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Properties of protein isolates from lupin (*Lupinus angustifolius* L.) as affected by the isolation method

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DANS LA VIE, RIEN N'EST À CRAINDRE, TOUT EST À COMPRENDRE
NOTHING IN LIFE IS TO BE FEARED. IT IS ONLY TO BE UNDERSTOOD

MARIE CURIE

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Contents

Preliminary Remarks

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The following peer reviewed publications were generated in the period of this work:

1. Muranyi, I. S., Otto, C., Pickardt, C., Koehler, P., & Schweiggert-Weisz, U. (2013). Microscopic characterisation and composition of proteins from lupin seed (*Lupinus angustifolius* L.) as affected by the isolation procedure. *Food Research International, 54*(2), 1419-1429. doi: 10.1016/j.foodres.2013.10.004

2. Muranyi, I. S., Volke, D., Hoffmann, R., Eisner, P., Herfellner, T., Brunnbauer, M., Koehler, P., & Schweiggert-Weisz, U. (2016). Protein distribution in lupin protein isolates from *Lupinus angustifolius* L. prepared by various isolation techniques. *Food Chemistry, 207,* 6-15. doi: 10.1016/j.foodchem.2016.03.073

3. Muranyi, I. S., Otto, C., Pickardt, C., Osen, R., Koehler, P., & Schweiggert-Weisz, U. (2016). Influence of the isolation method on the technofunctional properties of protein isolates from *Lupinus angustifolius* L. *Journal of Food Science*. doi: 10.1111/1750-3841.13515

Further scientific Poster Presentations resulted from the same period:

1. Muranyi, I., Sussmann, D. (2012) Influence of process parameters during protein isolation on lupine protein structures. EFFoST Annual Meeting, Le Corum, Montpellier, France, 20.–23.11.2012

2. Muranyi, I., Structure of lupin protein isolates and their application in food products. Poster at the 2nd Kiel Food Science Symposium, Max-Rubner-Institut, Kiel, 22.–23.5.201

List of Symbols and Abbreviations

Symbols	Term	International unit
Δ	Difference, increment	-
g	Gravitational acceleration	g/m²
G’	Elastic/storage modulus	Pa
G’’	Loss modulus	Pa
H	Enthalpy	J/g protein
η	Viscosity	Pa · s
λ	Wavelength	nm, m
M	Molar mass	g/mol
m	Mass	mg, g, kg, t
p	Pressure	mbar, bar
∑	Summation	-
S	Sedimentation coefficient	10^{-13} s
S_0	Initial slope, protein surface hydrophobicity	-
T	Peak temperature	°C
t	time	s, min, h
tanδ	Loss tangent, tanδ = G’’/G’	-
V	Volume	mL, L, m³

Abbreviations

a*	Redness
AA	Amino acid
AACCI	American Association of Cereal Chemists International
AN	Acetonitrile
ANOVA	Analysis of variance
ANS	8-Anilinonaphthalene-1-sulphonic acid
AOAC	Association of Official Analytical Chemists
AUC	Area under the curve
a_W	Water activity
BSA	Bovine serum albumin
CCD cts.	Counts of CCD (charge-coupled device)-Camera

cf.	Compare, *lat.* confer
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
cv.	Cultivar
D	Dimensional
em	Emission
et al.	And others, *lat.* et alii
exc	Excitation
DI water	Deionised water
DM	Dry matter
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EA	Alkaline extraction
EAS	Combined salt-induced and alkaline extraction
ES	Salt-induced extraction
HLB	Hydrophilic-lipophilic-balance
HPLC	High performance liquid chromatography
i.e.	That is, *lat.* id est
ILP	Isoelectric lupin protein isolate
IP	Isoelectric protein isolate
L. ang. L	*Lupinus angustifolius* L.
LDL	Low-density lipoprotein
MLP	Micellar lupin protein isolate
MOPS	3-(N-morpholino)propanesulphonic acid
MP	Micellar protein isolate
MUFA	Mono Unsaturated Fatty Acids
N	Protein nitrogen
n	Number of samples
OBC	Oil binding capacity
PAGE	Polyacrylamide gel electrophoresis
PD	Dilutive precipitation
PDI	Combined dilutive and isoelectric precipitation
PI	Isoelectric precipitation
p*I*	Isoelectric point
RFI	Relative fluorescence intensity
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SN	Supernatant
TCA	Trichloro-acetic acid
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
v/v	Volume per volume
VLDL	Very-low-density lipoprotein
WBC	Water binding capacity
w/v	Weight per volume
w/w	Weight per weight

GENERAL INTRODUCTION

Legumes play an important role in the traditional diets of many regions throughout the world (Joray, Rayas-Duarte, Mohamed, & van Santen, 2007; Yeheyis, Kijora, Wink, & Peters, 2011). The increasing demand for low cost and non-genetically modified vegetable proteins has pushed food scientists to explore different sources of protein. In particular lupin seeds are characterised by a virtually non-existent starch and high protein content (total protein content of approximately 34%) in comparison to other legumes such as beans and peas (Torres, Frias, & Vidal-Valverde, 2005). Because of the beneficial climate and soil conditions, lupins are mainly cultivated in Australia, Poland and Germany (FAOSTAT 2015, Römer 2007, Figure 1).

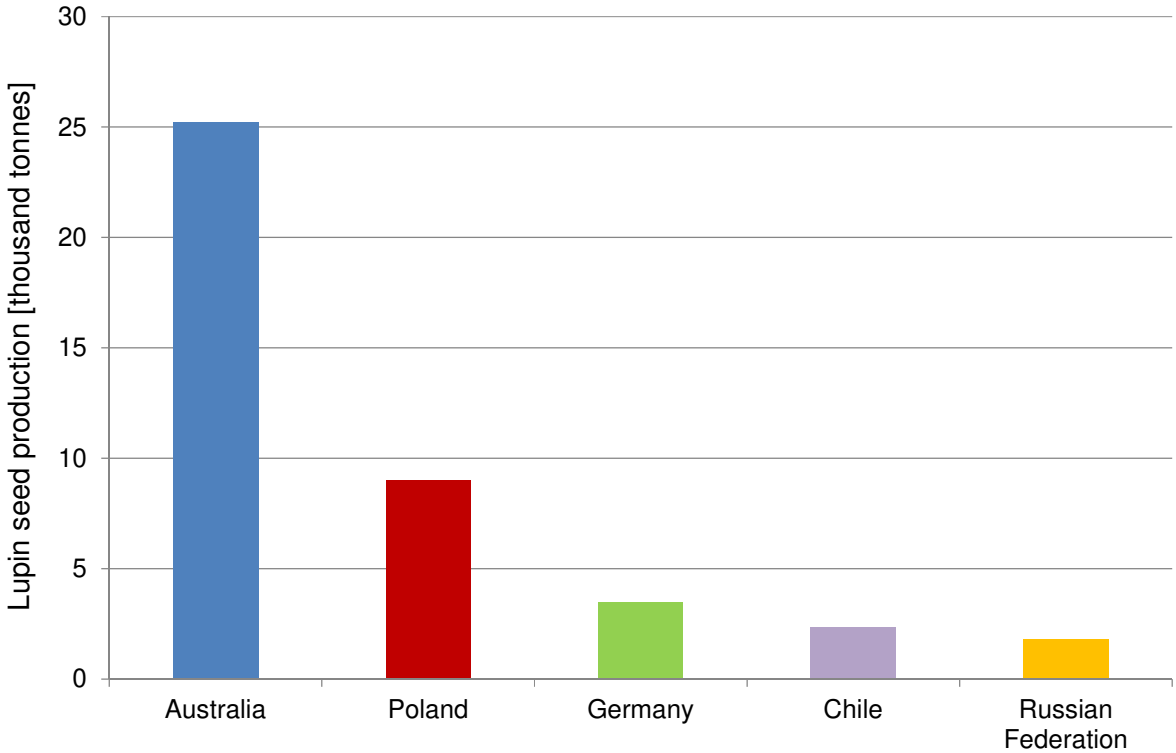

Figure 1: Top five lupin seed producers worldwide. Source: FAOSTAT 2015.

Lupins are financially rewarding because of their beneficial influence on the soil quality. Lupin roots are able to bind atmospheric nitrogen with the aid of nodule bacteria and use it for the biosynthesis of proteins. After harvest considerable amounts of nitrogen remain in the soil enhancing the soil quality for other plants.

Because of the high protein content and beneficial fatty acid profile lupin seeds became an inherent part of human nutrition, especially in Spain, Portugal and Brazil. Protein and amino acid contents of lupins are comparable to those in soy (Gueguen & Cerletti, 1994). However, amounts of anti-nutritive compounds such as isoflavones, are significantly lower in lupins compared to soy, and lupin protein isolates are essentially isoflavone free (Sirtori et al., 2004). As already proven for soy proteins, lupin proteins have shown to exhibit health-promoting effects. The most described effect is their ability to prevent arteriosclerosis by reducing the concentration of circulating LDL and VLDL (Duranti et al., 2004). A study from Bettzieche et al. (2008) on three different cultivars of the blue lupin showed the sweet cultivar *Lupinus angustifolius* L. Vitabor to exhibit the most beneficial effect on plasma lipids. Furthermore, the Vitabor cultivar showed highest crude protein and lowest seed alkaloid contents, compared to another 15 blue, yellow and white lupin cultivars (Yeheyis et al., 2012).

Besides the favourable nutritional effects, lupin proteins offer so-called technofunctional properties in food products. Important technofunctional properties of the proteins are for example hydration capacity, foaming and emulsifying properties, protein solubility and gelation which make this vegetable protein promising for food industry. Therefore, since the 20th century, fractionated lupin compounds are used for different foods to improve sensory attributes. Various intrinsic (amino acid sequence, protein conformation) and extrinsic factors (ionic strength, temperature, pH) influence the technofunctional properties of the proteins (Cheftel, et al., 1992) and the protein ingredients show different technofunctional properties. To know the influence of the isolation procedure on the microstructure, the chemical composition as well as the physical characteristics of the proteins is considered essential to develop tailor-made functional ingredients.

Lupin proteins of *Lupinus angustifolius* L. Vitabor represent the focus of the present study. The following section depicts the current state of research on lupins and proteins. Finally, hypotheses are presented describing an **interrelation between the isolation procedure and properties of protein isolates from lupin *(Lupinus angustifolius L.)***. In order to prove these hypotheses, scientific investigations were carried out and are discussed in the last section of this dissertation.

1 Lupin classification

The genus *Lupinus* L. (lupin) is a plant of the order Leguminosae (pulses). Among the family Fabaceae it is part of the tribe Genisteae and comprises up to 400 species, depending on the classification criteria (Holden & Egaas, 2005).

The nutrient-rich lupin seeds congenitally contain high amounts of bitter and toxic alkaloids such as lupinine and sparteine. Thanks to selective breeding (Duranti & Gius, 1997) edible lupins poor in bitter substances (less than 0.05% alkaloids), so called “sweet lupins”, are available since the end of the nineteen-twenties (Reinhard, Rupp, Sager, Streule, & Zoller, 2006; Torres, Frias, & Vidal-Valverde, 2005). Wild and partly cultivated lupins have been grown in the Mediterranean area already since millennia for human consumption, medical purposes or soil fertilising.

Four sweet lupins are of agricultural interest (Holden & Egaas, 2005):

- *Lupinus albus* L. (white lupin)
- *Lupinus luteus* L.(yellow lupin)
- *Lupinus angustifolius* L. (narrowleaf lupin)
- *Lupinus mutabilis* L. (Andean lupin)

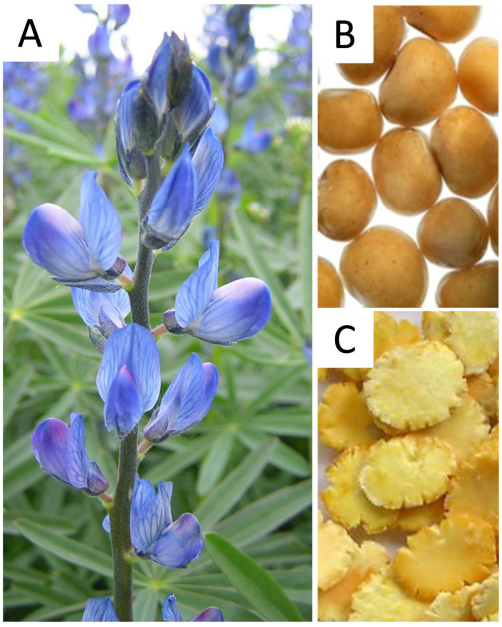

Figure 2: *Lupinus angustifolius* L. Vitabor. Flower (A), whole seed (B) and dehulled and flaked seed (C).

The flower and seeds of the sweet narrow-leaf or blue lupin cultivar *Lupinus angustifolius* L. are presented in Figure 2. Lupin seeds (Figure 2B) are built in a pod, which grow at the main stem of the lupin plant (Figure 2C). The seeds vary in size, colour and composition depending on the lupin species. *Lupinus angustifolius* L. is suited for all German climate conditions. It prospers also at low rainfalls and even on sandy soil from pH 5.0 to 6.8. Compared to the yellow lupin, the narrow-

leaf lupin has higher soil and water requirements; however is less in need of warmth and less frost-sensitive. Furthermore, it has a shorter growing period (120 to 150 days), a lower susceptibility to the lupins characteristic fungal disease called anthracnose and a higher potential yield (up to 45 dt/ha) compared to the yellow lupin (Römer, 2007; Entrup, 2003).

2 Morphology and composition of seeds from *Lupinus angustifolius* L.

The lupin seeds from *L. angustifolius* L. are basically composed of two parts: the cotyledon cells forming the kernel and the enclosing seed coat. The cotyledon cells are shown in Figure 3. Each cell is separated via a cell wall (cw) consisting of cellulose, hemicellulose, lignin and pectin.

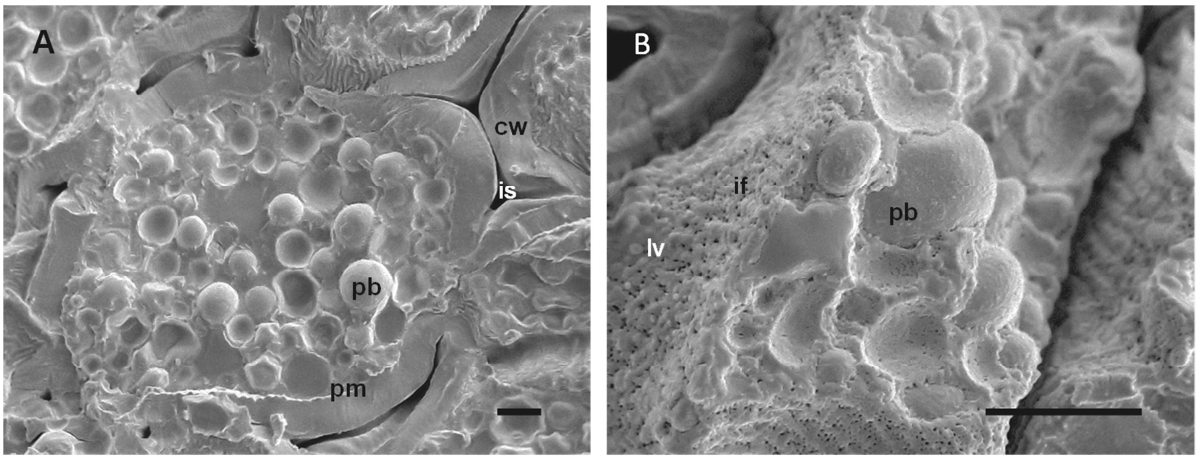

Figure 3: Cryo-scanning electron micrograph after freeze-fracture (A) and scanning electron micrograph (B) of seed from *L. angustifolius* L. Vitabor. The seed comprises cotyledon cells with cell wall (cw), plasma membrane (pm), intracellular fibre matrix (if), imbedded protein bodies (pb), lipid vesicles (lv) and intercellular space (is). The scale bars represent 5 µm.

The protein presents the main component of the lupin seed kernel (41%) and is stored in form of protein bodies (pb) in the cell interior of the cotyledons. In *L. angustifolius* L. the protein bodies show predominately globoid and paracrystalline inclusions (Pozuelo et al., 2001). An intracellular fibre matrix (if) covers the surface of the protein bodies and the inner site of the cell wall (cw). Small lipid vesicles (lv) are embedded in this intracellular fibre matrix and surround the protein bodies (cf. Figure 3B). Furthermore, the cell comprises oligo- and polysaccharides, starch and water (Lampart-Szczapa, 1996; Lampart-Szczapa et al., 2003; Pozuelo, et al., 2001). The main constituents of the lupin seed from *L.*

angustifolius L. and their content in the seed as well as in the dehulled kernels are presented in Table 1.

Table 1: Main components of the lupin seeds and dehulled kernels from *L. angustifolius* L. (Kingwell, 2005)

	Content in the seed [%]	Content in the kernel [%]
Seed coat	23	0
Water	9	12
Crude protein	32	41
Crude fat	6	7
Ash and lignin	4	4
Polysaccharides	22	28
Oligosaccharides	4	6

Lupin seeds are characterised by low fat and starch contents and high protein contents. The protein concentrations are higher than in other legumes such as lentils and beans, which range from 6–25% (Gueguen & Cerletti, 1994).

In addition to the nutritional components (proteins, lipids, carbohydrates, minerals and vitamins), some anti-nutritional components are present (cf. chapter 2.3) such as phytic acid and α-galactosides of the oligosaccharides (Martinez-Villaluenga, et al., 2009).

2.1 Lupin seed proteins

Lupin proteins are classified using two different analytical methods: 1) according to Osborne (Ternes, 2007) taking into account the solubility characteristics of the proteins in diverse solvents as shown in Table 2 and 2) according to their sedimentation coefficient.

The sedimentation coefficient reveals the migration speed of a protein inside a centrifugal field. To a certain degree the sedimentation coefficient of a specific molecule is proportionally dependent on its molecular mass. However, also density and volume are of importance. A big particle suffers a large friction force oppositely to the centrifugal force and therefore sediments slower than a small particle. Accordingly the sedimentation coefficient is high for big molecules and low for small molecules. The unit Svedberg (S) corresponds to 10^{-13} seconds. The higher the S-value, the slower a molecule is moving under influence of the centrifugal force (Svedberg & Peterson, 1940; Stryer, et al., 2007).

Table 2: Osborne classification of vegetable proteins (Ternes 2007)

Proteins	H_2O	NaCl	OH^-	H^+	EtOH
Albumins	+	+	-	-	-
Globulins	-	+	+	+	-
Glutelins	-	-	+	+	-
Prolamins	-	-	-	-	+

+ = soluble, - = insoluble

The seed proteins of *Lupinus angustifolius* L. consist mainly of the two glycosylated protein groups albumins and globulins with a mass ratio of the albumins to globulins of around 1 to 9. The content of further protein fractions such as prolamins and glutelins, is negligible (Chew, Casey, & Johnson, 2003; Gulewicz et al., 2008).

Albumins

Albumins are readily soluble in salt-free water and exhibit an isoelectric point (p*I*) within the pH range of pH 4.3 – 4.6. Many seed albumins serve as metabolic enzymes or – like lectins – play an important role within the plants defence mechanism (Duranti, Consonni, Magni, Sessa, and Scarafoni, 2008). Another important task is the storage and provision of sulphur during germination because of their high content of cysteine. The albumin fraction in lupins refers to conglutin δ.

Globulins

Globulins represent the main storage proteins in lupin seeds. In contrast to the albumins, globulins are insoluble in pure water but slightly soluble in salt solutions. All of the lupin globulins are glycosylated and are separated according to their electrophoretic mobility into conglutin α, β and γ (Blagrove & Gillespie, 1975). The legumin-like α-conglutin and the vicilin-like β-conglutin in lupin seeds represent the main globulins (Duranti, et al., 2008) and account with 76% and 16.4%, respectively, to total lupin seed protein content (Blagrove & Gillespie, 1975; Lqari, Pedroche, Giron-Calle, Vioque, & Millan, 2004; Plant & Moore, 1983).

Legumin is a hexameric protein built out of monomers which encompasses two peptide chains connected together via a disulphide bond. It is insoluble in water, but soluble in weak acids and alkalies.

Vicilin is usually a trimer and each monomer consists of one single peptide chain, with no cysteine and therefore no disulphide bonds. The vicilins are usually

glycosylated with carbohydrate chains such as glucose and mannose (Lqari, et al., 2004; Shutov, Kakhovskaya, Braun, Baumlein, & Muntz, 1995).

A detailed description of the individual conglutins in the lupin seeds of *L. angustifolius* L. is presented below. It should be noted, that the data about size and amount vary in the literature, depending for example on the separation method used for isolation of the proteins. Thus, for example according to the densitometric evaluation of fractionated globulins of *L. angustifolius* L. using Cellulose Acetate Electrphoresis, coglutin α accounts for about 51% of total globulins (Plant & Moore, 1983). However, after separation of the same proteins using ion exchange chromatography, conglutin α amounts of 76.6% of total globulins were found (Lqari, et al., 2004). Furthermore, conglutin contents and subunit associations are strongly dependent on the maturation state of the seed or simply on the pH during sample preparation (Freitas, Teixeira, & Ferreira, 2007; Ismond, Murray, & Arntfield, 1986a, 1986b; Lilley, 1986). Last but not least, size and amounts of the globulins are characteristic for each legume and vary within the lupin species (Freitas, et al., 2007).

Conglutin α is composed of three subunits connected non-covalently, with each exhibiting sizes of 64, 72 and 85 k (Johnson, Knight, & Gayler, 1985). The subunits are further divided into alkaline polypeptides of 21-24 and acidic polypeptides of 42-62 k linked together via intermolecular disulphide bonds. Lqari, et al. (2004) reported trimeric protein structures of around 216 k. Transition from a trimer to a hexamer occurs under limited proteolytic activity in the surrounding environment (Blagrove & Gillespie, 1975).

Conglutin β is the only lupin protein free of disulphide bonds, which leads to identic band profiles in SDS-PAGE under both reducing and non-reducing conditions. The prevalent form of the 150-170 k oligomer is trimeric but conglutin β also forms tetramers. The sizes of the subunits range from 20 to 70 k each of which being composed of 10 to 12 distinct and mostly glycosylated polypeptides.

Conglutin γ (also termed 7S protein) is an exceptional globulin soluble in both water and salt solution. In contrast to the other lupin conglutins, the isoelectric point of the 7S protein lies within the alkaline range (p*I* = 7.9; Arnoldi, et al., 2007; Duranti, et al., 2008; Duranti, Restani, Poniatowska, & Cerletti, 1981). The

glycoprotein shows very high sulphur contents, though it represents only 5% of total globulins. It exhibits a size of 100 k with 50 k subunits. The subunits consist of a light (17 k) and a heavy polypeptide chain (32 k), linked together via disulphide bonds (Johnson, et al., 1985; Kolivas & Gayler, 1993; Plant & Moore, 1983).

Conglutin δ accounts for 2.6% to the total protein content of the lupin seed (Salmanowicz, 2000). The molecular weight of conglutin δ varies depending on solvent composition and protein concentration. Conglutin δ_2 (14 k) is a monomer composed of a heavy (9.5 k) and a light (4.5 k) polypeptide chain. At neutral pH, it forms a dimer (conglutin δ_1, 28 k) that associates via disulphide bonds to oligomeric aggregates of up to 56 k (Lilley, 1986; Lqari, et al., 2004; Ternes, 2007).

The characteristics of the lupin proteins in seeds of *L. angustifolius* L. are summarised in Table 3.

Table 3: Most important properties of the conglutins in seeds of *lupins angustifolius* L.

	Conglutins							Conglutin subunits		Ref.
	Sedimentation coefficient	% among globulins	HS-SH bonds	Glycosy-lation	MW [k]	pI	Quaternary structure	Subunit character	Monomer size [k]	
α	11S (legumin) 7S (legumin)	51	yes	yes	216	5.6-5.9	Hexamer Trimer	acidic alkaline	42–62 21–24	[1,2,3,5]
β	7S (vicilin)	42	no	yes	150-170	5.9-6.2	Trimer Tetramer		20–80	[1,3,4]
γ	7S	5	yes	yes	100	7.9	Tetramer		32 17	[4,5,6]
δ	2S	2	yes	no	14	4.3	Dimer	Large Small	9.5 4.5	[2,7,8,9]

pI = Isoelectric point, MW = molecular weight. [1] Duranti, et al. (2008); [2] Sironi, Sessa, and Duranti (2005); [3] Lqari, et al. (2004); [4] Plant and Moore (1983) [5] Johnson, et al. (1985); [6] Kolivas and Gayler (1993); [7] Salmanowicz (2000); [8] Lilley (1986); [9] Ternes (2007).

The quantitative amino acid composition of the albumin and globulin fractions from the seed of *L. angustifolius* L. is presented in Table 4.

Table 4: Amino acid composition of albumin, globulins and the individual globulin fractions of lupin seeds from *Lupinus angustifolius* L. [mol %] (Lqari, et al., 2004).

	Albumin	Globulins	Globulin fractions		
			α-conglutin	β-conglutin	γ-conglutin
Aspartic Acid[a]	11.9 ± 1.8	12.1 ± 1.4	8.4 ± 0.9	13.5 ± 1.1	8.7 ± 1.3
Glutamic Acid[b]	22.1 ± 1.5	28.1 ± 2.7	20.2 ± 1.4	27.1 ± 3.2	9.0 ± 1.2
Serine	5.5 ± 0.1	6.2 ± 0.8	7.8 ± 0.0	6.6 ± 0.5	9.1 ± 1.3
Histidine	2.4 ± 0.8	2.3 ± 0.4	1.6 ± 0.2	1.6 ± 0.5	3.2 ± 0.7
Glycine	6.7 ± 0.2	4.0 ± 0.1	7.2 ± 0.1	5.9 ± 0.3	9.8 ± 0.9
Threonine	6.6 ± 0.7	3.5 ± 0.5	4.6 ± 0.2	3.4 ± 0.1	7.9 ± 0.4
Arginine	7.9 ± 0.5	12.3 ± 1.0	8.9 ± 1.0	9.2 ± 1.2	3.6 ± 0.1
Alanine	7.5 ± 1.2	3.1 ± 0.2	4.4 ± 0.6	3.9 ± 0.1	6.1 ± 0.3
Proline	7.7 ± 2.6	5.5 ± 1.3	3.3 ± 0.1	4.2 ± 0.7	1.3 ± 1.2
Tyrosine	3.2 ± 0.3	4.3 ± 0.5	4.3 ± 0.2	3.8 ± 0.2	3.5 ± 0.1
Valine	4.7 ± 0.5	3.5 ± 0.1	5.1 ± 1.9	3.3 ± 0.6	12.1 ± 6.1
Methionine	1.0 ± 0.2	0.3 ± 0.1	1.3 ± 0.0	0.2 ± 0.0	3.0 ± 0.0
Cysteine	2.6 ± 0.5	1.4 ± 0.1	0.9 ± 0.3	0.5 ± 0.2	1.5 ± 0.0
Isoleucine	3.6 ± 0.1	4.3 ± 0.1	4.4 ± 0.4	3.5 ± 1.5	3.9 ± 0.2
Leucine	6.9 ± 0.8	8.2 ± 0.4	10.8 ± 1.6	8.3 ± 0.5	9.0 ± 0.2
Phenylalanine	2.8 ± 0.8	4.3 ± 0.2	3.9 ± 0.4	2.9 ± 1.3	4.1 ± 0.1
Lysine	8.2 ± 0.2	4.2 ± 0.2	0.4 ± 0.0	3.5 ± 0.4	5.6 ± 0.1
Tryptophan	-	-	0.5 ± 0.0	0.5 ± 0.0	0.1 ± 0.0

[a]Aspartic Acid + Asparagine, [b]Glutamic Acid + Glutamine

The amino acids glutamic acid and glutamine predominate in all conglutins except of conglutin γ. All the essential (threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine and tryptophan) and semi-essential amino acids (arginine and histidine) are present in all conglutins, reflecting the high biological value of the lupin proteins. In particular leucine is abundant within the conglutins. The albumin conglutin δ is characterised by remarkably high amounts of the essential amino acid lysine. Both conglutins δ and γ, respectively, are characterised by high amounts of the essential amino acid threonine, compared to the other two conglutins. The high content of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine in conglutin γ makes this globulin promising for its isolated insertion within geriatric as well as sports medical nutrition (Burchardi & Larsen, 2004; Biesalski, et al., 2004).

Contents of the sulphur-containing amino acids methionine and cysteine are negligible in conglutin β, in contrast to the other conglutins, which explains the lack of disulphide bonding in this conglutin.

2.2 Further nutritional components

The carbohydrates of the lupin seeds of *Lupinus angustifolius* L. comprise parts of soluble and insoluble starch-free polysaccharides. The seed exhibits a negligible starch content of under 15 g/kg in the dry matter (DM). Free sugars are predominantly linked to glucose and galactose (30-40 g/kg in DM for each) and less to mannose (8-10 g/kg in DM). The polysaccharides (~400 g/kg in DM) cellulose, hemicellulose and pectin, are the major carbohydrate fractions in the lupin seeds and are mostly located in the seed coat. The lignin content in the whole seed amounts to 8.6 g/kg and in the kernel to 6.7 g/kg (Petterson, 1997). This is relatively low in comparison to lignin contents in other legumes such as soy (16 g/kg DM) and beans (20 g/kg DM); though at a similar level to the lignin content of peas (12 g/kg DM; Evans, Cheung, & Cheetham, 1993; Petterson, 2000; van Barneveld, 1999).

The seeds of *Lupinus angustifolius* L. exhibit a crude fat content of around 6%, which is composed of triglycerides (71.1%), phospholipids (14.9%), free sterols (5.2%), glycolipids (3.5%), sterols and wax esters (0.5%) as well as free fatty acids (0.4%) (cf. Table 5). The lipids of *Lupinus angustifolius* L. are higher in saturated (17.6%) and monounsaturated (38.5%) fatty acids and lower in polyunsaturated fatty acids (42.4%) compared to soybean (14.8%, 23.9% and 58.3%; Petterson, 2000; van Barneveld, 1999).

Table 5: Fatty acid composition [% of total fatty acids] of lupin and soybean (Petterson, 2000; van Barneveld, 1999).

Fatty Acid	*L. angustifolius* L. [%]	Soybean [%]
Palmitic acid (16:0)	11.0	10.3
Stearic acid (18:0)	3.8	4.5
Oleic acid (18:1 n-9)	38.2	23.9
Linoleic acid (18:2 n-6)	37.1	51.8
Alpha-linolenic acid (18:3 n-3)	5.3	6.5
Arachidic acid (20:0)	0.9	-
Gadoleic acid (20:1 n-9)	0.3	-
Behenic acid (22:0)	1.9	-
Erucic acid (22:1 n-11)	-	-

The minerals of the lupin seed are calcium (2.4 g/kg DM), magnesium, phosphorus (3.3 g/kg DM), potassium (8.9 g/kg DM), sodium and sulphur (2.5 g/kg DM). Furthermore, the seed of *Lupinus angustifolius* L. features a number of vitamins

such as vitamins B_1 (7.12 mg/kg in DM), B_2 (2.36 mg/kg in DM) and E (total tocopherols: 18.05 mg/kg in DM; Torres, et al., 2005).

