Review

Parameters for the evaluation of the thermal damage and nutraceutical potential of lupin-based ingredients and food products

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Foods based on sweet lupin proteins are gaining attention from industry and consumers because of their possible role in the prevention of cardiovascular disease. When promoting lupin-based foods for inclusion in a daily diet, the thermal damage suffered during processing is of relevance to the bioactive and nutritional quality of the food product. N-(2-furoylmethyl)-L-lysine (furosine) quantification demonstrates that currently available sweet lupin protein isolates have a thermal damage comparable to or lower than other traditional food ingredients, and are a good source of lysine in non-dairy products. In lupin-based foods claiming to have cholesterol-lowering potential, shotgun proteomics offers itself as a fast and effective screening method for assessing the biological availability of active peptides. Such a method is readily applicable to other legume-enriched food products.

Keywords: Bioactive peptides / Furosine / LC-ESI-MS/MS / *Lupinus albus* / Shot-gun proteomics

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1 Introduction

Following the definition of ILSI Europe, an industry-sponsored forum in which representatives from industry, academia and governments address nutrition issues, "a food can be regarded as functional when it is satisfactorily demonstrated to affect beneficially one or more target functions of the body, beyond adequate nutritional effects, in a way that is relevant to either an improved status of health and well-being and/or reduction of risk of disease" [1].

Functional foods marketed with claims to reduce heart disease focus primarily on the risk factors of blood cholesterol, hypertension, homocysteine, and hyperglycemia. In the past, health-related products aimed at reducing the intake of undesired components (*e.g.* saturated fats and sodium), though recently these products are being enriched in components that are believed to reduce the consumer's risk of suffering from certain diseases. The most common protective ingredients are fibers, ω-3 fatty acids, phytostanols/phytosterols, antioxidant vitamins, and soybean proteins [1], some of which may act synergistically. One example where the hypocholesterolemic property of the protein acting alone has been acknowledged is that of soybean proteins [2–4]. After being assessed in clinical studies [2, 3], the US Food and Drug Administration in 1999 approved a health claim for the reduced risk of heart disease to be put on foods that contain ≥6.25 g of soybean protein per serving [5]. Since that date, the market of functional foods based on soybean proteins has grown substantially, but in the interest of expanding the market, other vegetable proteins characterized by similar nutraceutical properties are being sought. To fulfill the requirements of an increasing number of consumers, these innovative proteins should be phytoestrogen-free [6], should provide the product with satisfactory sensory features, and preferably should not be genetically modified. A good candidate appears to be lupin, of which the four main domesticated species are *Lupinus albus* (white lupin), *L. angustifolius* (narrow-leaf lupin), *L. luteus* (yellow lupin), and *L. mutabilis* [7]. On average, lupin seeds have total protein content similar to soybean (34–43% of dry matter) and acceptable contents of essential amino acids [8]. Moreover, lupin has a lower content of the main anti-nutritional components, *i.e.* phytates, oligo-

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Abbreviations: **Furosine**, N-(2-furoylmethyl)-L-lysine; **LPI**, lupin protein isolate

saccharides, trypsin inhibitors, lectins, and isoflavones, than other grain-legumes do [9]. In lupin seeds, the globulins correspond to about 90% of the total protein content: the main acid globulins are vicilin-like proteins (or β-conglutin) with sedimentary coefficient 7S, and legumin-like proteins (or α-conglutin) with coefficient 11S, plus a minor fraction with 2S coefficient (δ-conglutin) [10]. Another interesting fraction is γ-conglutin, a basic globulin, with sedimentary coefficient 7S [10].

Foods based on sweet lupin proteins are gaining attention from industry and consumers because of their possible role in the prevention of cardiovascular disease. However, before promoting a certain food for inclusion in a daily diet, understanding the consequences of processing on the bioactivity of the proteins in question is important, as is the extent to which thermal processing treatments damage their nutritional quality. This review documents recent studies designed precisely to assess these aspects of lupin food quality.

