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Full Length Research Paper

Anti-inflammatory activities of enzymatic (alcalase) hydrolysate of a whey protein concentrate

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This study was designed to evaluate the anti-inflammatory effects of whey protein hydrolysate (WPH) in mice. Hydrolysis for this experiment was conducted in a pH-stat at 20% degree of hydrolysis. Carrageenan-induced paw edema, peritonitis induced by lipopolysaccharide (LPS) and acetic acid-induced writhing were tested to investigate anti-inflammatory activity. Overall, WPH inhibited carrageenan-induced mice paw edema significantly with doses of 30 mg/kg ($p < 0.001$), 100 mg/kg ($p < 0.01$) and 300 mg/kg ($p < 0.001$) at 3 h post carrageenan. The inhibitory values of edema were 60.34, 39.65 and 68.96%, respectively. After 4 h and an intraperitoneal injection of LPS (100 µg/kg), WPH further inhibited leukocyte recruitment to the peritoneal cavity of mice at a dose of 300 mg/kg ($p < 0.05$). In a number of the writhing episodes, WPH reduced contortions using a dose of 300 mg/kg. These results indicate that the hydrolysate obtained by treatment with alcalase demonstrated anti-inflammatory activity in the experimental models. All the anti-inflammatory actions obtained were also suggested to be due the presence of glutamine.

Key words: Whey protein, proteases, hydrolysate, anti-inflammatory activity, mice.

INTRODUCTION

Milk whey protein contains a multitude of proteins that remain soluble after the precipitation of caseins during the manufacturing of cheese. There are major components, such as β-lactoglobulin, α-lactalbumin, serum albumin and immune-globulins, and minor components, such as lactoferrin, lactoperoxidase and various growth factors, that make-up the remaining proteins. Protein hydrolysates or peptides are products from the hydrolyses of proteins by proteases, such as pepsin, trypsin, chymotrypsin and papain (Damodaram, 2000).

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Abbreviations: WPH, Whey protein hydrolysate; **LPS,** lipopolysaccharide.

Specifically, whey protein hydrolysate (WPH) has great health improvement potentials, including digestive, antimicrobial, immunomodulatory, anticholesteremic, antithrombotic, antihypertensive and anticancer activities (Pacheco and Antunes, 2009; Kim et al., 2007).

Many physiological effects for whey proteins have been identified including protective and anti-inflammatory activities (Beaulieu et al., 2007; Ward et al., 2002; Kano et al., 2002; Kimber et al., 2002). In particular, lactoferrin displayed anti-inflammatory properties in animal models through the regulation of cytokines (tumor necrosis factor, interleukin-6, interleukin-10 and pro-Th1 cytokines). This protein might also act as a potent anti-inflammatory at local sites of inflammation, including the respiratory and gastrointestinal tracts (Ward et al., 2002; Kano et al., 2002; Kimber et al., 2002; Shimizu et al., 2006).

Inflammation has been demonstrating to be the root of

almost all chronic diseases, such as cancer, cardio-vascular diseases and autoimmune diseases.

Considerable efforts and resources have been dedicating to the development of anti-inflammatory products; of particular interest are functional foods capable of modulating the expression of this activity (Tak and Firestein, 2001). The aim of this study was to investigate the anti-inflammatory activities of whey protein hydrolyzed by alcalase through the regression of experimentally induced inflammation in mice.

MATERIALS AND METHODS

Whey protein hydrolysate (WPH)

Whey protein concentrate (WPC) was obtained from Arla foods ingredients. Hydrolysis was conducted in a pH stat (model 716, Methron, Les Uleis, France) with a 10% (wt/vol) substrate concentration and a 20% degree of hydrolysis (DH). WPC was hydrolyzed by alcalase (0.6 L; Novo Nordisk, Bagsvaerd, Denmark) from *Bacillus licheniformis* at 50°C and pH 8.0. The DH was monitored by a pH stat method using NH_3OH (1 M) to hold the pH constant during the hydrolysis reaction (Adler-Nissen, 1986). The enzyme was inactivated at the end of the incubation period by heating at 85°C for 15 min, followed by freezing.

Analytical procedures

Composition

Crude protein (N x 6.38), ash and total solids and lipids were determined according to Association of Official Analytical Chemist standard methods (Horwitz, 2005), and lactose was determined according to the method of Acton (1977).

