was measured as the % of activated (with at least one cell of yeasts phagocyted) macrophages after a 100-cell count using an optical microscope.

double antibody sandwich enzyme-linked immuno sorbant assay (DAS-ELISA) for determination of IgA in the intestinal lumen

The small intestine from each mouse was recovered (by truncation at the stomach/duodenum junction and the ileum/ascending colon junction) and its contents were flushed with 5 ml of PBS. Particulate material was removed by centrifugation (10,000*g* for 10 min at 4 °C). The supernatant fluid was stored in triplicate at −20 °C. Total IgA antibodies were detected by standard DAS-ELISA. Briefly, affinity-purified monoclonal goat anti-IgA (α-chain specific) was added at 1.25 µg/well in 50 mM carbonate–bicarbonate buffer pH 9.6 to Costar 96-well, U-bottomed, high-binding polystyrene microplates (Corning Inc., NY, USA) and incubated at 37 °C for 1 h. The plates were then washed three times using an EL_X405 Auto Plate Washer (Bio-Tek Instruments, Inc., VT, USA) with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 1 h at 25 °C with 0.5% non-fat dry milk in PBS. Plates were washed five times with PBS-T and incubated for 2 h at 37 °C with triplicates of either 50 µl of standard kappa IgA, or 50 µl of serially diluted samples of intestinal fluid. Plates were washed seven times with PBS-T and incubated in the presence of horseradish-peroxidase conjugated anti-IgA specific antibodies at 1.25 µg/well for 1 h at 37 °C. Plates were again washed seven times and 100 µl of TMB reagent containing peroxide (BD Biosciences, Mississauga, Ont., Canada) was added to each well. Reactions were terminated with 100 µl H_2SO_4 (2 N) with gentle shaking. The optical density was read at 450 nm using a µQuant automatic microplate reader (Bio-Tek Instruments, Winooski, VT, USA). In IgA specific DAS-ELISA, all

antibodies, standards and buffers were purchased from Sigma Chemical Co., St Louis, MO, USA.

Histological studies of the gut

After intestinal content recovery, small intestine was processed for paraffin inclusion following the Sainte-Marie technique (Sainte-Marie, 1962). Serial paraffin-sections (4 μm) were stained with haematoxilin–eosin followed by light microscopy examination. The comparison of haematoxylin–eosin-stained histological sections of small intestine of control mice or animals that received the fermented FPC was carried out by three independent persons that performed the study on unidentified/codified samples.

Immunofluorescence test for B population (IgA + and IgG + cells) identification

Small intestine and the lungs were removed for histological preparation following Sainte-Marie technique (1962) for paraffin inclusion. The number of IgA producing (IgA +) cells was determined on histological slices of samples from the ileum region near Peyer's patches and on bronchial tissue by a direct immunofluorescence method (Vintini et al., 2000). The number of IgG + cells was also determined on histological slices of small intestine. The immunofluorescence test was performed using (α-chain specific) anti-mouse IgA FITC conjugate or (γ-chain specific) anti-mouse IgG FITC conjugate (Sigma-Aldrich, St. Louis, MO, USA). Histological slices were deparaffinized and rehydrated in a graded series of ethanol. Deparaffinized histological samples were incubated with the appropriate antibody dilution (1/100 for IgA or 1/50 for IgG) in PBS solution for 30 min at 37 °C. Then, samples were washed 2 times with PBS solution and examined using a fluorescent light microscope. The results were expressed as the number of IgA + or IgG + cells (positive: fluorescent cell) per 10 fields (magnification 100 ×). Data represent the mean of three histological slices for each animal, for each feeding period.

Cytokine assay

At the end of each feeding period, the small intestine was removed and processed for histological preparation as described above. IL-4, IL-6, IL-10, TNF-α and IFN-γ were studied by an indirect immunofluorescence method. Histological slices were deparaffinized and rehydrated in a graded series of ethanol, and then incubated for 30 min in a 1% blocking solution of BSA (Jackson Immuno Research, West Grove, PA, USA) at room temperature. Histological slices were then incubated for 60 min at 37 °C with rabbit anti-mouse IL-4, IL-6, IL-10, TNF-α or IFN-γ (Peprotech, Inc., Rocky Hill, NJ, USA) polyclonal antibodies. The incubation was followed by two washes with PBS solution and, finally, sections were treated for 45 min at 37 °C with a dilution of a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research). The results were expressed as the number of X-producing cells (positive: fluorescent cell) per 10 fields (magnification 100 ×). Data represent the mean of three histological slices for each animal, for each feeding period.

