

Elena Sironi · Fabio Sessa · Marcello Duranti

A simple procedure of lupin seed protein fractionation for selective food applications

Received: 7 December 2004 / Published online: 22 February 2005
© Springer-Verlag 2005

Abstract A simple procedure aimed at fractionating the main proteins of lupin seeds is proposed. The protocol consisted of alkali extraction of all proteins and isoelectric precipitation of two classical storage globulins, namely conglutins α and β , and one albumin, conglutin δ . The supernatant of this separation contained conglutin γ , the other main lupin protein. Conglutin γ could easily be purified further by selective Zn^{2+} precipitation, leading to a more than eightfold enrichment with respect to the amount in the flour. Resuspension of the isoelectric precipitate in saline water/ethanol solution allowed us to isolate conglutin δ , a 2S sulphur-rich lupin protein, at a very high purity level. Conglutins α and β could not be separated further by simple procedures. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis confirmed the identities of the isolated protein fractions. The distribution of each separated protein type is close to that of the corresponding proteins in the seed. The results are discussed in view of the applicability of the method and the potential use of each protein type as a food ingredient.

Keywords Lupin proteins · Isolation · Food use · Nutraceuticals · Conglutins

Introduction

Lupin seeds are a protein-rich source, with a protein content ranging from 31 to 35% in white lupin (*Lupinus albus* L.) [1]. As for many other legume seeds, the use of lupin seeds for human nutrition reached a minimum in the decades after World War II, also in the Mediterranean countries, where consumption was traditionally higher

than in other countries [2]. Among the reasons for this diminished consumption, some are related to the image of legume seeds as food (legumes are sometimes referred to as “poor people’s meat”) and the paucity of specific information on their properties; others are linked to their relatively low palatability, some discomfort upon ingestion, difficulties of seed handling, cooking, dressing, and the lack of integrated food formulations. Recently, several claims for the health benefits of legume intake, growing concern over some detrimental effects of animal protein sources, the availability of better varieties with a reduced content of antinutritional compounds, improved processing technologies and other factors are inducing vast sectors of the population to increase their consumption of legume seeds, despite the disadvantages mentioned [3–5].

In the light of these considerations, issues such as the essential amino acid complementarity between legume and cereal flours should be revisited in a more modern conceptual approach, including the development of ready-to-use, easy-to-cook and wholesome food commodities. An example of an integrated food formula, of pulse and cereal dishes, is pasta made of suitable blends of wheat flour and lupin protein concentrates/isolates [6]. Therefore, the need for developing strategies and processes for the best exploitation of legume, and in particular lupin, seed proteins and devising easy, low-cost and scalable procedures for isolation of single protein types for tailored uses in food formulations is acute. This is especially true if the exploitation of biological activities of single proteins is considered, as can be the case of the soybean 7S globulin α' subunit [7] and lupin conglutin γ [8] for their respective capabilities of modulating cholesterol and glucose hematic levels.

The bulk of lupin proteins consists of the typical 7S and 11S storage proteins, which resemble those of most other legume crops, although some relevant differences are noteworthy, such as the unusual glycosylation of the 11S globulin polypeptides [9] and the extensive endogenous proteolysis of the 7S globulin polypeptides [10]. In addition to the classical storage globulin fractions, lupin seeds contain two other relatively abundant proteins that

E. Sironi · F. Sessa · M. Duranti (✉)
Department of AgriFood Molecular Sciences,
State University of Milan,
Via Celoria 2, 20133 Milan, Italy
e-mail: marcello.duranti@unimi.it
Tel.: +39-2-50316817
Fax: +39-2-50316801

Table 1 Lupin seed protein classification

Protein type ^a	Protein family	Average sedimentation coefficient	Fraunhofer isolate ^b	Molecular mass range (kDa)	pI range
Conglutin α	Legumin-like	11S	Type E	300–430 ^c	5.6–5.9 ^e
Conglutin β	Vicilin-like	7S (acidic)		143–260 ^c	5.9–6.2 ^e
Conglutin δ	Sulphur-rich	2S		11–13, 23–26 ^d	4.3 ^c
Conglutin γ	–	7S (basic)	Type F	50 ^e	7.9 ^e