Determination of protein-bound amino acids and free amino acids

The amino acids released by acid hydrolysis (110°C, 22 h) were subjected to a phenyl isothiocyanate (PITC) pre-column, followed by separation using a reverse phase column on a Shimadzu HPLC and detected by UV (254 nm). Quantification was performed by internal calibration using α-aminobutyric acid (AAAB) as the standard. Quantification of the amino acids was based on a standard amino acid mixture (standard H/Pierce/P/N 20088).

Preparation of reference drug

WPH was administered in 30, 100 and 300 mg/kg doses after being suspended in the vehicle (1% sodium carboxymethylcellulose suspension in distilled water). The control group animals received the same experimental handling as the test group animals, except that the drug treatment was replaced with the appropriate volumes of dosing vehicle. Indomethacin (10 mg/kg) was used as the reference drug and was orally administered in a 10 ml/kg volume per body weight of the animal.

Pharmacological procedures

Animals

Adult, male, Swiss mice (25 ± 5 g) were obtained from the Central Animal Facility of the Federal University of Alfenas. Prior to this experiment, the animals were kept for at least seven days in the experimental laboratory at 23 ± 1°C under controlled light cycle conditions (12 h light and dark), receiving commercial rations and water *ad libitum*. All experiments were conducted according to Brazilian regulations for animal experimentation (COBEA), after approval by the Ethical Commission of Animal Experimentation at the Federal University of Alfenas (protocol 311/2010).

Evaluation of anti-inflammatory activity in mice

Carrageenan-induced mice paw edema: Pedal inflammation in mice (n = 6 per group) was produced as described by Johnson et al. (2010). Before the experiment, the animals were starved overnight with free access to water. Paw edema was measured with a plethysmometer (Model 7140, Ugo Basile, Italy). The basal volume of the right hind paw was determined before the administration of any drug. After determination of the basal volume, the animals were divided into the experimental groups, where the mean volumes of the different groups were similar. Vehicle, WPH or indomethacin was orally administered 1 h before the i.p injection of carrageenan (400 µg, 20 µl). The paw volume was measured 1, 2, 3 and 4 h after injection of the inflammatory stimulus. The results are presented as paw volume variations (µl) in relation to the basal values.

Peritonitis induced by lipopolysaccharide (LPS): To assess the possible effects of WPH on leukocyte recruitment to the peritoneal cavity, the animals (n = 6 per group) were orally pre-treated with vehicle and WPH. After 30 min, LPS from *E. coli* serotype 026:B6 (100 µg/kg i.p.) dissolved in pyrogen-free sterile saline was administrated. 4 h after the injection of LPS, the mice were killed by an inhalatory overdose of halothane, and the cells from the peritoneal cavities were harvested by injecting 5.0 ml of PBS, containing 0.5% sodium citrate. The abdomens of the mice were gently massaged and the blood-free cell suspensions were carefully aspirated with a syringe. Abdominal washings were placed into plastic tubes, and total cell counts were conducted immediately in a Neubauer chamber (Cunha et al., 1989).

Acetic acid-induced writhing in mice: Acetic acid (0.6% v/v, 10 ml/kg) was injected into the peritoneal cavities of the mice, which were placed in a large glass cylinder. The intensity of nociceptive behavior was quantified by counting the total number of writhes that occurred between 0 and 20 min after the stimulus injection, as described earlier Koster et al. (1959). Oral treatments with vehicle, indomethacin or WPH were administered 1 h prior to the injections of acetic acid (n = 6 per group). The writhing response consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as the writhing scores over a period of 20 min.

Statistical analysis

The data obtained were analyzed using the GraphPad software program (Version 4.0) and were expressed as mean ± S.E.M. Statistically, significant differences between the groups were calculated by the application of an analysis of variance (ANOVA), followed by a Newman-Keuls test. P-values less than 0.05 ($p < 0.05$) were considered significant.

RESULTS

The approximate percent composition and amino acid profile of WPC before and after hydrolysis with alcalase

Table 1. Approximate percent compositions of the whey protein concentrate (WPC) and whey protein hydrolysate (WPH).

Component	WPC	WPH
Protein (N content x 6.38)	83.86 ± 0.51	83.25 ± 0.09
Total Lipids	6.33 ± 0.14	6.29 ± 0.11
Ash	3.08 ± 0.09	3.64 ± 0.05
Lactose	6.73 ± 0.11	7.02 ± 0.10

Data are average values of three independent measurements.