2.3 Anti-nutritional factors

The low content of potential anti-nutritional factors is characteristic for lupin seeds and notably most of these are usually at levels considered non-influential. Anti-nutritional factors are developed by a variety of plants as a defence mechanism against pests. A high toxicity, bitterness and indigestibility for humans and animals are typical for those anti-nutritional components (Chew, et al., 2003; Lampart-Szczapa, et al., 2003). The characteristics and amounts of anti-nutritionals in the seeds of *L. angustifolius* L. in comparison to soy are summarised in Table 6.

Table 6: Anti-nutritional factors in *L. ang.* L. and soy. Adapted from Petterson (1997).

Anti-nutrient	*L. angustifolius* L. Whole Seed [g/kg]	*L. angustifolius* L. Kernel [g/kg]	Soybean meal (defatted) [g/kg]
Trypsin inhibitor	0.12	0.20	3.11
Alkaloids	< 0.20	< 0.25	0.01
Oligosaccharides*	41.00	68.00	68.00
Phytate	4.00	5.00	15.90
Saponins	0.60	0.60	6.70
Tannins	0.10	-	-

* Sum of raffinose, stachyose and verbascose

Minor compounds such as phytic acid were found to influence the solubility of the proteins. In the presence of phytic acid, protein-phytate interactions arise lowering the isoelectric point to lower values (Grynspan & Cheryan, 1989; Schwenke, Mothes, Marzilger, Borowska, & Kozlowska, 1987). Furthermore, complexes between phytic acids and metal ions such as calcium, iron, magnesium, phosphor and zink are known to reduce their intestinal resorption (Hurrell, 2003).

Notably, cyanogen compounds and lectins have not been detected in lupins in comparison to many other legumes. Furthermore, only small quantities or traces of peptidase inhibitors, saponins and flatulence factors have been determined in comparison to soy (Hondelmann, 1996).

3 Structure and physicochemical properties of proteins

Proteins are high molecular weight compounds built up out of 20 proteinogenic amino acids. Proteins feature optimal interactions with molecules in solution and arrange in characteristic conformations depending on the solvent. By definition, structure means the amino acid sequence inside the peptide chain (primary structure) and its specific three-dimensional structure (secondary, tertiary and quaternary structures).

The secondary structure reflects the local conformation skeleton of the polypeptide chain comprising for example the structural elements α-helix and β-sheet. It is built via inter- and intramolecular hydrogen bonds of the protein backbone. The three-dimensional arrangements of the secondary structure elements lead to the tertiary structure of a protein, which is stabilised by electrostatic forces, hydrogen bonds, disulphide bonds and hydrophobic interactions. Furthermore, proteins are able to construct quaternary structures via non-covalent association of the tertiary structures, exhibiting individual subunits (Belitz, Grosch, et al., 2009; Voet & Voet, 1992).

The term structure in combination with an order of magnitude (micro, macro) is often used to describe the structural properties of materials such as foods. For instance, often used orders of magnitudes are the molecular level and the microscale. However, the term of the order of magnitudes is often inconsistently used in literature (Jekle, 2012). In the present dissertation, food microscopy presents an important tool to investigate the structures among different protein isolates reaching scales from 100 nm to 100 µm. Within this scale the tertiary and quaternary structures of the proteins are encompassed which are termed the ‘protein microstructures’. Above 100 µm the ‘macroscopic scale’ is reached and defines already the transition to the textural or rheological properties of the material. At this scale, the properties of the molecular, nanoscopic, and microscopic scale are quantifiable in physical and dynamic measures. Hereby, the function of the structures becomes analysable (Jekle, 2012).

Globular associations into protein micelles are one example of a tertiary microstructure (Takekiyo, Yamazaki, Yamaguchi, Abe, & Yoshimura, 2012). The ability of proteins to form micellar aggregates is based on their surface activity given

by the amphiphilic character of the proteins. In an aqueous environment those micelles exhibit a hydrophobic core whereas the hydrophilic residues are in contact with the aqueous surrounding medium. This mechanism acts against the energetically unfavourable exposure of hydrophobic residues to water (Fisher & Oakenfull, 1977). Proteins generally form sub-particles or are arranged in supramolecular structures consisting of sub-particles depending on protein origin, concentration, size and milieu conditions (Westphal, Gerber, et al., 2003). A well-known example for this type of protein micelles of animal origin is the casein micelle (Corredig, Sharafbafi, & Kristo, 2011). Additionally, the globular storage proteins from vegetable sources show the ability to micelle formation (Ismond, Arntfield, & Murray, 1991; Ismond, et al., 1986a). The sub-particles are created by a limited number of monomers via hydrophobic forces and hydrogen bonds contrary to the electrostatic charge repulsions (Ismond, Georgiou, Arntfield, & Murray, 1990). Investigations of Cheftel, Cuq et al. (1992) revealed a preliminary unfolding and denaturation of proteins before the orientation into protein-protein interactions and ordered aggregations. Depending on the environmental modification, globular proteins associate aligned in row or randomised into gel-like aggregates.

Proteins are highly sensitive to shifts of their surrounding conditions. Differences in the extraction and precipitation method such as excessive dilution and regulation of ionic strength and pH, influence significantly the protein arrangement. The respective mechanisms are presented in the next chapter. The ability of proteins to adopt micellar structures seems therefore to be the result of perfectly matching milieu conditions. Protein micelles show to have a more complex character than detergent micelles, for example, as the hydrophobic and hydrophilic residues of the proteins are not uniformly orientated (Qi, 2007). In a study by Ismond, et al. (1991) a reciprocal interaction between the inconsistent oriented molecules is assumed to build extended protein micelle networks. This goes in line with data from Sathe (2002) where the non-covalent interactions of micelles were assessed to be responsible for the formation mechanism of protein gels. However, the mechanisms and interactions involved in the formation of the characteristic three-dimensional network of protein gels remain prospective.

4 Isolation of proteins based on extraction and precipitation

The solubility behaviour of proteins is determined via the hydration of proteins (Ternes, 2007). In solution, proteins are able to interact with the hydrating acting water molecules until a specific equilibrium condition. This equilibrium is characterised following the relation:

$$\text{protein} - \text{protein} \; + \; \text{solvent} - \text{solvent} \; \Longleftrightarrow \; \text{protein} - \text{solvent}$$

The dimension of the protein-protein or protein-water interactions among this equilibrium indicates which state the proteins adopt in food systems: The forms of a gel, insoluble precipitate, colloidal dispersion or solution (Westphal, et al., 2003). In case of energy release during the protein-solvent interaction, the equilibrium shifts towards the dissolved protein state.

Different process parameters influence the protein solubility such as particle size, pH, extraction time, solid/solvent proportion, temperature, type of salt and salt concentration (ionic strength). During extraction of the proteins from seeds of *L. angustifolius* L. the salt concentration and the pH showed the most important impact on protein solubility or extractability from a complex system such as a legume seed (Moure, Sineiro, Dominguez, & Parajo, 2006; Ruiz & Hove, 1976; Sussmann, Halter, Pickardt, Schweiggert-Weisz, & Eisner, 2013).

A precipitation of proteins is usually applied to recover previously dissolved proteins. The decrease in protein solubility for protein precipitation is induced by different physical and chemical alterations such as modification in temperature, pH, ionic strength, solvents, detergents, urea, metal ions or mechanical force. Every molecular alteration of the native protein state is called denaturation. Depending on the type and intensity of alteration or stress, proteins adopt various molecular conformations, which can be reversible or irreversible (Englard & Seifter, 1990). However, one should distinguish between irreversible denaturation and precipitation, as proteins can be precipitated without preliminary or subsequent denaturation. Vice versa, occurrence of irreversible protein denaturation is possible without subsequent protein precipitation. However, protein denaturation is usually accompanied by losses of natural protein functions and concerns the quaternary, tertiary and secondary protein structures. The primary protein structure, in contrast,

remains unaffected (Westphal, et al., 2003). The extraction and precipitation behaviour of proteins as affected by the adjustment of ionic strength or pH are described in more detail in the next chapters.

4.1 Influence of ionic strength on protein solubility

4.1.1 Salt-induced protein solubilisation

Salts influence the protein solubility individually via their ionic strength μ (Cheftel, et al., 1992) according to Equation 1:

Equation 1

$$\mu = \sum C_i Z_i^2$$

i [-] = Ion type
C [M] = Concentration
Z [-] = Valency of the ion

Sodium chloride at low concentrations (0.5 - 1 M) causes an attachment of sodium ions and chloride ions to the charged counterions of the peptide chains. As a consequence, the electrostatic protein-protein interactions are repressed and the association of the proteins disconnects (cf. Figure 4) leading to protein solubility. This raise in protein solubility was termed the ‘salting-in’ effect (Der, 2008). The dimension of the salting-in is dependent on the type of ions and their position inside the Hofmeister series (Hofmeister, 1888; Ternes, 2007; Der, 2008). The anions of sodium containing salts usually have a stronger effect on proteins, than the cations with the chloride anions exhibiting higher selective binding than the sodium cations due to their smaller hydrated radii (Thawornchinsombut & Park, 2004). The stabilising influence of the anions on the storage protein vicilin is ranged in the following decreasing order (Ismond, et al., 1986b):

citrate^{3-}	>	sulphate^{2-}	>	chlorine^{-}, acetate^{-}		>	bromine^{-}	>	iodine^{-}	>	thiocyanate^{-}
$C_6H_5O_7^{3-}$		SO_4^{2-}		Cl^-	$C_2H_3O_2^-$		Br^-		I^-		SCN^-

In a study from King, Aguirre, and Depablo (1985) an increase in protein solubility after addition of sodium chloride to alkali-extracted lupin protein isolates (at pH 8.6) was observed until the ionic strength of μ = 1. An interrelation between the ionic

strength influenced by salts and concurrent charges caused by pH shifts was shown: After acidic precipitation (pH 4.2-5.1) at this ionic strength, the isoelectric point of the proteins showed a shift towards lower pH values due to specific ion binding effects and the amount of solubilised proteins varied substantially (between 53 and 85%; Zayas, 1997).

4.1.2 Protein precipitation via modification of ionic strength

Raising the concentration of a neutral salt in water above 1 M leads to a reduction of the salting-in and consequently a decrease in protein solubility until protein precipitation (Figure 4). The 'salting-out' results from a competition between the added salt ions and the inherently charged protein molecules for free water molecules. Consequently, protein hydration is outcompeted and the proteins bind less water until the proteins aggregate (Englard & Seifter, 1990; Ternes, 2007).

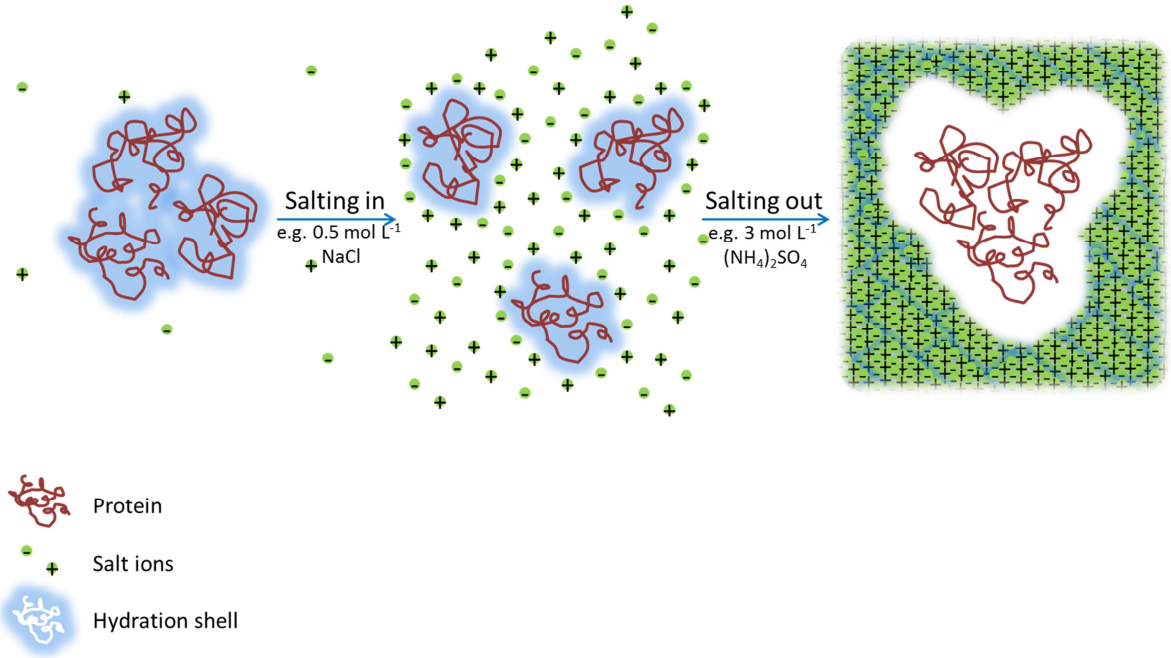

Figure 4: Molecular mechanisms during 'salting-in' and 'salting-out' of proteins. [Adapted from http://www.boku.ac.at/iam/edu/791.104_VO_Grundlagen_der_Bioprozesstechnik/Hahn_Bioprozesstechnik_Prazipitation_2007_08.pdf and http://faculty.ksu.edu.sa/Zaenab_Alzahrani/Documents/Experiment_no._5_handout_Final_copy.pdf]

Another method for protein precipitation amongst the variation of ionic strength is the 'hydrophobic-out' effect. Protein structures previously dissolved by the salting-in effect are precipitated by drastic reduction of this ionic strength towards a salt concentration of preferably 0 M. During adaptation to the new environmental

conditions with practically no salt content, the dissolved proteins form new low molecular weight protein associations in the thermodynamically most favourable orientation, i.e. in form of amphiphilic globular protein agglomerates or protein micelles. The polar residues are oriented outwards to the water, whereas the nonpolar residues are directed towards the hydrophobic core of the micelle (Murray, et al., 1978). The precipitated protein particles exhibit regular size and shape, but tend to coalescence at stationary conditions inside a diluted solution. Protein sedimentation to a viscous and gelatinous isolate mass is the result (Murray, Myers, et al., 1981).

4.2 Influence of pH on protein solubility

4.2.1 Protein solubilisation via pH modification

In aqueous solutions pH-shifts modify the charge state of a protein. At pH values above or below the isoelectric point the charged protein side chains accept or release hydrogen or hydroxide ions. Consequently, the protein exhibits a positive or negative excess net charge, enabling the water molecules to interact with those charges. Once the electrostatic repulsion exceeds the hydrophobic interactions of the proteins, protein solubility augments (Zayas, 1997). The pH-dependent dissociation and association mechanisms of the proteins are presented in Figure 5.

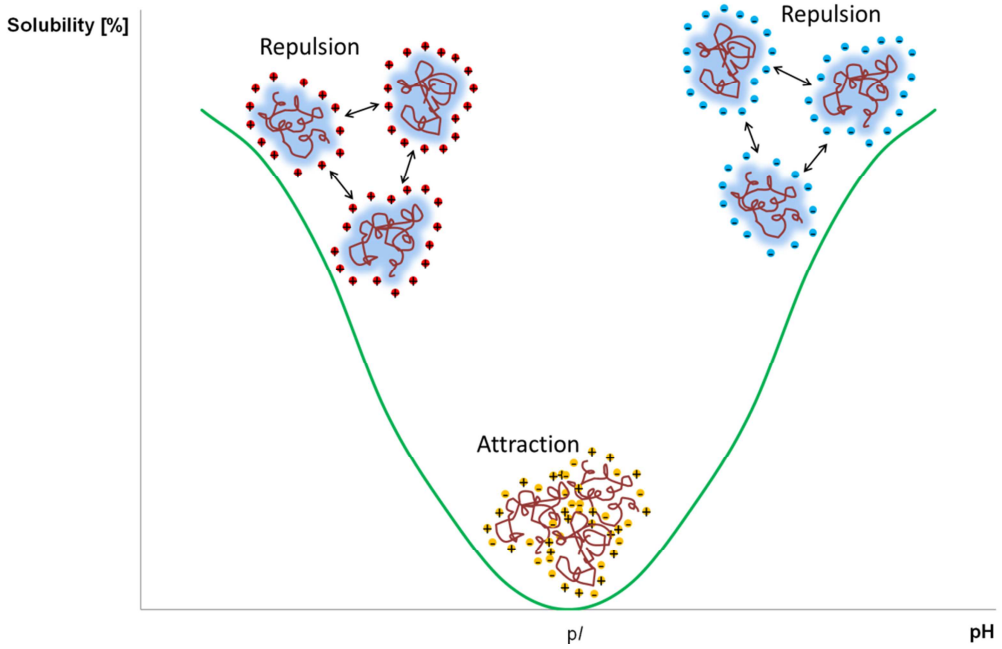

Figure 5: pH-Dependent molecular mechanisms of proteins. [Adapted from http://tu-freiberg.de/fakult2/bio/ag_mikrobio/lehre/skript_v2_methodenproteinbiochemie.pdf]

The solubility profile of the proteins from *L. angustifolius* L. as affected by the pH is demonstrated in Figure 6. The typical U-shaped run of the solubility curve highlights a solubility minimum (<20%) at the pH range 4-5. With increasing alkalinity until pH 11 up to 97% of the proteins get solubilised (Lqari, Vioque, Pedroche, & Millan, 2002; Ruiz & Hove, 1976).

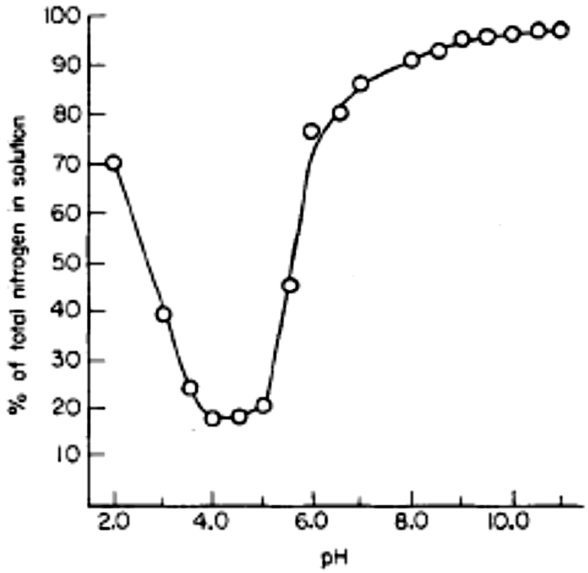

Figure 6: U-shaped solubility profile of proteins from *L. angustifolius* L. as affected by the pH (Ruiz & Hove, 1976).

Unfortunately, pH adjustment during protein extraction has shown to damage the proteins irreversibly. The endotherm peaks of Differential Scanning Calorimetry (DSC) thermograms enable the identification of total protein denaturation. Figure 7 shows thermograms of isoelectrically precipitated protein isolates from *Vicia faba* L. previously extracted in alkaline media of different pH values. With increasing alkalinity during extraction, the proteins show higher protein damage (Arntfield & Murray, 1981), i. e. less regulary packed structures.

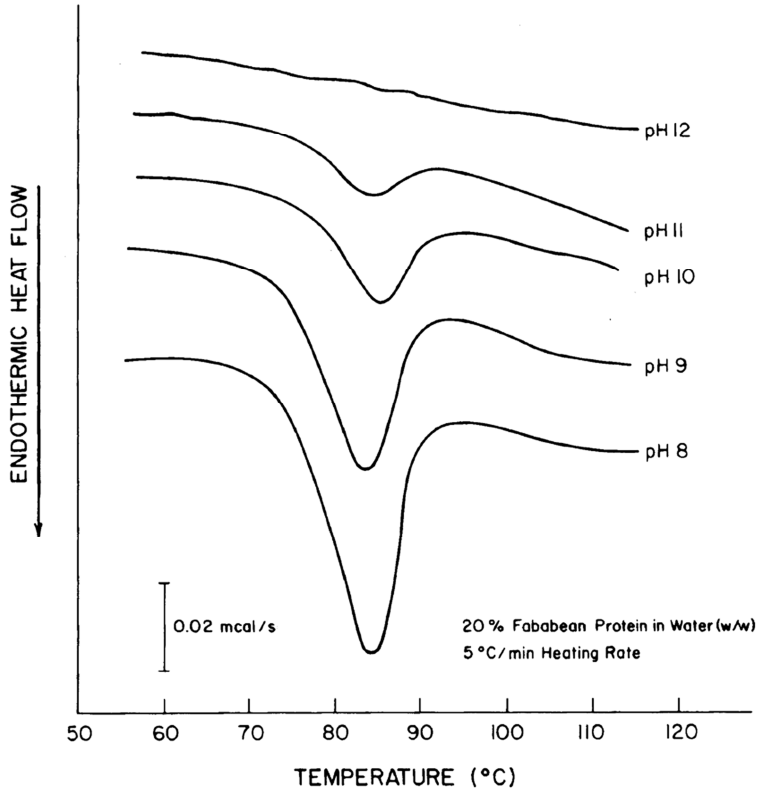

Figure 7: Effect of pH during alkaline extraction and isoelectric precipitation on the DSC thermogram of the resulting faba bean protein isolate (Arntfield & Murray, 1981).

4.2.2 Modification of pH for protein precipitation

Depending on the pH, the acidic or alkaline amino acids exhibit different charges which result in proteins with more or less positive or negative regions. As described in chapter 4.1, proteins with positive or negative excess charges are able to interact with unbound water molecules and become soluble. However, reaching the isoelectric point (p*I*), the number of negative and positive excess charges of the protein is equal. This leads to a neutral net charge of the protein and an electrophoretic mobility of zero (Figure 8).

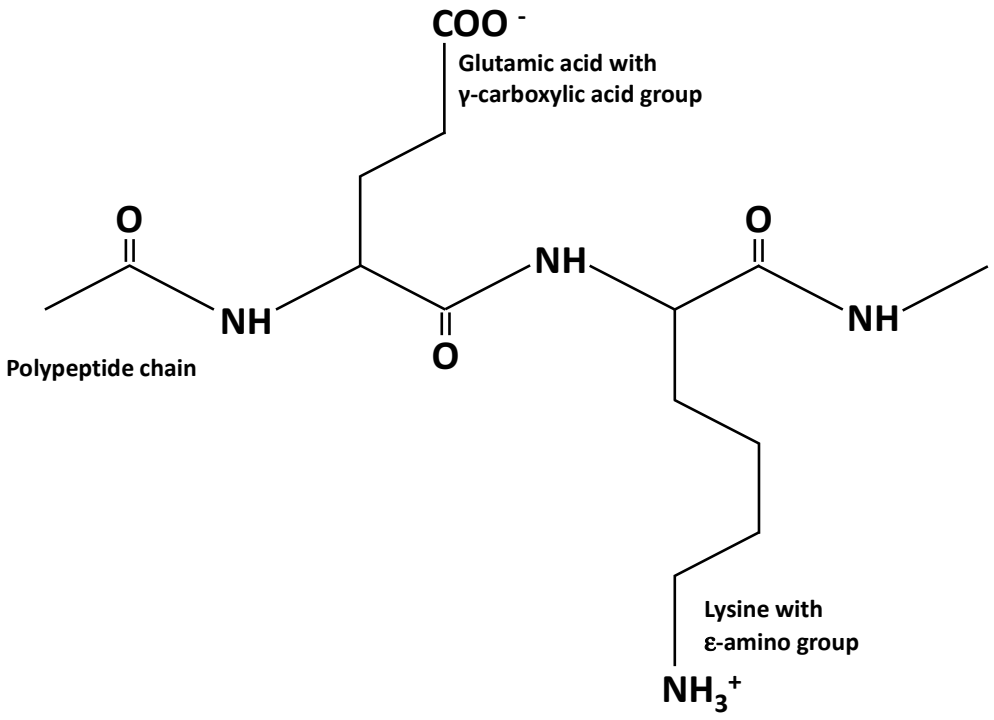

Figure 8: Section of an exemplary protein in aqueous solution at the isoelectric point.

Consequently, the globally neutral protein becomes unable to interact with the surrounding ions and finally aggregates. This effect of electrophoretic immobility at the p*I* is of avail for the isoelectric precipitation technique. The pH at which the protein exhibits its p*I* varies depending on the environment (salt ions, solvent) and is specific for each amino acid and protein under defined conditions.

5 Isolation of protein preparations from legume seeds

There are several possibilities to provide lupin protein preparations for human nutrition. Dependent on the protein content these preparations are classified into flour (<65% protein in dry matter), protein concentrate (45 - 80% protein in dry matter) and protein isolate (>90% protein in dry matter). An overview about the most important process steps for recovery of those three protein ingredients form legumes is presented in Figure 9.

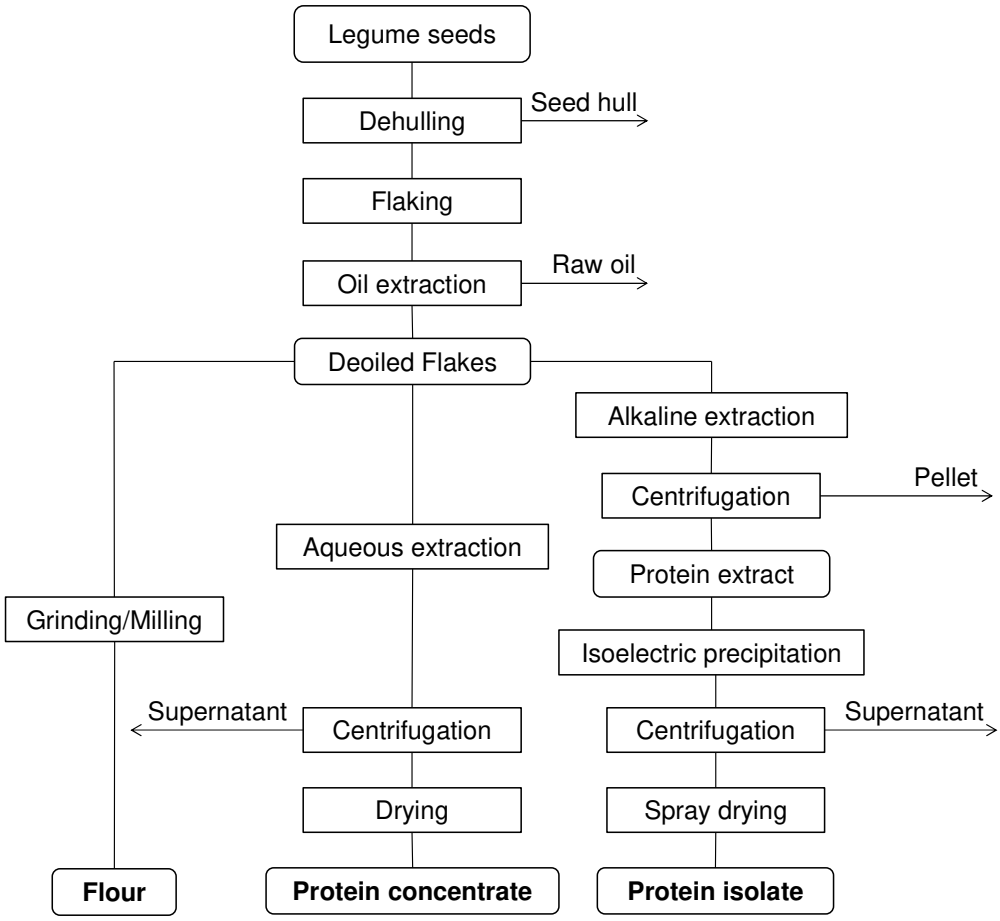

Figure 9: Production of different protein ingredients from legume seeds [modified from Belitz, Grosch and Schieberle, 2009]

The preparation of lupin flours is the simplest procedure. However, as there is no selective protein recovery, high amounts of most other lupin seed components such as fibres, oligosaccharides and potentially alkaloids remain present.

Protein concentrates are usually produced out of flaked and defatted (oil) seeds or meals, the residues of the oil extraction. Purification is performed in water or aqueous solution where the bulk of proteins (globulins) remain insoluble whereas minerals, oligosaccharides, low molecular weight nitrogen compounds and anti-nutritional factors are removed. For separation of those non-protein fractions the residue is centrifuged, washed and dried.

The elementary process for recovery of lupin protein isolates in the laboratory comprise three successive steps: extraction, precipitation and centrifugation (Sironi, et al., 2005). Generally, the solid-liquid extraction dissolves one or more components from a solid matter. Dry source products such as seed flakes (Figure 2C), soak the solvent after insertion into the liquid phase and permit the

target compound – here the lupin proteins – to be extracted (transition component). The crucial mass transport begins. After a slight accumulation of the transition component into the solvent the transient component is precipitated and builds small insoluble aggregates. These aggregates are finally separated from the solvent and concentrated via centrifugation (Keller, 1982). Dietary fibres and other compounds are notably removed during isolation procedure and the bean-like off-flavour, characteristic for lupin flours and lupin protein concentrates, is considerably reduced. However, among a broad range of research activities describing the use of protein isolates as functional food ingredients such as for soy, literature data investigating lupin seed proteins are scarce. Two important techniques of protein isolation are well-established: the alkaline extraction with subsequent isoelectric precipitation (Chew, et al., 2003; Lqari, et al., 2002; Lusas & Riaz, 1995) and the salt-induced extraction followed by dilutive precipitation (Rahma, Dudek, Mothes, Gornitz, & Schwenke, 2000). Isoelectric and micellar protein isolates are obtained, respectively. Thus, both named protein isolation procedures implement one of the two most important protein structure influencing parameters: modification of pH or ionic strength. The production of the isoelectric as well as the micellar protein isolate is described separately in the succeeding two chapters.

5.1 Production of isoelectric protein isolates

Due to the broad solubility range of the albumins, globulins, prolamins and glutelins there is no standard isolation method for legume proteins. Aqueous alkaline protein extractions in a pH range from pH 6 to pH 11 are established (Moure, et al., 2006; Ruiz & Hove, 1976). However, the occurrence of undesired modifications in protein structure at high pH needs to be considered because of associated changes in protein properties (Robbins & Ballew, 1982). As shown in Table 3, the p*I* of the three main lupin conglutins α, β and δ range between pH 4.3 and 6.2. Only conglutin γ, accounting for 5% to total lupin proteins, exhibits an alkaline p*I* of 7.9. For a highly abundant protein recovery of the solubilised proteins, a global isoelectric precipitation at pH 4.5 became standard for lupin proteins (Chew, et al., 2003; Lqari, et al., 2002; Ruiz & Hove, 1976). However, not all extracted proteins will be isolated if using this method. Particularly, proteins with high contents of sulphuric amino acids, which by nature are limited in lupins, cannot be precipitated

using the isoelectric precipitation (Oomah & Bushuk, 1983; Ruiz & Hove, 1976; Sgarbieri & Galeazzi, 1978). Furthermore, lowering the pH value to an acidic milieu bears the risk of negative effects on important functional properties such as gelation, or emulsification (Cheftel, et al., 1992; Egbert, 1997). Due to its aqueous extraction, the **i**soelectric **p**rotein isolate, hereinafter referred to as "IP", may be enriched with anti-nutrients such as phytic acid, and exhibit a reduced nutrient quality (Paredes-López, et al., 1991; Rahma, et al., 2000; Finot, 1997).