Statistical analysis

Data (% phagocytosis or the number of IgA or cytokine + cells per 10 fields) were analyzed using the one-way ANOVA procedure of SPSS software. The differences among means were detected by the Duncan's multiple range test (SPSS, 1996). Data were considered significantly different when $p < 0.05$.

Results

Fermentation extent of samples

A progressive increase in proteolysis was observed during the 24 h of fermentation of FPC (Fig. 1). Two different HPLC methods were used to determine the differences between the non-fermented and fermented products. Size-exclusion HPLC separates molecules in respect to their size, whereas RP-HPLC relies on the polarity of the compounds. Different size-exclusion HPLC elution profiles were observed between the non-fermented and fermented samples (Fig. 2). In particular, significant differences could be observed later in the chromatogram (from 32–62 min) indicating the release of smaller oligopeptides/peptides. In parallel, RP-HPLC analysis of fractions collected in this interval confirm the liberation of novel peptides that were not present beforehand (Fig. 3).

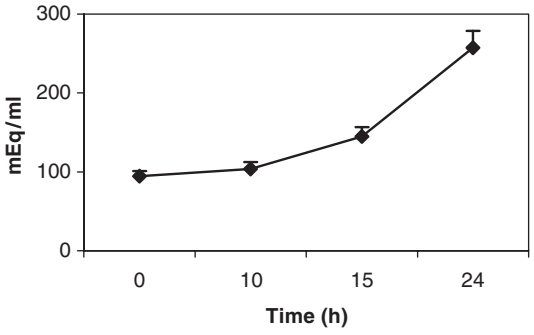

Fig. 1. Proteolysis degree of the FPC sample during the fermentation process.

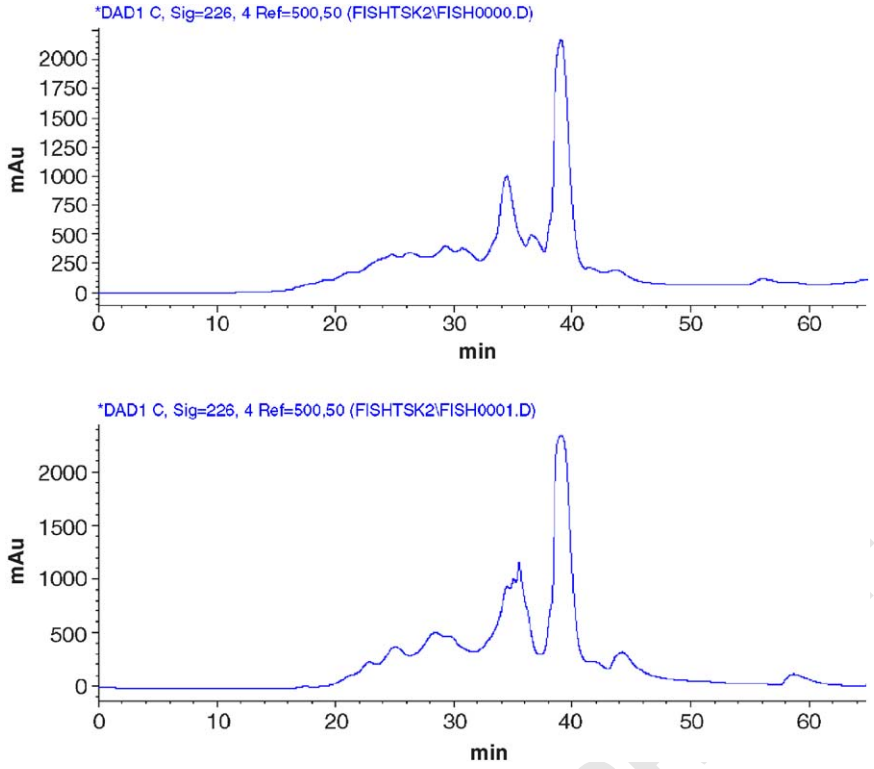

Fig. 2. Comparison of size-exclusion elution profiles at 226 nm of non-fermented (above) and fermented (below) samples. Samples were loaded on a TSK-G2000SW gel filtration column at a flow rate of 0.7 ml/min using an elution buffer consisting of 5 mM ammonium acetate pH 6.5.