2 Evaluating the added value of lupin-based food products

During processing, food undergoes some thermal damage, in part deriving from the Maillard reaction [11]. This reaction, which involves amino acids, proteins and reducing sugars, is responsible for an impairment of the nutritional quality: there is a depletion of essential amino acids and vitamins and a decrease of protein digestibility due to the unavailability of the sites of attack of proteases. In addition, the Maillard reaction may lead to the formation of antinutritional or toxic compounds [12, 13]. An estimate of the overall degree of thermal damage may be obtained by measuring specific molecular markers such as N-(2-furoylmethyl)-L-lysine (furosine), a stable derivative of the Amadori compound that is produced from the reaction between reducing sugars and lysine [14 – 16] and that is considered a reliable marker of the initial stage of the Maillard reaction. Furosine is mainly a marker of the glycosylation of protein-bound lysine, a process that impairs the bioavailability of the lysine. Although other markers have been proposed recently [17], furosine is still one of the most used, since it allows to calculate [18] the amount of unavailable lysine (blocked lysine) and in turn the available lysine and/or percent of lysine loss. Determining the available lysine content is an indicator of the protein quality and nutritional value of milk for example [19], where lysine is abundant and has a high biological quality. The quality of the raw materials and the effect of processing and/or storage can be equally assessed in pasta, eggs, soymilk and other products [20–22].

The effect of processing was specifically measured by D'Agostina *et al.* [23] when lupin protein isolates (LPI) were produced in a pilot plant. Initial trials produced two different isolates, named LPI-E and LPI-F that contained 25.7 and 53.6 mg furosine/00 g protein, respectively. As a very crucial stage of the production resulted to be the spray drying process, some small scale experiments were performed with progressive variation of the main parameters, *i. e.* in-let protein solution, in-let and out-let air. The application of the optimized technological parameters on a large scale gave an optimized trial in which the level of furosine in LPI-E was only 10.2 mg/100 g protein and loss in available lysine was only 1%; this increased to 110.5 mg/100 g protein in LPI-F due to a necessary prolonged pasteurization, yet loss of available lysine remained low at 2%. Despite the more extensive thermal damage of LPI-F, this did not detract from the very good foaming properties that in fact rendered LPI-F a techno-functional and not a nutritional ingredient [23].

Using the optimized protein isolate LPI-E in various amounts to substitute semolina (containing 27.4 ± 3.4 mg/100 g protein furosine) in spaghetti [24], differing results were obtained. Furosine content of semolina pasta is a reflection of the quality because it depends on the temperatures applied during drying: the plain semolina spaghetti contained 193.9 ± 0.21 mg furosine/100 g protein. Furosine content then increased proportionally up to a 15% addition by weight of the LPI-E, though the values remained in line with those reported in literature for traditional pasta [20, 25]. Beyond a 15% weight addition the furosine content remained constant.

It must be noted that the increase in furosine that was observed in the LPI-E-semolina spaghetti depended mainly on the lysine present in LPI-E becoming available for glycosylation, and it did not infer that there was a greater thermal damage. This was confirmed by the percentage lysine loss that increased only 3.6% (from 12.1 to 15.7%) when the lupin isolate was added. The subsequent plateau above a 15% weight substitution were probably due to a limited availability of reducing sugars for further lysine glycosylation.

The overall result of these interactions was that including LPI-E in spaghetti produced a food product with more available lysine than traditional semolina spaghetti [24].

The thermal damage was also evaluated in biscuits prepared with lupin flour (samples B and C), and LPI-E (10.2 mg furosine/100 g protein; samples E and F) [26]. Samples A and D were control biscuits made with 100% wheat flour. The results of the furosine analyses are reported in Table 1. In samples A – E, the furosine content was not significantly different, and was thus only minimally influenced by the composition changes: the slight decrease at the highest lupin protein concentration (sample F) was most probably due to a decrease in reducing sugars available for the Maillard reaction. Table 1 also reports the values of blocked and available lysine. As expected, the addition of LPI-E progressively increased the lysine content. However, as furosine, and therefore blocked lysine,

Table 1. Total protein content (%), lupin protein content (%), furosine content (mg/100 g protein), blocked lysine (mg/g protein), available lysine (mg/g protein) and loss of lysine (%) in six model biscuits (modified from Bez *et al.* [26])