Table 2. Amino acid profiles of the whey protein concentrate (WPC) and whey protein hydrolysate (WPH).

Amino Acid (g/100 g of protein)	WPC	WPH
Asp	10.75 ± 0.21	10.81 ± 0.13
Thr	6.88 ± 0.07	6.94 ± 0.09
Ser	5.53 ± 0.04	5.62 ± 0.02
Glu	17.81 ± 0.11	18.48 ± 0.17
Pro	5.97 ± 0.05	6.02 ± 0.04
Gly	1.79 ± 0.02	1.97 ± 0.02
Ala	4.75 ± 0.03	5.05 ± 0.03
Cys	2.45 ± 0.02	2.27 ± 0.02
Val	5.42 ± 0.06	4.94 ± 0.02
Met	2.46 ± 0.03	1.92 ± 0.02
Ile	5.69 ± 0.04	5.45 ± 0.04
Leu	10.55 ± 0.12	10.80 ± 0.19
Tyr	3.14 ± 0.02	3.06 ± 0.04
Phe	3.44 ± 0.01	3.46 ± 0.03
Lys	9.97 ± 0.07	9.80 ± 0.05
His	2.54 ± 0.03	1.81 ± 0.04
Arg	1.66 ± 0.02	1.80 ± 0.03

Data are average values of two independent determinations. Trp was destroyed by acid hydrolysis prior to analysis.

(WPH) are shown in Tables 1 and 2, respectively. In both, WPC and WPH contained high concentrations of glutamine, which was derived from L-glutamic acid. The protein content of WPC and the hydrolysate are shown in Table 1. WPH showed a slightly higher content of minerals (ash) and other similar components, as compared to WPC.

Carrageenan-induced mice paw edema

Figure 1 shows that WPH significantly inhibited carrageenan-induced mice paw edema at doses of 30 mg/kg ($p < 0.001$), 100 mg/kg ($p < 0.01$) and 300 mg/kg ($p < 0.001$) and 3 h post carrageenan induction, as the inhibitory values of edema were 60.34, 39.65 and 68.96%, respectively. Indomethacin (10 mg/kg) gave a percent inhibition of 65.52%.

Peritonitis induced by lipopolysaccharide

In agreement with previous studies, LPS-induced peritonitis was followed by a significant increase in the number of leukocytes in the peritoneal cavity of the mice when compared to the control group treated only with vehicle [14]. As shown in Figure 2, 4 h after the intraperitoneal injection of LPS (100 µg/kg), the WPH significantly inhibited leukocyte recruitment into the peritoneal cavity of the mice at a dose of 300 mg/kg ($p < 0.05$). The percent inhibition of leukocyte recruitment at 4 h post-LPS was 23.51% for 300 mg/kg of the extract. No