^a Gillespie and Blagrove [31]^b Wäsche et al. [16]^c Restani et al. [27]^d Esnault et al. [32]^e Duranti et al. [11]

are both rich in sulphur-containing amino acids, namely conglutin γ and conglutin δ [11–13]. Conglutin γ showed a high homology with soybean Bg7S [12] and conserved positions of the cysteine residues with a heterogeneous family of plant xylanase inhibitors [14]. Conglutin δ belongs to the 2S sulphur-rich albumin family and has the highest homology with α -amylase inhibitor CIII of *Triticum aestivum* [15]. Owing to the complexity of legume and lupin seed protein nomenclature, Table 1 reports the correspondence among main lupin seed proteins and the canonical legume seed protein families, as well as some of their relevant physicochemical properties.

In view of the potentially increasing demand of food ingredients based on vegetable proteins, lupin protein fractionation technology has already been investigated by Wäsche and coworkers [16, 17]. In these works, the preparation of two types of lupin protein isolates, namely Fraunhofer type E and type F, whose properties are customised for specific applications and formulations in food production, is described.

In the present paper, we propose a protocol which is partly based on this previous procedure, but it is aimed at further isolating lupin seed protein types. The protocol is characterised by its simplicity and, thus, scalability. The properties of the purified/enriched protein fractions are discussed in view of their exploitation in specific areas of food production.

Materials and methods

Materials

Dry mature seeds of white lupin (*L. albus* L. var. Multitalia) were kindly provided by Massimo Fagnano, University of Naples, Italy. All chemicals were of analytical grade.

Methods

Lupin flour preparation

Whole seeds were ground in a coffee grinder. The resulting flour was defatted in a Soxhlet apparatus by extraction with *n*-pentane at 37 °C for 4 h.

Isolation procedure of lupin seed proteins

Defatted lupin flour was suspended in water (1:20 w/v) brought to and kept at pH 8.0–8.5 with a diluted NaOH solution, under stirring for 2–3 h at room temperature. The slurry was centrifuged at 10,000g for 30 min and the pellet was discharged. The supernatant was precipitated by addition of dilute HCl down to pH 4.5. Upon centrifugation at 10,000g for 30 min, a pellet (pellet A), containing conglutins α , β and δ , and a supernatant (supernatant A), containing raw conglutin γ , were obtained. The raw conglutin γ was further purified by selective precipitation with Zn²⁺. The pH of supernatant A was readjusted to 7.0, by dilute NaOH solution, and ZnCl₂ was added at a final concentration of 20 mM. The pellet recovered after centrifugation (pellet C) corresponded to the conglutin γ isolate. Pellet A was resuspended in 0.1 M CH₃COONa, pH 4.0, containing 0.4 M NaCl and 40% ethanol, and stirred for 1 h at room temperature. This treatment allowed us to bring conglutin δ in solution, and this remained in the supernatant upon centrifugation as described earlier (supernatant B), whereas conglutins α and β were in the pellet (pellet B). Recovery of conglutin δ in a solid form was accomplished by cold precipitation, keeping supernatant B at -20 °C overnight. After centrifugation at 10,000g for 30 min at 0 °C, a pellet (pellet D), corresponding to the conglutin δ isolate, was obtained.

Protein assay

The protein concentration was determined by the Bradford method [18] using bovine serum albumin as a standard protein.