5.2 Production of micellar protein isolates

As described in chapter 4.1 neutral salts at low concentrations (0.5-1.0 mol·L^{-1}) increase protein solubility ('salting-in'). The use of salt solutions for protein extraction has been investigated for a number of raw materials such as safflower, sunflower, canola, faba bean, soy, pea and lupin (El-Adawy, Rahma, El-Bedawey, & Gafar, 2001; Ismond, et al., 1990; Ismond, et al., 1986a, 1986b; Pickardt, Neidhart, Griesbach, et al., 2009). The salt concentration in the extract has an important impact on protein yield. In a study from Sussmann, Pickardt, Schweiggert, and Eisner (2011), 0.5 mol·L^{-1} NaCl in the extraction solution showed the highest protein extractability of lupin proteins from *L. angustifolius* L. After protein extraction, the ionic strength is drastically reduced using deionised water, inducing protein precipitation. This effect for protein precipitation was first published by Murray et al. (1978).

Because of a reported formation of protein micelles, the production of dilutive protein isolate is often called "micellisation" and the deriving product MP, **m**icellar **p**rotein isolate (see chapter 4.1; Ismond, et al., 1990; Murray, et al., 1981). Up to now the formation of protein micelles applying the micellisation procedure was solely published by Ismond, et al. (1990) using faba bean as the raw material. In named study light microscopy was used to investigate the formation of micellar gel networks depending on the environments using different agents. However, the formation procedure of the protein micelle itself still remans unexplored. The lack of progress in structure studies of protein particles as yet is largely due to the complexity of the protein character and the limited availability of techniques for protein characterisation without modifying the specific protein constitution. Proteins are highly sensitive to modifications of their surrounding media (Cooper, 1988).

Alcohols, acids, salts or simply water, usually used as staining agents, tend to interfere with the protein conformation and can cause severe surface distortion or irreversible protein denaturation. Another major problem for the surface characterisation of protein isolates is the exposure to physical parameters such as temperature modification, elimination of water or mechanical stress. In addition, the composition of the protein isolates (high molecular weight and high water content) limits the applicability of imaging (e.g. scanning electron microscopy) or spectroscopic (e.g. nuclear magnetic resonance) techniques.

To corroborate the development of protein micelles and because of the high potential for food applications (Rodriguez-Ambriz, Martinez-Ayala, Millan, & Davila-Ortiz, 2005), detailed structural investiagtions need to be accomplished and presents a main task in the present dissertation.

5.3 Combination of different precipitation methods

The use of salts or modifying the pH of proteins is known to influence the specific molecular protein arrangement. At the same time different protein isolates are known to show characteristic technofunctional properties. However, the reason for differences in technofunctional properties of the different protein isolates is highly complex and still remains unexplored. Most likely, the specific molecular protein arrangement affects the technofunctional properties of the protein isolate, but more detailed investigations to check the interrelation between isolation procedure, protein microstructures and technofunctional properties are needed to verify this assumption.

Up to now, it has not been attempted to combine different protein precipitation techniques with each other. However, combining both common protein isolation processes was assumed to lead to innovative protein preparations disclosing important information about the influence of systematic milieu conditions on characteristic protein structure formations.

6 Technofunctional properties of protein isolates and their application in food products

In general, protein isolates feature specific physico-chemical (description in chapter 3) and technofunctional properties. The term ‘technofunctional’ has been established to create a clear dissociation from the biological definition ’functional’ used by medicines (Schwenke, 2001; Siebert, 2003). The technofunctional properties are understood as being the capability of the protein to interact specifically with surrounding substances determining their application potential as a food ingredient. As published by Schwenke (2001) the protein technofunctionality is divided into 1) properties related to the molecular protein structure i.e. the viscosity behaviour, 2) properties related to the “interaction capacity” such as the protein solubility as well as 3) “interfacial” properties including the emulsifying capacity (Cordero-de-los-Santos, Osuna-Castro, et al., 2005; Lampart-Szczapa & Jankowski, 1998; Schwenke, 2001). Accordingly, depending on the type of function, the technofunctionality of a protein isolate is not only influenced by one specific protein property but either essentially by the protein conformation, the chemical composition or the steric properties of the protein inside a defined environment (Schwenke, 2001).

6.1 Technofunctional properties of isoelectric (IP) and micellar protein isolate (MP)

Various studies compared the technofunctional properties of the isoelectrically precipitated and dilutive precipitated protein products from different sources such as amaranthus, flax seed, soybean, chickpea, mung bean and lupins (Cordero-de-los-Santos, et al., 2005; El-Adawy, et al., 2001; Krause, Schultz, & Dudek, 2002). Table 7 reports property differences of isoelectric (IP) and dilutive (MI) precipitated protein isolates from different raw materials.

Table 7: Technofunctional properties of isoelectric (IP) and dilutive (MP) protein isolates from various sources.

Technofunctional property	IP	MP	Protein source	Reference
Protein solubility (NaCl) [%]	-	+	lupin, flaxseed, chickpea	1, 3, 5
Water absorption capacity [g/100g isolate]	-	+	lupin, safflower, chickpea	1, 4, 5
Fat absorption capacity [g/100g isolate]	-	+	lupin, safflower, chickpea	1, 4, 5, 6
Emulsification activity [%]	-	+	flaxseed	3
Emulsification capacity [ml oil/g isolate]	-	+	lupin	1
Emulsification stability [%]	-	+	safflower, chickpea	4, 5
Foam capacity [% volume increase]	-	+	lupin, safflower	1, 4
Foam stability [% volume increase]	+	-	chickpea	5
Foam expansion [%]	+	-	chickpea	5
Isoelectric point p/	-	+	lupin, amaranth	2, 6
Storage modulus G' compared to G''	+	-	flaxseed	3

- lower, + higher;
[1] El-Adawy, et al. (2001), [2] Cordero-de-los-Santos, et al. (2005), [3] Krause et al. (2002), [4] Paredes-López & Ordorica-Falomir (1986), [5] Paredes-López, et al. (1991), [6] Rodriguez-Ambriz, et al. (2005).

The IP exhibits higher foam stability and foam expansion but lower protein solubility, fat absorption and emulsification capacity compared to MP. Furthermore, IP is capable of forming protein networks as opposed to MP (Cordero-de-los-Santos, et al., 2005; Rodriguez-Ambriz, et al., 2005).

The differences in the technofunctional properties of both protein isolates might be attributed to the different isolation procedures. Process parameters such as pH adjustment or the addition of detergents can affect the proteins irreversibly and lead to protein unfolding or a loss in functionality (Schwenke, 2001). Some functional properties such as surface hydrophobicity require a partial protein unfolding (Pozani, Doxastakis, & Kiosseoglou, 2002). During protein denaturation, the hydrophobic regions have a lower ability to maintain hydrophobic protein-protein interactions and align towards the protein surface (Schwenke, 2001). In contrast, a low denatured and highly flexible character of the protein is required for an optimal emulsifying capacity (Damodaran, 2005). Furthermore, protein solubility requires lowly protein denaturation for increased protein-solvent interactions (Sousa, Morgan, Mitchell, Harding, & Hill, 1996).

6.2 Application of lupin protein isolates in food products

The beneficial technofunctional properties of legume proteins make them interesting for the use as food additives to improve texture properties of different products, including bakery products, pasta and sausages (Archer, Johnson,

Devereux, & Baxter, 2004; Arozarena, Bertholo, Empis, Bunger, & de Sousa, 2001; Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002; Mubarak, 2001; Torres, Frias, Granito, Guerra, & Vidal-Valverde, 2007). However, end products containing soy protein are perceived highly controversial by the consumer as more than 50% of soybean crops are genetically modified. Therefore, the consumption of deriving products is refused by a significant number of consumers (Moses, 1999; Schyver & Smith, 2005). In contrast, the application of pea is limited due to adverse sensory properties. Lupin seeds are a potential alternative because of similar nutritional properties and as lupin crops are neither genetically modified nor is their use limited due to adverse sensory and nutritive properties (Duranti, et al., 2008; Resta, Boschin, D'Agostina, & Arnoldi, 2008; Rodriguez-Ambriz, et al., 2005; Sirtori et al., 2004). In a study from Lásztity, Khalil, Haraszi, Baticz, & Tömösközi (2001) the enrichment of fruit- and vegetable-based baby foods with lupin protein isolates resulted in products with excellent organoleptic properties. However, lupin allergens such as Lup-2 show sequence homology with Ara h 3 from peanut and glycinin G1 from soybean (Guillamon et al., 2010). Therefore, lupin is one of the 14 major allergens that must be declared when applied in food products (European Directives 2003/89/EC and 2006/142/EC).

In addition to the replacement of soy (King, et al., 1985; Kiosseoglou, Doxastakis, Alevisopoulos, & Kasapis, 1999; Mavrakis, Doxastakis, & Kiosseoglou, 2003), lupin preparations can serve as substitutes for egg (Arozarena, et al., 2001) or various other ingredients in candy, meat and bakery industry (Drakos, Doxastakis, & Kiosseoglou, 2007) to improve sensorial and textural properties in the different food products.

Preservation of the fat-like texture in fat-reduced products is important for current researches, the industry and a wide range of consumers. Lupin protein isolates showed to be of great interest for the development and production of energy-reduced food products featuring simultaneously the functionality of a fat substitute (Kiosseoglou, et al., 1999). Furthermore, MLP was discovered to feature fat-like properties and its application in a low-fat formulation of truffle fillings provided a tasty praline with appropriate sensorial properties (Sussmann, Pickardt, Schweiggert-Weisz, & Eisner, 2010). Due to the supplemented lupin protein, corresponding food products cause higher satiety than the products without lupin

protein supplementation (Drakos, et al., 2007; Lee, Mori, Sipsas, Barden, et al., 2006). Moreover, the increasing number of obese consumers due to the excess consumption of saturated fats and cholesterol of animal origin shows the general need for vegetable proteins in human nutrition.

7 Aims of the study

In recent years, lupin proteins have been commercially applied in food products, the goal being to improve final product qualities. The main focus was placed on isoelectrically precipitated lupin protein isolates (ILP) featuring high protein yields and favourable technofunctional properties.

Based on a Canadian patent from the seventies (Murray, Myers et al., 1978), Sussmann, et al. (2010) developed a lupin protein isolate via dilutive precipitation exhibiting fat-like properties. Due to the assumption of micellar aggregate formation the product is often described as micellar lupin protein isolate (MLP, Ismond, et al., 1990).

There are a number of indications for process-dependent differences in protein properties among both protein isolates ILP and MLP. The composition of individual protein fractions and of minor compounds seems similar in both protein isolates, but proportions differ (Rodriguez-Ambriz, et al., 2005). Furthermore, the consistency of ILP is rough, compact and curdy, while MLP is smooth, pasty, and mellifluent at room temperature, and features a fat-like texture (Sussmann, et al., 2010). The reasons for these differences are still unclear but might arise from different protein microstructures. However, the formation of isolation-dependent protein aggregates remains uncertain, as detailed scientific investigations have not been conducted up to now. Studying the microstructures in detail in their natural and unmodified appearance is considered crucial to understand and control the production of tailored protein isolates with desired technofunctional properties (Cordero-de-los-Santos, Osuna-Castro et al. 2005, Lampart-Szczapa & Jankowski, 1998).

The aim of the present study was therefore to investigate the influence of the isolation procedure on the formation of the microstructure, the chemical composition as well as on physical protein properties of protein isolates derived from seeds of the sweet blue lupin *Lupinus angustifolius* L. Vitabor.

According to that goal, a number of working hypotheses and analytical approaches were investigated in the present dissertation. This knowledge was assumed to permit deeper insight into the precipitation behaviour of proteins and their interaction with technofunctional properties.

Hypothesis 1) The isolation procedure is assumed to influence the protein yield and composition of the lupin protein isolates. The isoelectric precipitation method is reported to show a higher protein yield than the dilutive precipitation procedure. However the reason remains unknown. Determining the protein yield of protein isolates systematically dependent on the isolation procedure was anticipated to disclose the most important process steps within the extraction or precipitation to influence the protein recovery. On the basis of these results optimal protein recovery in combination with specific microstructure formation was aimed to release.

For this purpose, besides ILP and MLP another seven protein isolates were investigated in this study combining the alkaline with the salt-induced extraction, as well as the isoelectric with the dilutive precipitation. Then, total protein yields as well as the protein distribution within the individual isolation steps were compared. Furthermore, the quantitatively most important compounds such as dry matter, protein, ash, and fat contents were determined in order to investigate the differences in the chemical composition of the protein isolates.

Hypothesis 2) The formation of specific protein microstructures is expected to depend on the isolation process. The main objective was to investigate the mechanism of the micelle formation of MLP known to exhibit fat-like properties (Sussmann, et al., 2010). The insight into the complex folding mechanism of proteins through a microscope as affected by their isolation procedures was expected to lead to a better understanding of the behaviour of different isolation media and procedures on the technofunctional and textural characteristics of different protein isolates.

To prove this hypothesis, different non-invasive microscopic tools were used in the present dissertation to characterise the protein microstructures. In order to monitor structural proteins formations, intrinsic fluorescences were identified and compared using reflected-light fluorescence microscopy. An insight into the superior three-

dimensional structure of protein arrangements (tertiary and quaternary structures) recovered by micellisation in comparison to the isoelectric precipitation technique was provided by cryo-scanning electron microscopy (cryo-SEM). In addition to the screening and statistical evaluation of the microstructures, the behaviour of precipitate formation was studied “on-line” by means of optical microscopy, i.e. by reproducing the micellar isolation procedure under microscopic surveillance.

Hypothesis 3) The protein composition is presumed to vary within the protein isolates, as the different protein families show different solubility characteristics. The heterogeneous protein composition in lupin seeds indicates that the globulins might preferably be extracted by salty conditions whereas the albumins might rather be extracted in water than by salt solution, for example. At the same time, the different p*I*s of the individual lupin protein fractions are assumed to lead to distinct pH-mediated precipitation conditions. If the distribution of the protein fractions would vary among the protein isolates, the different protein properties of the protein isolates could be attributed to the protein composition. In case of an identical protein composition, however, the technofunctional properties of the protein isolates might rather be explained by physical or structural features.

This task has yet to be investigated by compositional studies such as investigating the amino acid composition by ion exchange chromatography, fractionating the proteins via high performance liquid chromatography (HPLC) and determining the molecular weights by gel electrophoreses.

Hypothesis 4) Structural features of the protein isolates are assumed to interact with physico-functional properties. Both physical attributes, the microstructures and the physical agility of the proteins, might be a result of specific isolation conditions. Especially the protein denaturation may vary within the protein isolates depending on the harshness of the isolation procedure and lead to further physical protein properties.

To assess the physical protein differences dependent on the isolation procedure, important physico-functional properties of the different protein isolates such as surface hydrophobicity and denaturation behaviour were investigated. Based on this knowledge, explanations for specific technological functions of different protein isolates were aimed to provide.

Hypothesis 5) Technofunctional properties of both protein isolates IP and MP, respectively, are known to differ. However, the reasons for the differences remain unexplored. Due to the pronounced differences in the textural character **an interrelation predominantly between the microstructures of selected protein isolates and their technofunctional properties is expected.** In this dissertation this interrelation was aimed to prove to enable optimal use of the technofunctional properties of the proteins. On the basis of this knowledge tailored protein isolates were aimed to be made available, meeting the requirements of the versatile food products on the market.

Hypothesis 6) Furthermore, interactions between different technofunctional properties have been reported (Egbert, 1997) always on the basis of a fixed pH. However, the pH is known to influence the protein microstructures which might in turn affect the technofunctional behaviour. This encourages elucidating the **interrelation between technofunctional properties depending on the protein microstructures, and independent of the pH.** Therefore, the industrially most important technofunctional characteristics such as rheological properties, protein solubility, emulsifying behaviour and oil and water binding capacities were determined and related to the isolation procedure as well as to each other.

CHAPTER 1

Microscopic characterisation and composition of proteins from lupin seed (*Lupinus angustifolius* L.) as affected by the isolation procedure

The precipitation behaviour and structure formation of lupin protein isolates upon application of different procedures was investigated. The focus was placed on a specific lupin protein isolate showing fat-like properties. For production of the protein isolates, different extraction (alkaline and salt-induced protein extraction) and precipitation parameters (isoelectric and dilutive protein precipitation), known to influence the protein microstructures, were combined.

The protein microstructures were assessed using bright field light microscopy, fluorescence microscopy and cryo-scanning electron microscopy. The studies on structure formation revealed that precipitation at pH 4.5 always led to an unfolding of the protein indicating an irreversible denaturation. Applying the dilutive precipitation, formation of flexible but well-ordered globular aggregates was observed. This effect persisted after combining dilutive and pH mediated precipitation procedures. Only the isoelectric lupin protein isolate showed autofluorescence properties at three wavelengths. A number of indications evidenced that the physical protein structure was responsible for differences in protein behaviour rather than variations in the chemical composition of the protein isolates. The formation of protein micelles upon dilutive precipitation was held responsible for the fat-like sensation of this protein isolate.

I. Muranyi designed the study, collected microscopic test data, wrote the manuscript and interpreted the results. C. Otto collected microscopic test data and assisted in interpreting the results. C. Pickardt managed the supported research project, assisted in interpreting the results and contributed to writing of the manuscript. Furthermore, contributions to writing were made by P. Koehler and U. Schweiggert-Weisz.

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Microscopic characterisation and composition of proteins from lupin seed (*Lupinus angustifolius* L.) as affected by the isolation procedure

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ABSTRACT

Two important techniques for protein isolation from vegetable sources are well-established, the alkaline extraction with subsequent isoelectric precipitation and the salt-induced extraction followed by dilutive precipitation. Both techniques provide protein isolates with different properties. In the case of lupin the isoelectric protein isolate is commercially applied as an egg-substitute in diverse food products, whereas a specific dilutive lupin protein isolate exhibits fat-like properties and shows the high potential of lupin proteins for fat replacement. However, the reason for this behaviour has not been investigated up to now. Therefore, the influence of common precipitation parameters on structure formation of proteins from *Lupinus angustifolius* L. Vitabor was investigated using bright field light microscopy, fluorescence microscopy and cryo-scanning electron microscopy. The structure formation upon application of nine different procedures revealed that precipitation at pH 4.5 always led to an unfolding of the protein indicating an irreversible denaturation. Applying the dilutive precipitation, formation of flexible but well-ordered globular aggregates was observed. This effect persisted after combining dilutive and pH mediated precipitation procedures. Only the isoelectric lupin protein isolate showed autofluorescence properties at three wavelengths. A number of indications evidenced that the physical protein structure was responsible for differences in protein behaviour rather than variations in the chemical composition of the protein isolates.

1. Introduction

Legumes play an important role in the traditional diets of many regions throughout the world (Joray, Rayas-Duarte, Mohamed, & van Santen, 2007; Yeheyis, Kijora, Wink, & Peters, 2011). In particular, lupin seeds are characterised by a virtually non-existent starch and high protein content (total protein content of approximately 34%) in comparison to other legumes such as beans and peas (Torres, Frias, & Vidal-Valverde, 2005).

The seed proteins of *Lupinus angustifolius* L. consist mainly of the two glycosylated protein groups albumins and globulins. The mass ratio of albumins to globulins is around 1 to 9. The albumins are also referred to as δ-conglutin according to their electrophoretic separation (Salmanowicz, 2000). The legumin-like α-conglutin and the vicilin-like β-conglutin in lupin seeds represent the main globulins (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). The content of further protein fractions, such as prolamins and glutelins, is negligible (Chew, Casey, & Johnson, 2003; Gulewicz et al., 2008).

Two important techniques of protein isolation are well-established: the alkaline extraction with subsequent isoelectric precipitation (Chew et al., 2003; Lqari, Vioque, Pedroche, & Millan, 2002; Lusas & Riaz, 1995) and the salt-induced extraction followed by dilutive precipitation (Rahma, Dudek, Mothes, Gornitz, & Schwenke, 2000). While the chemical composition of both products is comparable (Paredes-López & Ordorica-Falomir, 1986), the protein isolates differ considerably in protein yield (Cordero-de-los-Santos, Osuna-Castro, Borodanenko, & Paredes-Lopez, 2005; Rodriguez-Ambriz, Martinez-Ayala, Millan, & Davila-Ortiz, 2005), their techno-functional properties (Cordero-de-los-Santos et al., 2005; El-Adawy, Rahma, El-Bedawey, & Gafar, 2001; Krause, Schultz, & Dudek, 2002), visual appearance and rheological properties (Krause et al., 2002).

Among the techno-functional properties of lupin proteins water binding capacity, foaming activity, and emulsification properties are most important. Therefore, they are applied for improving the texture of different food products, e.g. bakery products, pasta and sausage (Archer, Johnson, Devereux, & Baxter, 2004; Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002; Torres, Frias, Granito, Guerra, & Vidal-Valverde, 2007). The utilisation of lupin seeds for the production of

Abbreviations: ILP, isoelectric lupin protein isolate; MLP, micellar lupin protein isolate; ES, salt-induced extraction; EAS, combined salt-induced and alkaline extraction; EA, alkaline extraction; PD, dilutive precipitation; PI, isoelectric precipitation; PDI, combined dilutive and isoelectric precipitation; 5.5, protein isolates adjusted to pH 5.5; SEM, scanning electron microscopy.

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protein isolates has the advantage that lupin crops are neither genetically modified, such as soybean crops, nor is their use limited due to adverse sensory properties, such as is the case for peas, for example (Duranti et al., 2008; Resta, Boschin, D'Agostina, & Arnoldi, 2008; Rodriguez-Ambriz et al., 2005).

The consistency of the isoelectric lupin protein precipitate is rough, compact and curdy, while the product obtained by dilutive precipitation is smooth, pasty, and mellifluent at room temperature and features a fat-like texture (Sussmann, Pickardt, Schweiggert-Weisz, & Eisner, 2010). In a recent study from Sussmann et al. (2010) the application of the dilutive lupin protein isolate in a low-fat formulation of truffle fillings exhibited a tasty praline with appropriate sensorial properties. Preservation of the fat-like texture in fat-reduced products is the focus of current researches all over the world, industry and last but not least a wide range of consumers. This specific protein isolate is often described as micelle-protein (Ismond, Georgiou, Arntfield, & Murray, 1990); however, the formation of micellar protein aggregates remains uncertain as detailed scientific investigations have not been conducted up to now.

The lacking progress in structure studies of protein particles is largely due to the complexity of the protein character and the limited availability of techniques for protein characterisation without modifying the specific protein constitution. Proteins are highly sensitive to modifications of their surrounding media (Cooper, 1988). Alcohols, acids, salts or simply water usually used as staining agents tend to interfere with the protein conformation and could cause severe surface distortion or even surface damages. Another major problem for the surface characterisation of protein isolates is the exposure to physical parameters, such as temperature modification, elimination of water or mechanical stress. Besides, the composition of the protein isolates (high molecular weight and high water content) limits the applicability of imaging (e.g. scanning electron microscopy) or spectroscopic (e.g. nuclear magnetic resonance spectroscopy) techniques.

Therefore, the aim of the present study was to investigate the influence of isolation conditions on formation of the microstructure of protein isolates derived from seeds of the sweet blue lupin *L. angustifolius* L. Vitabor. In order to determine the physicochemical changes during production of protein isolates, different milieu conditions, which are known to influence the conformation of proteins, were applied. Thus, nine protein isolates were produced by combining the production parameters from the alkaline extraction with isoelectric precipitation and the salt-induced extraction with dilutive precipitation, and the protein microstructures were investigated by different microscopic techniques. Structural characteristics were identified using reflected-light fluorescence microscopy. An insight into the three-dimensional structure of protein arrangements recovered by micellisation in comparison to the isoelectric precipitation technique was provided by cryo-scanning electron microscopy (cryo-SEM). In addition, the formation of protein micelles was studied "on-line" by means of optical microscopy, i.e. reproducing the micellar isolation procedure under microscopic surveillance, to gain insight into the behaviour of precipitate formation.

2. Experimental

2.1. Materials

Lupin seeds of the sweet narrowleaf lupin cultivar *L. angustifolius* L. (cv. Vitabor, 2005) were obtained from Saatzucht Steinach (Steinach, Germany). Bovine serum albumin (BSA, Fraction V), glycerol and NaCl were obtained from VWR International (Darmstadt, Germany). Antifadent mounting medium AF2 was purchased from Citifluor Ltd. (London, United Kingdom) and Phloroglucin from Sigma-Aldrich (Steinheim, Germany). Deionised (DI) water was used throughout. All reagents used were of analytical grade or stated otherwise.

2.2. Pre-treatment of the lupin seeds

The seeds were dehulled with an underflow peeler (Streckel & Schrader, Hamburg, Germany) and classified in an air-lift system (Alpine Hosokawa AG, Augsburg, Germany). The dehulled kernels were flaked using a roller mill (Streckel & Schrader, Hamburg, Germany) to obtain lupin flakes, which were used as raw material for the production of the protein extracts and protein isolates, respectively.

2.2.1. Preparation of Isoelectric Lupin Protein isolate (ILP) by aqueous alkaline extraction and isoelectric precipitation

Based on the production process for preparation of isoelectric lupin protein isolates described in literature (Lqari et al., 2002; Ruiz & Hove, 1976) full-fat lupin flakes were suspended in DI water at a ratio of 1:8 (w/v). For alkaline protein extraction the pH value was adjusted to pH 8 with 0.5 mol L^{-1} NaOH and this suspension stirred for 1 h at room temperature to maximise the extend of solubilised proteins. After separation through a sieve (mesh size: 1.5 mm) removing the fibre fraction, the suspension was centrifuged at 3300 *g* for 10 min. The supernatant was filtrated through a rayon-polyester filter (pore size: 22–25 μm) for separation of fine suspended solids from the extract. The supernatant was acidified to pH 4.5 with 0.5 mol L^{-1} HCl. For an exhaustive protein precipitation, the crude protein suspension was left for 18 h at 1 °C. The precipitate was recovered by centrifugation and the supernatant was discarded. The protein precipitate was washed with DI water (ratio of 1:10 (w/v)) to eliminate effectively surplus salts. After a last centrifugation step the precipitated ILP was stored at −20 °C for a minimum of 24 h until microscopic analysis. For chemical analysis the protein isolate was directly frozen at −50 °C and lyophilised in a freeze-drier (Beta 1–8, Christ GmbH, Osterode, Deutschland).

2.2.2. Preparation of Micellar Lupin Protein isolate (MLP) by salt-induced extraction and dilutive precipitation

The micellar protein isolate MLP was prepared according to a method described by Sussmann, Pickardt, Schweiggert, and Eisner (2011) with some modifications. Briefly, full-fat lupin flakes were suspended in 0.5 mol L^{-1} NaCl solution at a ratio of 1:8 (w/v). After stirring for 1 h at room temperature, the suspension was separated through a sieve (mesh size: 1.5 mm) to remove the fibre fraction and centrifuged at 3300 *g* for 10 min. The supernatant was filtrated through a rayon-polyester filter (pore size: 22–25 μm) for separation of fine suspended solids from the extract. In order to precipitate the MLP the clear supernatant was diluted with DI water at room temperature at a ratio of 1:3 (v/v) while stirring continuously. The salty protein dispersion was left for 18 h at 1 °C. The precipitate was recovered by centrifugation and the supernatant was discarded. The pasty precipitate was washed with DI water (ratio of 1:10 (w/v)). After a final centrifugation step the pH value was measured and resulted in pH 5.5. The protein isolate was stored at −20 °C for a minimum of 24 h until microscopic analysis. For chemical analysis the protein isolate was directly freezed at −50 °C and lyophilised.

2.2.3. Preparation of hybrid protein isolates by either aqueous alkaline or salt-induced extraction, followed by isoelectric precipitation, isoelectric dilutive precipitation, or dilutive precipitation

Different "hybrid" protein precipitates were produced by combining the parameters of both isolation techniques, ILP and MLP, as described above resulting in a total of 9 different protein isolates presented in Table 1. Additionally, two protein isolates were prepared (protein isolates 4a and 7a) with pH adjustment of the extracts prior to dilution to the naturally arising pH of MLP (pH 5.5) using 0.5 mol L^{-1} HCl in order to equalise the conditions to the dilutive precipitation technique.

For all protein isolates prepared by alkaline extraction (abbreviated by 'EA'), the flakes-water suspension (1:8) was adjusted to pH 8 with 0.5 mol L^{-1} NaOH. For all protein isolates prepared by salt-induced

Table 1
Process steps for the production of individual lupin protein isolates.

Protein isolates	Extraction		Precipitation	
	Salt induced in 0.5 mol L^{-1} NaCl	Alkaline (pH 8) using NaOH	1:3 dilution with dest. H_2O	Isoelectric point adjustment (pH 4.5) using HCl
1 ES-PD = MLP		+	+	
2 ES-PI	+			+
3 ES-PDI	+		+	+
4 EAS-PD	+	+	+	
4a EAS-PD5.5	+	+	+[a]	
5 EAS-PI	+	+		+
6 EAS-PDI	+	+	+	+
7 EA-PD		+	+	
7a EA-PD5.5		+	+[a]	
8 EA-PI = ILP		+		+
9 EA-PDI		+	+	+

+ Process step was carried out.
ES = salt-induced extraction, EAS = combined salt-induced and alkaline extraction, EA = alkaline extraction, PD = dilutive precipitation, PI = isoelectric precipitation, PDI = combined dilutive and isoelectric precipitation, 5.5 = protein isolates adjusted to pH 5.5.
[a] Adjustment to pH 5.5 (naturally arising pH after micellisation procedure of investigated lupin seeds).

extraction (abbreviated by 'ES'), the full-fat lupin flakes were suspended in 0.5 mol L^{-1} NaCl solution (ratio flakes:salt-solution 1:8). For all protein isolates prepared by a combination of alkaline and salt-induced extraction (abbreviated by 'EAS'), the full-fat lupin flakes were suspended in 0.5 mol L^{-1} NaCl solution (ratio flakes: salt-solution 1:8). This suspension was adjusted to pH 8 with 0.5 mol L^{-1} NaOH. All suspensions were stirred for 1 h at room temperature. After the individual extraction processes all suspensions were separated as described for ILP and MLP. The supernatant of all protein isolates precipitated isoelectrically (abbreviated by 'PI') was acidified to pH 4.5 with 0.5 mol L^{-1} HCl. The supernatant of all protein isolates yielded by dilutive precipitation (abbreviated by 'PD') was diluted with DI water at room temperature at a ratio of 1:3 (v/v) while stirring continuously. The supernatant of all protein isolates precipitated by a combination of isoelectric and dilutive precipitation (abbreviated by 'PDI') was first diluted with DI water (ratio 1:3 (v/v)) before adjusting the pH to 4.5 with 0.5 mol L^{-1} HCl. After the individual precipitation procedures, all crude protein suspensions were left for 18 h at 1 °C, the precipitates were treated as described above, and the final protein isolates were stored at −20 °C for a minimum of 24 h until microscopic analysis or lyophilised for chemical analyses. Table 1 reports the process steps for production of each protein isolate.