Sample	Total protein (%)	Lupin protein (%)	Furosine (mg/100 g protein)	Blocked lysine (mg/g protein)	Available lysine (mg/g protein)	Loss of lysine (%)
A	10.0	0	189.8 ± 1.6	2.7	20.5	11.6
B	12.4	3	176.1 ± 7.6	2.5	26.6	8.6
C	14.8	6	168.9 ± 8.3	2.4	30.7	7.3
D	9.5	0	171.7 ± 5.2	2.4	20.8	10.3
E	16.0	6.75	185.7 ± 4.8	2.6	30.9	7.8
F	22.3	13.5	138.3 ± 5.0	2.0	35.9	5.3

remained practically constant, available lysine was much higher in lupin-containing samples than in the control biscuits, in particular when protein isolates were used.

It was clear that in both food products the addition of lupin proteins (as flour or an isolate) gave positive results for the nutritional quality. The higher protein content of lupin compared with wheat provides products with increased protein content, and the additional lysine contributed by the lupin boosts the available lysine content of the products.

Determining the thermal damage in a lupin beverage was an important trial performed by Resta *et al.* [27] because of the abundance of high-biological quality lysine provided by cow's milk. In a dairy-free diet where a lupin or soybean beverage may be chosen, the nutritional intake of the individuals should not suffer. A pasteurized lupin beverage produced in a pilot plant was determined to contain 15 mg furosine/100 g protein. This value is comparable with those found in other special milks, for example pasteurized goat's or ewe's milk aimed at those intolerant to cow's milk [28]. When compared to infant formulas the furosine content of the lupin beverage is substantially lower. Values ranging from 200 – 1000 mg furosine/100 g protein were found in ten infant formulas [29]. The authors conclude that such high values of furosine indicate that the Maillard reaction had had a considerable effect on the amount of lysine provided by the formulas. This was not the case with the lupin beverage that had a minimal lysine loss (0.15%).

3 The potential hypocholesterolemic effect of lupin proteins

Beyond the incorporation of sweet lupin proteins into food products to boost the protein content, there is rising evidence showing that food products rich in sweet lupin proteins may have a hypocholesterolemic, as well as beneficial cardiovascular effects [30]. When a total seed protein extract (from white lupin) was administered in an established rodent model of hyperlipidemia [31], it led to the reduction of total and LDL cholesterol levels. A marked hypocholesterolemic effect of blue lupin was also observed in rats fed flour and protein fractions for 10 days [32]. Whole blue lupin seeds, included in the diet of pigs for 3 weeks, again exerted a marked hypocholesterolemic effect [33], though some involvement of the phytosterols was hypothesized. Lastly, in a clinical study that used a lupin beverage, it was effective in reducing total and LDL cholesterol levels [34].

Similar hypocholesterolemic activity has been associated with the consumption of other legume seeds [35 – 45], though not in all cases in protein the enty active component, as is also true for lupin [46].

Among legumes, soybean is without doubt the most studied. Experimental studies on its globulin proteins now highlight peptides deriving from the α′ subunits of the β-conglycinin protein as being the most active in the cholesterol-lowering effect of soybean proteins [47 – 50], and a necessary involvement of isoflavones has been rejected. Since lupins are naturally low in isoflavones, and their storage proteins are analogous to those in soybean, specific peptides deriving from the lupin proteins may also be exerting the cholesterol-lowering effects already observed. Following this logic, preliminary studies were carried out by Wait *et al.* [51] after 2-D electrophoretic separation of a total protein extract and a protein isolate that were prepared in a pilot plant: gel spots were digested and submitted to MS. Forty-two fragments were found to belong to β-conglutin, the lupin protein analogous to soybean β-conglycinin. Sequence alignment by the same authors also highlighted that the lupin β-conglutin (gi|46451223) (Monteiro *et al.*, submitted) possesses identity level 53%, similarity level 73% and gaps 2%, when compared with the apparently most active subunit (α′) of soybean β-conglycinin (gi|9967361) [52]. The same results are obtained if the comparison to soybean is repeated with the more recently deposited lupin vicilin-like protein sequence (gi|89994190) (Scarafoni *et al.*, submitted), the identity level being 57%, similarity level 79% and gaps 2%.