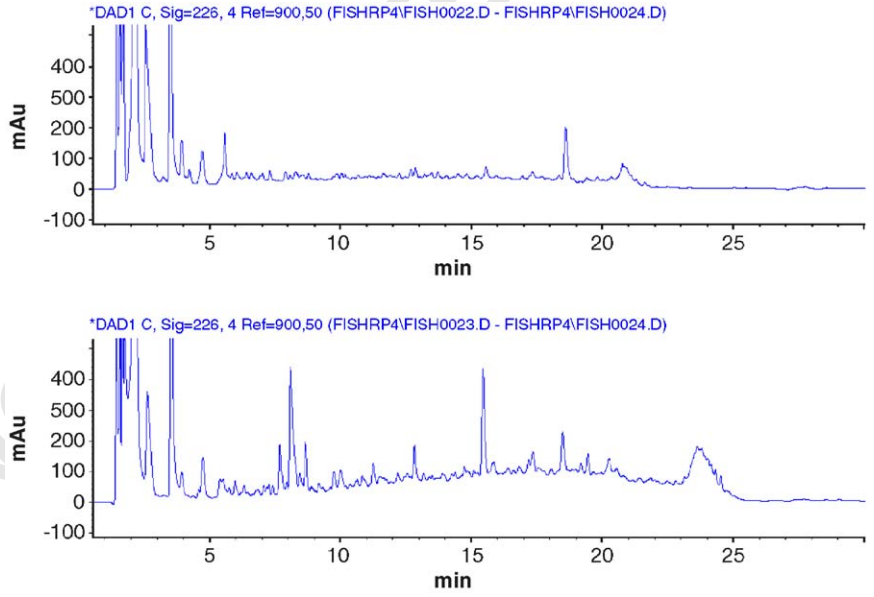

Fig. 3. Comparison of RP-HPLC elution profiles at 226 nm of non-fermented (above) and fermented (below) samples. Samples were loaded on a Zorbaz SB-C18 column that had been equilibrated with solvent A (0.115% trifluoroacetic acid; TFA) at a flow rate of 1 ml/min. Peptides were eluted with a solvent B (60% acetonitrile in 0.1% TFA) as follows; 0–30 min: 0–60% B; 30–35 min: 60–100% B; 35–42 min: 100–0% B.

Determination and characterization of the immune response induced

We analyzed the effects of different doses of FPC on the immune response. The lowest concentrations of FPC assessed (0.20 and 0.25 mg/ml) as well as all the concentrations of the non-fermented samples assessed (0.20, 0.25 and 0.30 mg/ml) were not able to modify the number of IgA+ cells in the small intestine lamina propria (data not shown) and were, then, no longer considered in this study. The immune response obtained with 0.3 mg/ml of FPC supplementation was further characterized by cytokine analysis.

The histological study of the small intestine (haematoxilin–eosin) revealed no differences in the morphological architecture between control mice and animals that received FPC. No lymphocyte infiltrates, or the presence of oedema or mucosal atrophy were observed. No significant morphological changes in the overall architecture of the small intestine were observed when compared to control mice.

Fig. 4 shows the phagocytic activity of peritoneal macrophages of mice that received FPC (0.3 mg/ml) for 2, 5 or 7 consecutive days. A significant increase of phagocytosis in the peritoneal macrophages was observed only after 7 days of FPC administration.

A significant increase was observed in the number of IgA+ cells in the lamina propria of small intestine (Fig. 5) as well as in the content of secretory-IgA (S-IgA) (Fig. 6) in the small intestine lumen of mice that received FPC (0.3 mg/ml) for 7 consecutive days. No significant increase of IgA+ cells was observed in the bronchial tissue (Fig. 5) of animals that received FPC for 2, 5 or 7 consecutive days, nor was an increase observed in the number of IgG+ cells in the small intestine lamina propria of these animals following FPC administration (Fig. 5).

The profiles of cytokines after FPC administration for the different feeding periods are displayed in Fig. 7. A

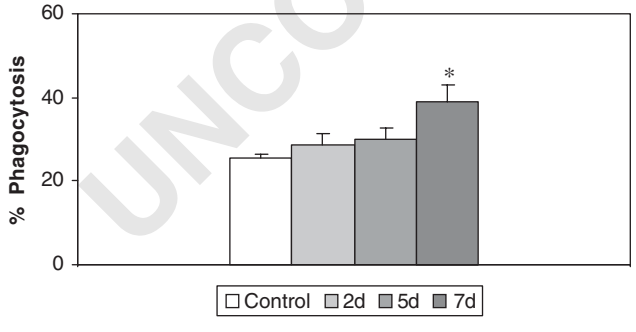

Fig. 4. Effect of the oral administration of 0.3 mg/ml of FPC for 2 (▒), 5 (▓) or 7 (■) consecutive days on the percentage of phagocytosis of peritoneal macrophages. *Significantly different from control (□) ($p<0.05$). Data are the mean of five animals per time period with standard deviation.