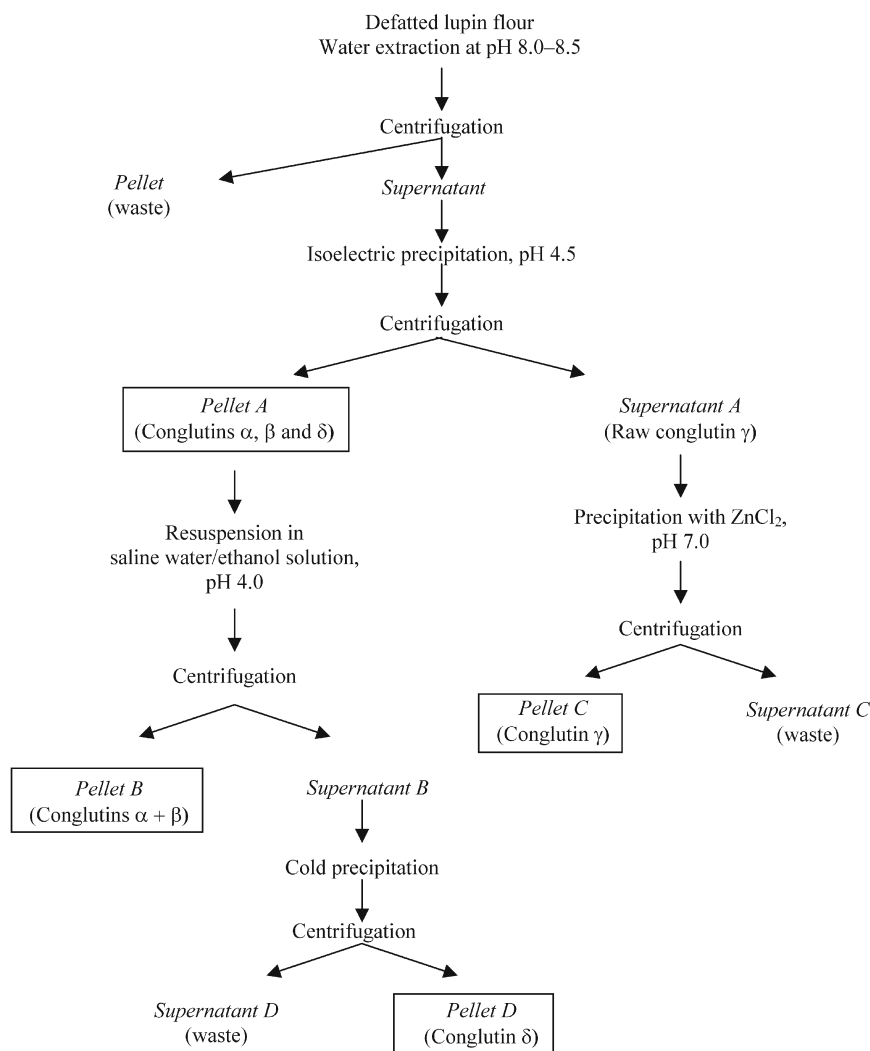
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

An aliquot of each sample obtained using the protocol described by Laemmli [19] was denatured solution (1:1 v/v) in the presence or absence of 2-mercaptoethanol.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed in NuPAGE Novex [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane 10% gels using a XCell SureLock Mini-Cell (Invitrogen, Milan, Italy). NuPAGE MES SDS running buffer and SeeBlue Plus2 prestained standard (Invitrogen, Milan, Italy) were used. This procedure ensured the visualisation of polypeptides in the range of M_r from 200 to 3,000 Da. The gels were stained with SimplyBlue SafeStain (Invitrogen, Milan, Italy).

The SDS-PAGE images were acquired using a scanner, CanoScan 8000F (Canon, Milan, Italy), interfaced with a personal computer and densitometric scan was done with ImageMaster 1D software (Amersham Pharmacia Biotech, Milan, Italy).

Fig. 1 Flow sheet of the procedure of lupin protein fractionation



Results and discussion

Lupin flour is widely considered an excellent raw material for supplementing various food products owing to its high protein content [1]; however, the use of flours at a level of substitution higher than 5% can have detrimental effects on the food quality [20] because of the presence of other constituents, such as fibres, oligosaccharides, etc. In addition, the exploitation of specific properties of individual proteins for given food applications is becoming more and more desirable. For these main reasons, we decided to set up an easy protocol for single protein fractionation from lupin seeds. The flow sheet of the procedure is reported in Fig. 1 and the experimental details are available in the “Methods”. In general, all steps involve only solubilisation, precipitation and centrifugation operations which can easily be performed at laboratory, pilot-plant or industrial level. In particular, the first step, i.e. alkaline water extraction and isoelectric precipitation, did not differ significantly from the conventional preparation of any seed protein isolate. It consisted of an alkaline extraction aimed at solubilising the bulk of the

seed proteins. The solubility curve of lupin proteins as a function of pH is sharper than that of soybean protein, with a minimum between 4.0 and 5.0 and a sudden rise at neutral pH values. The highest solubility of lupin proteins has been measured around pH 8.0 [16]. This allowed the avoidance of too alkaline pH values, with positive effects on the preservation of the native structure of the protein and therefore on the solubility. Isoelectric precipitation of the main globulins at pH 4.5 is a classical step of legume protein fractionation. The first step of the procedure allowed the recovery of the vast majority of lupin proteins, namely conglutins α, β and δ, as can be seen in Fig. 2 (lanes 5). These proteins together represented more than 90% of the total seed proteins [11]. In this step conglutin γ, which has a neutral isoelectric point, remained soluble and could be separated by centrifugation. The crude conglutin γ preparation was still contaminated by other proteins (Fig. 2, lanes 2), with a twofold increase of its relative concentration, from 6% in the flour to 12% in this soluble fraction, as judged by densitometric scans. This fraction closely resembled the type F Fraunhofer isolate [17]. In order to improve the purity of this protein, a