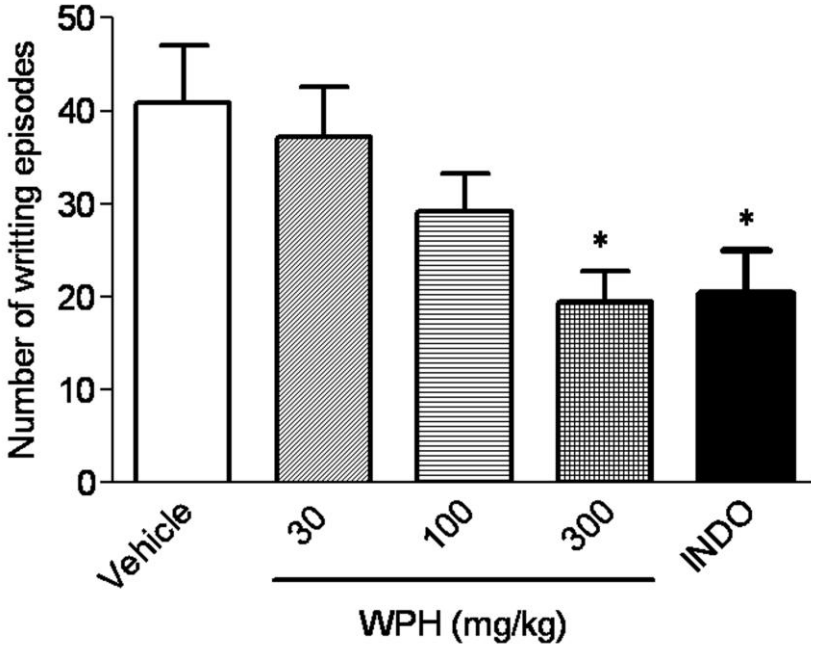

Figure 1. Whey protein hydrolysate (WPH) administered orally, verses acetic acid induced writhing movements in mice. Animals were pretreated orally with vehicle (1% sodium carboxymethylcellulose (CMC), WPH (doses 30, 100 and 300 mg/kg) and indomethacin (INDO; 5 mg/kg). Each column represents the mean and the S.E.M. for six mice in each group. The asterisks denote the significance levels when compared with the control group (CMC): *p < 0.05.

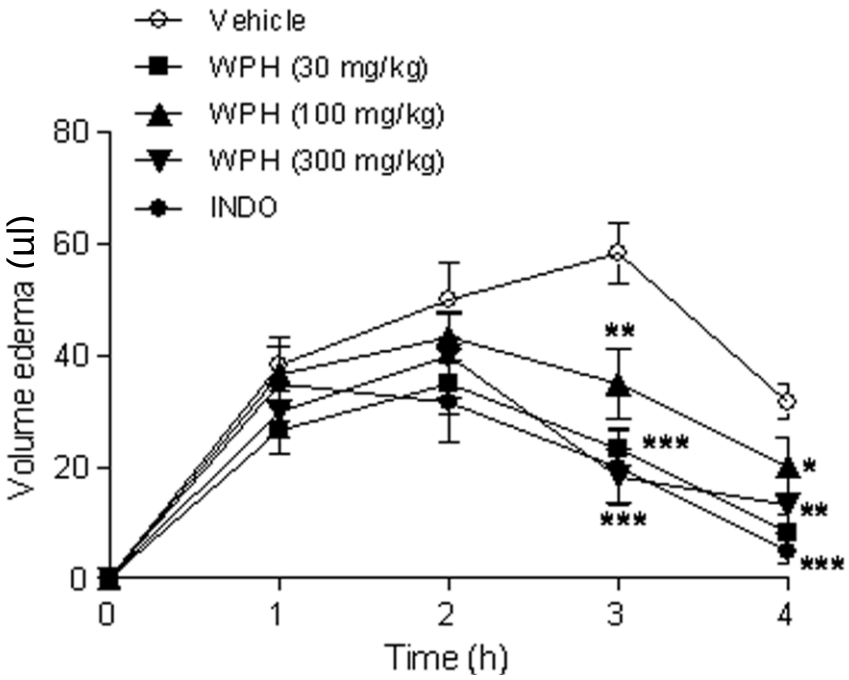

Figure 2. Effects of orally administered vehicle (1% sodium CMC), whey protein hydrolysate (WPH; 30, 100 and 300 mg/kg) and indomethacin (5 mg/kg) on paw edemas induced by intraplantar carrageenan injection (1 mg/paw). Each line represents the mean and the S.E.M. for six mice in each group. The asterisks denote the significance levels when compared with the control group (one-way ANOVA followed by Newman–Keuls test): *p < 0.05, **p < 0.01 and ***p < 0.001.