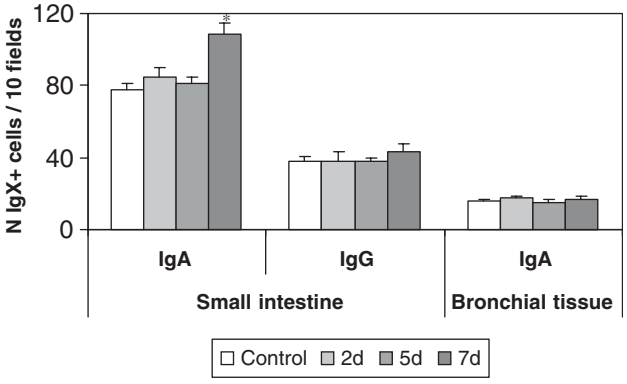

Fig. 5. Effect of the oral administration of 0.3 mg/ml of FPC for 2 (▒), 5 (▓) or 7 (■) consecutive days on the number of IgA+ and IgG+ cells on histological slices of small intestine and on the number of IgA+ cells on histological slices of bronchial tissue. *Significantly different from control (□) ($p<0.05$). Data are the mean of five animals per time period with standard deviation.

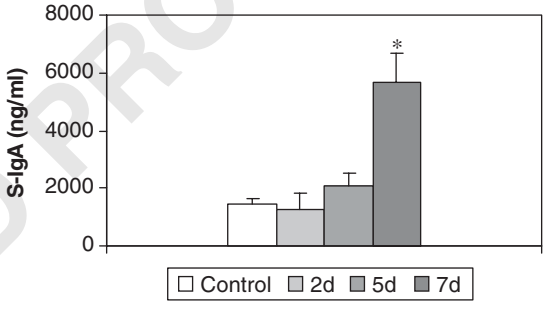

Fig. 6. Secretory IgA (S-IgA) in the luminal content of small intestine of animals that received 0.3 mg/ml of FPC for 2 (▒), 5 (▓) or 7 (■) consecutive days, compared to the control group. *Significantly different from control (□) ($p<0.05$). Data are the mean of five animals per time period with standard deviation.

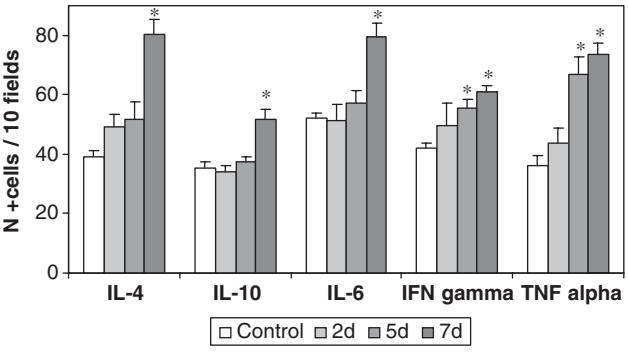

Fig. 7. Effect of the oral administration of 0.3 mg/ml of FPC for 2 (▒), 5 (▓) or 7 (■) consecutive days on the number of IL-4+, IL-10+, IL-6+, IFNγ+ and TNFα+ cells on histological slices of small intestine. *Significantly different from control (□) ($p<0.05$). Data are the mean of five animals per time period with standard deviation.

significant increase in the number of IL-4+, IL-10+ and IL-6+ cells was observed after 7 days of FPC administration. For IFNγ+ and TNFα+ cells, a significant increase was observed after both 5 and 7 days of FPC feeding.