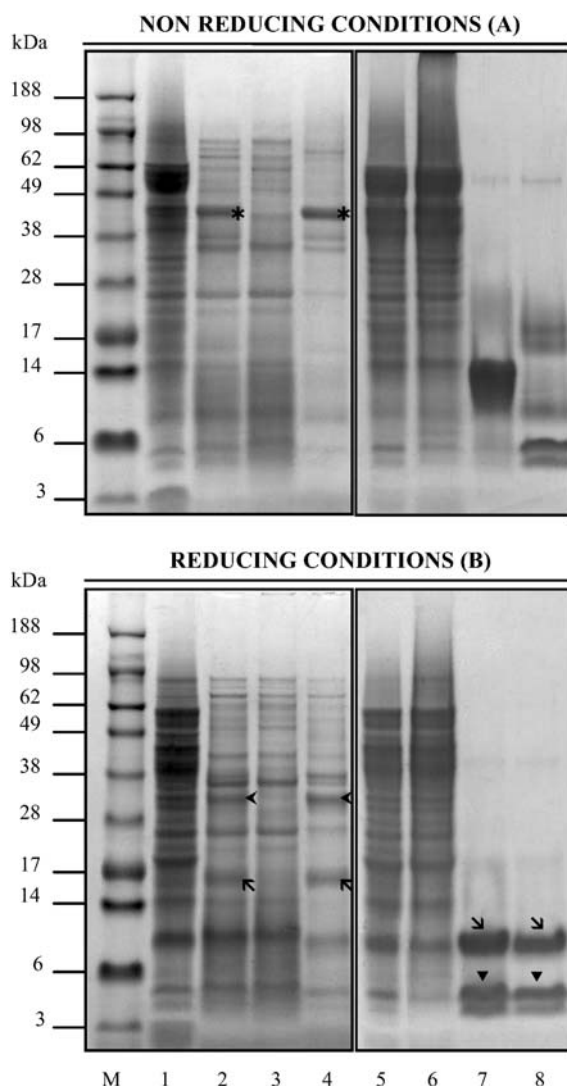


Fig. 2 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis under nonreducing (A) and reducing (B) conditions of lupin protein fractions isolated according to the protocol of Fig. 1. 1 Lupin total protein alkaline extract; 2 supernatant of the isoelectric precipitation of total proteins (supernatant A); 3 ZnCl_2 supernatant (supernatant C); 4 ZnCl_2 pellet (conglutins γ) (pellet C); 5 pellet of the isoelectric precipitation of total proteins (pellet A); 6 saline water/ethanol pellet of the isoelectric precipitate (conglutins α and β) (pellet B); 7 saline water/ethanol supernatant of the isoelectric precipitate; 8 cold insoluble fraction (conglutins δ) (pellet D); stars unreduced 50-kDa conglutins γ protomer; arrowheads reduced 30-kDa conglutins γ subunit; arrows pointing up reduced 17-kDa conglutins γ subunit; arrows pointing down reduced 9-kDa conglutins δ subunit; triangles reduced 3-kDa conglutins δ subunit; M marker proteins

specific insolubilisation step, involving metal ion aggregation [21], using ZnCl_2 precipitation dramatically increased the degree of purity of conglutins γ (Fig. 2a, lane 4), with a more than eightfold enrichment with respect to its relative amount in lupin flour. The Zn^{2+} content of the freeze-dried conglutins γ preparation was extremely high, likely due not only to the specific metal chelation properties of the protein [22], but also to physical entrapment

of the metal solution. A washing step of the precipitated protein is therefore recommendable.