4 Shotgun proteomics as a tool to screen for bioactive peptides

Availability of these peptides after processing should be a relevant issue for the quality of functional foods based on sweet lupin proteins. This problem was demonstrated for

Table 2. Spectrum intensities of common peptides in raw seeds, LPI-E, and lupin beverage. Only those peptides with score greater than 10 and SPI higher than 70% were included. See Locati *et al.* [54] for experimental details

	Peptide spectrum intensity ($\times 10^7$)		
	Lupin seeds	LPI-E	Lupin beverage
NTLEAFNTR	11.70	16.60	7.02
YEEIQR	–	–	–
ATITIVNPDRR	4.27	13.21	–
DQQSYFSGFSR	8.01	1.71	4.21
HSDADYVLVVLNGR	6.10	14.84	3.87
IVEFQSKPNTLILPK	28.60	11.00	22.30
LAIPINNPGYFYDFYPSSTK	13.27	–	6.20
IPAGSTSYILNPDDNQK	2.93	2.65	–
QAYNLEYGDALR	7.77	13.05	5.26
LENLQNYR	29.79	15.90	3.91
SNEPIYSNK	–	8.52	–
INEGALLLPHYNSK	4.35	5.18	–
YGNFYEITPDR	–	9.42	–
Sum of spectrum intensities vs. number of peptides ($\times 10^7$)	11.07	10.46	7.54

soybean by Gianazza *et al.* [53], using a soybean concentrate shown to be effective in LDL cholesterol reduction and an isolate known to be only moderately effective. In the case of the isolate, the majority of the stainable components derived from the 11S globulin, and not from the more active 7S globulin.

The same problem was also investigated by Locati *et al.* [54] using the innovative tool shotgun proteomics, a technique that enables single peptides within a complex mixture to be identified [55]. The authors analyzed industrially relevant products, including the protein isolate LPI-E, a lupin beverage and the raw seed. Focus was on the identification of peptides common to the lupin β-conglutin precursor, the lupin vicilin-like protein and the α′-subunit of soybean β-conglycinin. Of the 13 common peptides (listed in Table 2), 11 were found in LPI-E, 10 in total protein extract (TPE)-WF, and 7 in the lupin beverage.

A useful characteristic of the Spectrum Mill software used by Locati *et al.* [54] is that it assigns a spectrum intensity to each peptide starting from the MS/MS data. This parameter allows a semi-quantitative label-free evaluation of the peptide concentration in different samples. Table 2 lists the average spectrum intensities of each identified peptide and the total spectrum intensity of each sample (previously unpublished data). This latter parameter, being related to the concentration of potentially hypocholesterolemic peptides deriving from vicilin-like proteins, could be considered as an index of the potential biological activity. As such, shotgun proteomics could be readily applied as a fast and effective screening method for lupin-based food products and ingredients with alleged hypocholesterolemic activity, eliminating those that have suffered excessive thermal damage and investigating further those rich in bioactive peptides.

5 Concluding remarks

Some very recent papers have provided important information in this field. In particular, the chemical composition of different lupin lines has been investigated in detail [8] and the nutritional quality of a lupin protein isolate from *L. albus* has been assessed on rats [56].

Most of the past doubts about a more extensive use of lupin in human nutrition were based on two issues: the possible presence of toxic alkaloids [57] and the allergenic potential [58]. The former issue has been resolved by the selection of sweet lines that respect the minimum risk level fixed at 200 mg/kg [57]. Two of these lines, Arés and Typ Top, were used in the preparation of the foods submitted to the investigations described in this study. The latter issue remains certainly critical, although restricted to a very small part of the population. Recent investigations with suitable models have, however, demonstrated that the allergenic potential of lupin is lower than that of other grain legumes, such as peanut [59]. Important progress made in recent years thus highlight the strengths of sweet lupin proteins in food applications.

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