Discussion

Bioactive peptides from foods are endowed with multiple physiological activities, such as morphinomimetic, immunomodulant, opioid, antihypertensive and antithrombotic effects (Meisel and Bockelmann, 1999; Hoerr and Bostwick, 2000). Among dietary proteins, fish proteins are likely to contain a large amount of bioactive sequences and constitute a potential reservoir for potent immunomodulators. Previous studies demonstrated that the supplementation of the diet with proteins derived from fish had been correlated with lower levels of total cholesterol (Wergedahl et al., 2004), with the re-establishment of the aminoacid profile in haemodialysis patients (Tietze and Pedersen, 1991) and even with enhanced longevity (Mimura et al., 1992). Potent bioactive peptides, such as antihypertensive peptides, have been isolated from fish (Yamamoto et al., 2003). Although, biologically active peptides have been localized in the primary structure of plant and animal proteins, the marine resources constitute a potential reservoir for potent immunomodulators. Short chain peptides derived from sardines have been reported to show a potent angiotensin I converting enzyme (ACE) inhibitory activity (Kawasaki et al., 2000). It seems likely that antihypertensive biopeptides could act as immunomodulating agents. Indeed, ACE inhibitory peptides might exert an indirect immunostimulation by enhancing the activity of bradykinin, a vasodilating nanopeptide that may mediate increased immunostimulation and neurotransmission. Bradykinin is also able to stimulate macrophages, enhance lymphocyte migration and increase secretion of lymphokines (Paegelow and Werner, 1986).

In the present study, fish powder concentrate, as a dietary supplement, was prepared by 24 h fermentation of fish protein by yeast. During the fermentation process, the metabolic activity of yeast yielded the release of smaller oligopeptides, as revealed by the HPLC analysis. OPA analysis indicated a progressive increase in proteolysis throughout the fermentation, with the highest values observed after 24 h (Fig. 1). Since the OPA reagent binds to the amino-terminal portion of proteins, this proteolysis can be correlated to the degradation of larger proteins into peptides.

Size exclusion and RP-HPLC analysis confirmed the liberation of small peptides following fermentation (Fig. 2). These peptides can be seen as increased peaks in fermented samples that were not present beforehand (compare Figs. 2C and D). Differences between various fermentation batches were insignificant (data not shown) denoting stability of the process and product and reproducible results between batches. Size exclusion HPLC elution profiles (Fig. 3) along with the increase of the degree of proteolysis (over 50%) confirm the occurrence of fish protein breakdown during fermentation and thereby the generation of new small oligopeptides. Some structural features are attributed to potentially bioactive peptides, such as enhanced hydrophobicity and small molecular weight ranging from 10,000 to 1000 kDa specific for peptides from 3 to 15 amino acids (Kitts and Weiler, 2003).

The introduction of an intestinal immunomodulating food or supplement in the diet must be accompanied by the absence of side effects such as a strong inflammatory response (Perdigón et al., 2001), thus the risk of induction of this kind of response should be evaluated carefully. The pharmacokinetic properties of a potential dietary supplement should be studied to determine for how long the product has to be administered to the host to induce a beneficial effect (Pavan et al., 2003). The balance between mucosal IFNγ and IL-10 is essential in the maintenance of intestinal homoeostasis (Agnholt and Kaltoft, 2001). In this study, we observed an increase in the number of some pro-inflammatory cytokine-producing cells as well as in the number of the regulatory IL-10-producing cells, showing that different accumulative doses of FPC did not induce any inflammatory immune response and the normal morphology of the small intestine was not affected, as revealed in the haematoxylin–eosin studies.

Macrophages are considered the first line of defence in immune response to foreign invaders. Oral administration of viable probiotic microorganisms or the products of their metabolism (e.g. peptides) represent ways to stimulate the host non-specific immunity by enhancing the systemic immune response or by modulating the functions of immunocompetent cells (Perdigón et al., 1986). In this work, phagocytic activity of peritoneal macrophages was enhanced following FPC administration at 0.3 mg/ml for 7 consecutive days, compared to the untreated control, meaning that the FPC induced the release of cytokines able to activate immune cells distant from the site of induction of the immune response (small intestine).

We analyze the effects of FPC on the gut, where the secretory-IgA plays a major function exerting immune exclusion by intimate cooperation with the innate non-specific defence mechanisms (Macpherson et al., 2001). When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propria but also in other

