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# Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway

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ABSTRACT

Lupine protein hydrolysates (LPHs) were obtained from a lupine protein isolate (LPI) by enzymatic hydrolysis using two proteases, Izyme AL and Alcalase 2.4 L, and their potential anti-inflammatory capacities were studied by determining their *in vitro* inhibition of the following enzymes that are involved in the inflammatory process: phospholipase A2 ($PLA_2$), cyclooxygenase 2 (COX-2), thrombin, and transglutaminase (TG). The strongest inhibitory activities toward $PLA_2$ and TG were found in the hydrolysates obtained by hydrolysis with Izyme and subsequently with Alcalase, with more than 70% inhibition obtained in some cases. All of the hydrolysates tested inhibited more than 60% of the COX-2 activity. In no case did the percentage of thrombin activity inhibition exceed 40%. The best inhibitory activities were found in the LPH obtained after 15 min of hydrolysis with Alcalase and in the LPH obtained after 60 min of hydrolysis with Izyme followed by 15 min of hydrolysis with Alcalase. Enzyme kinetic analyses were conducted to determine the $K_m$ and $V_{max}$ parameters of these two hydrolysates using the Lineweaver–Burk equation. Both hydrolysates competitively inhibited the thrombin and $PLA_2$ activities. In the case of COX-2 and TG, the inhibition appeared to be the mixed type.



## 1. Introduction

Chronic inflammation is involved in the onset and development of numerous chronic diseases, such as cardiovascular diseases, cancer, diabetes, obesity, arthritis, neurological diseases, pulmonary diseases, psychological diseases and autoimmune diseases (Sung, Prasad, Gupta, Patchva, & Aggarwal, 2012). Inflammation is one of the complex biological responses of the immune system of an organism. In the initial steps, the inflammatory response is mediated by phospholipase A2 ($PLA_2$), which is elevated in many inflammatory disorders. $PLA_2$ hydrolyses the sn-2 ester bond of glycerophospholipids to produce free fatty acids, particularly arachidonic acid (AA), and lysophospholipids (Yedgar, Cohen, & Shoseyov, 2006). AA is metabolised mainly via the cyclo-oxygenase (COX) and lipo-oxygenase enzymatic pathways to produce diverse families of eicosanoids, including prostaglandins (PGs), thromboxanes, and leukotrienes. These eicosanoids are involved in the development of many pathological conditions, particularly inflammation-related processes (Yedgar et al., 2006).

There is a close relationship between inflammation and coagulation. Inflammation-induced coagulation is due to tissue factor, which produces thrombin generation (Levi & van der Poll, 2005). Thrombin is a trypsin-like serine protease that has a central role in haemostasis and thrombosis. Once formed, thrombin cleaves fibrinogen to produce the fibrin mesh of the blood clot (Cirino et al., 1996). Thrombin also activates transglutaminase or factor XIII, which cross-links fibrin molecules via the formation of interchain ε-(γ-glutamyl)-lysine isopeptide bonds and stabilises the clot by preventing its hydrolysis by proteases (Moreno, 2006).

Diet is thought to be one of the major risk factors for the development of chronic diseases. Thus, modifying the diet could prevent or delay these diseases (Prasad, Sung, & Aggarwal, 2012). Some dietary agents have shown the potential to inactivate inflammatory molecules by direct binding. Recent studies have provided evidence that peptides can modify crucial regulatory functions of inflammatory processes (Fernandes, Schmidhuber, & Brain, 2009). There are many reports about synthetically derived anti-inflammatory peptides (Selvatici, Siniscalchi, & Spisani, 2013). However, few studies have examined the effect of plant protein hydrolysates on inflammatory markers. It has been reported that soybean protein hydrolysates reduce the level of some of these markers (Vernaza, Dia, González de Mejía, & Chang, 2012).

Lupine (*Lupinus angustifolius* L.) is an herbaceous plant typical of the Mediterranean region. Lupine seeds can be incorporated as a protein source in both animal feed and in a variety of human foods.

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Lupine flour is rich in protein (20–40%), and this content can be increased to more than 90% in protein isolates (Lqari, Vioque, Pedroche, & Millán, 2002). Lupine protein hydrolysates (LPHs) have shown bile-acid binding activity, so these may have potential applications for reducing cholesterol in hypercholesterolemic patients (Yoshie-Stark & Wäsche, 2004). More recently, lupine proteins have been shown to exert hypotensive and hypotriglyceridemic effects *in vivo* (Cam & Gonzalez de Mejia, 2012), but there are no reports of their anti-inflammatory activity.

The objective of this work was to determine the effect of LPHs on the activity of four enzymes implicated in the inflammatory process: $PLA_2$, COX-2, thrombin, and TG. LPHs were obtained by hydrolysis of lupine protein isolate (LPI) with Izyme and Alcalase, two food-grade proteases produced by Novozymes. Izyme has trypsin-like activity whereas Alcalase is a non-specific endoprotease. Both trypsin and Alcalase have been previously used for the generation of bioactive peptides (Korhonen & Pihlanto, 2006). Samples were collected after different periods of incubation, and the activity of the above mentioned enzymes was tested in presence of LPHs with different degrees of hydrolysis. The LPHs that exhibited a higher inhibitory activity were selected, and their enzyme kinetic parameters ($K_m$, $V_{max}$) were determined.