The isoelectric precipitate, containing conglutins α , β and δ , was resuspended in aqueous ethanol in the presence of 0.4 M NaCl. This treatment brought conglutins δ in solution (Fig. 2, lanes 7), but it did not solubilise conglutins α and β . Recovery of conglutins δ in a solid form could be accomplished by cold precipitation at -20°C . The result of this step is clearly visible in lanes 8 of Fig. 2: conglutins δ were extremely pure, as also quantitatively shown by densitometric scanning. Similar results were obtained by resuspending the isoelectric precipitate in a solution of 80% ammonium sulphate, as suggested by Lilley [23]. However, this procedure did not yield solid conglutins δ and the high salt concentration should drastically be reduced before any food use. Denaturation of conglutins δ under nonreducing conditions prior to SDS-PAGE analysis caused a rearrangement of disulphide bonds, as already shown for other storage proteins by Inquello et al. [24]. Reduction of the sample with 2-mercaptoethanol restored the normal electrophoretic pattern of conglutins δ , with two main polypeptides of 4 and 9 kDa.

Various unsuccessful attempts to separate conglutins α from conglutins β without using costly and time-consuming chromatographic separations [11, 25] were made. The lack of separation between these two globulins may be due to their similar pI/s (Table 1), extreme heterogeneity [26], common glycoprotein nature [9], and, in the case of lupin vicilins, to the extensive endogenous cleavage which they undergo during seed maturation [10]. Therefore these two globulins were not separated further. This conglutins α and conglutins β fraction resembled the type E isolate produced by Fraunhofer [16], but it lacks conglutins δ , which in our protocol is recovered separately.

All the isolated protein fractions at various degrees of purification (Fig. 1, Table 2) obtained by this protocol could be recovered in a solid form, which makes their further processing and stabilisation easier and relatively inexpensive.

As already mentioned, Fig. 2 shows the SDS-PAGE separation of the samples produced in the various steps of the protocol. The gels were run both under nonreducing and reducing conditions in order to help identification of the polypeptides. While the subunits of conglutins γ and δ were clearly distinguishable (see labels in Fig. 2), for their characteristic molecular size and properties [13, 21], those of conglutins α and β gave a very complex pattern [27], which could efficiently be resolved only by 2D isoelectric focusing/SDS-PAGE, as reported in ref. [28].

Ethanol treatment of conglutins α and β caused the formation of some high molecular weight aggregates, visible in Fig. 2a (lane 6), most, but not all, of which were abolished by reduction, Fig. 2b (lane 6), indicating that they were at least in part mediated by disulphide formation/exchange.

Table 2 reports some quantitative data relevant to the procedure proposed. The quantitative distribution of the protein fractions recovered is in good agreement with

Table 2 Quantitative data of lupin protein isolation process

Protein type	Protein yield (g protein/kg flour)	Experimental distribution of the recovered proteins (%)	Calculated distribution of the proteins (%) ^a	Purification level (%)
Conglutins α and β	230.0	94.3	81.1	nd
Conglutin γ	10.3	4.3	6.3	53
Conglutin δ	3.5	1.4	12.6	83

nd not determined

^a From Duranti et al. [11]

previous data based on independent approaches. Only the conglutin δ percentage appeared quite dissimilar from the previously reported one. This may be due to the fact that in the previous work the conglutin δ fraction included a number of other protein components, as deduced by the SDS-PAGE pattern of that fraction [27]. As a matter of fact the purity of our conglutin δ , as assessed by densitometric scanning of lanes 7 and 8 of Fig. 2b, was extremely high, as shown in Table 2. The purification fold of conglutin γ , calculated as the ratio between its purity level and its amount in the lupin seed [11], was at least eightfold. However it cannot be excluded that the final purity level is underestimated (see Fig. 2a, lane 4).

The overall protein yield, i.e. about 25% with respect to the flour, could be enhanced if the extraction conditions which are known to be critical, such as particle size, solution to solid ratio, time of extraction and temperature, were optimised. This aspect should be regarded as a technological refinement of the procedure and was not among the goals of the present work.

The mild conditions used in our protocol are not expected to significantly alter the native structures of the proteins, thus preserving their original properties, especially solubility. In this line, the use of acidic treatments, except for the initial alkaline extraction, seemingly helps to prevent disulphide formation/interchange, which is among the reasons for protein covalent aggregation and loss of solubility, as can be seen in the SDS-PAGE unreduced patterns where conglutins α and δ are present.