mucosal sites such as the respiratory, urogenital or mammary tissues or salivary glands. This migration is known as the IgA cycle. While not fully understood, it is known that there exists a common mucosal immune system whereby immune cells stimulated in one mucosal tissue can spread and relocate through various mucosal sites. This concept implies that oral immune stimulation can induce immunity in distal intestinal mucosal sites (Phillips-Quagliata and Lamn, 1988). In this work, we observed that the oral administration of FPC, derived from a fermentative process, increased the number of IgA+ cells in the small intestine lamina propria of mice as well as the S-IgA content in the intestinal lumen. The local response observed in the small intestine lamina propria, since no increment of IgA+ cells was observed in the bronchial tissue, led us to conclude that the immunostimulation with FPC is important for the induction of a local immune response, and may be due to the kind of stimulus administered (soluble antigens). These findings are not in disagreement with the enhanced activity of peritoneal macrophages since the mechanisms that regulate macrophage activation and leukocyte traffic (IgA+ cells) are completely different from each other. The translocation of IgG to the lumen depends on passive intercellular diffusion and increases during intestinal inflammatory processes (Mielants and Veys, 1996; Rodrigues et al., 1998). However, in addition to responses to inflammation, serum IgG can also translocate towards the lumen by a physiological mechanism associated with the normal catabolism of Igs. Additionally, it appears that mucosal IgG-positive B cells participate in specific local immune protection. In this sense, some other functions of local IgG could be to specifically control mucosal invasion of pathogens, to complement the activity of locally synthesized S-IgA, and to participate in a IgA-dependent transcytosis of subepithelial immune complexes (Bouvet and Fischetti, 1999). In this study, we observed no modification in the number of IgG+ cells following the administration of FPC. This fact supports the absence of any inflammatory process at the mucosal level, which agrees with the histological studies by haematoxylin–eosin stain.

When we compared the effects of fermented and non-fermented fish powders on the gut immune response in vivo, we noticed that the oral administration of low doses (0.20–0.25 mg/ml) of FPC or the administration of up to 0.30 mg/ml of the non-fermented product did not modify the number of IgA+ cells in the small intestine lamina propria. A threshold dose of FPC of 0.30 mg/ml had to be reached to induce an immune response. A comparable dose of the dried yeast alone was also administered and did not result in any positive result when compared to the administration of FPC (data not shown), ruling out the possibility that the observed effects were a response to the yeast by-products independent of the protein source. This observation is in agreement with a previous report where no macrophage activation or modulation of the intestinal immune response was observed when a strain of *S. cerevisiae* was administered as a pure culture to mice: only when the product fermented by this yeast strain was used, immunomodulating effects were observed (Koh et al., 2002).

The enumeration of IgA+ cells and increased production of S-IgA in the small intestine lumen, along with the concomitant results of the phagocytic activity of macrophages and the histological studies of the gut, allowed us to establish a dose of FPC of 0.30 mg/ml, which was administered for 7 consecutive days to induce gut immune stimulation in our experimental model. Gill (1998), Donnet-Hughes et al. (1999) and Perdigón et al. (1991, 2001) stated that defining the effective dose for any strain or product for diet supplementation is an important step for future studies in this field.

B cell immunoglobulin switching and differentiation to plasmocytes secreting IgA occurs upon interactions with T cells in the lamina propria in an environment rich in IL-4, IL-5, IL-10 and TGFβ (Blum et al., 1999), while IL-6 promotes terminal differentiation of B cells into plasma cells (Christensen et al., 2002). In this study, IL-4, IL-6 and IL-10 were all significantly increased in the lamina propria of the small intestine of animals that received FPC, which is in accordance with the increase in the number of IgA+ cells observed in the small intestine. At the same time, some pro-inflammatory cytokines such as IFNγ and TNFα also increased after the oral administration of FPC. It can be stated that the increased levels of IL-10 minimized the inflammatory effects induced by IFNγ and TNFα, as confirmed by the haematoxylin–eosin studies. The enhanced IgA levels found in the intestinal environment following the administration of FPC at a functional dose could be used as an approach in the protection towards intestinal infections (Macpherson et al., 2001). Even when the cytokines measured are mainly involved in innate immunity, we cannot ignore the participation of the Th1 population. The ability to launch a Th1 profile is important for the activation of cell-mediated immune defense mechanisms to combat viral or intracellular pathogen challenge and to enhance tumouricidal activity (Cross et al., 2004).

Proteolysis of fish proteins by the fermentative yeast appeared to effectively increase the yield of functional peptides in this work, as was also demonstrated in previous works (Tietze and Pedersen, 1991; Mimura et al., 1992; Wergedahl et al., 2004,). The immunomodulatory activity shown through this study might be largely correlated to the release of novel and potentially bioactive peptides by promoting the "unfolding" of larger proteins. In this study, hydrolyzed proteins from white fish have been correlated with better immunomodulation of the intestinal mucosa than the non-

fermented product, which is in accordance with a previous finding (Qin et al., 2001). We conclude that oral administration of FPC enhances gut-associated non-specific immunity without an inflammatory outcome and that this effect would be induced by the products appeared during the fermentation process. FPC is an immunomodulating food with a demonstrated capacity to enhance non-specific host defense mechanisms.

Uncited Reference

Perdigón et al., 1999.

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