2.3. Chemical analysis

The protein content in all protein isolates was calculated based on the nitrogen content (N × 5.7) determined according to the Dumas combustion method, AOAC method 968.06 (AOAC International, 1990c), using a Nitrogen Analyser FP 528 (Leco Corporation, St. Joseph, MI, USA). The total fat contents were measured in a fat determination system (B-815/B-820, Büchi Labortechnik AG, Flavil, Switzerland) according to Caviezel following the method of DGF K-I 2c (00) (2004). This method comprised fat determination by gas chromatography after extraction in *n*-butanol and saponification using potassium hydroxide pellets (Pendl, Bauer, Caviezel, & Schulthess, 1998). For determination of the dry matter the samples were dried to weight constancy at 105 °C in a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) according to the AOAC method 925.10 (AOAC International, 1990b). The ash contents were determined in the same system by combustion at 950 °C until weight constancy according to AOAC method 923.03 (AOAC International, 1990a). Protein, fat and ash contents were reported on a dry matter basis.

2.4. Rapid lignin test and quantitative determination of acid-insoluble lignin

To screen lignin out of being responsible for autofluorescence behaviour of ILP, the presence of lignin was determined in both protein isolates ILP and MLP, respectively. Qualitative occurrence of lignin in the lupin protein isolates was investigated with phloroglucinol-HCl staining (Speer, 1987). Approximately 0.2 g ILP and MLP, respectively, were spread across microscope slides and 2 mL phloroglucinol solution [5% (w/v) phloroglucin, 50% (v/v) ethanol (99.9%) and 50% (v/v) hydrochloric acid (37% w/w)] was added to each protein isolate. After incubation for 15 min the microscope slides were washed briefly in DI water. As a control ILP and MLP were treated the same way but without phloroglucinol addition. Photographic documentation and colour analysis were performed with the DigiEye system including DigiEye Software 2.53b from VeriVide Ltd. (Leicester, United Kingdom). For the evaluation of the red staining Δa^* values were calculated by subtracting the a^* value (redness) of the control from the a^* value of the sample treated with phloroglucinol.

Quantitative determination of acid-insoluble (Klason) lignin (AIL) was performed using a Fibre Therm Analyser (FIBRETHERM FT 12, C. Gerhardt GmbH & Co.KG, Königswinter, Germany) following the standardised ASTM method D-1106 (ASTM Standard, 2000). The lignin contents were finally determined gravimetrically in a 5-fold determination for ILP and a 6-fold determination for MLP and were reported to the dry matter of each protein isolate.

2.5. Optical microscopy of protein suspensions

The precipitation procedures to simulate the production of ILP, MLP and a combination thereof, were reproduced under an Olympus AX70 Provis optical microscope (Olympus Optical Co. Europa GmbH, Hamburg, Germany). The required amounts of precipitation media (HCl and H_2O) for the reaction under the microscope were converted according to the proportions in the laboratory scale. Therefore, 1 µL of 0.05 mol L^{-1} HCl for isoelectric precipitation or 4 µL of DI water for dilutive precipitation was added to 2 µL of each corresponding extract. All samples were prepared on a microscope slide at room temperature, covered with a coverslip and snapshots of 1000× magnification were taken after 5 s incubation. Digital images were documented using the documentation software U-MCB.

2.6. Image analysis

Micrographs of MLP obtained with optical microscopy were analysed using ImageJ software (ImageJ 1.44p, USA) to estimate the size distribution of protein micelles. Standardised modifications of the images comprised formation of an 8-bit image, preset background correction, conversion to a black-and-white image using the appropriate threshold and removal of 2.0 pixel outliers in black and white, respectively.

2.7. Reflected-light fluorescence microscopy of ILP and MLP

About 0.5 g of the protein isolate ILP or MLP was mounted onto a microscope slide, coated with anti-fade mounting medium AF2 and covered with a cover slip. To localise the protein isolate, a bright field image was taken at the edge of the sample. Then, fluorescence records of ILP and MLP were taken at the same position of the sample with 40-fold magnification and an exposure time of 200 ms to identify autofluorescence areas. Excitation wavelengths and standard filters designed for Cy™2 (λ_{exc}: ~440–505 nm, max.: 489 nm; λ_{em}: ~490–506 nm, max.: 506 nm), Cy™3 (λ_{exc}: ~500–575 nm, max.: 550 nm; λ_{em}: ~550–570 nm, max.: 570 nm), AMCA (λ_{exc}: ~305–385 nm, max.: 345 nm; λ_{em}: ~345–441 nm, max.: 441 nm) and Cy™5 (λ_{exc}: ~595–685 nm, max.: 646 nm; λ_{em}: ~645–700 nm, max.: 664 nm) were used. The acquisitions were performed with a fluorescence microscope (BX61WI, Olympus Optical Co., Hamburg, Germany) and digital pictures were taken with a high-resolution black-and-white 12-bit monochrome CCD camera (F-View II, Soft Imaging System, Münster, Germany). Images of the protein isolates were analysed using Olympus CellP Software.

2.8. Cryo-scanning electron microscopy of ILP and MLP

About 5 mg of the protein isolate ILP or MLP was mounted onto a sample carrier and plunged into slush nitrogen at atmospheric pressure (−210 °C). To study the microstructure, the frozen specimen was transferred to a Polaron SC 7600 preparation stage (Quorum Technologies Ltd, U.K.) and freeze-fractured at −150 °C within high vacuum. The surface water was slightly sublimated at −80 °C for 20 min and the fractured sample then sputter coated at −150 °C with 5 nm of platinum. The platinum coated sample was transferred into a scanning electron microscope (Zeiss SUPRA 40VP, EMITECH K1250X) using the transportable cryo stage and observed with 5 kV accelerating voltage maintaining the sample below −130 °C.

2.9. Statistical evaluation of the data

Values in the tables are given as mean values and standard deviations. For chemical analysis (protein, fat, ash, dry matter and lignin) a number of 3–6 experiments were conducted. The precipitation behaviour of proteins monitored by light microscopy was approved by a 3-fold repetition rate. Image analysis determining the particle size distribution was performed with a number of 16 images. The number of experiments was abbreviated by an "n". Three or more corresponding groups with each comprising a minimum of three individual values were compared by analysis of variance (ANOVA, $\alpha = 0.05$) with subsequent testing of significant differences by means of the Scheffé-Test using the software WinSTAT® (Microsoft, USA).

The Feret diameter of the micelles in MLP determined with ImageJ software was used for determination of particle size distribution. The data is presented as cumulative distribution (Excel®, Microsoft, USA).

3. Results and discussion

Prior to the microscopic studies, the chemical composition of the protein isolates was investigated, since the occurrence of other ingredients might influence their microscopic behaviour. Water, protein, fat and ash contents of each protein isolate were determined. Beginning from a macroscopic overview, deeper insights into the structure were provided by fluorescence and cryo-scanning electron microscopy. Particular attention was paid to the mechanism of protein structure formation according to each precipitation procedure.

Table 2
Chemical composition of lupin flakes (raw material) and protein isolates from *Lupinus angustifolius* L. Vitabor.

Sample	Code designation	Dry matter [%]	Protein* [%]	Ash* [%]	Fat* [%]
0	Lupiri flakes	67.0	43.4	4.2	9.5
1	ES-PD (MLP)	44.4 ± 0.6	95.1 ± 0.6^{a}	1.3 ± 0.3^{a,b,c}	3.2 ± 0.2^{a}
2	ES-PI	38.7 ± 0.8	91.3 ± 0.7^{b,c}	1.8 ± 0.5^{a,b,c,d}	4.8 ± 02^{b}
3	ES-PDI	50.1 ± 0.7	94.4 ± 0.4^{a,d}	2.0 ± 0.1^{c,d}	3.0 ± 0.1^{a}
4	EAS-PD	–	–	–	–
4a	EAS-PD5.5	43.1 ± 0.7	95.7 ± 0.9^{a}	1.2 ± 0.1^{a,b}	3.6 ± 0.8^{a}
5	EAS-PI	40.6 ± 0.3	92.9 ± 0.4^{c,d}	1.9 ± 0.3^{b,c,d}	3.7 ± 0.4^{a}
6	EAS-PDI	41.6 ± 1.8	95.1 ± 0.6^{a}	1.3 ± 01^{a}	3.6 ± 0.1^{a}
7	EA-PD	–	–	–	–
7a	EA-PDS.5	44.2 ± 1.0	94.9 ± 0.6^{a}	1.1 ± 0.1^{a}	5.0 ± 0.5^{b,c}
8	EA-PI (ILP)	43.1 ± 1.0	90.2 ± 0.9^{b}	2.5 ± 0.0^{d}	5.5 ± 0.1^{b,c}
9	EA-PDI	44.2 ± 1.1	90.7 ± 1.1^{b}	2.4 ± 0.1^{d}	5.7 ± 0.7^{c}

ES = salt-induced extraction, EAS = combined salt-induced and alkaline extraction, EA = alkaline extraction, PD = dilutive precipitation, PI = isoelectric precipitation, PDI = combined dilutive and isoelectric precipitation, 5.5 = protein isolates adjusted to pH 5.5. Different letters (a, b, c, d) indicate that mean values in the same column are significantly different ($P < 0.05$). n = 3-5.

* Values are related to dry matter; determination of protein content using lupins' specific N-factor of 5.7.

3.1. Influence of the isolation procedure on chemical composition of lupin protein isolates

Moisture, protein, ash and fat contents of the lupin flakes used for protein isolation and all deriving protein isolates are summarised in Table 2.

At first, only seven protein isolates out of nine possible combinations resulting from the three extraction (salt-induced, alkaline, and combined) and three precipitation modes (dilution, pH adjustment, and combined) were obtained. A protein precipitation after dilution of the alkaline protein extracts no. 4 (EAS-PD) and 7 (EA-PD) could not be observed, probably because of the high solubility of the lupin proteins at pH 8 present during precipitation. In contrast, a pH of 5.5 resulted for MLP after dilutive precipitation. Therefore, the pH of both mentioned protein isolates was gently adjusted to pH 5.5 prior to dilution, as described in the Experimental, Section 2.2.3, to equalise the conditions to the dilutive precipitation. Since the pH had an important impact on structure formation of the proteins, the step of pH adjustment to the typically occurring pH of MLP was important to facilitate the precipitation, resulting in a total of 9 protein isolates, as shown in Table 2. Because of the similarity in production of the protein isolates 4 and 7 the two adjusted protein isolates were named 4a (EAS-PD5.5) and 7a (EA-PD5.5).

The protein isolates showed protein contents between 90.2 and 95.7% based on dry matter, whereas the lupin flakes contained 43.4% of protein, justifying the protein isolation procedure. In contrast, the ash content was higher in the raw material (4.2%) as compared to the protein isolates (1.1 to 2.5%). Fat contents of the protein isolates ranged between 3.0 and 5.7% and were lower as of the lupin flakes (9.5%). Protein isolates 1 (ES-PD = MLP) and 3 (ES-PDI) revealed the lowest fat-contents of 3.2 and 3.0%, respectively. This seemed to be affected by the combination of salt-induced extraction and a dilutive precipitation, as both protein isolates were extracted in NaCl solution and precipitated after reducing ionic strength with water. Unexpectedly, ES-PI, which was also salt-induced extracted but precipitated at the isoelectric point, exhibited significantly higher fat content. We assume that the lack of dilution might be responsible for the remaining lipid compounds. However, this phenomenon needs more experiments and could not be explained entirely up to now.

In contrast, extraction in alkaline solution (samples 7a, 8 = ILP and 9) resulted in products with the highest fat-contents (5.0 to 5.7%) independent of the precipitation parameters. Alkaline aqueous media

are not able to extract lipid compounds, but in the presence of NaOH the fat components can be saponified and form emulsions (Rosenthal, Pyle, & Niranjan, 1996), which consequently might be partially included in the protein structure. Thus, fats of the lupin seed possibly may have been saponified during steady mixing of the "flakes-water-NaOH-conglomerate" for 1 h in the extraction step. As a consequence, these emulsions may have emulsified more fats into the aqueous phase and thus may have increased the total fat content of the protein isolates.

The lower protein and higher ash contents in ILP, as compared to the other protein isolates, were probably caused by the addition of NaOH during extraction and HCl during precipitation facilitating the formation of NaCl, which remained in the final product (El-Adawy et al., 2001). The salt content had an important impact on the electrostatic forces within the proteins and will be discussed in detail in the light microscopy part below, Section 3.2.1.

Comparing solely the procedures of ILP and MLP isolation, it becomes obvious that the alkaline extraction and isoelectric precipitation (ILP) resulted in a product with lower protein content than the salt-induced extraction and dilutive precipitation (MLP). This goes in line with the results obtained by Paredes-López, Ordorica-Falomir, and Olivares-Vazquez (1991) for chickpea protein isolates and by Rahma et al. (2000) for mung bean protein isolates. Paredes-López et al. (1991) postulated that the micellar protein isolates have higher protein contents due to the described favoured protein–protein association when ionic strength of the extracted sample was reduced. As no differences have been found, when EA, EAS or ES processes were applied, the final pH 5.5 appeared as the major controlling factor for protein content in the lupin protein isolates. In a study from El-Adawy et al. (2001), however, the opposite effect was observed with the isoelectric protein isolate exhibiting higher protein contents, as compared to the micellar protein isolate. This might be due to slight differences in the isolation procedure, as El-Adawy and co-authors used Na_2SO_3 during alkaline extraction for preparation of the isoelectric and 1 mol L^{-1} instead of 0.5 mol L^{-1} NaCl for salt-induced extraction for preparation of the dilutive protein isolate. As shown in a study from Sussmann et al. (2011) salt concentration in the extract has an important impact on protein yield, with 0.5 mol L^{-1} NaCl showing the highest protein extractability of lupin proteins from *L. angustifolius* L, investigated in the present study.

ILP belonged to the protein isolates with lowest protein contents while MLP showed the highest protein content. Examining all protein isolates, it appears that the precipitation procedure is the key for regulating the protein content. The protein content was higher in protein isolates precipitated by dilution in water (samples 1, 4a and 7a; 94.9–95.7%) compared to the protein isolates produced by isoelectric precipitation (samples 2, 5 and 8; 90.2–92.9%). The higher solubility of ingredients under alkaline conditions and co-precipitation during isoelectric precipitation could be an explanation for the lower protein content of ILP.

In conclusion, the protein contents of all protein products investigated in this study were higher than 90% indicating a protein isolate in contrast to a protein concentrate with about 70% of protein (Doxastakis, 2000). Furthermore, these high protein contents emphasise that microscopic figures of the protein isolates demonstrated in the following are primarily formed by the naturally present protein.

3.2. Influence of the isolation procedure on macroscopic and microscopic characteristics of the lupin protein isolates

Photographic records of the nine freshly produced protein isolates were taken immediately after isolation and are presented in Fig. 1. Only MLP (sample 1, ES-PD) showed an oily and highly viscous texture. All the other protein isolates were rough and friable and exhibited an appearance similar to that of ILP (sample 8, EA-PI).

3.2.1. Optical microscopy of protein suspensions

After extraction of lupin flakes in aqueous alkaline and in NaCl solution the individual protein precipitations were investigated "on-line" with a light microscope and are itemised in Fig. 2. The precipitation behaviour of the proteins extracted by means of alkaline extraction is shown in the upper row (B, C) and those of the salt-induced protein extract in the lower row (b, c).

All nine protein isolates were investigated by light optical microscope (micrographs not shown). After pH adjustment to pH 4.5 for the isoelectric precipitation the protein aggregates showed large-area and disordered structures, as indicated upon precipitation in Fig. 2C. Combining the salt-induced extraction with both the dilutive and the isoelectric precipitation resulted in a mixture of micelles and extended unfolded protein areas partially incorporating the micellar aggregates, as could be initially observed during the precipitation, Fig. 2c. Only extracts previously extracted with NaCl solution showed globular structures after dilutive precipitation, corresponding to the micelle formation visualised in Figs. 2b and c.

Besides some small air bubbles both, the alkaline extract (Fig. 2A) and the salty extract (Fig. 2a), respectively, containing the extracted proteins did not show any particles. After the addition of water to the alkaline extract, no change was observed (Fig. 2B), whereas a sudden and extensive development of globular precipitates up to 6 μm in diameter was observed in the salty extract (Fig. 2b = MLP). Indeed, the precipitation demonstrated in Fig. 2B corresponds to the isolation procedure no. 7 without pH adjustment, corroborating the results presented before (cf. Section 2.2.3, Table 1). The alkaline extract showed irregular protein aggregates after the combined precipitation with the acid solution (Fig. 2C = ILP). Presumably, intermolecular hydrogen bonds have been repelled irreversibly by the addition of HCl and the protein structures consequently have been unfolded. Similar observations were proven in the salty extract combining the dilution and isoelectric precipitation techniques; Fig. 2c presents a mixture of isoelectrically generated irregular protein aggregates and again a large number of protein structures arranged in micellar entities up to 2 μm in diameter due to the water insertion (Fig. 2c = intermediate isolate, combined precipitation).

The typical emergence of these ordered spherical structures of lupin proteins was visually demonstrated for the first time. The fact that micelle formation always requires the preliminary application of salt during extraction emphasises the ionic strength as an important milieu factor. Therefore, the salt content in the protein isolates is assumed to contribute to the physico-chemical structure differences of the protein isolates ILP and MLP, respectively (El-Adawy et al., 2001). Indeed, ILP showed the highest ash percentage (Section 3.1, Table 2) reflecting the high final NaCl content resulting from the isolation procedure applying both, NaOH and HCl. The differences in NaCl content influence the ionic strength and electrostatic forces within the proteins which, in turn, are important mediators for association properties of proteins (Rodriguez-Ambriz et al., 2005).

The rapid reduction of a certain ionic strength shows to be a prerequisite for dilutive precipitation and micelle formation, respectively. The ions of sodium chloride in solution (Na^+ and Cl^-) during extraction are supposed of being attached to the charged counterions of the peptide chains. As a result, the electrostatic protein–protein interactions would be diminished leading to dissolved protein chains. This increase in protein solubility in low molar salt concentrations (0.5 to 1.0 M) is based on the salting-in effect, which can be rated using the Hofmeister series (Der, 2008). When the ionic strength is reduced for dilutive precipitation the dissolved protein-salt aggregates undergo a set of dissociation reactions to conform to the new milieu conditions with the salt content near 0 mol L^{-1}. A rapid decrease in molecular weight of the incoherent protein aggregates and formation of numerous new uniform low-molecular protein–protein associations is the consequence (Murray, Myers, Barker, & Maurice, 1981). This effect could be spotted during the dilutive precipitation (Fig. 2b).

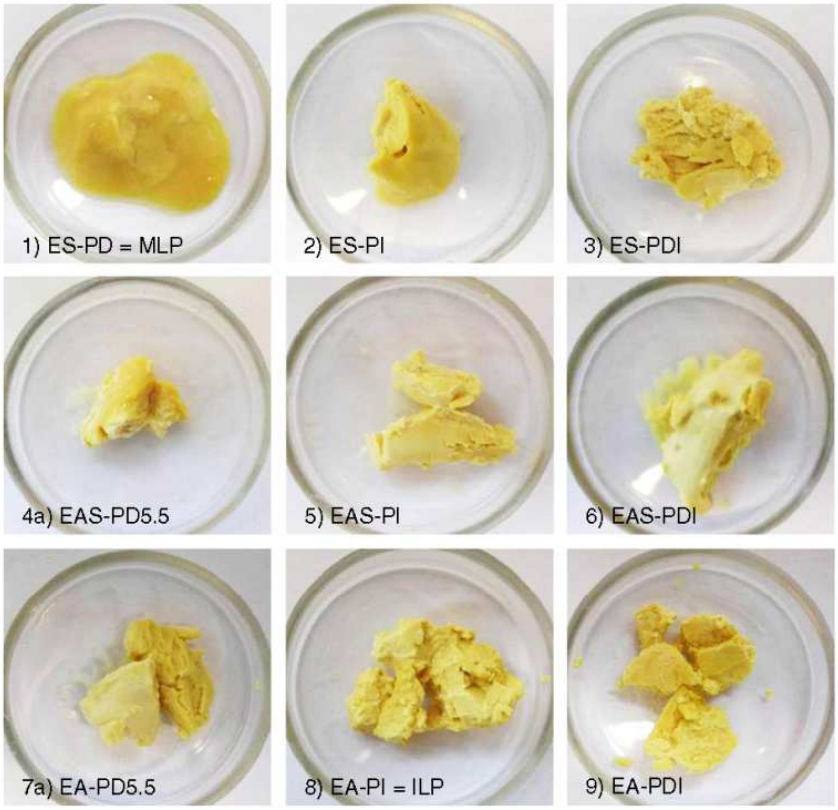

Fig. 1. Photographs of the nine different protein isolates of *Lupinus angustifolius* L. for optical comparison. ES = salt-induced extraction, EAS = combined salt-induced and alkaline extraction, EA = alkaline extraction, PD = dilutive precipitation, PI = isoelectric precipitation, PDI = combined dilutive and isoelectric precipitation, 5.5 = protein isolates adjusted to pH 5.5.

Besides the ionic strength, the hydrophobic effect is assumed to influence the three-dimensional structure of the proteins. The pH of MLP after dilutive precipitation naturally sets at 5.5 which is close to the isoelectric point (pH 4.5) of the lupin seed proteins from *L. angustifolius* L. (Ruiz & Hove, 1976). Therefore, at this pH during dilutive precipitation the electric net charges of the proteins are assumed to be at their minimum and electrostatic attraction maximised. Consequently, water molecules are excluded, thus, improving protein–protein bonds and facilitating protein arrangement. The sum of hydrophobic and hydrophilic residues pursues a

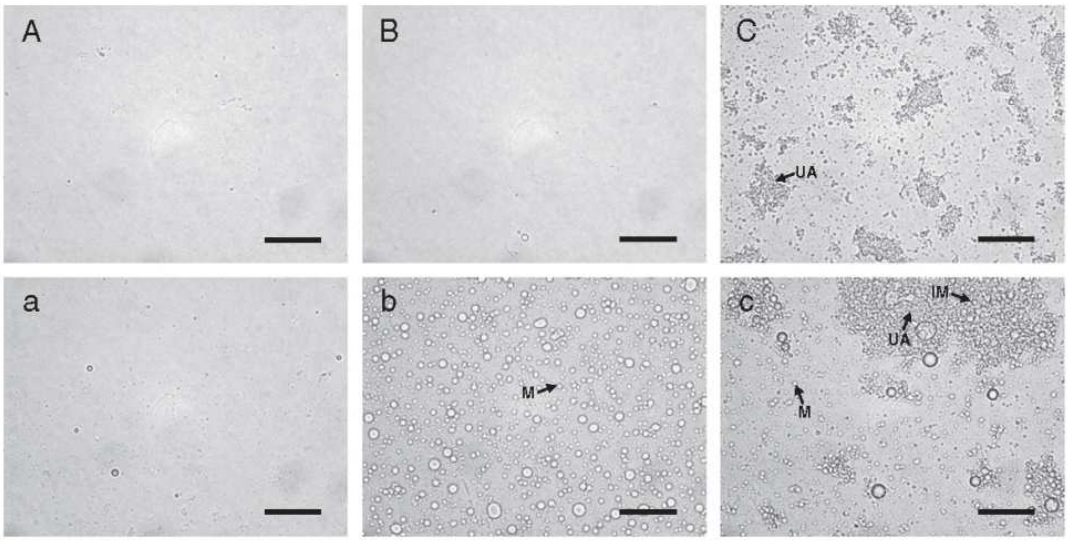

Fig. 2. Precipitation behaviour of proteins monitored by light microscopy. Upper row (upper-case letters) = alkaline extract and precipitation from alkaline extracts; lower row (lower-case letters) = salt-induced protein extract. (A, a) Extracts without any additions; (B, b) extracts after addition of DI water (1:3 dilution); (C, c) extracts after addition of diluted HCl solution = combined dilutive and isoelectric precipitation; the scale bars correspond to 25 μm for all images.

thermodynamically stable orientation. Self-aggregation to thermodynamically favourable associations inside an aqueous environment as a result of a specific hydrophilic–hydrophobic balance, and equilibrium of repulsive and attractive forces is suggested of being responsible for the generation of a micellar response (Tanford, 1980). In an aqueous environment hydrophobic moieties are likely to be concentrated in an inner core of those micelles, whereas hydrophilic residues are in contact with the aqueous surrounding medium. This mechanism acts against the energetically unfavourable exposure of hydrophobic residues to water (Fisher & Oakenfull, 1977). The amphiphilic protein micelles observed after dilutive precipitation in this study are, therefore, supposed to exhibit a hydrophilic hull encapsulating hydrophobic protein domains in the core. A well—known example for this type of protein micelles of animal origin is the casein micelle (Corredig, Sharafbafi, & Kristo, 2011).

Proteins generally form sub-particles or are arranged in supramolecular structures consisting of sub-particles depending on protein origin, concentration, size and milieu conditions. The sub-particles are created by a limited number of monomers via hydrophobic forces and hydrogen bonds contrary to the electrostatic charge repulsions (Ismond et al., 1990). Reaching a critical protein concentration, proteins are able to build aggregates in micellar forms.

Legumin-like 11S and vicilin-like 7S proteins, i.e. α- and β-conglutin, respectively, are the main storage proteins of lupin seeds (Nadal, Canela, Katakis, & O'Sullivan, 2011). Legumin and vicilin themselves are known to build spherical aggregates. The size of those aggregates and the degree of coalescence were observed to decrease with pH increase from 6.0 to 8.0 (Ismond, Arntfield, & Murray, 1991), which would corroborate the fact that we could not observe micelle structures upon dilution at pH 8, whereas the dilution at pH 5.5 leads to micelle formation. The globular structure of legumin-like proteins is stabilised by hydrophobic interactions (Plietz, Drescher, & Damaschun, 1987). The complex equilibrium of local attractive and repulsive forces stabilises mostly the globular structure of the storage proteins and is the precondition for a micellar arrangement. The concomitance of a small quantity of electrostatic repulsion at pH 5.5 may have permitted the still flexible proteins to rearrange in this compact vicilin and legumin-like oligomeric structure. Finally, the lupin proteins were able to reorganise in the thermodynamically favourable ordered micellar agglomerates.

In contrast, the extreme conditions of pH adjustment during production of ILP might have led to a lack of appropriate reorientation of the lupin proteins. Cordero-de-los-Santos et al. (2005) reported a dependency of the denaturation properties on the isolation procedure. Amaranth protein isolates produced by micellisation showed lower protein denaturation as amaranth protein isolates produced by isoelectric precipitation. Presumably, the isoelectrically precipitated lupin proteins have been denatured to higher extents during the isolation process and might therefore not be flexible enough to rearrange into the energetically favourable micelle form.

Please notice that incubation time and -temperature of this micellar "on-line" product precipitated under the optical microscope (Fig. 2b) varied from the parameters used for the product produced at laboratory scale (cf. Section 2.2.3, Table 1). The micelle sizes might therefore differ in both protein products, i.e. before and after an incubation time (see Experimental, Section 2.2.3). The focus of the present chapter was on the mechanism of the micelle formation, whereas all further investigations described in this paper dealt with the characterisation of the micelles inside the final product produced at laboratory scale.

3.2.2. Statistical size distribution of micelles in micellar lupin protein isolate (MLP)

The micelle size distribution of light micrographs from MLP produced at laboratory scale (figures not shown) was analysed with ImageJ software and is presented in Fig. 3.

To visualise the data without artefacts, the size intervals in the range between 0.3 and 5.0 μm were chosen. Because of the low resolution in optical microscopy, particles below 0.3 μm might have represented definition lack pixels and were therefore excluded for graphic account. The total particle sizes reached a maximum of 17.4 μm, however, the range from 5.1 to 17.4 μm accounted for less than 5% of the overall statistical distribution.

The particle sizes showed two maxima, at 0.6 and 0.8 μm, each of which accounted for 7% to the total particle size distribution. The majority of the particles showed to be smaller, than 1.5 μm (56%). Based on the visual evaluation of the images obtained with light microscopy, formation of larger agglomerates by assembling of micelles was excluded. The particle size of 0.3 to 1.5 μm is therefore assumed to be the most energetically favourable and therefore predominant micelle form.

3.2.3. Upright wide-field fluorescence microscopy of ILP and MLP

Fluorescence microscopic investigations of both protein isolates ILP and MLP, respectively, showed fundamental differences. Autofluorescence could only be detected in ILP and not in MLP at excitation wavelengths from 345 nm to 550 nm. The sample position was optically selected in the bright field mode at the edge of the sample facilitating the precise identification of fluorescence active areas. The areas filled

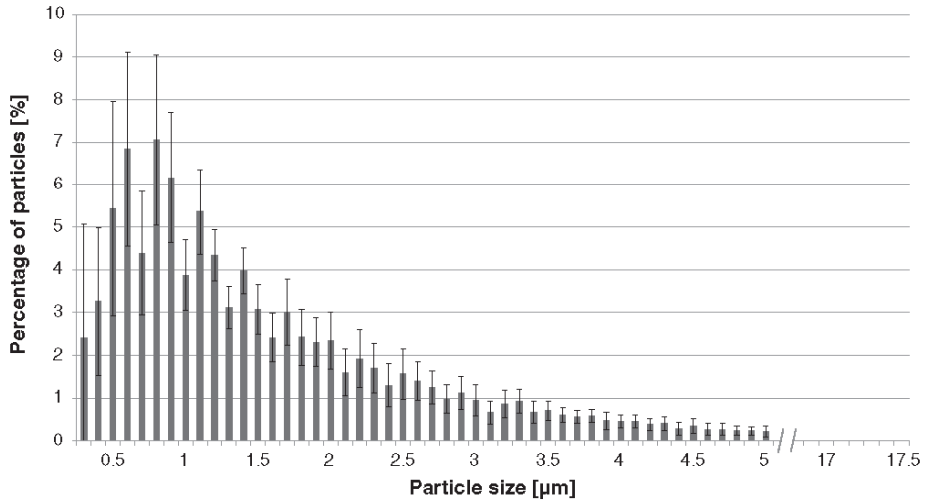

Fig. 3. Statistical particle size distribution [%] of the micelles in micellar lupin protein isolate (MLP) light micrographs (n = 16) using the statistical evaluation software ImageJ.

with the isoelectric protein isolate showed uniform autofluorescence. At an excitation wavelength of 673 nm, both protein isolates did not exhibit fluorescence behaviour. The highest fluorescence emission of ILP was detected with the AMCA channel (λ_{exc} max: 345 nm, λ_{em} max: 441 nm), followed by Cy3 channel (λ_{exc} max: 550 nm, λ_{em} max: 570 nm) and finally with Cy2 channel (λ_{exc} max: 489 nm, λ_{em} max: 506 nm). The corresponding Fluorescence micrographs of ILP are shown in Fig. 4.