In a previous work, acidic water was used to directly mash defatted lupin flakes without solubilisation of the globulins [16]. This process was found to be particularly effective if full-fat lupin flours or flakes were used, in relation to the alkaline activation of lipoxygenase with consequent off-flavour generation (A. Wäsche, personal communication). However, although this treatment had some technical advantages, the mild alkaline extraction prior to acidic precipitation used in this work allowed a greater degree of purification of the proteins (Table 2).

When the properties of lupin protein concentrates and isolates are taken in consideration, there is a wide agreement on the excellent results which can be obtained in supplemented foods or new formulas from the nutritional, techno/functional and organoleptic properties. These isolates have been proposed and experimented as animal protein replacements, including milk and egg proteins. The type E isolate from Fraunhofer, which contains conglutins α , β and δ , has been proposed for use in fat-reduced spreads, spread cheese, emulsified meat

products, convenience foods and salad dressings because of its excellent emulsifying properties [16]. In salad dressings and formulations with low and medium oil content, lupin protein is capable of completely replacing egg yolk or caseinates [29]. The removal of conglutin δ by our protocol should not change significantly the techno/functional properties of this globulin fraction. On the other hand, the availability of an isolated sulphur-rich protein which also contains the very reactive thiolic group of Cys⁴⁰ of the large subunit [13] may be useful to specifically target its use as a food ingredient such as in bread, baked food and pasta where disulphide formation/interchange plays a key role on the quality of the end product. This is especially relevant in products for individuals with coeliac disease, where replacement of gluten remains a significant technological challenge.

Conglutin γ enriched Fraunhofer type F, which resembles the supernatant of the isoelectric precipitation in our procedure (Fig. 2, lanes 2), offers pH-stable functional properties, especially foaming capacity, which proved to be as good as that of ovalbumin [29]. A further conglutin γ enrichment, as proposed in this work (Fig. 2, lanes 4), should help the calibration of its use as a tailored food ingredient, making it suitable as an egg yolk replacement in a number of food preparations, including confectionery, marshmallows, egg glazes and ice creams.

Although the analysis of the costs of the process devised was not a goal of this work, we are aware that the isolation of single protein components may results in additional costs with respect to the other procedures of concentrate and isolate production. However, these costs could largely be compensated by the added value exploitation of single protein types. This is especially evident if not only techno/functional and nutritional aspects, but also the biological activities of the isolated proteins or the replacement of other protein ingredients in food formulas for specific groups of population, such as hypercholesterolemic and hyperglycemic individuals, are considered. Indeed, recent studies have shown that lupin protein extracts are capable of reducing rat total and very low density lipoprotein plus low-density lipoprotein cholesterol concentrations by 21 and 30%, respectively, and in particular, conglutin γ displayed a remarkable up-regulatory effect for low-density lipoprotein uptake and degradation in human hepatome cells [30]. More recently, a specific in vitro interaction of conglutin γ with insulin was measured and this binding paralleled a significant reduction of glycemia in glucose-overloaded rats [8]. Although the mechanism(s) underlying these effects

is(are) not understood yet, these results represent a gateway to a targeted nutraceutical use of the lupin protein.

A further aspect which is worth mentioning concerns the allergenic potential of single lupin protein fractions, currently under investigation [28]. The specificity of protein isolation by our method can also be seen as an effective way of removing undesirable protein components, once their detrimental effects on human health have been proved.

Acknowledgements This work was supported by grants from MIUR of Italy (Progetto autonomo FIRB 2002 RBAU01JS5C_002) and from the EC, Fifth Framework Programme, Quality of Life and Management of Living Resources Programme, Healthy-Profood, QLRT 2001-002235. The authors thank Salvatore Ciappellano for Zn^{2+} determinations.