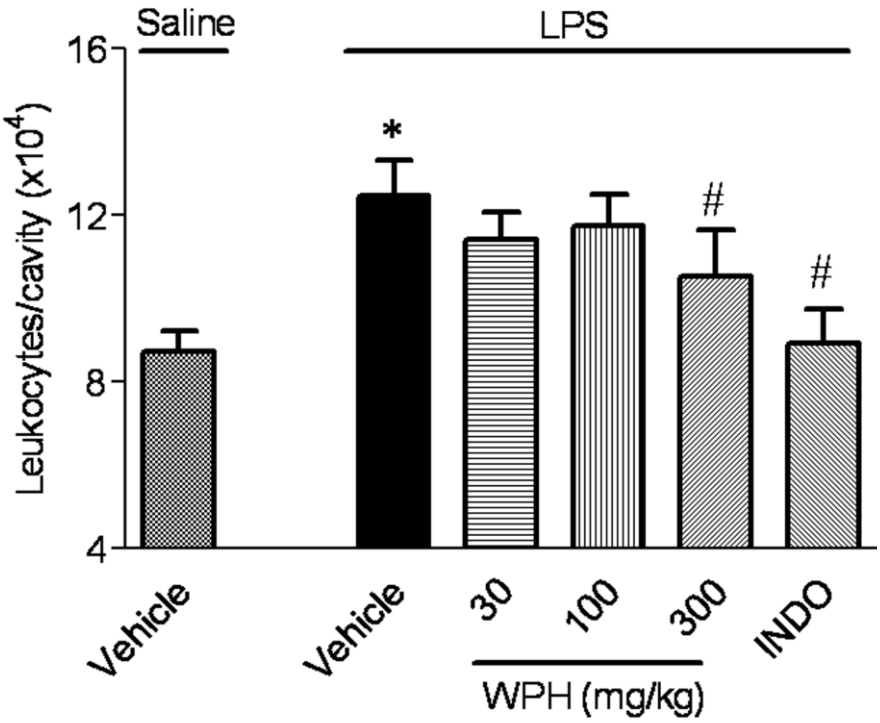

Figure 3. Effects of orally administered vehicle (1% sodium CMC), whey protein hydrolysate (WPH; 30, 100 and 300 mg/kg) and indomethacin (INDO; 5mg/kg) on lipopolysaccharide-induced recruitment of leukocytes to the peritoneal cavity of mice. Each column represents the mean ± S.E.M. of eight animals per group. *$p < 0.05$; saline + vehicle group, #$p < 0.05$.

significant inhibitions were observed with WPH at doses of 30 to 100 mg/kg. However, indomethacin (10 mg/kg) induced a significant percent inhibition ($p < 0.05$) of 31.56%.

Acetic acid-induced writhing in mice

Results of the acetic acid writhing test are shown in Figure 3. WPH at a dose of 300 mg/kg ($p < 0.05$) significantly reduced the number of writhes by 52.32%. Conversely, no significant reductions were observed with WPH at doses of 30 and 100 mg/kg. Indomethacin (10 mg/kg) exhibited a significant percent reduction of 50.12% ($p < 0.05$) during the acetic acid-induced writhing.

DISCUSSION

The anti-inflammatory properties of the whey protein hydrolyzed (WPH) by alcalase was studied. In carregeenan peritonitis and acid acetic tests, the WPH attenuates the injury and we suggested that glutamine, which is a major amino acid component is the key for attenuation of injury.

The anti-inflammatory properties of WPHs hydrolyzed by alcalase were studied in this work. The anti-inflammatory effect of WPH was evaluated in carrageenan-induced paw edema, a widely employed animal model for the screening of anti-inflammatory compounds. These experimental models exhibit a high degree of reproducibility. Particularly in mice, the inflammatory response induced by carrageenan is characterized by a biphasic response with marked edema formation resulting from the rapid production of several inflammatory mediators, such as histamine, serotonin and bradykinin (first-phase). Subsequently, prostaglandins and nitric oxide (second-phase), produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively, are released to sustain the model and peak at 3 h (Seibert et al., 1994). In this study, previous oral treatment with WPH was effective in reducing the edematogenic response evoked by carrageenan in the mice, between the third and the fourth hours after the injection. This evidence allows us to suggest that the anti-inflammatory actions of WPH were related to the inhibition of intracellular signaling pathways involved in the effects of second-phase inflammation.

The anti-inflammatory activity of the WPH was also evaluated using peritonitis induced with LPS and carrageenan-induced mice paw models. Neutrophils are a class of blood cells that are part of the immune system and are one of five major types of leukocytes. Neutrophilia is one of the hallmarks of inflammation and

when LPS, the major component of the cell walls of Gram-negative bacteria was administered, a marked recruitment of neutrophils from marrow and blood to tissues was observed (Bozza et al., 1994). *In vivo*, LPS may interact with different macrophage surface receptors, especially with the complex CD14/Toll receptor 2, leading to intracellular signaling events and consequent secretion of inflammatory mediators, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα). These inflammatory mediators are essential neutrophil chemoattractants in LPS-induced inflammation (Yang et al., 1998). WPH inhibited the leukocyte migration induced by the i.p injection of LPS. A possible mechanism associated with this activity could be the inhibition of the syntheses of many inflammatory mediators.