The difference in autofluorescence behaviour of both protein isolates was probably based on the different microstructures and in consequence different abilities of light to be absorbed and emitted by fluorescence active amino acid side chains, such as tryptophan.

Emission wavelengths of tyrosine and phenylalanine are below the lowest analysed emission wavelength of 345 nm (tyrosine: λ_{em} max: 303 nm, phenylalanine: λ_{em} max: 285 nm). Only tryptophan (λ_{em}: 340–370 nm) shows a fluorescence emission after excitation within the violet–blue wavelength investigated amongst others (Timperman, Oldenburg, & Sweedler, 1995). However, the chemical composition seems not to be the reason for the differences in fluorescence. As investigated by El-Adawy et al. (2001) comparable percentages of fluorophoric compounds such as tryptophan and phytic acid were present in both, an isoelectric (0.88%) and a micellar protein isolate (0.74%) produced from the white lupin variety *Lupinus albus* L. These observations militate against the fluorophore contents being responsible for the strong differences in intrinsic fluorescence.

Presence of lignin deposited in the cell walls of the lupin kernel as well as the hull is already well documented (Evans, Cheung, & Cheetham, 1993; Glencross et al., 2008); however, occurrence of lignin in protein isolates has not been determined so far. Depending on the various types of lignin-derivatives, lignin is known to exhibit maximum autofluorescence from λ 350 nm to 380 nm (Albinsson, Li, Lundquist, & Stomberg, 1999) and could therefore be responsible for the strong violet–blue fluorescence emission demonstrated in Fig. 4B. To prove this assumption first a qualitative rapid lignin test and later on a quantitative determination of Klason lignin was carried out as well. Both results are presented further down in this section. It should be noted that both, tryptophan and lignin, show autofluorescence emission in the violet–blue spectra. An additive effect could therefore be the reason for the strong fluorescence observed at the corresponding wavelengths. However, a positive lignin staining was observed for both protein isolates ILP and MLP, respectively. The intensity of the red staining being an indicator for the quantity of lignin present, showed a minor concentration of red staining in both protein isolates, though, indicating even a lower lignin content in ILP ($\Delta a^* = 4.15$) than in MLP ($\Delta a^* = 4.38$). The quantitative lignin test by the fibre bag method confirmed the presence of lignin in both protein isolates with MLP exhibiting significantly higher lignin contents ($3.95 \pm 1.67\%$) as compared to ILP ($0.11 \pm 0.07\%$). However, only minor total lignin contents were detected. In summary, the very small lignin contents might possibly contribute to the autofluorescence observed in ILP, however, the marked difference in fluorescence behaviour of both protein isolates cannot be explained by the occurrence of lignin since this would have the opposite intensity effect.

Small quantities of fat, present in both protein isolates (Section 3.1, Table 2), indicate that concomitant constituents, such as lipophilic compounds, might be present and could contribute to measured yellow autofluorescence (Nagovitsyn, Chudinova, Savranskii, & Komissarov, 2004). Carotenoids are known to show fluorescence quenching of chlorophyll. Besides the effect of fluorescence reduction a shift of detected fluorescence wavelength is a characteristic (Karapetyan, 2008). To confirm these results, more detailed investigations are required.

Since MLP showed no fluorescence emission at all, it seems that structural features of the protein isolates were the main cause for the differences in autofluorescence and not the chemical composition of the protein isolates ILP and MLP. The fluorescent side chain of the amino acid tryptophan, the other mentioned naturally occurring fluorophores, such as phytic acid and lignin, or simply fluorescent particles with delocalised electrons inside the macromolecular

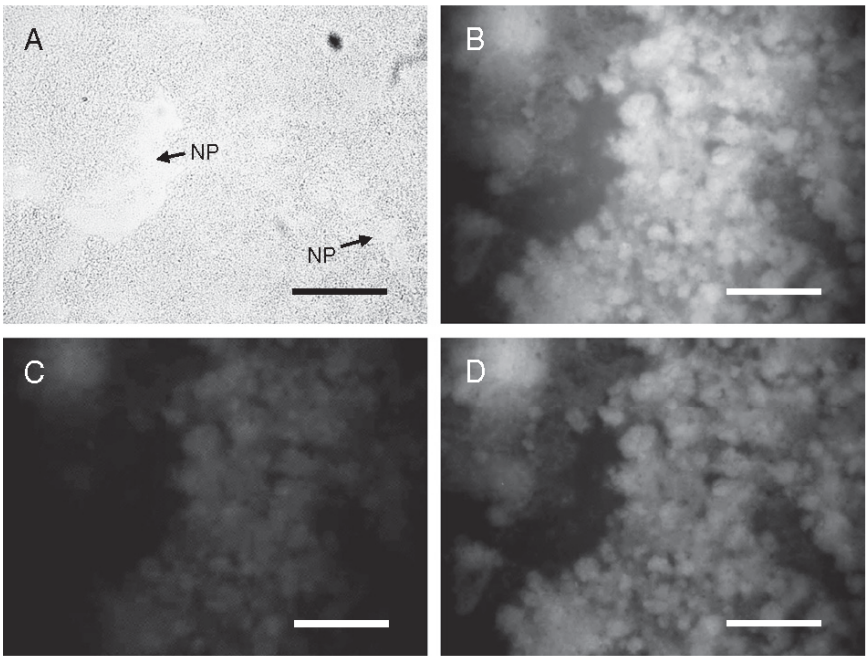

Fig. 4. Fluorescence micrographs of isoelectric lupin protein isolate (ILP). A) bright-field illumination; B) very strong violet–blue autofluorescence (λ_{em}: 345–441 nm) with the AMCA channel at λ_{exc} max: 345 nm; C) slight green autofluorescence (λ_{em}: 490–506 nm) with the Cy2 channel at λ_{exc} max: 489 nm; D) strong yellow autofluorescence (λ_{em}: 550–570 nm) with the Cy3 channel at λ_{exc} max: 550 nm; no fluorescence was observed with the Cy5 channel (λ_{em}: 645–700 nm) at λ_{exc} max: 646 nm (image not shown). Areas with no protein (NP) are designated with an arrow. Scale bars represent 200 μm.

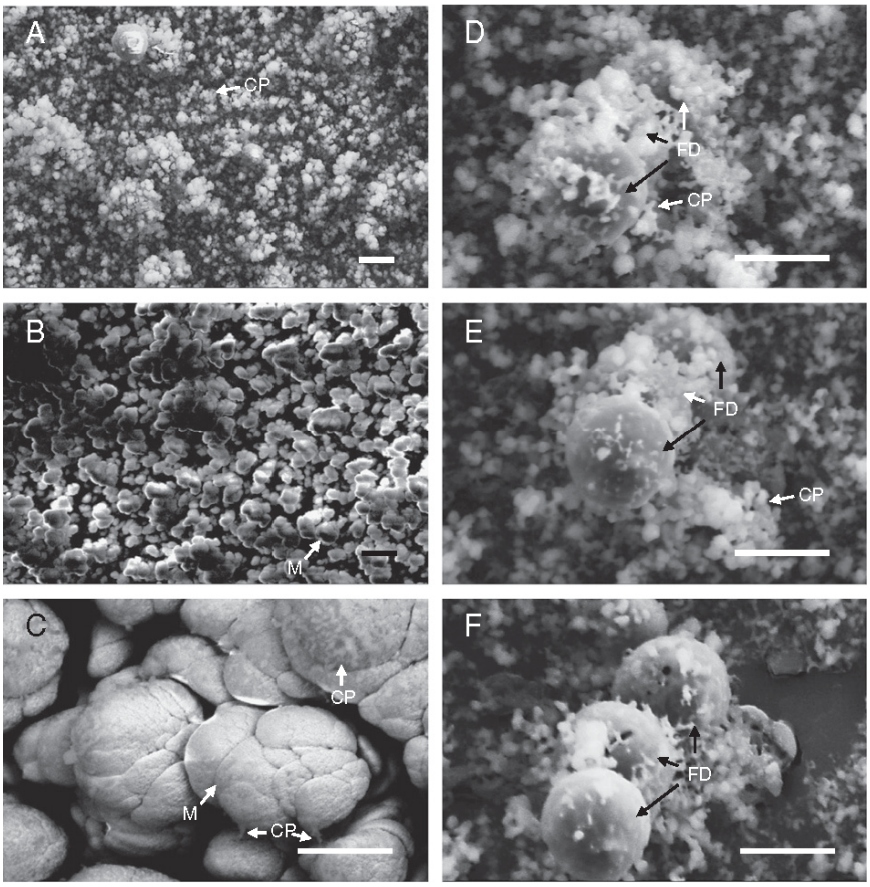

Fig. 5. Cryo-SEM images of isoelectric lupin protein isolate (ILP: A, D, E, F) and micellar lupin protein isolate (MLP: B, C). Except image C all micrographs are taken after freeze-fracture, showing therefore the aqueous interior of the protein isolates. Image C shows MLP at the sample surface, therefore at the interface between the aqueous interior and the hydrophobic air phase. Images D, E and F show a progressive uncovering of oil droplets in ILP during cryo-SEM investigation, with D) t = 0 min, E) t = 1:37 min and F) t = 4:27 min. Cross-linked protein structures (CP), micelles (M) and fat droplets (FD) are designated with an arrow. All scale bars represent 2 μm.

protein geometry, might be exposed in ILP because of its unfolded structure and may lead to the strong autofluorescence of ILP. In contrast, the same fluorescent molecules in MLP might be excited by the light; however, emission may be quenched because of the compact and imbedding globular structure of MLP resulting in no fluorescence.

3.2.4. Cryo-scanning electron microscopy of ILP and MLP

Cryo-scanning electron micrographs of ILP and MLP are shown in Fig. 5. All images, except Fig. 5C, were taken after freeze-fracture, thus, showing the aqueous interior of the sample.

Fig. 5A and B shows both, the particle size distribution previously analysed by light microscopy (cf. Sections 3.2.1 and 3.2.2, Figs. 2 and 3) and the surface structure of ILP and MLP, respectively analysed by cryo-scanning electron microscopy. After freeze-fracture ILP showed cross-linked and superfine protein structures (Fig. 5A). In contrast, separate colloidal protein aggregates from 0.3 μm to 1 μm were observed in MLP (Fig. 5B).

In addition to the images taken within the aqueous interior, an additional one was taken at the surface of the MLP isolate. Fig. 5C shows the surface of MLP in contact with the air phase (no freeze-fracture during sample preparation). The globular structure observed in Fig. 5B could be confirmed; however, larger connected protein globules from approximately 3.5 μm up to extended spherical chains (Fig. 5C) were detected, confirming the still flexible behaviour of the MLP proteins acting into an energetically favourable form of a connected barrier against the hydrophobic air phase.

At the interface of the hydrophilic protein environment and the hydrophobic air phase the previously described hydrophilic–hydrophobic balance for micelle formation may have broken down. As a consequence, the globular protein arrangements may have flattened out and built a more continuous "protein layer". On top of the surface, the protein globules showed some cross-linked protein structures similar to "tiny hairs". These cross-linked protein structures probably indicate a partial protein unfolding, which locally might has caused a loss of protein flexibility.

During microscopic investigation it was considerably more difficult to focus ILP than MLP. The embossed knolls of ILP visible in Fig. 5A represented some fat droplets overlaid with the protein. Already within a short time the electron ray treatment of ILP with 5 kV leads to a marked damage of the protein isolate. Three successive cryo-SEM micrographs of ILP after exposure times of 0 min, 1:37 min and 4:27 min, respectively, are presented in Fig. 5D, E and F, showing a progressive uncovering of an oil droplet. This sensibility to the electron rays made it impossible to determine an exact size of the protein aggregates. In contrast, a modification of MLP could not be observed even after an electron irradiation for 30 min at the same voltage. The compact globular structures of MLP possibly may protect the proteins from external impacts, such as the electron rays and heat, to a certain extent. However, the previously described "tiny hairs" on top of the protein micelle surface were as well sensible to the electron rays and decreased after electron irradiation of 1 to 2 min. Possibly these fragile "protein hairs" have a similar behaviour to those in ILP protruding from the flexible arranged protein micelles due to a denatured character.

4. Conclusions

The interaction of process parameters, in particular pH and salt-content, and structure formation of lupin proteins were elaborated. Throughout this study isoelectric precipitation was characterised by an unfolding of protein molecules, whereas formation of regular spherical structures was typical for the dilutive micellisation procedure; however the latter always required a preliminary salt-induced extraction. Both characteristic protein formations — emphasised by optical microscopy — are therefore able to serve as an indicator for previous protein treatment. Vice versa a prediction about the structure formation of proteins using investigated protein treatments can be reached. The smooth and pasty macroscopic peculiarity of MLP was accentuated by microscopic investigation: MLP was the only protein isolate containing exclusively micelles and no unfolded protein structures within all studied variations. The micelle formation is probably the major cause of the fat-like properties of MLP. However, also differences in composition are in discussion since ILP showed autofluorescence at characteristic wavelengths of substances like lignin and chlorophyll in contrast to MLP. Determination of slightly higher lignin content in MLP in comparison to ILP, however, could not confirm this supposition. More detailed chemical investigations about the protein isolates are in progress and will be published in another study.

The typical emergence of these ordered spherical structures of lupin proteins was visually demonstrated for the first time using light microscopy. Furthermore, a breakthrough of detailed structure analysis of protein micelle surface was achieved in this paper with cryo-scanning electron microscopy. For the first time microstructure of ILP and MLP could be recorded in high resolution at a 20.000-fold magnification. The hypothesis of micellar formation in MLP has now been optically confirmed. Besides, size distribution of demonstrated micelles has been statistically evaluated.

In the present study, structure-specific susceptibilities of proteins and limits of imaging methods have been identified. For a screening and orientation of the structure of aqueous plant proteins light microscopy showed to be the most expedient. For detailed structure information cryo-SEM turned out to be indispensable.

The presence of micellar particles was shown to be responsible for the fat-like character of MLP. Especially, in a hydrophilic environment, where hydrophilic–hydrophobic balance is sustained, these colloidal particles presumably slide from one another similar to the natural functionality of oil. Since exclusively agents safe to use (NaCl and water) are needed for the preparation of MLP, the application of MLP in fat-reduced formulations is assumed to be expanded in the near future.

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CHAPTER 2

Protein distribution in lupin protein isolates from *Lupinus angustifolius* L. prepared by various isolation techniques

The protein distribution of various protein isolates from *Lupinus angustifolius* L. Vitabor was identified as affected by the isolation procedure (alkaline and/or salt-induced extraction followed by isoelectric and/or dilutive precipitation).

Differences in the protein composition were assessed by ion exchange chromatography, reversed-phase high performance liquid chromatography (HPLC), sodium dodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE) and reducing 2D electrophoresis. Protein isolates extracted in alkaline solution showed higher protein yields (26.4–31.7%) compared to salt-induced extraction (19.8–30.0%) or combined alkaline and salt-induced extraction (23.3–25.6%). Chemical variations among the protein isolates especially occurred within the albumins. Protein isolates precipitated isoelectrically showed the highest, whereas protein isolates precipitated by dilution showed the lowest contents of conglutin δ. Furthermore, the content of the alkaline subunits of conglutin α and conglutin γ decreased during alkaline extraction compared to salt-induced extraction. Compared to the flakes, the protein isolation procedure was characterised by a decrease in the contents of protein-bound polar and basic amino acids and an increase in the number of nonpolar, aliphatic, aromatic, hydroxylated and sulfur-rich amino acids, independent on the method used. The prevalence of sulphur-rich conglutins α and γ in MLP might be associated with protein micelle stabilisation in the micellar lupin protein isolate.

I. Muranyi designed the study, collected test data, wrote the manuscript and interpreted the results. D. Volke and R. Hoffmann conducted 2D electrophoresis. M. Brunnbauer assisted in interpreting the HPLC results. P. Eisner, T. Herfellner and U. Schweiggert-Weisz contributed to writing of the manuscript. P. Koehler assisted in study design, data interpretations and writing.

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Protein distribution in lupin protein isolates from *Lupinus angustifolius* L. prepared by various isolation techniques

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ABSTRACT

Differences in the protein distribution of various protein isolates from *Lupinus angustifolius* L. Vitabor were identified as affected by the isolation procedure (alkaline and/or salt-induced extraction followed by isoelectric and/or dilutive precipitation). Protein isolates extracted in alkaline solution showed higher protein yields (26.4–31.7%) compared to salt-induced extraction (19.8–30.0%) or combined alkaline and salt-induced extraction (23.3–25.6%). Chemical variations among the protein isolates especially occurred within the albumins. Protein isolates precipitated isoelectrically showed the highest contents, whereas protein isolates precipitated by dilutive showed the lowest contents of conglutin δ. Furthermore, the alkaline subunits of conglutin α and conglutin γ decreased during alkaline extraction compared to salt-induced extraction. A decrease in protein-bound polar and basic amino acids was shown after protein isolation. In contrast, the amounts of nonpolar, aliphatic, aromatic, hydroxylated and sulfur-rich amino acids were higher in the lupin protein isolates compared to the lupin flakes. However, the functional side chains could not be related to the specific molecular arrangements of the protein isolates, as a similar amino acid composition was found among the protein isolates.

1. Introduction

Lupin proteins offer excellent techno-functional properties such as water binding capacity as well as foaming and emulsification properties. Consequently, the industrial use of lupin proteins in food and feed applications has increased in the last years (Sanchez et al., 2005). However, the recovery procedure strongly affects the techno-functional properties of the protein products (El-Adawy, Rahma, El-Bedawey, & Gafar, 2001).

Both alkaline extraction with subsequent isoelectric precipitation and salt-induced extraction followed by dilutive precipitation are important techniques for protein recovery from vegetable sources (Chew, Casey, & Johnson, 2003; Lqari, Vioque, Pedroche, & Millan, 2002; Rahma, Dudek, Mothes, Gornitz, & Schwenke,

Abbreviations: AN, acetonitrile; ANOVA, analysis of variance; AOAC, Association of Official Analytical Chemists; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; cv., cultivar; D, dimensional; et al., and others, *lat.* et alii; DI water, deionised water; DM, dry matter; DTT, dithiothreitol; EA, alkaline extraction; EAS, combined salt-induced and alkaline extraction; ES, salt-induced extraction; HPLC, high performance liquid chromatography; ILP, isoelectric lupin protein isolate; MLP, micellar lupin protein isolate; MOPS, 3-(N-morpholino)propanesul phonic acid; N, protein nitrogen; n, number of samples; PAGE, polyacrylamide gel electrophoresis; PD, dilutive precipitation; PDI, combined dilutive and isoelectric precipitation; PI, isoelectric precipitation; p*I*, isoelectric point; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)-aminomethane; v/v, volume per volume; w/v, weight per volume.

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2000). Microscopic analyses of protein isolates from the seeds of *Lupinus angustifolius* L. Vitabor prepared via these two methods showed fundamental differences in the microstructural protein organization (Muranyi, Otto, Pickardt, Koehler, & Schweiggert-Weisz, 2013). The isoelectric protein isolate (ILP) shows an unfolding and irreversible denaturation of the proteins, while after dilutive precipitation the formation of flexible well-ordered regular spherical structures (micellar arrangement) was observed. Therefore, the latter is often called "micellar protein isolate" (MLP) and the corresponding isolation procedure "micellization" (Ismond, Georgiou, Arntfield, & Murray, 1990). Thus, precipitation and extraction procedures play a decisive role within the microstructural orientation. Besides microstructure formation, the protein composition is presumed to be strongly dependent on the isolation condition because of the different extractability behaviour of lupin proteins (Van der Borght et al., 2006).

The seed proteins of *Lupinus angustifolius* L. mainly consist of two glycosylated protein types, the albumins and the globulins (mass ratio 1:9). The content of further protein fractions such as prolamins and glutelins is negligible (Gulewicz et al., 2008).

The water soluble sulfur-rich albumins (conglutin δ) account for 2.6% of the total protein content of the lupin seed (Salmanowicz, 2000). The molecular weight of conglutin δ varies depending on solvent composition and protein concentration. Conglutin δ_2 (14 kDa) is a monomer composed of a heavy (9.5 kDa) and a light (4.5 kDa) polypeptide chain. At neutral pH, it forms a dimer (conglutin δ_1, 28 kDa) that associates via disulfide bonds to oligomeric aggregates of up to 56 kDa (Lilley, 1986; Lqari, Pedroche, Giron-Calle, Vioque, & Millan, 2004; Ternes, 2007).

The legumin-like conglutin α and the vicilin-like conglutin β of lupin seeds present the main globulins (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008) and account with 76% and 16.4%, respectively, of total lupin seed protein content (Blagrove & Gillespie, 1975; Lqari et al., 2004; Plant & Moore, 1983). Conglutin α is composed of three subunits connected non-covalently, with each exhibiting sizes of 64, 72 and 85 kDa (Johnson, Knight, & Gayler, 1985). The subunits are further divided into alkaline polypeptides of 21–24 kDa and acidic polypeptides of 42–62 kDa linked together via intermolecular disulfide bonds. Lqari and coworkers found trimeric protein structures of around 216 kDa (Lqari et al., 2004). Transition from a trimer to a hexamer occurs under limited proteolytic activity due to shifts in the environment, such as a decrease in pH (Blagrove & Gillespie, 1975).

Conglutin β is the only lupin protein free of disulfide bonds, which leads to identical band profiles in SDS-PAGE under both reducing and non-reducing conditions. The prevalent form of the 150–170 kDa oligomer is trimeric but conglutin β also forms tetramers. The sizes of the subunits range from 20 to 70 kDa each of which is composed of 10 to 12 distinct and mostly glycosylated polypeptides (Duranti, Gorinstein, & Cerletti, 1990 and Duranti, Sessa, & Carpen, 1992).

Conglutin γ (also termed 7S protein) is an exceptional globulin soluble in both water and salt solutions. In contrast to the other lupin conglutins, the isoelectric point of the 7S protein lies within the alkaline range (7.9, Arnoldi et al., 2007; Duranti, Restani, Poniatowska, & Cerletti, 1981; Duranti et al., 2008). The glycoprotein shows a very high sulfur content, although it represents only 5% of total globulins. It exhibits a size of 100 kDa with 50 kDa subunits. The subunits consist of a light (17 kDa) and a heavy polypeptide chain (32 kDa), linked together via disulfide bonds (Johnson et al., 1985; Kolivas & Gayler, 1993; Plant & Moore, 1983).

The solubility characteristics of the conglutins indicate that different isolation procedures (modifying the pH or ionic strength) might lead to protein isolates with different protein subunits. As the proteins interact specifically with their surrounding medium, the three-dimensional protein arrangement within a defined solution might vary among the protein isolates dependent in the subunit characteristics. However, up to now, influences of different repartition of proteins or protein subunits on the three-dimensional protein structure have not been well investigated. Structural investigations of plant protein isolates are limited to physical approaches investigating the effect of environmental conditions on electrostatic repulsion or hydrophobic properties of the protein surfaces (Hu et al., 2013; Ismond et al., 1990).

Therefore, the aim of the present study was to investigate the influence of the isolation procedure (alkaline extraction/isoelectric precipitation and salt-induced extraction/dilutive precipitation) on the protein distribution, protein subunits, and amino acid composition of protein isolates derived from seeds of *Lupinus angustifolius* L. Vitabor and to get more insight and understanding of the microstructural orientation and techno-functional properties of the protein isolates as affected by the isolation procedure.

2. Materials and methods

2.1. Materials

Seeds of *Lupinus angustifolius* L. (cv. Vitabor, 2005 harvest) were received from Saatzucht Steinach (Steinach, Germany). NaCl was obtained from VWR International GmbH (Darmstadt, Germany). 3-(N-morpholino)propanesulfonic acid (MOPS) was purchased from Sigma Aldrich Co. LLC., SDS, NaOH and HCl from Merck KGaA (Darmstadt, Germany). Acrylamide and 4× stacking gel buffer were from Amersham Biosciences AB (Uppsala, Sweden). Phast Gel blue R (bromphenol blue) was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Sodium sulfite and norleucine were obtained from Dionex Corporation (Sunnyvale, USA). Trifluoroacetic acid (TFA), iodoacetamide, urea and thiourea were purchased from Fluka (Neu-Ulm, Germany). 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). IPG strips, Ready Prep 2D clean up kit, Bio-Lyte 3–6, 5–8, 7–10 were from Bio-Rad Laboratories GmbH (Munich, Germany). Dithiothreitol (DTT), acetonitrile (AN), glycerol and coomassie brilliant blue were from Carl-Roth GmbH & Co. KG (Karlsruhe, Germany), and Tris(hydroxymethyl)-aminomethane (Tris) from AppliChem GmbH (Darmstadt, Germany). Deionized (DI) water was used throughout. For HPLC, water over 16 $M\Omega \cdot cm^{-1}$ was prepared by Milli-Q Ultrapure Water System (Millipore, Milford, MA, USA). All reagents used were of analytical grade unless stated otherwise.

2.2. Pre-treatment of the lupin seeds

The seeds were dehulled with an underflow peeler (Streckel & Schrader, Hamburg, Germany) and classified into kernels and hulls in an air-lift system (Alpine Hosokawa AG, Augsburg, Germany). The dehulled kernels were flaked using a roller mill (Streckel & Schrader, Hamburg, Germany). The full-fat lupin flakes were used as raw material for the recovery of protein isolates.

2.3. Preparation of protein isolates

The different protein isolates from the lupin seed flakes of *Lupinus angustifolius* L. Vitabor were prepared according to the methods described in a previous study (Muranyi et al., 2013).

2.3.1. Extraction

For all protein isolates prepared by alkaline extracts (EA), the lupin flakes were suspended in DI water at a ratio of 1:8 (w/v). The pH value was adjusted to pH 8 with 0.5 $mol \cdot L^{-1}$ NaOH. Preparation of salt-induced extracts (ES) comprised a suspension of the

lupin flakes in 0.5 mol·L^{-1} NaCl solution at a ratio 1:8 (w/v). For combining the alkaline and the salt-induced extraction (EAS) the lupin flakes were suspended in 0.5 mol·L^{-1} NaCl solution (ratio of flakes : salt-solution = 1:8). This suspension was adjusted to pH 8 with 0.5 mol·L^{-1} NaOH. All suspensions were stirred for 1 h at room temperature. After the individual extraction processes, all suspensions were separated through a sieve (mesh size: 1.5 mm) and centrifuged at 3300g for 10 min. The supernatants were filtrated through a rayon-polyester filter (pore size: 22–25 μm).

2.3.2. Precipitation

For isoelectric precipitation (PI) the protein extracts were acidified with 0.5 mol·L^{-1} HCl to pH 4.5. Dilutive precipitation (PD) comprised the dilution of the clear extracts with DI water (ratio 1:3 v/v) at room temperature while stirring continuously. Because of the high solubility at pH 8 of the lupin proteins extracted according to EA and EAS, the pH of the named extracts needed to be adjusted to 5.5 using 0.5 mol·L^{-1} HCl, which is the naturally arising pH of ES-PD (MLP). Thereby, the precipitation conditions were equalized to the dilutive precipitation technique (PD5.5). The extracts of all protein isolates precipitated by a combination of isoelectric and dilutive precipitation (PDI) were first diluted with DI water at a ratio of 1:3 (v/v) before adjusting the pH to 4.5 with 0.5 mol·L^{-1} HCl. After a final centrifugation step, the pH values of the precipitates were measured and resulted in pH 5.5 for protein isolates 1, 4a and 7a, and in 4.5 for all other protein isolates. For chemical analysis the protein isolates were frozen at −50 °C and lyophilized in a freeze-drier (Beta 1–8, Christ GmbH, Osterode, Deutschland).

2.4. Determination of the protein content and the protein yield

The protein content of all raw materials and protein isolates was calculated based on the nitrogen content determined according to the Dumas combustion method (AOAC International, 1990) using a Nitrogen Analyzer FP 528 (Leco Corporation, St. Joseph, MI, USA). The lupin-specific conversion factor of N × 5.8 was used, as reported by Mossé, Huet, and Baudet (1987). Based on these values the protein yields were assessed.

2.5. Amino acid quantitation by ion exchange chromatography

Amino acid analysis was carried out according to the method described by Miedel, Hulmes, and Pan (1989) with slight modifications. Samples were dissolved in 6 mol·L^{-1} HCl (final concentration: 1 mg protein/ml). Sodium sulfite was added as an oxygen-scavenger and norleucine as an internal standard. Acid hydrolysis was carried out in a vacuum (20 mbar) for 24 h at 110 °C. Aliquots of 20 μl were subjected to an ICS 3000 Amino Acid Analyzer (Dionex Corporation, Sunnyvale) equipped with an Amino Pac® PA10 column and an electrochemical detector. Amino acids were quantitated by calculating the concentration of each specific amino acid to external standards, and normalized to the internal standard norleucine. All amino acids were determined except for tryptophan, as it is completely degraded during the acidic hydrolysis. As the tryptophan content in legumes only amounts up to 1% (El-Adawy et al., 2001) it plays a minor role in lupin protein isolates and was not accounted for in calculations of the amino acid contents. The values for asparagine and aspartic acid were summed up and termed aspartic acid. Analogously, glutamine and glutamic acid were termed together as glutamic acid. The amounts of amino acids were given as mean values deriving from two independent hydrolyses with three injections each of the respective protein samples.

2.6. Separation of protein fractions by reversed-phase high performance liquid chromatography (RP-HPLC)

Protein fractionation was performed on a BIO-TEK HPLC instrument (BIO-TEK® Instruments GmbH, Neufahrn, Germany) comprising a pump, 522, and a UV-detector, 535. Separation was achieved on a C_8 column (Nucleosil, 200 × 4.6 mm, particle size: 5 μm, mean pore diameter: 30 nm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and analysis with Geminyx Software (Goebel Instrumentelle Analytik GmbH, Au i. d. Hallertau, Germany). For sample preparation, protein isolates were lyophilized and finely ground. Sample dispersions of 0.02% (w/v) in 50 mmol·L^{-1} MOPS/Tris/1% SDS buffer (pH 6.9) were dissolved by sonication for 3 min. After filtration through a 0.45-μm mini-filter into amber coloured glass vials, a volume of 100 μL protein solution was injected. The mobile phase comprised two solvents, A: water/TFA (99.9:0.1 v/v) and B: acetonitrile (AN)/TFA (99.9:0.1 v/v). The gradient profile was 0–5 min: 70–60% A, 5–7 min: 60–58% A, 7–12 min: 58–58% A, 12–25 min: 58–48% A, 25–26 min: 48–10% A, 26–35 min: 10–10% A, 35–36 min: 10–70% A and 36–45 min: 70–70% A. The flow rate was 1 mL·min^{-1} at 40 °C. Detection was performed at λ = 210 nm. The recovery rate of the proteins was always higher than 86%. The number of experiments was n = 13 for both protein isolates.