References

- Gueguen J, Cerletti P (1994) Proteins of some legume seeds: soybean, pea, fababean and lupin. In: Hudson BJJ (ed) *New and developing sources of food proteins*. Chapman and Hall, London, pp 145–193
- Petterson DS (1998) Composition and food uses of lupins. In: Gladstones JS, Atkins CA, Hamblin J (eds) *Lupin as crop plants: biology, production and utilisation*. CAB International, Wallingford, Oxon, pp 353–384
- Champ M (2001) Benefits of pulses in human diet. In: Cracow AEP (ed) *Proceedings of 4th European Conference of Grain Legumes: Toward the sustainable production of healthy food, feed and novel products*, pp 109–113
- Muzquiz M, Pedrosa MM, Cuadrado C, Ayet G, Burbano C, Brenes A (1998) Variation of alkaloids, alkaloid esters, phytic acid and phytase activity in germinated seed of *Lupinus albus* and *L. luteus*. In: Jasman AJM, Hill GD, Huisman J, van der Poel AFB (eds) *Recent advances of research in antinutritional factors in legume seeds and rape seeds*. EAAP Publication no. 93. Wageningen Press, Wageningen, The Netherlands, pp 387–390
- Evans AJ, Cheung PC-K, Cheetham NWH (1993) *J Sci Food Agric* 61:189–194
- Duranti M (2004) European Patent EP 0 997 078 A2
- Duranti M, Lovati MR, Dani V, Barbiroli A, Scarafoni A, Castiglioni S, Ponzzone C, Morazzoni P (2004) *J Nutr* 134:1334–1339
- Magni C, Sessa F, Accardo E, Vanoni M, Morazzoni P, Scarafoni A, Duranti M (2004) *J Nutr Biochem* 15:646–650
- Duranti M, Horstmann C, Gilroy J, Croy RRD (1995) *J Protein Chem* 14:107–110
- Duranti M, Sessa F, Carpen A (1992) *J Exp Bot* 43:1373–1378
- Duranti M, Restani P, Poniatowska M, Cerletti P (1981) *Phytochemistry* 20:2071–2075
- Scarafoni A, Di Cataldo A, Vassilevskaia TD, Bekman EP, Rodrigues-Pousada C, Ceciliani F, Duranti M (2001) *Biochim Biophys Acta* 1519:147–151
- Salmanowicz BP, Weder JKP (1997) *Z Lebensm Unters Forsch A* 204:129–135
- Fierens K, Brijs K, Courtin CM, Gebruers K, Goesaert H, Raedschelders G, Robben J, Van Campenhout S, Volckaert G, Delcour JA (2003) *FEBS Lett* 540:259–263
- Lilley GG, Inglis AS (1986) *FEBS Lett* 195:235–241
- Wäsche A, Müller K, Knauf U (2001) *Nahrung/Food* 45:393–395
- Luck T, Mueller K, Holley W, Kamal H, Wäsche A, Borcherting A (2000) Method for treating and processing alkaloid-, oil- and protein-containing lupin seed. Patent no. WO0054608
- Bradford MM (1976) *Anal Biochem* 72:248–254
- Laemmli UK (1970) *Nature* 227:680–685
- Pollard NJ, Stoddard FL, Popineau Y, Wrigley CW, MacRitchie F (2002) *Cereal Chem* 79:662–669
- Duranti M, Scarafoni A, Di Cataldo A, Sessa F (2001) *Phytochemistry* 56:529–533
- Duranti M, Di Cataldo A, Sessa F, Scarafoni A, Ceciliani F (2002) *J Agric Food Chem* 50:2029–2033
- Lilley GG (1986) *J Sci Food Agric* 37:20–30
- Inquello V, Raymond J, Azanza JL (1993) *Eur J Biochem* 217:891–895
- Melo TS, Ferreira RB, Teixeira AN (1994) *Phytochemistry* 37:641–648
- Casero P, Duranti M, Cerletti P (1983) *J Sci Food Agric* 34:1113–1116
- Restani P, Duranti M, Cerletti P, Simonetti P (1981) *Phytochemistry* 20:2077–2083
- Magni C, Ballabio C, Restani P, Sironi E, Scarafoni A, Poiesi C, Duranti M (2005) *J Agric Food Chem* 53:(in press)
- Wäsche A, Müller K, Schäfer C (2001) *Int Food Inged* 3:30–33
- Sirtori CR, Lovati MR, Manzoni C, Castiglioni S, Duranti M, Magni C, Morandi S, D'Agostina A, Arnoldi A (2004) *J Nutr* 134:18–23
- Gillespie JM, Blagrove RJ (1975) *Aust J Plant Physiol* 2:29–39
- Esnault MA, Pichereau V, Klingler J (1997) *Bot Acta* 110:164–171