

EFFECT OF PROCESSING PARAMETERS ON *LUPINUS ANGUSTIFOLIUS* PROTEIN ISOLATE: BIOCHEMICAL AND BIOLOGICAL APPROACHES

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ABSTRACT

Lupin proteins are a promising ingredient in functional foods because of their purported hypocholesterolemic activity. In this study a lupin protein isolate from *L. angustifolius* cv. Boregine (LUPI-E) was treated thermally and mechanically and the effect of these treatments on the protein content were determined. After treatment, the soluble proteins were extracted and the degradation behaviour was studied. 2D-electrophoresis and differential scanning calorimetry (DSC) were used to determine the content of native/degraded proteins, coupled with HPLC-Chip-MS/MS that enabled the fingerprinting of the available peptides.

The results indicated that lupin protein isolate partially or completely lost their protein integrity, even if some spots with high intensity were present on 2D-gels. However, specific peptides derived from the tryptic digestion of the treated samples were still available after a prolonged heating.

KEYWORDS

Lupinus angustifolius, thermal processing, bioactive peptides, legumes, storage protein

INTRODUCTION

During the last decade functional foods have gained attention from the Food Industry because of their nutraceutical properties and the role in the maintenance of good health. This opens the door to a wider use of vegetable sources of protein, including lupin.

Lupin seed proteins have proven to be an interesting model to study protein thermal conformational stability under different processing conditions, from both biochemical and techno-functional points of view (Duranti *et al.* 2000; Chango *et al.* 1993). Considering the presence in lupin seeds of specific bioactive components with hypocholesterolemic properties (Marchesi *et al.* 2008; Martins *et al.* 2005; Sirtori *et al.* 2004), it is interesting to evaluate the integrity of the bioactive proteins subjected to different technological treatments and how much they are altered by processing. Therefore, the present study was designed to evaluate the effect of different industrial processing conditions – thermal and mechanical stress –

on the integrity of lupin protein content. A combined proteomic approach allowed the study of the protein profile at the different conditions and the identification of the available peptides (peptides fingerprint), still presented after mild and harsh treatments.

MATERIALS AND METHODS

TOTAL PROTEIN EXTRACT (TPE)

The industrial lupin protein isolate (LPI), from *Lupinus angustifolius* cultivar Boregine, was extracted with 100 mM Tris-HCl and 0.5 M NaCl, pH 8.2, for 2 hrs at room temperature (RT). The slurry was centrifuged at 10,000 rpm, 4°C for 20 min and the extracted proteins present in the supernatant were dialysed against 30 mM Tris-HCl, pH 8.2.

BIDIMENSIONAL ELECTROPHORESIS (2D-E)

20 µL of TPE (about 7 µg/µL) was diluted in 130 µL of IEF solubilisation buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM Tris-HCl pH 8.8, 0.1 mM EDTA); the proteins were reduced with 10 mM DTT for 1 hr at RT and alkylated with 20 mM IAM for 1 hr at RT. Isoelectric focusing was performed on 7 cm, pH 3-10 non linear IPG strips*. Strips were focused for 20,000 V/hrs, with a maximum of 3,000 V using the Protean IEF Cell*. Prior to the second dimension, strips were rehydrated in equilibration buffer (6 M urea, 30% glycerol, 1% SDS, 0.1 mM EDTA, 50 mM Tris-HCl pH 8.8) for 25 min at RT. The separation was performed on 13% SDS-PAGE gels, using the Mini Protean 3 Dodeca Cell (all items were supplied by Biorad Laboratories Inc., USA). Gels were scanned in a VersaDoc 3000*, and the PDQuest software* was used to compare the different 2D-maps.

TPE TRYPTIC DIGESTION

The TPE were denatured with 6 M urea, reduced with 1 M DTT (in the ratio 50 mol of DTT vs 1 mol of Cys), and alkylated with 1 M IAM (in the ratio 200 mol of IAM vs 1 mol of Cys); each step was performed for 1 h at RT. The proteins were then digested with sequencing-grade trypsin (0.5 µg/µL) (Promega, USA) in the ratio 1:50 (w/w) enzyme/protein for 18 h at 37°C.