2.7. Determination of molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The molecular weights of the proteins were investigated by non-reducing SDS-PAGE which was carried out at least in triplicate using the vertical gel unit Hoefer SE 600 Ruby (Amersham Biosciences, Germany). Acrylamide concentration in the stacking gel was 4% and the resolving gradient gel ranged from 5% to 20%. The freeze-dried and finely ground protein samples were prepared according to the method described by Magni et al. (2005). In order to determine the molecular weights of the protein fractions a molecular weight standard from 10 kDa to 250 kDa (Precision Plus Protein Kaleidoscope™ Standard, Bio-Rad Laboratories GmbH, Germany) was used and added on at least two lanes on the gel. Gels were stained using Coomassie Blue R 250, and scanned in colour. The molecular weight of each band was related to the molecular weight standard (Precision Plus Protein Kaleidoscope™ Standard) using the Image Quant TL Software (Amersham Biosciences, Germany).

2.8. Determination of molecular weight and isoelectric point by reducing 2D electrophoresis

For 30 min at room temperature, 30 mg of EA-PI (ILP) or ES-PD (MLP) were dissolved in 1 mL multi-chaotropic solution (3.5 mol·L^{-1} urea, 1 mol·L^{-1} thiourea, 1 mmol·L^{-1} DTT, 2% CHAPS). Both samples were centrifuged for 1 min at 12,000g. The supernatant was treated with the 2D-clean up Kit from Bio-Rad Laboratories Inc. to the manufactures instructions. 5 μL of the sample were transferred to 120 μL of multi-chaotropic solution containing 0.2% Bio-Lyte 3-10 and 0.0002% bromophenol blue when IPG strips 3–6 or 5–8 where used. Where IPG strips 7–10 where used, the multi-chaotropic solution contained 0.2% Bio-Lyte 7–10 and 0.0002% bromophenol blue.

Afterwards, active rehydration was performed for 12 h at 50 V and 20 °C, followed by focussing at 250 V for 15 min, rising to 4000 V until 20,000 Vh was reached at 20 °C. Strips were equilibrated for 15 min at room temperature in DTT solution (6 mol·L^{-1} urea, 0.375 mol·L^{-1} Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) followed by iodoacetamide-solution (6 mol·L^{-1} urea, 0.375 mol·L^{-1} Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide). Self-cast polyacrylamide gels (T 12%, C 2.67%,

7 cm × 10 cm) were made using a Mini-Protean 3 multi-casting chamber (Bio-Rad Laboratories GmbH, Munich, Germany) and polymerized overnight. The equilibrated IPG-strips were fixed with molten agarose (0.5% in running buffer with bromophenol blue) on the gel surface. Separation was done in a Mini-Protean 3 Dodeca Cell at 200 V, and cooled to 15 °C. Gels were washed three times for 5 min with deionized water, stained with colloidal coomassie for 1 h at room temperature, destained and stored in deionized water. For image acquisition a CCD-camera Fusion FX7 from Peqlab Biotechnologie GmbH (Erlangen, Germany) was used.

2.9. 2.9.Statistical evaluation of data

All values in the tables and figures are given as mean values and standard deviations. The number of experiments was abbreviated by an "n". Three or more corresponding groups were compared by analysis of variance (ANOVA, $\alpha = 0.05$) with subsequent testing of significant differences by means of the Scheffé-Test (WinSTAT®, Microsoft, USA).

3. Results and discussion

The isolation procedures for production of EA-PI (ILP) and ES-PD (MLP) are important techniques for the recovery of proteins from vegetable sources. As both protein isolates show different structural and technofunctional properties, the extremely different isolation parameters were held responsible for the product differences. All protein investigations within this study were approved with both protein isolates. Investigations showing qualitative differences were repeated with the protein isolates prepared by combining the isolation procedures of both products to identify the influence of the isolation procedure on the protein composition.

3.1. Influence of the isolation procedure on protein yields

The protein yields of the lupin protein isolates are presented in Table 1.

Protein yield was highest for the combined extraction in alkaline salt solution (EAS), whereas protein yields from the alkaline (EA) and the salt-containing protein extracts (ES) alone were similar. The majority of the protein (approx. 60%) remained undissolved within the lupine flakes during extraction.

After salt-induced extraction, the combination of dilutive and isoelectric precipitation caused the highest protein yield (ES-PDI). However, after alkaline or combined alkaline and salt-induced extraction, the protein yields were comparable for all three precipitation methods.

The micellar protein isolate (ES-PD) showed the lowest protein yield among all protein isolates, while the protein yield of the isoelectric protein isolate (EA-PI) was particularly high. The results agree with the significantly higher protein recovery for isoelectric lupin protein isolate (ILP, corresponding to EA-PI: 60%) reported by Rodriguez-Ambriz, Martinez-Ayala, Millan, and Davila-Ortiz (2005), compared to the micellar lupin protein isolate (MLP, corresponding to ES-PD: 30%). However, the yield difference of 30% between both protein isolates was higher than determined for the protein isolates in the present study (9%). The reason might be the differences in pH adjustment. Rodriguez-Ambriz and coworkers used pH 9 for ILP extraction and adjusted the pH to 7 during salt-induced extraction for the production of MLP. In contrast, in the present study alkaline extraction was performed at pH 8 and salt-induced extraction without pH adjustment. Rodriguez-Ambriz and coworkers assumed the higher levels of protein recovery to result from its more complete protein extraction including solubilization of more proteins from the lupin seed flour when using the alkaline extraction. However, this assumption could not be confirmed in this study, as protein contents in both the 'EA' and the 'ES' extracts were similar despite fundamentally different extraction parameters. The high ionic strength during salt-induced extraction is assumed to favour the lupin globulins to become solubilized. During alkaline extraction however, the acidic albumins were presumed to be dissolved, whereas some alkaline globulins may have remained insoluble inside the lupin flakes.

Table 1
Protein yields of lupin seed flakes, lupin protein extracts and -isolates from *Lupinus angustifolius* L. as affected by the isolation procedure. Protein isolates showing the same extraction method are arranged together in three-group blocks.

Sample	Code designation	Protein yield* [%]	
		Extracts	Isolates
0	Lupin flakes	100	
1	ES-PD (MLP)	37.8 ± 0.4^{a}	19.8 ± 0.1^{a}
2	ES-PI	37.8 ± 0.4^{a}	$21.4 \pm 0.0^{a,c,e}$
3	ES-PDI	37.8 ± 0.4^{a}	$30.0 \pm 2.9^{b,d,f}$
4	EAS-PD	40.5 ± 0.3^{b}	–
4a	EAS-PDpH5.5	40.5 ± 0.3^{b}	$25.6 \pm 1.7^{c,e,g}$
5	EAS-PI	40.5 ± 0.3^{b}	$23.3 \pm 0.2^{a,d,e}$
6	EAS-PDI	40.5 ± 0.3^{b}	24.5 ± 1.8^{e}
7	EA-PD	38.0 ± 0.4^{a}	–
7a	EA-PDpH5.5	38.0 ± 0.4^{a}	$26.4 \pm 1.7^{b,e,g}$
8	EA-PI (ILP)	38.0 ± 0.4^{a}	$29.2 \pm 0.1^{f,g}$
9	EA-PDI	38.0 ± 0.4^{a}	31.7 ± 0.8^{f}

ES = salt-induced extraction, EAS = combined salt-induced and alkaline extraction, EA = alkaline extraction, PD = dilutive precipitation, PI = isoelectric precipitation, PDI = combined dilutive and isoelectric precipitation, 5.5 designates protein isolates adjusted to pH 5.5.

* Values are related to dry matter; determination of protein content using Dumas combustion method (N × 5.8); Mean values marked with the same letter (a, b, c, d) are not significantly different from each other in the same column (Scheffé-Test, $p < 0.05$). Mean values ± standard deviation of four experiments (n = 4).

Protein isolates ES-PI, EAS-PI and EA-PI, each of which extracted differently but precipitated via isoelectric precipitation at pH 4.5, showed significantly different total protein yields. Thus, the mentioned qualitative differences only became visible after isoelectric precipitation of both extracts at pH 4.5.

Protein yields for isolates prepared by the combined salt-induced and alkaline extraction were similar for all three precipitation methods (protein isolates 4a, 5 and 6). Using NaCl during alkaline extraction allowed the salt-soluble globulins to dissolve, in addition to the acidic albumins. The total protein amount was, therefore, enhanced after extraction facilitating hydrophobic interactions (Cooper, 1988). The reduced amount of proteins to be precipitated at pH 4.5 might be caused by a shift in overall p*I* of the EAS extracted proteins (presumably between 4.5 and 5.5) protein precipitation .

To explain the differences in protein yields, the distribution of the lupin protein fractions was determined by RP-HPLC and gel electrophoresis.

3.2. Influence of the isolation procedure on lupin protein composition

3.2.1. Quantitative protein composition (RP-HPLC)

As determined by RP-HPLC, both protein isolates EA-PI (ILP) and ES-PD (MLP) exhibited three distinct groups of eluted proteins at comparable retention times (fractions 1 to 3, Fig. 1). However, to investigate any quantitative differences in the protein distribution between both protein isolates, the normalized absorbance areas of the fractions were compared. Minor quantitative differences were identified in the individual fractions. Both protein isolates exhibited the highest content of the proteins in fraction 1 and least of

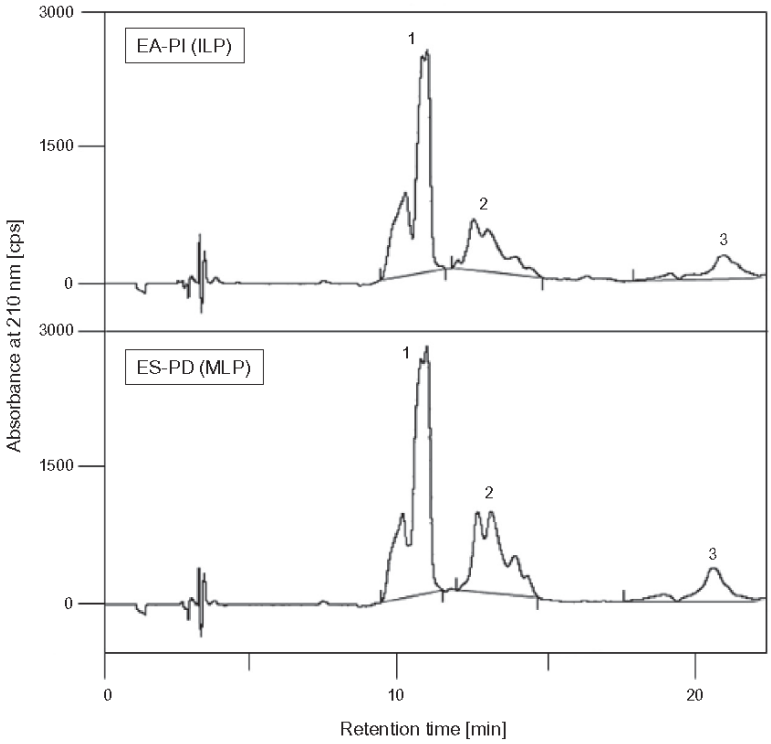

Fig. 1. Separation of lupin proteins in EA-PI (isoelectric lupin protein isolate, ILP) and ES-PD (micellar lupin protein isolate, MLP) by reversed-phase high performance liquid chromatography (210 nm). Protein fractions are marked with 1 to 3.

those in fraction 3. EA-PI showed a higher content of the proteins in fraction 1 (EA-PI: 63.0 ± 1.0%, ES-PD: 57.2 ± 1.1%) relative to the initial protein amount, whereas ES-PD showed higher contents of the proteins in fraction 2 (EA-PI: 25.1 ± 1.0%, ES-PD: 29.6 ± 1.9%). Fraction 3 was comparable between both protein isolates (EA-PI: 11.9 ± 1.4%, ES-PD: 13.2 ± 1.2%). Thereafter, protein quality was comparable between EA-PI and ES-PD as all detected protein fractions were present in both protein isolates.

3.2.2. Qualitative protein composition (electrophoresis)

To investigate the differences in protein composition between EA-PI (ILP) and ES-PD (MLP), 2D electrophoresis was applied. Results are shown in Fig. 2. Globulins are an extremely heterogeneous class of proteins. Often series of spots (same molecular weight but varying p*I*) instead of single spots were found, due to different isoforms of the same protein with different degrees of post-translational phosphorylation and glycosylation. The results were in line with the total protein extract from *Lupinus angustifolius* L. published by Sirtori, Resta, Brambilla, Zacherl, and Arnoldi (2010). In that study, the individual lupin protein spots were identified by tryptic in-gel digestion and subsequent fragmentation using a HPLC-Chip-Ion Trap. After protein database searches with the Spectrum Mill software, the conglutins were located within the 2D-map. The same reducing methodology for sample preparation was used to localize the individual conglutins and conglutin subunits within the protein isolates EA-PI and ES-PD in the present study.

To understand the dynamic allocation of the lupin proteins dependent on the isolation procedure, and to investigate the chemical differences among the lupin protein isolates, non-reducing 1D SDS-PAGE of all isolate variations was performed. Data are shown in Table 3 and a list showing the properties of the conglutins is in Table 2. Because of an overlap of protein spots with similar molecular weights in α-conglutin and β-conglutin, 1D SDS was insufficient to identify the distribution differences of according polypeptides among all lupin protein isolates. However, successful identification of α-conglutin and β-conglutin was achieved for EA-PI and ES-PD samples, as well as of γ-conglutin and δ-conglutin, for all protein isolates on the basis of the assembled results from both electrophoresis experiments. Results for both SDS-PAGES are discussed in the sections for the individual conglutins.

Conglutin α. ES-PD showed a cluster of high-intensity spots at p*I* 4–6 and within the molecular weight range 40–70 kDa (cluster No. 1), identified as the N-terminal acidic alpha chain of α-conglutin (Sirtori et al., 2010). In contrast, the amount of the acidic alpha chain was significantly reduced in EA-PI. Analogously, spots in the region of 20 40 kDa (19, 31, 36, 42, 46 kDa, at pI 7) corresponding to the C-terminal basic beta chain of α-conglutin (p*I* = 6.7 8.6, Magni et al., 2005) were most abundant in ES-PD (cluster No. 2 and 3), in contrast to EA-PI. Conglutin α consists of four subunits with sizes of 53, 60, 66 and 70 kDa and exhibits a p*I* of 5.1–5.8 (Melo, Ferreira, & Teixeira, 1994). The beneficial pH after dilutive precipitation (pH 5.5–6.0) was hold responsible for the higher amounts of α-conglutin in ES-PD, compared to the isoelectrically precipitated protein isolate (pH 4.5). In contrast, the pH value during alkaline extraction (pH 8) reduced the occurrence of this protein fraction.

Conglutin β. Starting at p*I* 5, the protein profile of β-conglutin was highly heterogeneous even though it contains no disulfide bridges (Restani, Duranti, Cerletti, & Simonetti, 1981). Especially the larger subunits (cluster No. 4, 5; 54, 62, 72 kDa, Melo et al., 1994) showed spots spread out nearly throughout the entire pH range which is due to the high extent of glycosylation, characteristic for many β-conglutin subunits (Duranti et al., 1990, 1992). The sizes of the

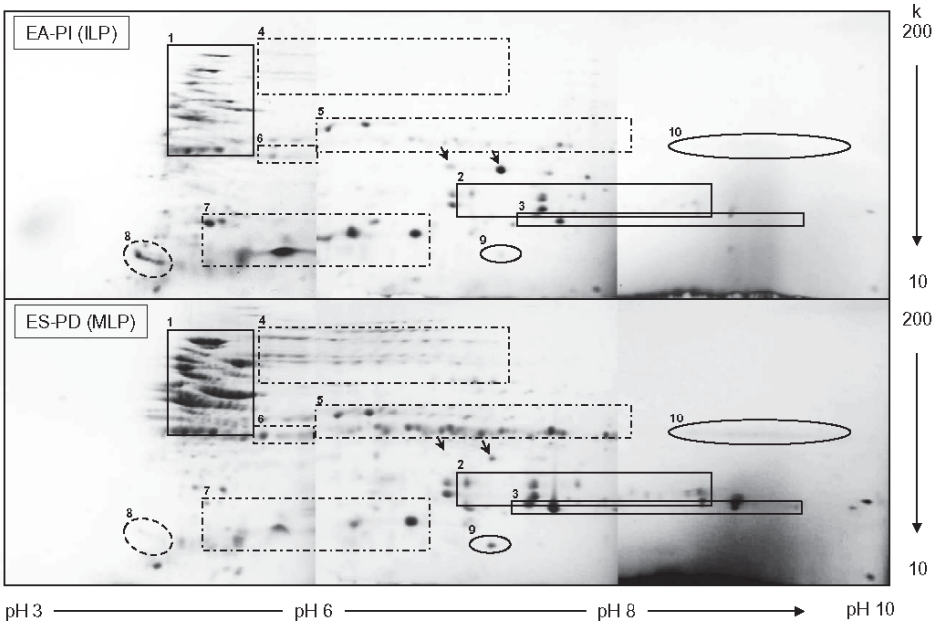

Fig. 2. 2D-map of reduced EA-PI (isoelectric lupin protein isolate, ILP) and ES-PD (micellar lupin protein isolate, MLP) with protein assignment adapted from Sirtori et al. determined by Spectrum Mill software. Solid line rectangles (—) designate the acidic and basic subunits of α-conglutin (spot clusters 1–3), the dash-dotted rectangles (—·—·—) β-conglutin (spot clusters 4–7). The dotted oval (- -) indicates the heavy chain of δ-conglutin (spot cluster 8) and the solid line ovals the large and small subunits of γ-conglutin (spot clusters 9–10). The arrows designate putative triacylglycerol factor protein (Sirtori et al., 2010).

Table 2
Molecular weights of the conglutins enumerated in Fig. 2 under reducing and non-reducing conditions.

Protein identification[a]			pI	Molecular weight[a] [kDa]	
Cluster No.	Conglutin	Subunit		Reduced	Non-reduced
1	α	Acidic α-chain	5.1–5.8[b]	40–70	62–74
2, 3		Basic β-chain	6.7–8.6[b]	20–24, 36	
4	β		5.9–6.2[c]	54–72[b]	54–72[b]
5				34–48[b]	34–48[b]
6			5.0–6.0[d]	27–31[b]	27–31[b]
7				15–21[b]	15–21[b]
8	δ	Large subunit	4.3[c]	9	13
		Small subunit		4	
9	γ	Small subunit	7.9[c]	17	47
10		Large subunit		29	

[a] Sirtori et al. (2010).
[b] Melo et al. (1994).
[c] Duranti et al. (1981).
[d] Duranti et al. (2008).

small subunits are 15, 18, 21, 27, 31, 34, 38, 42 and 48 kDa (Melo et al., 1994) and show less glycosylation (Fig. 2, cluster Nos. 6 and 7). EA-PI predominantly contained the acidic polypeptides in the size range 15–21 kDa (cluster No. 7) and only traces of the larger subunits showing a slightly higher p*I*. In contrast, ES-PD comprised especially the larger subunits (cluster Nos. 4 and 5) and lower amounts of the smaller subunits. The subunits of β-conglutin exhibit a p*I* of 5.0–6.0 (Duranti et al., 2008). Subunits featuring lower p*I* were favoured to become precipitated into EA-PI and those with higher p*I* (exhibiting higher molecular weight) into ES-PD. Therefore, similar to the repartition of α-conglutin, the different amounts in both protein isolates were attributed to the shifted final pH of both protein isolates (EA-PI: pH 4.5, ES-PD: pH 5.5–6.0).

Conglutin γ. ILP contained only traces of the small 17 kDa subunit of γ-conglutin and the large 47 kDa subunit described by Sirtori et al. (2010) and could barely be shown (Fig. 2). In contrast, ES-PDshowed a significant dot (cluster No. 10) belonging to the small subunit of γ-conglutin and traces of the large subunit (cluster No. 11). Conglutin γ is the only lupin protein fraction exhibiting a slightly alkaline p*I* (p*I* 7.9, Duranti et al., 2008). During alkaline extraction, this protein is assumed to remain insoluble inside the lupin flakes and thereby will be lost during separation of the soaked solids. This assumption was corroborated by the low amounts in all protein isolates extracted in alkaline solution (47 kDa in protein isolates 7–9: 2.9–3.4%, Table 3) compared to isolates extracted in salt solution (47 kDa in protein isolates 1–3: 3.6–4.4%) or alkaline salt solution (47 kDa in protein isolates 4–6: 4.1–4.7%). The low amount of γ-conglutin in ILP was therefore attributed to its lower p*I*. Thus, a similar behaviour to the alkaline subunit of α-conglutin was found: in contrast to their extraction into the salt-containing extracts, both conglutin γ and conglutin α remained insoluble during alkaline extraction and were lost within the soaked lupin flakes during the first extraction step.

Table 3
Molecular weight distribution of the lupin protein isolates and the additional protein isolate prepared by isoelectric precipitation of the MLP supernatant (MLP-SNIP) as determined by non-reducing SDS-PAGE [% of total protein content]. The relative contents were determined by software-based semi-quantitative evaluation of bitmaps of 1D-SDS electropherograms. For each sample, the total area of all protein zones was set to 100%.

Molecular weight	Protein isolates									
	1 ES-PD (MLP)	2 ES-PI	3 ES-PDI	4a EAS-PD	5 EAS-PI	6 EAS-PDI	7a EA-PD	8 EA-PI (ILP)	9 EA-PDI	MLP-SNIP
[kDa]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
71	3.3	3.6	3.5	3.3	3.9	4.0	3.1	2.7	2.8	1.0
67	2.6	2.7	2.3	2.6	2.8	2.5	3.1	2.3	2.3	0.6
62	4.2	4.9	3.6	4.4	4.5	4.5	3.5	4.1	2.8	1.6
58	4.8	4.1	4.3	3.4	4.1	5.1	4.1	3.5	4.1	2.1
54	6.1	6.3	5.1	6.9	5.6	6.3	5.6	4.8	5.7	3.0
50	5.9	6.9	6.5	6.5	7.8	7.1	7.2	7.0	6.0	5.0
47	4.3	3.6	4.4	4.3	4.7	4.1	3.4	3.3	2.9	3.4
45	3.0	3.0	2.6	2.7	3.6	3.1	3.1	3.1	2.9	2.5
42	4.0	4.0	3.7	3.1	4.1	3.6	3.4	3.7	4.2	3.4
38	4.3	4.1	2.8	4.5	4.4	4.0	4.4	4.1	3.6	2.8
36	3.3	4.5	2.9	3.3	3.9	3.4	3.0	2.9	3.0	3.2
34	3.8	3.8	3.4	4.0	3.1	2.9	3.1	2.9	3.5	4.0
33	5.2	6.0	5.7	3.7	5.2	5.6	5.5	5.1	5.6	4.4
31	8.6	7.4	9.0	10.2	7.8	7.8	8.5	9.3	9.0	9.1
28	4.3	4.4	3.6	3.5	4.2	3.7	3.8	3.7	3.6	5.2
24	4.3	3.0	3.4	2.9	3.1	2.7	2.6	2.4	3.8	5.2
21	4.2	4.4	5.4	5.1	3.1	3.9	4.3	4.5	3.7	5.6
17	4.8	4.1	3.8	3.7	4.0	5.0	4.1	3.1	3.3	3.6
16.5	3.6	3.3	4.0	4.0	2.9	2.6	4.1	4.0	3.4	5.3
16	2.8	2.2	2.9	1.9	2.1	1.9	2.3	2.5	2.9	3.2
15	5.6	3.6	5.7	6.4	5.1	6.5	8.2	8.9	7.5	7.5
13	6.8	9.9	11.4	9.8	10.2	9.9	9.7	12.3	13.3	18.1

ES = salt-induced extraction, EAS = combined salt-induced and alkaline extraction, EA = alkaline extraction, PD = dilutive precipitation, PI = isoelectric precipitation, PDI = combined dilutive and isoelectric precipitation, 5.5 designates protein isolates adjusted to pH 5.5.

Conglutin δ. While EA-PI contained all four spots at 13 kDa (cluster No. 8), identified by Sirtori and coworkers corresponding to δ-conglutin, ES-PD lacked the lupin albumin fraction (heavy polypeptide chain of conglutin δ_2, Lilley, 1986; Lqari et al., 2004). Furthermore, all the three protein isolates prepared by dilutive precipitation contained the lowest amounts of conglutin δ compared to the other protein isolates (13 kDa in protein isolates 1, 4a and 7a: 6.8–9.8%, all other protein isolates: 9.9–13.3%, Table 3). The reason is the high solubility of albumins in water (Ternes, 2007). A precipitation of conglutin δ during dilutive precipitation (ES-PD) was impeded because of its high solubility in the aqueous solution. Consequently, the low molecular weight proteins remained soluble in the discarded supernatant. In contrast, the pH was set to 4.5 for isoelectric precipitation. As conglutin δ exhibits a p*I* of 4.3 (Duranti et al., 1981) the isoelectric precipitation condition was highly beneficial for isolation of this conglutin, and explains its presence in all the protein isolates prepared by isoelectric or combined dilutive and isoelectric precipitation (Table 3). In addition, all the other conglutins show p*I* values at higher pH ranges (5.6–7.9, Sironi, Sessa, & Duranti, 2005) and were primarily precipitated during the dilutive isolation procedure, where pH sets were at 5–6. Hence, a predominance of the globulins further reduced the protein percentages at 13 kDa of protein isolates ES-PD, EAS-PD and EA-PD (Table 3).

To confirm, whether the heavy chain of conglutin δ remained soluble in the supernatant in protein isolates recovered by dilutive precipitation, the ES-PD supernatant was adjusted to pH 4.5 with HCl in an additional set of experiments. Using SDS-PAGE, the isoelectrically precipitated supernatant of ES-PD (MLP-SNIP) was proved to contain three times more of the 13 kDa fraction compared to ES-PD, and half that of proteins in the range between 40 and 70 kDa corresponding to conglutin α (protein isolate 'MLP-SNIP', Table 3).

The different quality of the isolates might influence their macromolecular orientation as shown in Muranyi et al. (2013). Protein isolates containing low amounts of the water soluble conglutin δ bind the small proteins in such an energetically favorable way, that hydrophobic protein residues are encapsulated in the core and the albumins are directed towards the aqueous surrounding medium (Fisher & Oakenfull, 1977), resulting in the formation of protein micelles. However, as the albumin content increases, the bound albumin might extensively enhance the protein surface by unfolding of the protein. This is corroborated by the unfolded protein structures of protein isolates 8 (ILP) and 9 (Muranyi et al., 2013) most abundant in conglutin δ (Table 3).

The reduction or lack of conglutin γ in protein isolates extracted in alkaline solution might further influence the structural organization of the protein isolates, as it had the most significant sulfur content among the conglutins. The disulfide-bridged polypeptide chains fulfil important stabilizing functions within the globular structures, such as structure conservation and non-specific subunit interactions (Plietz, Drescher, & Damaschun, 1987). The presence of micelles only in protein isolates 1 (ES-PD) and 3 (ES-PDI), determined by different microscopic approaches, might be explained by the structure-stabilizing effect of both protein isolates enriching conglutin γ during salt-induced extraction. The reason for the lack of micelles in the third protein isolate extracted in salt solutions, namely protein isolate 2 (ES-PI), is of course the missing extreme reduction of dilutive precipitation, which is a prerequisite for micelle formation (Muranyi et al., 2013).

In addition to the conglutins, especially in EA-PI, two spots in the vicillin area were detected. Sirtori and coworkers identified both spots to correspond to putative triacylglycerol factor protein. This protein is a dehydrogenase-like enzyme and found to be responsible for the accumulation of seed cells with triacylglycerol (Francki, Whitaker, Smith, & Atkins, 2002). The p*I* of the putative TAG factor protein (around 7) was closer to the pH of ES-PD and near to the pH 8, used for alkaline extraction within the EA-PI procedure. Nevertheless, EA-PI showed higher contents. The reason is most probably the reduction of disulfide bonds during sample preparation for SDS-PAGE delivering certain polypeptides

Table 4
Percentage of amino acids on total amino acid content in the lupin seed flakes, EA-PI (isoelectric lupin protein isolate, ILP) and ES-PD (micellar lupin protein isolate, MLP). [mol-% of total protein], Mean values ± standard deviation of six experiments (n = 6).

Amino acid	Lupin flakes [mol-%]	EA-PI (ILP) [mol-%]	ES-PD (MLP) [mol-%]	Polar	Non polar	Aliphatic	Aromatic	Basic	Sulfur-rich	Hydroxylated
Arginine	20.09 ± 4.09[a]	10.29 ± 0.06[b]	9.97 ± 0.21[b]	x				x		
Aspartic acid[′]	11.25 ± 0.42[a]	11.90 ± 0.00[b]	12.92 ± 0.25[c]	x						
Cysteine	0.47 ± 0.03[a]	0.62 ± 0.02[b]	0.82 ± 0.01[c]	x					x	
Glutamic acid[″]	19.78 ± 2.12[a,b]	18.98 ± 0.11[a]	21.43 ± 0.50[b]	x						
Histidine	4.37 ± 0.16[a]	3.67 ± 0.05[b]	4.01 ± 0.14[c]	x			x	x		
Lysine	4.40 ± 0.33[a]	4.80 ± 0.01[b]	4.06 ± 0.23[c]	x				x		
Serine	3.46 ± 0.09[a]	4.14 ± 0.01[b]	3.80 ± 0.17[c]	x						x
Threonine	2.95 ± 0.05[a]	3.43 ± 0.00[b]	3.02 ± 0.16[a]	x						x
Tyrosine	2.91 ± 0.14[a]	4.03 ± 0.06[b]	3.91 ± 0.20[b]	x			x			x
Alanine	4.80 ± 0.22[a]	4.90 ± 0.05[a]	4.23 ± 0.17[b]		x	x				
Glycine	6.34 ± 0.24[a]	6.66 ± 0.06[b]	6.31 ± 0.14[a]		x	x				
Isoleucine	3.19 ± 0.45[a]	4.94 ± 0.05[b]	4.75 ± 0.05[b]		x	x				
Leucine	4.56 ± 0.49[a]	6.51 ± 0.08[b]	6.37 ± 0.27[b]		x	x				
Methionine	0.11 ± 0.08[a]	0.27 ± 0.01[b]	0.03 ± 0.01[c]		x	x			x	
Phenylalanine	2.32 ± 1.02[a]	3.65 ± 0.02[b]	4.06 ± 0.61[b]		x		x			
Proline	5.53 ± 0.16[a]	6.95 ± 0.05[b]	6.60 ± 0.46[b]		x	x				
Valine	3.71 ± 0.13[a]	4.60 ± 0.07[b]	4.12 ± 0.08[c]		x	x				

Mean values marked with the same letter (a, b, c) are not significantly different within each row [Scheffé-Test, $p < 0.05$].
[′] Aspartic acid + asparagine.
[″] Glutamic acid + glutamine.

(Melo et al., 1994). The putative TAG factor proteins were assumed to have been linked to acidic proteins such as δ-conglutin, or the small fractions of β-conglutin which were more abundant in EA-PI, releasing the TAG factor proteins after protein reduction.