This work shows that WPH p.o produced significant antinociception according to the assessments for the abdominal writhes elicited by acetic acid, a model used to evaluate the potential analgesic activities of drugs. It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons (Collier et al., 1968). In the acetic acid-induced abdominal writhing model, which is a visceral pain model, the process releases arachidonic acid via cyclo-oxygenase (COX). In other words, the biosynthesis of these prostaglandins plays an important role in the nociceptive mechanism (Duarte, et al., 1988). This method is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), narcotics and other centrally acting drugs (Collier et al., 1968; Santos et al., 1998).

The slightly higher ash content of the hydrolysates can be attributed to the alkali (NaOH) pH adjustment before the hydrolysis. Other components of the hydrolysates were similar, as expected. The amino acid compositions of WPC and WPH were predominantly glutamine, aspartic acid, leucine, isoleucine, lysine, histidine, and sulfur-containing amino acids. Some characteristics of the amino acid profile of WPC have been emphasized in the literature as important based on several aspects. In particular, the high content of branched chain amino acids, particularly leucines, seems to be important for tissue regeneration in multiple traumas and burns. Additionally, the high content of sulfur-containing amino acids and the ideal 1:1 ratio of methionine to cysteine make WPC similar to human milk, which is useful for the development of humanized milk formulas for infants. As expected in our study, this excellent amino acid profile of WPC was maintained in the whole hydrolysates. Glutamine (Gln), the most abundant amino acid of WPC, is shown to be a key pharmaco-nutrient in the body's response to stress and injury, as have been the sulfur-containing amino acids. Gln exerts its protective effects via multiple mechanisms, including direct protection of cells and tissue from injury, attenuation of inflammation and preservation of metabolic function (Weitzel et al., 2010). An experimental model of sepsis induced by cecal ligation and punctures with a single dose of Gln improved survival and arterial oxygenation, prevented pulmonary mechanic deteriorations and minimized histological changes as well as attenuating epithelial cell apoptosis of the lungs and distal organs. These findings suggested that Gln might modulate the inflammatory process, reducing the risk of lung and distal organ injury (Oliveira et al., 2009). In other models of LPS-induced sepsis, Gln prevented neutrophil recruitment and infiltration, protected the alveolar barrier and attenuated inflammation injury (Zhang et al., 2009). In many conditions, plasma and muscle glutamine concentrations were decreased, leading to impaired immune response (Guadagni and Biolo, 2009). Glutamine supplementation is associated with improved immune defenses in critical illnesses (Newsholme, 2001; Castell and Newsholme, 2001; Walsh et al., 1998; Calder and Yaqoob, 1999) and possibly in other conditions (Guadagni and Biolo, 2009). *In vivo* and *in vitro* experiments have shown that Gln regulates immune response, attenuates the release of TNF-α, IL-6 and IL-8 in response to oxidative stress and prevents lung injury in acute respiratory distress (Coeffier et al., 2001; Wischmeyer et al., 2003; Singleton et al., 2005; Jun et al., 2009). After its uptake into the cells, Gln is converted to glutamate and ammonia. Glutamate is a necessary substrate for the synthesis of glutathione (GSH); therefore, Gln is also a precursor of GSH (Zhang et al., 2009). In neonatal rats, Gln was shown to be unique by reversing the effects of the septic liver oxidative process (Babu et al., 2001).

Moreover, this literature provided information on the relationship to Gln and COX (Cole et al., 2003; Argenzio et al., 1993). It is also known that the Gln acts in macrophages as a precursor to arginine, which is a precursor to the synthesis of NO in a reaction catalyzed by enzyme inducible nitric oxide synthase (iNOS) (Murphy and Newsholme, 1998). Furthermore, Gln can increase the transport of arginine through the plasma membrane of macrophages, and it can suppress the activity of the arginase enzyme, which can increase the intracellular concentration of arginine. Alternatively, Gln could modulate the expression (at a transcriptional level) and the activity of the iNOS enzyme (Bellows and Jaffe, 1999).

The data from this present study suggested that WPH obtained by alcalase hydrolysis displays considerable anti-inflammatory activity by alleviating the paw edema and reducing the peritonitis LPS-induced. In addition, the mechanism of these effects could be due to the presence glutamine amino acids. WPH might have potential therapeutic value related to its use in the treatment of inflammatory disorders.

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