## 2. Materials and methods

### 2.1. Materials

The *L. angustifolius* seeds were provided by Koipesol Semillas, S.A. (Seville, Spain). Izyme AL (564 EU/ml) and Alcalase 2.4 L (2.4 AU/g) were provided by Novozymes (Bagsvaerd, Denmark). $PLA_2$ from bovine pancreas, albumin (human, essentially fatty acid-free), thrombin from bovine plasma, antithrombin III (AT III), tosyl-gly-pro-arg-*p*-nitroanilide acetate salt (Chromozym), heparin sodium salt, protamine sulphate salt from salmon, TG from guinea pig liver, monodansylcadaverine (dansyl-CAD), dextran-coated charcoal, DL-dithiothreitol (DTT), 2-mercaptoethanol, cystamine dihydrochloride, COX-2 human, AA from porcine liver, N,N,N′,N′-tetramethyl *p*-phenylenediamine (TMPD), flunixin meglumine, and hematin (porcine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-Palmitoyl-2-pyrenedecanoyl phosphatidylcholine (10-pyrene-PC) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bovine milk casein was obtained from Calbiochem (Merck Group, Darmstadt, Germany). Iodoacetamide was purchased from Merck (Darmstadt, Germany).

### 2.2. Methods

#### 2.2.1. Obtaining the lupine protein isolate

The LPI was obtained using the method of Lqari et al. (2002). Briefly, defatted lupine flour was extracted with 0.25% $Na_2SO_3$ (w/v) at pH 10.5, for 1 h. After centrifuging the extract at 7500 rpm for 15 min, the supernatant was recovered, and the pellet was extracted again. Both of the supernatants were adjusted to the isoelectric point of lupine proteins (pH 4.3). The resulting precipitate was washed with distilled water adjusted to pH 4.3 and centrifuged to remove residual salts and other non-protein compounds. Finally, the precipitated proteins were lyophilised and stored at room temperature.

#### 2.2.2. Hydrolysis of the lupine protein isolate

Hydrolysis was conducted in a bioreactor under stirring at a controlled pH and temperature. The LPI was suspended in distilled water (10% w/v), and two types of hydrolysis were performed: one with Izyme followed by Alcalase and one using only Alcalase. The following conditions were used.

Hydrolysis with Izyme and Alcalase: First, the LPI was hydrolysed with Izyme for 1 h at pH 10, 50 °C, and E/S = 100 EU/g protein. Then, a second hydrolysis with Alcalase at pH 8, 50 °C, and E/S = 0.3 AU/g protein was conducted for 1 h.

Hydrolysis with Alcalase: The LPI was hydrolysed with Alcalase for 1 h at pH 8, 50 °C, and E/S = 0.3 AU/g protein.

Samples were taken at different times, and enzymes were inactivated by heating at 85 °C for 15 min. The supernatants obtained from centrifugation at 8000 rpm for 15 min constituted the LPHs. The LPHs obtained using Izyme followed by Alcalase were designated 0I, 15I, 30I, 60I, 60I + 15A, 60I + 30A, and 60I + 60A. The LPHs obtained using Alcalase were designated 0A, 5A, 10A, 15A, 20A, 25A, 30A, and 60A. The number indicates the time of hydrolysis in minutes.

#### 2.2.3. Determination of the degree of hydrolysis (DH)

The DH, defined as the percentage of peptide bonds cleaved, was determined by the TNBS method according to Adler-Nissen (1979). The total number of amino groups was determined in a sample that had been 100% hydrolysed at 110 °C for 24 h in 6 N HCl.

#### 2.2.4. Determination of protein and peptide content

The protein concentrations were determined by elemental microanalysis as % nitrogen content × 6.25 using a Leco CHNS-932 analyser (St. Joseph, MI, USA).

#### 2.2.5. $PLA_2$ activity assay

The $PLA_2$ activity was evaluated *in vitro* by measuring the fluorescence produced by its hydrolysis of the 10-pyrene-PC substrate (Cunningham, Maciejewski, & Yao, 2006). $PLA_2$ was dissolved in a solution of 50 mM Tris, 0.1 M NaCl, and 2 mM $CaCl_2$ with 0.25% fatty acid-free albumin (pH 7.4) added to a final concentration of 0.5 mg/ml. The substrate (supplied in chloroform) was dried under a nitrogen stream, dissolved in absolute ethanol, and stored at −20 °C prior to use. A 5 μM solution of substrate was prepared in the same buffer used for the enzyme, but without albumin. The fluorescence at 355 nm excitation/420 nM emission was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA). The reactions were evaluated for 60 min at 25 °C. The concentration of the LPHs was 0.6 mg/ml. A negative control without LPHs and a positive control (0.6 mg/ml flunixin meglumine in the well) were used.

#### 2.2.6. COX-2 activity assay

The COX-2 activity was determined according to the method described by Somvanshi et al. (2007) with some modifications. An aliquot of 0.35 μl of commercial COX-2 was incubated with 5 μl of LPHs (at 70 μg protein/μl), and 95 μl of 100 mM Tris, 1 mM EDTA, and 2 μM hematin pH 8.3 for 1 h at room temperature. The reaction was initiated by adding 50 μl of 1.6 mM arachidonic acid and 50 μl of 0.8 mM TMPD dissolved in the same buffer, but without hematin and preincubated for 30 min at room temperature. A control without LPHs and a positive control (1.24 mg/ml flunixin meglumine in the well) were used. TMPD oxidation was determined spectrophotometrically by the absorbance at 610 nm using a Multiskan Spectrum microplate reader (Thermo Labsystem). The initial rate of the reaction was measured during the first 21 s, and the percentage inhibition was calculated.

#### 2.2.7. Thrombin activity assay

The thrombin activity was determined by following the increase in the absorbance at 405 nm that accompanies hydrolysis of the chromogenic substrate Chromozym (Ialenti et al., 2001). Fifty microlitres of LPH (at 70 μg protein/μl) was preincubated for 1 h with 5 μl of enzyme (0.136 μg/ml), at room temperature.