3.2.3. Amino acid composition

The functional side chains of amino acids feature important characteristics for specific molecular protein arrangements in the two- and three-dimensional structure organization (Marrero-Ponce et al., 2015). To identify a possible influence of the amino acid composition on the structural differences of the protein isolates, amino acid composition was compared. However, as both corner products EA-PI (ILP) and ES-PD (MLP) showed a similar amino acid composition, the analysis was limited to both protein isolates. The amino acid amounts in the lupin seed flakes as well as in EA-PI and ES-PD are presented in Table 4.

All three samples showed highest concentrations of the amino acids arginine (9.97–20.09%), glutamic acid + glutamine (18.98–21.43%) and aspartic acid + asparagine (11.25–12.92%) and lower contents of both sulfur-containing amino acids cysteine and methionine with each <1%, which is characteristic for vegetable proteins (Petterson, 1997). Contents of arginine were notably higher in the lupin seed flakes, compared to both protein isolates EA-PI and ES-PD, respectively.

Summing up, the amounts of amino acids according to their individual functional side chains showed a reduction in polar (EA-PI: 61.54%, ES-PD: 63.54%) and an enrichment in nonpolar amino acids (EA-PI: 38.46%, ES-PD: 36.46%; Table 4) after the isolation procedure (lupin flakes, polar: 69.43%, nonpolar: 30.57%). This might be due to the specific amino acid distribution of the individually occurring conglutins such as conlgutin α which contains the highest amount of leucine and conglutin γ exhibiting the highest portion of methionine (Lqari et al., 2004).

In a recent study from Benjamin, Silcock, Beauchamp, Buettner, and Everett (2014) the lower percentage of nonpolar amino acids was in line with a lower surface hydrophobicity of a lupin protein isolate compared to corresponding protein isolates from other raw materials. The content of polar and nonpolar amino acids was therefore supposed to influence the mouth-feel of both protein isolates, and a higher content of nonpolar residues might facilitate the formation of protein micelles inside the aqueous surrounding medium. However, this assumption could not be confirmed, as both protein isolates showed more or less the same quantity of nonpolar amino acids (total of nonpolar amino acids in EA-PI: 38.46 ± 0.01–0.08%, in ES-PD: 36.46 ± 0.01–0.61%).

Furthermore, both isolation procedures led to a considerable reduction of amino acids with basic side chains (Lupin flakes: 28.86%, EA-PI: 18.76%, ES-PD: 18.04%). This reduction was attributed to the acidic pH of both protein isolates after precipitation (EA-PI: pH 4.5, ES-PD: pH 5.5–6.0). In contrast, the content of amino acids with aliphatic (Lupin flakes: 28.24%, EA-PI: 34.83%, ES-PD: 32.41%), aromatic (Lupin flakes: 9.60%, EA-PI: 11.35%, ES-PD: 11.08%), hydroxylated (Lupin flakes: 9.32%, EA-PI: 11.60%, ES-PD: 10.73%) and sulfur-rich side chains (Lupin flakes: 0.58%, EA-PI: 0.89%, ES-PD: 0.85%) rose after the isolation procedures, with the last group showing the most important increase.

Among both protein isolates, the amino acid amounts generally showed the same trend. Correspondingly, the total amounts of amino acids within the same functional groups, such as the basic (Lys, Arg, His), aliphatic (Ala, Gly, Iso, Leu, Met, Pro, Val), aromatic (His, Phe, Trp,Tyr), sulfur-rich (Cys, Met), and hydroxylated (Ser, Thr, Tyr) side chains, were similar among both protein isolates. Therefore, the influence of individual amino acids is considered insignificant on the formation of the fundamentally different microstructures of both protein isolates EA-PI and ES-PD, as shown by Muranyi et al. (2013). Not the average hydrophobicity of the proteins itself but the hydrophobicity of the protein surface is instrumental in governing the functional properties of legume proteins (Kato & Nakai, 1980).

4. Conclusion

Influences of the isolation procedure on protein yield as well as on the quantitative distribution of individual protein fractions were shown.

The protein yields after salt-induced or alkaline extraction were similar. In contrast to the similar protein amount also yielded after combined dilutive and isoelectric precipitation, the protein yields were significantly higher for both the dilutive and isoelectric precipitation after alkaline, than after salt-induced extraction. Therefore, a different protein microstructure of EA-PD5.5 was assumed to differ from the micellar ES-PD. This goes in line with the lack in micelles shown microscopically in a previous study in contrast to the protein micelles in ES-PD.

Conglutin δ was extracted independently on the extraction method (using the alkaline or salt-induced extraction); however only isoelectric precipitation was found effective to yield the lupin albumin exhaustively. These higher amounts might contribute to an enlarged surface area of the proteins by facilitating their interaction with the aqueous phase. This would corroborate the unfolded protein structures of the isoelectrically precipitated protein isolates, as shown by microscopic approaches in the former investigations.

Furthermore, there might be an interrelation between the albumin contents and the individual protein yields, as the higher the albumin content, the higher the protein yield. Protein denaturation is known to enhance protein yields but also to unfold protein structures. To investigate the reasons for the yield differences in more detail, correlation statistics as well as denaturation studies should be investigated in another study.

All the other lupin conglutins occurred in all protein isolates, however with important differences in their amounts. The higher amount of the sulfur-rich conglutin α and conglutin γ in ES-PD were ascribed to the stabilized micellar protein arrangement of this micellar lupine protein isolate.

The amino acid composition and protein composition investigated by high performance chromatography were similar among the protein isolates. Therefore, electrophoresis seemed more appropriate to identify differences in the protein composition, than chromatographic investigations alone.

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CHAPTER 3

Influence of the isolation method on the technofunctional properties of protein isolates from *Lupinus angustifolius* L.

The technofunctional properties of ILP and MLP from *Lupinus angustifolius* L. Vitabor were investigated. Focus was placed on the influence of the protein microstructure on technofunctional properties.

Both protein isolates were characterised determining their viscoelastic properties by rheological investigations, protein solubility profile, water and oil binding capacities, emulsifying capacity, surface hydrophobicity and denaturation behaviour using differential scanning calorimetry. ILP showed a significantly higher degree of protein denaturation and lower denaturation temperatures than MLP. ILP revealed higher firmness and a viscoelastic solid-like behaviour, in contrast to MLP that showed viscoelastic, liquid-like properties. Protein solubility of MLP was higher compared to ILP and solubility minima were slightly different for both protein isolates. ILP showed higher water binding and lower oil binding capacities than MLP. The surface hydrophobicity was pH-dependent and lowest at the individual p*I*. Even though both protein isolates were investigated at their p*I*, emulsifying capacity varied significantly: MLP showed forefront emulsifying capacity, while the one of ILP was below the detection limit. This highlighted the influence of structural properties such as a high molecular flexibility on technofunctional properties. The results obtained revealed different putative application fields of both lupin protein isolates as food ingredients, with ILP as a moisture enhancer and MLP as a “natural” emulsifier in mixed food systems.

I. Muranyi designed the study, wrote the manuscript and interpreted the results. C. Otto collected test data and assisted interpreting the results. C. Pickardt managed the subventioned project, assisted interpreting the results and contributed to writing of the manuscript. Furthermore, contributions to writing were made by P. Koehler, U. Schweiggert-Weisz, and R. Osen.

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Influence of the Isolation Method on the Technofunctional Properties of Protein Isolates from *Lupinus angustifolius* L

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Abstract: The technofunctional properties of 2 protein isolates from *Lupinus angustifolius* L. Vitabor isolated by different procedures were investigated. The lupin protein isolate prepared by aqueous alkaline extraction with subsequent isoelectric precipitation (ILP) showed a significantly higher degree of protein denaturation and lower denaturation temperatures than the one obtained by aqueous salt-induced extraction followed by dilutive precipitation (MLP) as determined by differential scanning calorimetry. Rheological investigations revealed higher firmness and a viscoelastic solid-like behavior of ILP, in contrast to MLP that showed viscoelastic, liquid-like properties. Protein solubility of MLP was higher compared to ILP and solubility minima were slightly different for both lupin protein isolates. The protein isolates exhibited different technofunctional properties with ILP showing higher water binding capacity, lower oil binding capacity and lower emulsifying capacity than MLP. This reflects the different putative application of both lupin protein isolates as food ingredients, for example for ILP as a moisture enhancer and for MLP as a "natural" emulsifier in mixed food systems.

Keywords: denaturation, emulsification, extraction, functional properties, rheological properties

Practical Application: Due to their superior technofunctional properties and nutritional value, legume proteins become more and more important for food industry. In particular, lupins are an underestimated source of proteins, which offer a great potential for the utilization as functional ingredients. Two different methods could be applied for the isolation of proteins—an alkaline extraction followed by isoelectric precipitation and a salt-induced extracted combined with a dilutive precipitation. It has been shown in this study, that the protein products derived thereof differ considerably in their technofunctional properties. Applying the appropriate isolation conditions, tailor-made protein products with specific properties such as a high emulsifying or high water binding capacity could be obtained.

Introduction

In the last years, an increasing demand for plant proteins has been observed in the food industry. Soy is one of the most important plant protein sources worldwide. However, soy has been associated with some disadvantages such as the deforestation of rainforest or the utilization of genetically modified organisms. Hence, the search for alternative plant protein sources is steadily increasing (Lawal and others 2007). One promising but still underestimated raw material with high protein content in the seed is the lupin, a member of the legume family.

The high protein content makes lupin seeds attractive for human nutrition (El-Adawy and others 2001). Furthermore, several health-promoting effects of the lupin proteins and fibers have been reported in literature (Fechner and others 2013; Baehr and others 2015). Besides the nutritional benefits, lupin proteins exhibit technofunctional properties, which enable their application as food ingredients.

The production of lupin protein isolates for application in food systems is usually based on 2 different isolation procedures. The common alkaline extraction has been in the focus of recent studies. Usually, extraction by dilute NaOH is followed by isoelectric precipitation using HCl to adjust the pH. This lupin protein isolate (ILP) exhibits a curdy mouth-feel and has gelation properties similar to egg (Noll 2001; Bez and others 2005). The protein microstructure of ILP shows denatured, unfolded and large protein agglomerates (Muranyi and others 2013).

Another isolation method is based on salt-supported extraction followed by a dilutive precipitation. In a study from Sussmann and others (2013) relevant processing parameters and different raw materials have been investigated systematically to obtain high yields of protein preparations with characteristic fatlike textural properties. In the case of full-fat lupin flakes, a protein yield of 38% was achieved. The sensory profile of the lupin protein isolate (MLP) showed unique creamy, smooth and fatlike characteristics due to micellar aggregate formation (Ismond and others 1990; Muranyi and others 2013).

Other authors have described specific characteristics for both types of isolates from amaranth, flax seed, safflower, soybean and chickpea (Paredes-López and others 1991; El-Adawy and others 2001; Krause and others 2002). The protein isolates obtained by isoelectric precipitation exhibit higher foam stability and foam expansion but lower protein solubility, fat absorption and emulsification capacity compared to the micellar protein isolates. Furthermore, ILP is capable of forming protein networks as opposed to the micellar protein isolates (Cordero-de-los-Santos and others 2005; Rodriguez-Ambriz and others 2005).

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The differences in the technofunctional properties of both protein isolates could be attributed to the different isolation procedures. Process parameters such as pH adjustment or the addition of detergents can affect the proteins irreversibly and lead to protein unfolding or a loss in functionality (Schwenke 2001). Changes of some functional properties such as surface hydrophobicity require a partial protein unfolding (Pozani and others 2002). During protein denaturation, the hydrophobic regions have a lower ability to maintain hydrophobic protein–protein interactions and align toward the protein surface (Schwenke 2001). In contrast, a low degree of denaturation and a highly flexible character of the protein are required for an optimal emulsifying capacity (Damodaran 2005). Furthermore, protein solubility requires low protein denaturation for increased protein–solvent interactions (Sousa and others 1996).

Although several studies have investigated the technofunctional properties of individual protein isolates as stated above, literature data revealing correlations between the isolation procedure and the technofunctional properties of the final product are scarce. As this knowledge is a prerequisite for the production of tailor-made functional ingredients, the objective of the present study was to characterize the technofunctional properties of 2 lupin protein isolates obtained by alkaline extraction followed by isoelectric precipitation or by salt-induced extraction followed by dilutive precipitation. The viscoelastic behavior, the protein solubility as well as water binding, oil binding and emulsification capacities of both isolates were investigated. The findings obtained in the current study are discussed in the context of the chemical composition (Muranyi and others 2016) and—even more important—the microscopic appearance of both isolates (Muranyi and others 2013). An interrelation between the isolation, these characteristics, and the technofunctional properties of the individual proteins should be elaborated.

Experimental

Materials

Lupin seeds of the sweet narrowleaf lupin cultivar *Lupinus angustifolius* L. (cv. Vitabor, 2005) were obtained from Saatzucht Steinach (Steinach, Germany). A commercial soy protein ingredient Wilpro I200 was obtained from Yihai Kerry (Qinhuangdao) Protein Industries Co., Ltd (China) and was used as a reference for the determination of technofunctional properties. The pH of this product was 7.2. Bovine serum albumin (BSA) and NaCl were purchased from VWR International (Darmstadt, Germany), phosphate buffer (NaH_2PO_4 and Na_2HPO_4) and ANS (8-anilinonaphthalene-1-sulphonic acid) probe were from Sigma-Aldrich Chemie GmbH (Munich, Germany), NaOH and HCl from Merck KGaA (Darmstadt, Germany) and Mazola corn oil was obtained from Unilever GmbH (Hamburg, Germany). Deionized (DI) water was used during all experiments. All reagents used were of analytical grade or stated otherwise.

Pretreatment of the lupin seeds

The seeds were dehulled with an underflow peeler (Streckel & Schrader, Hamburg, Germany) and classified in an air-lift system (Alpine Hosokawa AG, Augsburg, Germany) to remove the hulls. The dehulled seeds were flaked using a roller mill (Streckel & Schrader, Hamburg, Germany) to obtain lupin flakes, which were used as raw material for the production of the protein extracts and protein isolates, respectively.

Preparation of protein isolates

The protein isolates were prepared according to the methods described by Muranyi and others (2013). A brief description of the preparation process is given in the following.

Isoelectric lupin protein isolate (ILP): An aqueous alkaline extract of the lupin flakes was prepared in DI water at a ratio of 1:8 (w/v) at pH 8.0 using 0.5 mol/L NaOH to adjust the pH value. In order to obtain the lupin protein isolate, the proteins were precipitated by the acidification of the extract to pH 4.5 using 0.5 mol/L HCl.

Micellar lupin protein isolate (MLP): An aqueous salty extract of the lupin flakes was prepared using a NaCl solution with a final concentration of 0.5 mol/L at a ratio of 1:8 (w/v). In order to obtain the lupin protein isolate, ionic strength was reduced by addition of DI water at room temperature (ratio 1:3 [v/v]) to precipitate the proteins.

Both protein suspensions (ILP and MLP) were stored at 1 °C for 18 h to facilitate protein precipitation and finally centrifuged at 3300 × *g* for 10 min to recover the individual protein isolates. Further treatments of the isolates as preparation for specific analytical methods are mentioned at the respective analytical sections.

Differential scanning calorimetry

The denaturation properties of the protein isolates were analyzed by differential scanning calorimetry (DSC) equipped with a cooling system (DSC Q2000 and RCS 90; TA Instruments, Lukens Drive, New Castle, Del., U.S.A.) according to the method of Sousa and others (1995). The samples (12 ± 0.01 mg) were heated in sealed standard aluminum pans (TA Tzero Hermetic, Stadt, Land) on 2 successive cycles from 303 to 393 K (30 to 120 °C) at a linear heating and cooling rate of 2 °K/min.

Denaturation enthalpies and peak denaturation temperatures were evaluated using the Software TA Universal Analysis (TA Instruments). Enthalpy values were calculated based on the protein content of each sample [J/g protein].

Dynamic oscillatory rheology

Prior to oscillatory strain sweep tests, the freshly prepared protein isolates were conditioned to 23 °C for 10 min. Measurements were performed using a shear stress controlled rheometer CVO 100 (Bohlin Instruments, Pforzheim, Germany) according to the procedures described by Krause and others (2002) with some modifications. An aliquot of 1.5 g sample was placed between parallel plates ($d = 20$ mm, gap size = 1mm) and covered around the plate edges by a low-viscosity silicon oil to avoid water evaporation.

Viscoelastic properties of the protein isolates were studied using dynamic measurements. Amplitude sweep tests were performed to determine the linear viscoelastic region (LVR) of the samples at a frequency of 1 Hz at increasing strain ranging from 0.01% to 100% (logarithmic scale). A frequency sweep test was carried out at a constant strain of 1% at decreasing frequency ranging from 55 to 0.1 Hz (logarithmic scale) to evaluate the storage modulus G' [Pa] and the loss modulus G'' [Pa].

Protein solubility

The pH-dependent protein solubility of the freshly produced protein isolates at 0.1 mol/L NaCl from pH 2 to 9 was determined according to the method of King and others (1985) using 0.5 mol/L HCl or 0.5 mol/L NaOH to adjust pH values.

Technofunctional properties

For the determination of water and oil binding capacities as well as emulsifying capacity, both protein isolates were frozen at –50 °C and lyophilized in a freeze-drier (Beta 1–8, Christ GmbH, Osterode, Deutschland). The dried protein isolates were milled using a Retsch mill (sieve insert: 0.5 mm) and stored at 14 °C.

Water binding capacity. The water binding capacity [mL water/g protein] was determined according to the AACC International standard method 56-30 with DI water after centrifugation at 20 °C and 1000 × *g* for 15 min.

Oil binding capacity. Aliquots of 3.0 g protein isolate were dispersed with 20 mL corn oil in a graduated 25 mL centrifuge tube for 1 min at ambient temperature. After centrifugation of the suspension for 15 min at 700 × *g* and 20 °C, the volume of unbound oil (supernatant) was determined. Finally, oil binding capacity (OBC) [mL oil / g protein] was calculated as follows:

$$\text{OBC (mL/g)} = \frac{V_1 - V_2}{m_1}$$

where V_1 is the total oil volume; V_2 is the volume of unbound oil; and m_1 is the weighed sample mass.

Emulsifying capacity. The emulsifying capacity was determined according to the method described by Wäsche and others (2001) using a 1-L-reactor equipped with a stirrer and an Ultra-Turrax (IKA-Werke GmbH & Co. KG, Staufen, Germany).

Spectrophotometric determination of surface hydrophobicity

Protein surface hydrophobicity (S_0) was determined according to the method published by (Kato and Nakai 1980) with some modifications. Samples ILP and MLP were dispersed (0.019% w/v) in 10 mmol/L phosphate buffer and diluted to 5 concentrations between 0.0019% and 0.019% at pH 3, 4.5, 5.5, and 8. After addition of 20 μL 8-anilinonaphthalene-1-sulphonic acid (ANS) to 1 mL of each protein sample, solutions were mixed for 15 min in the dark. The signal intensity measurement of 100 μL of each protein suspension was determined with a BIO TEK Synergy H4 Performance Reader (BIO TEK Instruments GmbH, Bad Friedrichshafen, Germany) with excitation/emission wavelengths of 360/490 nm. S_0 measurements were recorded as mean values deriving from 2 independent serial dilutions of the respective protein samples injected 3 times into the microplate reader. Surface hydrophobicity derived from the slope of the linear regression intensity signal ($r^2 > 0.95$) using solution of ANS in phosphate buffer as a blank without protein addition.

Statistical evaluation of the data

Values are given as mean values and standard deviations. For all analyses a number of 3 to 5 experiments were conducted. Average values of 2 corresponding groups were compared using the unpaired Student's *t*-test. Significant differences between 2 corresponding groups ($P < 0.05$) are marked in the tables by an asterisk (*).

Results and Discussion

The chemical composition of both lupin protein isolates obtained from the full fat lupin flakes was described in detail in Muranyi and others (2013). The pH values in the freshly produced final products (dry matter ILP: 43.1%, MLP: 44.4%) were pH 4.5 for ILP and 5.5 for MLP. In other studies, the pH is standardized prior to technofunctional characterization to reduce the number of influencing factors and to enhance comparability among the protein isolates. However, pH modifications are known to alter the protein microstructures by modifying the charges within the entire protein (Paulson and Tung 1987). As the aim of our study was to attribute the technofunctional properties to the protein microstructures as revealed by Muranyi and others (2013), the pH was intentionally not adjusted.

Protein denaturation

The salt-induced extraction followed by dilutive precipitation is assumed to be a "mild" process (Paredes-López and others 1991; Cordero-de-los-Santos and others 2005) in contrast to the alkaline protein extraction followed by isoelectric precipitation. To prove this assumption, the denaturation properties of both isolates were examined.

The thermogram of ILP (Figure 1) showed 2 endothermic transitions with peak temperatures of 78.94 ± 0.24 and 91.85 ± 0.31 °C and denaturation enthalpies of ΔH_2 = 4.52 ± 0.26 and ΔH_3 5.92 ±0.25 J/g protein. These temperature values corresponded to the denaturation temperatures reported for the 7S (conglutins γ and β) and the 11S (conglutin α) lupin proteins (Sousa and others 1995).

In contrast, MLP showed 3 endothermic transitions with 2 major peaks at 87.27 ± 0.14 and 101.12 ± 0.62 °C and a minor peak at 70.00 ± 0.16 °C. The peak temperatures were assumed to correspond to the 2S (conglutin δ), 7S (conglutins γ and β), and the 11S (conglutin α) lupin protein fractions (Sousa and others 1995). The peak for conglutin δ did not appear in the ILP sample, although a previous study has shown that ILP contains around 5% more of this albumin fraction than MLP (Muranyi and others 2016). Hence, a complete irreversible denaturation of the lupin albumin fraction during the isolation procedure was assumed.

The enthalpy values of the 3 peaks in MLP amounted to ΔH_1 = 0.85 ± 0.14 J/g, ΔH_2 = 9.42 ± 0.10 J/g and ΔH_3 = 7.54 ± 0.09 J/g protein (Figure 1). The higher enthalpy of the MLP sample measured during the heating cycle could be attributed to a lower degree of protein denaturation during the isolation process of MLP compared to ILP. Arntfield and Murray (1981) described the alkaline extraction and isoelectric precipitation as extreme isolation conditions. This might be due to the immediate changes in the electrostatic repulsion of the proteins

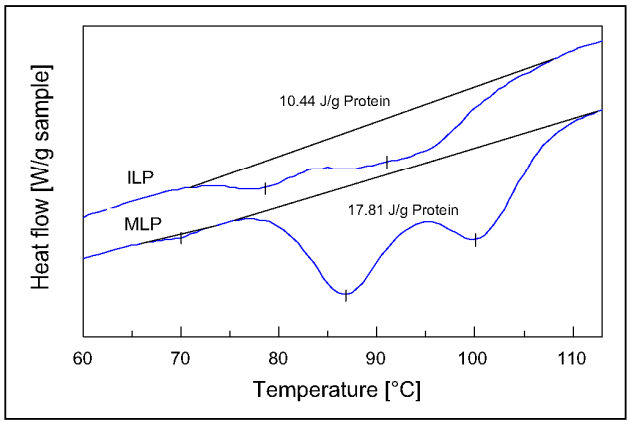

Figure 1–Heat flow graphs of isoelectric (ILP) and micellar lupin protein isolate (MLP) including total denaturation enthalpies [J/g protein].

when changing from alkaline to acidic conditions. In contrast, the mild conditions during production of MLP largely maintained the native molecular state of the proteins (Sousa and others 1995).

The denaturation temperatures of the proteins were 10 °C higher for MLP than for ILP. Nagano and others (1994) ascribed the differences in thermal stabilities of proteins to strong hydrophobic interactions between the globulin subunits and correlated an increase in denaturation temperature with a raise in hydrophobic forces. Correspondingly, it could be assumed that due to pronounced hydrophobic interactions, the globular protein structures in MLP were more resistant against the thermal impact than the partially unfolded proteins in ILP. Presumably, in MLP so called subparticles are arranged in compact supramolecular structures protecting the encapsulated side chains (Boulet and others 2000). This is in accordance with the macroscopic and microscopic investigations in a previous study, where only MLP consisted solely of the homogeneous and compactly arranged micellar protein structures (Muranyi and others 2013). In contrast, the more denatured ILP proteins revealed an inhomogeneous and unfolded structure with weaker hydrophobic interactions. This structure, furthermore, resulted in a larger contact surface for external influences such as thermal stress facilitating structural changes (Cooper 1988). As a consequence, the denaturation temperatures were reduced.

Furthermore, the broadening of the peaks for ILP are associated with the disruption of hydrogen bonds and the existence of intermediate forms different from the native state (Wang and Damodaran 1991; Nagano and others 1994) and confirmed its inhomogeneous and unfolded structure in contrast to MLP.

Viscoelastic properties

In the present study, the viscoelastic properties of the protein isolates were determined by fundamental rheology using an oscillation test. The storage modulus, reflecting the elastic component of the lupin protein samples, was characterized by G' and the loss modulus, reflecting the viscous component, by G''. The amplitude sweep curves and the frequency sweep curves are shown in Figure 2.

Both ILP and MLP exhibited a similar curve pattern of a viscoelastic material that is independent of a strain up to a critical flow point. ILP showed a storage modulus of $G' = 64300 \pm 6049$ Pa and a loss modulus of $G'' = 50980 \pm 9545$ Pa in the linear viscoelastic range (LVR) until a deformation rate of 1% (Figure 2A). A similar result was achieved for flax seed protein isolates in a study of Krause and others (2002), in which dispersions of micellar protein exhibited lower modulus values at pH 3 and 8 compared to the dispersions of isoelectrically recovered protein isolates at the same pH values.

ILP is characterized by a viscoelastic solid-like behavior ($G' > G''$, (Xu and Mohamed 2003). In contrast, MLP exhibited viscoelastic liquid-like properties ($G'' > G'$) in a wider LVR until a deformation rate of 4% with significantly lower moduli ($G' = 378 \pm 116$ Pa and $G'' = 1593 \pm 353$ Pa; $P < 0.05$, unpaired Student's t-test). As the dry matter and protein contents of both protein solutions used for analysis were similar, their rheological behavior was attributed to complex interactions among several variables such as protein size and shape as well as protein–solvent interactions (Krause and others 2002).

MLP is proposed to consist of large micellar protein particles of an average size of 0.6 μm as determined by laser diffraction (data not shown). The core is hydrophobic and the surface hydrophilic (Ismond and others 1990; Muranyi and others 2013). In contrast, ILP comprises aggregates of denatured proteins of an average size of 1.0 μm showing irregular shapes and sizes without micellar microstructures. Following this hypothesis, the different rheological behaviors of the proteins were explained by the size, shape and net-charge effects as follows:

Under low frequency conditions, the intermolecular interactions between the compact MLP micelles are assumed to be mainly based on surface charge effects with low steric interactions between the micelles due to their regular spherical shape. This would allow the micelles to slide against each other and would explain the viscoelastic liquid-like behavior. In contrast, the irregular shape and size of the ILP aggregates result in a comparatively loosened packing of particles that are stabilized by steric effects under low shear conditions. These unfolded proteins in ILP are assumed to present obstacles to each other and raise the resistance towards deformation (raise in G'). Consequently, a viscoelastic-solid-like character as well as a higher elastic modulus is recorded for ILP compared to MLP. Simultaneously, sensitivity towards deformation is higher and ILP starts to flow at a lower deformation level than MLP (Figure 2A) which is reflected by the lower yield point of ILP compared to MLP.

The frequency sweep curves in Figure 2B showed a greater slope for MLP than for ILP showing higher frequency dependency for MLP compared to ILP (Xu and Mohamed 2003).

These results are in accordance with those reported by Xu and others (2006), who compared the rheological behavior of diafiltrated lupin proteins with an isoelectrically precipitated lupin

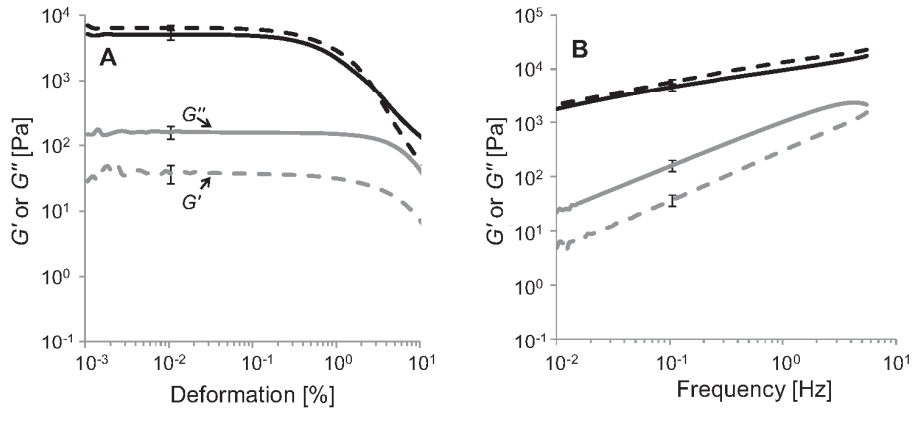

Figure 2–Storage modulus G' [Pa] (dashed line) and loss modulus G'' [Pa] (solid line) of isoelectric lupin protein isolate (ILP, black) and micellar lupin protein isolate (MLP, gray). (A) Amplitude sweep at a frequency of 1 Hz (6.28 rad/s), (B) frequency sweep at a constant strain of 1%. Mean values ± standard deviation of 3 experiments are given ($n = 3$).

protein. The latter showed solid-like behavior while the former exhibited viscoelastic liquid-like behavior with a higher frequency dependency, which was assumed to be associated with lower degrees of protein entanglements in the diafiltrated lupin proteins. Therefore, the lower frequency-dependency of ILP in comparison to MLP is supposed to arise from its partial denaturation and irreversible aggregation of the proteins into irregular protein tangles.

Protein solubility

The protein solubility profiles of ILP and MLP as affected by the pH-value are presented in Figure 3. MLP exhibited a wider solubility range from 0% to 100% and generally higher protein solubility (except at pH 5 to 6) compared to ILP with a solubility range of 0% to 86%. The differences were distinctive in the lower acidic as well as in the neutral to alkaline range whereas both isolates exhibited very low solubilities (near 0) at their isoelectric points which were slightly different with pH 4.5 to 5.0 for ILP and pH 5.0 to 5.5 for MLP. Similar solubility profiles were shown for isoelectric and micellar soy protein isolates showing their solubility minima between pH 4 and 6. In accordance with our study, the micellar soy protein isolate reached protein solubility of 100%, whereas maximum solubility of isoelectric soy protein isolate was below 90% (Rodriguez-Ambriz and others 2005).

Protein solubility is dependent on the accessibility of polar and nonpolar amino acid side chains at the surface of the protein molecules (Schwenke 2001). Therefore, the irreversible unfolding of hydrophobic side chains towards the proteins surface during the isolation procedure reduced the protein solubility of ILP.