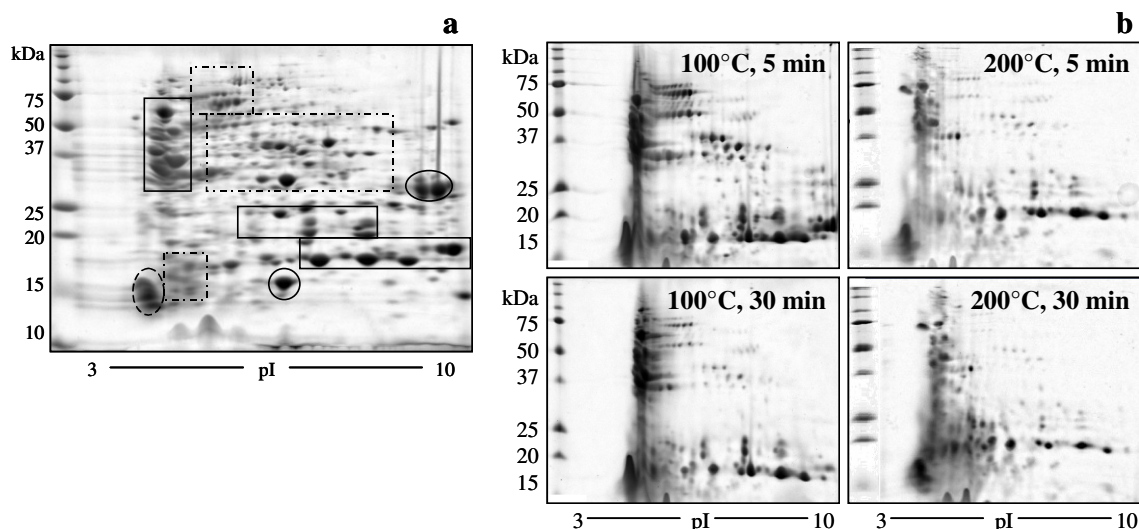


Fig. 1. a) 2D-gel of untreated LPI. Solid line rectangles (—) indicate the acidic and basic subunits of legumin protein. Dash-dotted rectangles (— · — ·) indicate vicilin protein. The dotted oval (· ·) highlights the heavy chain of δ -conglutin. The solid line ovals enclose the large and small subunits of γ -conglutin.; b) exemplary 2D-gels of thermal treated samples, at different time of exposure. Standard markers are indicated (in kDa) on the left.

Table 1. Sequences of the peptides obtained after tryptic digestion of the samples treated at 100° and 200°C, for 30 min. The common peptides are in bold.

Sample	Protein identification	Peptide sequence
100°C, 30 min	Conglutina beta [NCBIInr149208401]	LENLQNYR IVEFQSNPNTLILPK HSDADYILVVLNGR KGKPSSESGPFNLR LLGFGINANENQR ELTFPGSAQDVER NQQQSYFANAQPQQK
	Conglutin alpha [NCBIInr 2313076]	HNIGQSTSPDAYNPQAGR TLTSLDFPILR WLGLAAEHGSIYK
	Legumin-like protein [NCBIInr 85361412]	QQPQENECQFQR RPFYTNAPQEIIYQQGR FLVPPPQSQLR
	Conglutin delta-2 large chain [NCBIInr 116181]	ICGFGPLR ALQQIYESQSEQCEGR QQEQQLEGELEKLPR
	Conglutina beta [NCBIInr 149208401]	NTLEATFNTR LLGFGINANENQR NFLAGSEDNVISQLDR ELTFPGSAQDVER NQQQSYFANAQPQQK
200°C, 30 min	Conglutin alpha [NCBIInr 2313076]	HNIGQSTSPDAYNPQAGR TLTSLDFPILR
	Legumin-like protein [NCBIInr 85361412]	RPFYTNAPQEIIYQQGR FLVPPPQSQLR
	Conglutin delta-2 large chain [NCBIInr 116181]	SSQESESEELDQCCEQLNELNSQR ALQQIYESQSEQCEGR QQEQQLEGELEKLPR

DIFFERENTIAL SCANNING CALORIMETRY (DSC) ANALYSIS

DSC was carried out according to Sousa *et al.* (1995). The pre-treated, lyophilised protein isolates were dissolved with demineralised water at a percentage of 20% (w/w) by stirring for 10 minutes. A small amount of the protein solutions was weighed in the Tzero Hermetic pans (TA Instruments – Waters LLC, USA) and the pans were sealed hermetically. Thermograms were obtained heating from 313 K (40°C) to 393 K (120°C) at a heating rate of 2 K/min, using a DSCQ 2000 system (TA Instruments – Waters LLC, USA). All samples were immediately re-scanned after cooling down to 40°C to investigate reversibility. Besides the onset point temperatures of the protein denaturation peak of the samples, the software calculated the enthalpy ΔH (in J/g) from the peak areas. As reference an empty pan was used.

RESULTS AND DISCUSSION

EFFECT OF THERMAL TREATMENT ON LPI

With the aim of mimicking industrial processing of functional foods, the LPI was treated thermally and the effects on the protein content were determined. Heating was performed in an oven from 100 to 200°C at different time of exposure (5, 15, 30 min). After treatment, the soluble proteins were extracted and the degradation behaviour of the major storage proteins (legumin, vicilin, δ -conglutin, and γ -conglutin) was studied.

The loss of proteins during processing has been determined from 2D-gels of the treated samples (Fig. 1). A long thermal treatment denaturates the majority of the proteins. However, after the heavy treatment (200°C), both basic and acidic subunits of legumin, and the 2S globulins were still clearly visible on the 2D-gels, even though their intensity after 15 and 30 min heating were reduced. On the contrary, vicilin (7S protein) are more heat labile and after only a short heat treatment the corresponding spots appeared faintly. This difference reflects the 3D structure of 7S proteins which are held together by weak non-covalent bonds, whereas legumins have disulphide bridges between their acidic and basic subunits, which require more energy to break.

EFFECT OF MECHANICAL STRESS ON LPI

The mechanical stress was applied using an Ultra-turrax (8,000, 13,500, and 24,000 min^{-1}) and a homogeniser (100, 300 and 1000 Bar), in order to simulate different mixing and extrusion devices. The gels of the treated samples, both with Ultra-turrax and homogeniser showed no obvious differences vs. untreated sample and the spots intensity were also constant (data not shown), in agreement with the DSC results (Fig. 2). According to Chapleau *et al.* (2003) lupin proteins are very sensitive to pressure ranging from 200 to 600 MPa (= 2,000-6,000 Bar), whereas the samples analysed were treated less severely (1000 Bar).

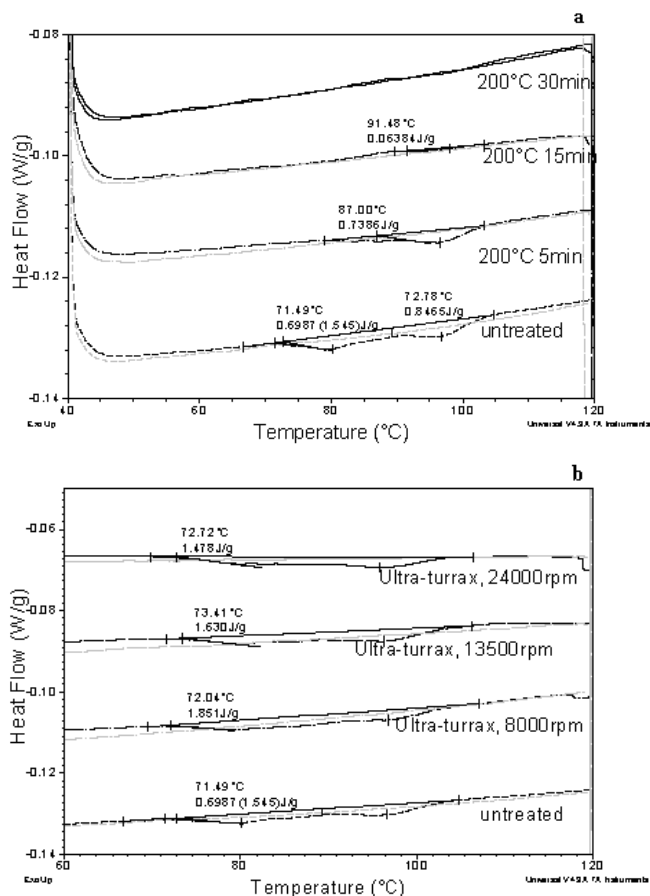


Fig. 2. Exemplary DSC graphs of the thermal and mechanical treated samples, with the untreated LPI as reference. Denaturation enthalpy and onset point data of protein denaturation are shown.

Application of high pressure on globular proteins, such as lupin proteins, was shown to involve in the stabilisation of the quaternary structure of proteins. Pressure higher than 200 MPa modified electrostatic and hydrophobic interactions which lead to structure modification as far as protein aggregation induced by tertiary and secondary structure changes (Masson, 1992).

PEPTIDES FINGERPRINTING

In order to identify the peptides/proteins resistant to processing, the treated samples were digested with trypsin and analysed via HPLC-Chip-MS/MS. The peptides sequences are reported in table 1.

According to the previous results observed in the 2D protein profile study, the majority of the identified peptides belong to the legumin and delta-conglutin proteins with a high coverage level, but also some traces of vicilin were present. These proteins were completely or partially dissociated, but were still able to release intact putative bioactive peptides, also after extensive industrial processing conditions.

DSC ANALYSIS

DSC experiments were used to determine the content of native/degraded proteins in treated samples, both thermally and mechanically. The presence of two peaks in the graphs indicates the TPE major storage proteins: legumin (native protein, 400 kDa) and vicilin (200 kDa). The results showed a decreasing denaturation enthalpy of the LPI (decreasing content of native protein) with increased intensity of the thermal treatment (Fig. 2). The absence of native protein in the treated LPI, in particular in the vicilin peak area, was in agreement with the protein profile trend observed in the 2D-gels. On the contrary, mechanical treatments did not show any significant difference.

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