In addition to the pH dependency, salt ions at low concentrations are able to increase the protein solubility, also known as the "salting in" (Hofmeister 1888). The proteins are surrounded by the salt counter ions, which neutralize surface charges and lead to decreasing electrostatic interactions between the protein molecules. As a consequence, protein solubility increases (Vojdani 1996). NaCl shifts the p*I* to a more acidic pH due to specific ion binding effects with the chloride anions exhibiting higher selective binding than the sodium cations due to their smaller hydrated radii (Thawornchinsombut and Park 2004). The higher salt content of ILP (3%) compared to MLP (1%, Muranyi and others 2013) might, therefore, affect the isolate properties and lower the p*I* due to excess negative charge at the ILP-proteins. These findings go in line with observations (Pawar and others 2001) where the region of minimum solubility of sunflower proteins (pH 4 to 6) was shifted to lower pH values in salt solutions.

The goal of the present study was to characterize the protein isolates by maintaining their characteristic protein microstructures. Therefore, the pH was not adjusted prior to the functional measurements. Please not, that according to the minimum solubility determined for ILP (pH 4.5 to 5.0) and MLP (pH 5.0 to 6.0) in this section indicating that all investigations within this study were approved at their individual isoelectric points.

Technofunctional properties

The technofunctional properties of ILP and MLP are presented in Table 2. The water–oil binding index was developed by Dekanterewicz and others (1987) to describe the relative hydrophilic–lipophilic character of proteins and to predict their emulsifying properties. The water–oil binding index is calculated relating the water binding capacity to the oil binding capacity.

ILP exhibited a higher water binding capacity than MLP. The water binding capacity of a protein isolate depends on the availability of polar amino acids for protein–water interactions (Paredes-López and others 1991). Furthermore, the water binding capacity depends on the denaturation level of the respective protein and is higher for a denatured protein because of an increase in surface area to mass ratio due to protein unfolding (Sumner and others 1981; Damodaran 1996). The differences in water binding capacity could therefore be ascribed partially to the higher denaturation of ILP (Table 1). The addition of salts at low concentrations (<0.2 mol/L) increases the water binding capacity of proteins due to binding of the hydrated salt ions to the charged protein groups (Damodaran 1996). As the salt content of ILP was higher (2.5%) than the one of MLP (1.3%, Muranyi and others

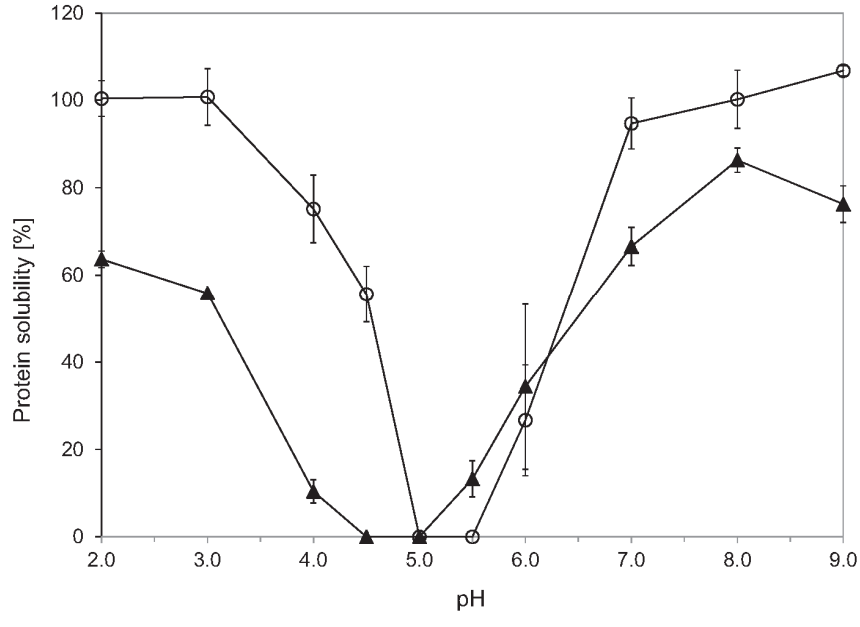

Figure 3–Protein solubility of ILP (triangle) and MLP (circle) between pH 2.0 and 9.0. Mean values ± standard deviation of 3 experiments are given ($n = 3$).

Table 1–Thermodynamic properties of ILP and MLP assessed by differential scanning calorimetry.

	Peak area 1		Peak area 2		Peak area 3		Total enthalpy
	T_1 [°C]	H_1 [J/g Protein]	T_2 [°C]	H_2 [J/g Protein]	T_3 [°C]	H_3 [J/g Protein]	H_Σ [J/g Protein]
ILP		–	78.9 ± 0.2	4.5 ± 0.3	91.9 ± 0.3	5.9 ± 0.3	10.4 ± 0.3
MLP	70.0 ± 0.2	0.9 ± 0.1	87.3 ± 0.1*	9.4 ± 0.1*	101.1 ± 0.6	7.5 ± 0.1*	17.8 ± 0.1*

Mean values marked with an asterisk (*) are significantly higher than corresponding values in the same column (Student's *t*-test, $P < 0.05$). Mean values ± standard deviation of 3 experiments are given ($n = 3$).

Table 2–Technofunctional properties of ILP and MLP.

Protein isolate	Water binding capacity [mL water/g protein]	Oil binding capacity [mL oil/g protein]	W/O binding index [mL H_2O/mL oil]	Emulsifying capacity [mL oil/g protein]
ILP	0.85 ± 0.06*	0.85 ± 0.03	1.00	–
MLP	0.45 ± 0.03	1.20 ± 0.05*	2.67	472.5 ± 15*

Mean values marked with an asterisk (*) are significantly higher than corresponding values in the same column (Student's *t*-test, $P < 0.05$). Mean values ± standard deviation of 3 experiments are given ($n = 3$).

2013), the higher water binding of ILP might also be the result of an enhanced amount of hydrated salt ions bound to the proteins.

Oil binding capacity was higher for MLP than for ILP, which is in accordance to the findings from El-Adawy and others (2001) and Paredes-López and others (1991). They found a higher fat absorption capacity of micellar protein isolates compared to isoelectric lupin protein isolates. Furthermore, Sumner and others (1981) investigated isoelectric protein isolates and observed that a higher degree of denaturation also lowered oil binding capacity, which is in accordance to our study.

The binding mechanisms are not clear until now, but the lower degree of protein denaturation determined for MLP might be associated with improved oil binding capacity. Presumably, due to the high molecular flexibility, the proteins of MLP are able to reorient appropriately their functional side chains exposing lipophilic amino acids and hiding hydrophilic amino acids from the oil. In contrast, reorientation of the ILP-proteins might be limited due to the higher degree of protein denaturation. However, the underlying oil binding mechanisms have to be investigated in further studies.

Conformational factors are primarily related to the differences in the surface activity of proteins. Therefore, the surface hydrophobicity (ANS S_0) was also measured (Figure 4).

S_0 of both protein isolates was pH-dependent. S_0 of ILP ranged from 207.0 to 10157.5 and that of MLP from 401.0 to 12601.5. The results confirmed minimum electric net charge of both individual lupin protein isolates at their respective pH values prevailing upon precipitation (pH 4.5 for ILP and pH 5.5 for MLP). This pH-dependency was in connection with the repulsive and attractive forces caused by the protein's characteristic electric net charge at the different pH values. S_0 of ILP was lowest at pH 4.5 and S_0 of MLP lowest at pH 5.5, which corresponds to the individual solubility minima of both protein isolates presented in Figure 3 (p*I* of MLP: 5.5, p*I* of ILP: 4.5). At the minimum electric net charge, hydrophobic side chains are hidden and reoriented towards the inner core of the protein aggregates (Muranyi and others 2013).

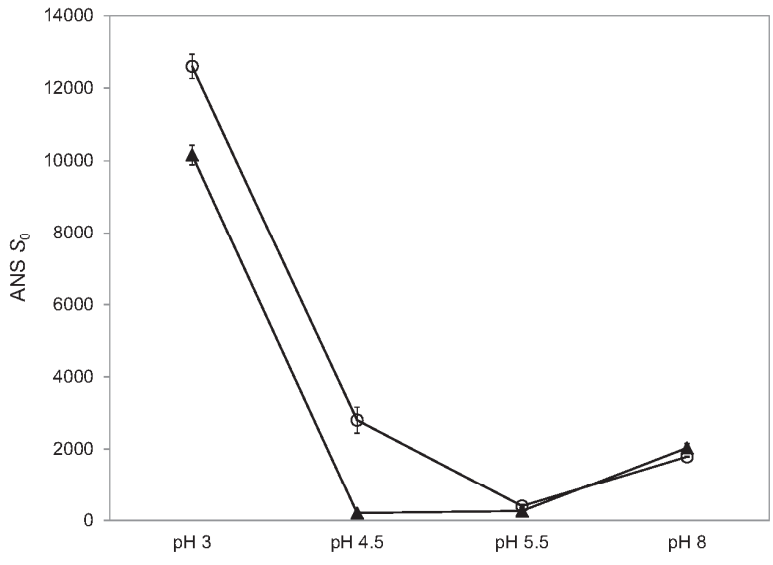

Figure 4–Surface hydrophobicity (S_0) of ILP (triangle) and MLP (circle) at different pH values (pH 3.0, 4.5, 5.5, 8.0); $n = 2$.

Indeed, the exposure of hydrophobic side chains, which is reflected by S_0, essentially influences the emulsifying properties (Kato and Nakai 1980). However, the emulsifying capacity in this study was investigated at the S_0 minima of both protein isolates ($S_0 = 401$ for MLP at pH 5.5 and $S_0 = 207$ for ILP at pH 4.5), as focus was placed on the protein functionality at the individual pH values arising after precipitation. Therefore, the results were independent of the electric net charge of the proteins and dependency of the molecular flexibility on emulsifying capacity became visible. The results go absolutely in line with the findings of Damodaran (2005), were "Molecular flexibility represents the susceptibility of a protein's conformation to altered environment. Rapid conformation change at an interface is essential for the protein to reorient its hydrophobic and hydrophilic residues toward oil and aqueous phases and also to maximize the exposure and partitioning of these residues toward the 2 phases" (Damodaran 2005).

The higher water–oil binding index of MLP (2.67 mL water/mL oil) compared to ILP (1.00 mL water/mL oil) went in line with the lower degree of protein denaturation and higher protein flexibility as determined by DSC (Section "Protein denaturation"). A water–oil binding index of 2 is correlated with highest emulsifying capacity, whereas values below or above 2 result in decreased emulsifying capacity (Dekanterewicz and others 1987). Indeed, the measured emulsifying capacity of MLP was 472.5 ± 15.0 mL oil/g protein, whereas emulsifying capacity of ILP was below the detection limit (≤125.0 mL oil/g protein). Similar differences between the emulsifying capacities of isoelectric and micellar lupin protein isolates were observed by El-Adawy and others (2001).

Compared to a commercial soy protein isolate (Wilpro I200) showing the ability to bind 8 mL water/g protein, both protein isolates exhibited significantly lower water binding. However, similar functionality of MLP was shown for oil binding (Wilpro I200: 1.3 mL oil/g protein) and emulsifying capacity (Wilpro I200: 542.5 mL/g protein) highlighting its generally high potential to be used as a plant-derived emulsifying ingredient. Besides the molecular flexibility, protein solubility is described to be a prerequisite for good emulsifying properties by providing hydrophobic side chains (Kato and Nakai 1980). As protein solubility of both protein isolates increased significantly at pH values distant from the p*I* the emulsifying capacity is expected to further be increased at lower or higher pH values. Therefore, the pH-dependency of the emulsifying characteristics of MLP should be investigated in further studies to provide more information regarding its application potential in food systems that require pH values lower or higher than 5.5.

Conclusions

An influence of alkaline extraction and isoelectric precipitation compared with salt-induced extraction followed by dilutive precipitation on the technofunctional properties of the respective lupin protein isolates (ILP and MLP) was found. It was shown by means of DSC measurement that MLP had a lower protein denaturation than ILP. Further studies by circular dichroism could be applied to investigate the extent of denaturation in more detail. The viscoelastic properties of the lupin protein isolates can be attributed to their microstructural organization. Accordingly, the micellar proteins revealed viscoelastic liquid-like properties due to the formation of homogeneous protein micelles and showed a higher resistance towards deformation. The unfolded proteins in ILP led to a viscoelastic solid-like character of the protein isolate. With the protein micelles sliding against each other, the physical function of oil droplets is mimicked corroborating the fat-like properties found in sensory tests. Among the investigated technofunctional properties, MLP showed excellent emulsifying capacity in contrast to ILP. Therefore, besides its high potential for the application as a natural fat replacer, MLP is expected to be especially suitable for the application as a vegetable emulsifier in mixed food systems such as mayonnaise or salad dressings. Due to the higher water binding capacity, ILP might be more suitable to enhance the moisture content of for example bakery products. Finally, the study shows that the isolation procedure has a considerable impact on the specific characteristics of the protein isolates. Depending on the desired properties of the products an appropriate isolation procedure must be chosen.

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Author Contributions

I. Muranyi designed the study, wrote the manuscript and interpreted the results. C. Otto collected test data and assisted interpreting the results. C. Pickardt managed the subventioned project, assisted interpreting the results and contributed to writing of the manuscript. Furthermore, contributions to writing were made by P. Koehler, U. Schweiggert-Weisz, and R. Osen.

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GENERAL DISCUSSION AND CONCLUSIONS

Besides the production of ILP (protein yield: 29%), the isolation procedures of protein isolates ES-PDI (protein yield: 30%) and EA-PDI (protein yield: 32%) showed economically most favourable for the purpose of a maximal protein recovery. The higher albumin contents might contribute to the higher protein yields shown for isoelectrically precipitated protein isolates. Therefore, **the isoelectric precipitation and combined isoelectric- and dilutive precipitation are considered useful for food producers to yield the albumin fraction abudantly.** Thereby, **Hypothesis 1** of this dissertation was supported. Besides, protein denaturation was also higher after isoelectric precipitation and is known to enhance protein yields, but also to unfold protein structures. To investigate the reasons for the yield differences in more detail, correlation statistics between the protein composition, protein denaturation and resulting protein yields should be investigated in a subsequent study.

The assumption that formation of specific protein microstructures depends on the isolation process **(Hypothesis 2)** was approved. Using lupin, the dilutive protein presipitation could be applied to create a protein isolate (MLP) entirely consisting of protein micelles. The identification of the microstructures in MLP presented the main focus within the detailed microscopic characterisation of the protein isolates. The reason was that this protein isolate was assumed to feature protein micelles causing its characteristic fat-like texture sensation (Sussmann, et al., 2010). Light microscopy showed to be most expedient for a screening and identification of the microscopic structures of aqueous plant proteins. For detailed structure information, cryo-scanning electron microscopy proved indispensable. Environmental scanning electron microscopy and conventional scanning electron microscopy at atmospheric pressure were detected unsuitable due to major artefact formation in the protein microstructures. Comparing the results from all microscopic approaches, the microstructures determined within ILP and MLP were coherent and the microscopic methodology proved appropriate to monitor the structural features of the protein isolates. Furthermore, **the hypothesis of protein micelles described in literature was corroborated by the analytical methods.** The hydrophilic proteins were

predominately situated within the hull, while encapsulating hydrophobic protein domains in the core, as determined by the pH-dependent protein surface hydrophobicity. The particle sizes of MLP were analysed after statistical particle sizes determination with ImageJ using the MLP micrographs. The optical evaluation program ImageJ is considered to be an appropriate and precise analysis method, however, aligning the microscopic agglomerates digitally to the program requirements was significantly more time consuming than determination of the particle sizes by laser diffraction. Therefore, the determination of particle sizes by a Mastersizer is considered more appropriate and should be compared in another study. In addition, the comparison of both protein isolates ILP and MLP displayed by the laser diffraction method is assumed to corroborate the important structural features presented in the present dissertation. Accordingly, the irregular and disrupted protein microstructures of ILP might be displayed by a diffuse curve progression, whereas the regular and well-defined protein microstructures of MLP might be corroborated by a sharp and pointed curve progression reflecting a clear particle size distribution of uniform micelle particles in MLP.

An influence of the isolation procedure on quantitative distribution of individual protein fractions was shown and the microstructural organisation of the protein isolates was attributed amongst others to the protein composition. Therefore, **Hypothesis 3** proved right. Conglutin δ was extracted independently of the extraction method (using the alkaline or salt-induced extraction); however, only isoelectric precipitation was found effective to yield the lupin albumin exhaustively. These higher amounts might contribute to an enlarged protein surface of the proteins by facilitating their interaction with the aqueous phase. This would corroborate the unfolded protein structures of the isoelectrically precipitated protein isolates, as shown by the microscopic approaches. All the other lupin conglutins always occurred in all protein isolates, however with important differences in their amounts. The higher amount of the sulfur-rich conglutin α and conglutin γ in ES-PD (MLP) were ascribed to the stabilised micellar protein arrangement of this micellar lupin protein isolate.

Surface hydrophobicity showed to be influenced by the electric net charges of the proteins and corresponded to the pH-dependent protein solubility of the protein isolates. In the investigated pH-range both, the micellar and the isoelectric protein

isolates, showed comparable surface hydrophobicities. Therefore **Hypothesis 4**, describing an interaction between the protein denaturation and the surface hydrophobicity, remains unclear. Comparing the surface hydrophobicity of proteins denatured systematically to different extents seems promising to eludidate an impact of the protein denaturation on physico-functional properties.

An interrelation between the protein microstructures and technofunctional properties was shown. Therefore, **Hypothesis 5** was corroborated. Besides its high potential for the application as a "natural" fat replacer, MLP is expected to be especially suitable for the application as a vegetable emulsifier in mixed food systems such as mayonnaise or salad dressings. Due to the higher water binding capacity, ILP might be more suitable to enhance the moisture content for example of bakery products. Variations in the isolation procedure to obtain lupin protein ingredients with desired functional properties are considered promising: **Using method EAS-PD5.5 provided a protein isolate showing a highly favourable combination of diverse technofunctional properties.** This product was produced by combining salt-induced and alkaline extraction and precipitating the proteins subsequently by dilutive precipitation. Protein isolate EAS-PD5.5 seems to outperform the product qualities of ILP and MLP as determined in preliminary results. Meeting various requirements at the same time, this protein isolate is considered highly promising for food technologists and the food industry to be inserted into complex food systems to enhance the final product quality such as for sensory optimisation of energy-reduced dietary food products, for quality increase of meat analogues or for the protein-enrichment of sports drinks. Therefore, further application experiments seem promising to develop an "all-rounder" among tailored protein isolates based on lupin proteins.

The protein denaturation, the protein microstructures and the technofunctional properties were all found to depend on the molecular flexibility of the proteins. Furthermore, an interrelation was found between these properties, supporting **Hypothesis 6.** The molecular flexibility showed to be an appropriate indicator for the firmness of the protein precipitates determined by rheological measurements. Especially in a hydrophilic environment, where hydrophilic-hydrophobic balance is sustained, a certain micelle quantity and flexibility enables these colloidal particles to slide away from each other, similar to

the physical functionality of oil. The freshly produced MLP (dry matter: 44%) showed minimal resistance due to the formation of highly compact and stable fat-like micelles (physical mimickry). MLP showed the lowest total protein denaturation. The pH adjustments during extraction or precipitation raised protein denaturation and lowered the denaturation temperatures reflecting a decreased thermal stability of the proteins. Generally, the precipitation method presented a higher impact on protein denaturation than the extraction method presumably due to additive effects in protein strain. With a reduction in the denaturation temperature of the 11S proteins, presenting the main proteins in the lupin seed, the quantity of unfolded protein branches increased, the number of micelles decreased and sample firmness rose. The protein unfolding went along with higher water binding capacity, while high protein flexibility was required for oil binding and emulsifying capacity. The reason for the excellent emulsifying capacity of MLP was, that at an oil-water interface the flexible proteins were able to rearrange towards the individual interfaces and formed emulsions, whereas the highly denatured proteins in ILP were not. Thereafter, it is considered essential to use MLP at its native pH to prevent irreversible protein denaturation and to take advantage of its technofunctional properties.

Further considerations

Spectroscopic analysis has the potential to disclose important structural changes in molecules such as shifts in the two-dimensional protein structure due to salts or denaturation stress (Lichan, 1994). According to the lower degree of denaturation of MLP compared to ILP shown in this dissertation, MLP should show a higher content of α-helical structures and lower contents of antiparallel β-sheet arrangements than ILP. A comparative study between the Raman spectra of both protein isolates ILP and MLP seems warranted in future studies to corroborate analytically the important differences in three-dimensional structures and molecular protein denaturation of both lupin protein isolates.

The fundamental differences in protein conformation of the protein isolates might result in different allergenic potentials. Taking the example of both “corner products”, the unfolded protein structures in ILP might expose more allergenic sites

(linear epitopes) than the well-organised and firmly arranged micellar proteins of MLP. The conformation itself might as well present an allergic mediator (conformational epitopes), as the IgE response within lupin allergenicity was found to depend on the intrinsic properties of the conglutins (amino acid sequence, protein conformation; Foss, Duranti, Magni, & Frokiaer, 2006). Besides the structural differences, the protein composition of the protein isolates might represent a major risk for individuals suffering from allergy. The reason is the small molecular weight of the lupin albumin conglutin δ (10 k) highly abundant in ILP compared to MLP. Small proteins are known to facilitate the activation of immune active cells that initiate allergic responses. In case of a reduced allergic potential in MLP an innovative and simple method (micellar vs. isoelectric isolation procedure) to produce food products low in allergic potential could be provided to pharma and food industry. An immuno assay combined with Western Blot, for example, would present an adequate and fast method to estimate the allergenic potential of the lupin protein isolates on serums of allergy suffering patients.

In this dissertation the protein isolation procedure per se was characterised by an enrichment of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine (Burchardi & Larsen, 2004; Biesalski, et al., 2004) in the proteins of the isolates. Therefore, independent of their individual technofunctional properties, the lupin protein isolates are suitable for manufacturing products for geriatric as well as sports medical nutrition to increase nutritional protein quality. Investigations on the application of the lupin protein isolates in personalised products are assumed promising.

Based on the recipes worked out for fat-reduced mayonnaises, MLP could be readily applied by food technologists into food products. However, the sensorial tasting section showed a remaining optimisation potential. The increasing sensation of the attribute “legume-like” at high MLP contents might limit at present the consumer acceptance. Detailed research activities to reduce the responsible sensorically responsible substances are still missing, however, are considered essential to enable an industrial implementation of lupin protein ingredients in the future.

The application of MLP was at present only tested in moderately heated model foods (chocolate fillings, Sussmann, et al., 2010). The reason is the irreversible

protein modification of the protein micelles at temperatures exceeding 70 °C. Intense heat treatments such as boiling and baking would lead to strong textural and physical modifications of the micelles and of most other food proteins. Accordingly, the thermal stability of the protein micelles is another promising issue in the application of MLP as a “natural” fat-replacer in food products. In literature, a number of physical, chemical and enzymatic treatments are described to improve the physicochemical properties of proteins (Babiker, 2000; Chapleau & de Lamballerie-Anton, 2003; Mounsey, O'Kennedy, & Kelly, 2005; Puppo, et al., 2005; Tang, Chen, Li, & Yang, 2006; Wang, et al., 2008). Enzymatic modifications using microbial transglutaminase as well as physical modifications through high-pressure treatment are described to enhance the thermal stability of proteins, and might protect the micelles in MLP from losing their flexible protein behaviour during thermal stress. Therefore, investigations on the influence of physical and enzymatic modifications on protein stabilisation in MLP are assumed to extend the application field of MLP to thermally processed food products while maintaining its particular fat-like and technofunctional properties.

In the present dissertation, lupin protein isolates were shown appropriate as a food ingredient to increase product quality by their favourable technofunctional properties. However, important nutritional risks based on the sweet lupin *lupinus angustifolius* L. were highlighted by a study from Rahman (2000). In named study, the lupin proteins were assumed to be partly responsible for osteoblastic and or osteoclastic acivities that may limit their use in baby food. Furthermore, the lupin albumin fraction appeared highly toxic to growing rats. Even though conglutin δ was present in low concentrations in MLP, studies on post-prandial effects of feeding different lupin seed protein fractions and the metabolomic effects are missing entirely. Therefore, prior to industrial implementation of the lupin protein isolates, in particular nutritional investigations by human intervention studies should be carried out to ensure consumer safety.

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SUMMARY

Alkaline extraction with subsequent isoelectric precipitation and salt-induced extraction followed by dilutive precipitation are well-established techniques to isolate proteins from vegetable sources. Both techniques provide protein isolates with different technofunctional properties. In the case of lupins, the isoelectric protein isolate (ILP) is applied as an egg-substitute in diverse food products, whereas the specific dilutive protein isolate (MLP) exhibits fat-like properties. However, the reason for this behaviour has not been investigated well up to now.

The focus of the present dissertation was to elucidate the influence of the isolation procedure on the formation of the microstructure as well as the impact on chemical and physical characteristics of protein isolates derived from seeds of the sweet blue lupin *Lupinus angustifolius* L. Vitabor. In order to determine the physicochemical changes during the production of protein isolates, different milieu conditions, which are known to influence the conformation of proteins, were applied. A total of nine protein isolates were produced by combining the production parameters from the alkaline extraction with isoelectric precipitation and the salt-induced extraction with dilutive precipitation.

The protein microstructures of the protein isolates were investigated by light microscopy and cryo-scanning electron microscopy. Application of the dilutive precipitation led to the formation of flexible but well-ordered globular aggregates. This effect persisted after combining dilutive and pH mediated precipitation procedures. However, any pH adjustment (pH 8.0 during protein extraction or pH 4.5 during precipitation) unfolded the proteins to different extents indicating irreversible protein denaturation. In contrast to ILP, MLP lacked autofluorescence properties corroborating the compact globular microstructure of the MLP-proteins and proving protein unfolding of the ILP-proteins.

The isoelectric precipitation showed significantly higher protein yields, compared to the protein isolates precipitated by dilution. Chemical investigations using ion exchange chromatography showed the amino acid distribution of the protein isolates being independent on the isolation procedure. However indeed, an

influence of the isolation procedure on the portion of the individual conglutins was found, as determined by different gel electrophoreses. Conglutin δ was extracted independently of the extraction method (using the alkaline or salt-induced extraction); however, only isoelectric precipitation was found effective to yield the lupin albumin exhaustively. The other lupin conglutins always occurred in all protein isolates, however with important differences in their content. The higher concentration of the sulphur-rich conglutins α and γ in MLP were ascribed to the stabilised micellar protein arrangement of this lupin protein isolate.

Within the investigated pH range from pH 3 to 8, measurements of surface hydrophobicity and protein solubility showed similar curve progressions dependent on the electric net charges of the proteins. Determination of the denaturation behaviour permitted evaluating the harshness / mildness of the isolation procedure. Protein flexibility was inversely reflected by the extent of denaturation and production of MLP featured the mildest isolation procedure. Rheological investigations of the freshly produced isolates (dry matter: 43-44%) revealed viscoelastic liquid-like properties for MLP (G'' > G'), while the emulsifying capacity was particularly high. In contrast, due to the harsh isolation procedure, ILP showed particularly high protein denaturation and a viscoelastic solid-like behaviour (G'' < G'), whereas emulsifying capacity was below the detection limit. A microstructure-dependent interrelation trend between the thermal stability and the firmness of the lupin protein isolates was shown: Firmness rose with decreasing thermal stability of the 11S proteins.

Finally, besides its high potential as a “natural” fat replacer, the micellar protein isolate MLP was expected to be especially suitable for the application as a vegetable emulsifier in mixed food systems.

ZUSAMMENFASSUNG

Pflanzenproteine werden aufgrund ihrer ernährungsphysiologischen und technofunktionellen Wirkungen in Form von Isolaten als Zutat in Lebensmitteln eingesetzt. Die alkalische Extraktion mit anschließender isoelektrischer Fällung sowie die salzinduzierte Extraktion mit anschließender Verdünnungsfällung sind die in der Literatur am häufigsten untersuchten Methoden zur Proteingewinnung. Die daraus resultierenden Proteinisolate weisen unterschiedliche technofunktionelle Eigenschaften auf. Während das isoelektrisch gefällte Proteinisolat aus der Lupine (ILP) als Ei-Ersatz Verwendung findet weist das verdünnungsgefällte Proteinisolat (MLP) fettartige Eigenschaften auf. Die Struktur von MLP soll aus Proteinmizellen bestehen, wobei die chemischen und physikalischen Ursachen für die Ausbildung der mizellaren Struktur bisher nicht bekannt sind.

Die Zielstellung der Doktorarbeit war es, den Einfluss des Herstellungsverfahrens von Proteinisolaten aus *Lupinus angustifolius* L. Vitabor auf die Proteinstruktur sowie die Auswirkungen auf chemische und physikalische Eigenschaften zu untersuchen. Dazu wurden durch ausgewählte, die Struktur beeinflussende Variationen in der Proteinisolierung, insgesamt neun unterschiedliche Proteinisolate aus der Lupinensaat hergestellt und charakterisiert.

Licht- und kryo-Rasterelektronenmikroskopie dienten der mikrostrukturellen Untersuchung der Proteinisolate. Unter allen Isolaten zeigte nur MLP ausschließlich sphärische Strukturen. Ferner wurden nach Kombination der isoelektrischen Fällung mit der der Verdünnungsfällung Proteinmizellen ausgebildet, jedoch führte eine pH-Einstellung während der Extraktion oder Fällung zu einer Auffaltung der Proteinstrukturen, was auf eine irreversible Proteindenaturierung hindeutete. Im Gegensatz zum ILP wies MLP keine Autofluoreszenz auf, was die Theorie der kompakten und globulären Mizellenstruktur der MLP-Proteine stütze und die Auffaltung der ILP-Proteine belegte.

Die isoelektrische Fällung ergab signifikant höhere Proteinausbeuten, als die Verdünnungsfällung. Während die Aminosäurezusammensetzung der Isolate ähnlich war, zeigte die chemische Untersuchung mittels unterschiedlicher

elelektrophoretischer Methoden eine Abhängigkeit der Gehalte einzelner Conglutine vom Herstellungsverfahren. Der Gehalt an Conglutin δ war von der Extraktionsmethode unabhängig, jedoch ergab die isoelektrische Fällung prozentual die doppelte Menge des Albumins im Gegensatz zum Verdünnungsverfahren. MLP zeigte im Vergleich zu ILP höhere Gehalte der schwefelreichen Conglutine α und γ.

Im untersuchten pH-Bereich zwischen pH 3 und 8 wiesen die Oberflächenhydrophobität und die Proteinlöslichkeit ähnliche Kurvenverläufe auf. Beide Eigenschaften waren abhängig von der elektrischen Gesamtladung der Proteine. Über die Ermittlung der Denaturierungseigenschaften der Proteinisolate war es möglich, das Ausmaß der Proteinbeanspruchung durch das jeweilige Herstellungsverfahren abzuschätzen. Die Proteinflexibilität stand im umgekehrten Zusammenhang mit der Proteindenaturierung und beeinflusste wesentlich die technofunktionellen Eigenschaften. Ein strukturabhängiger Zusammenhang zwischen der thermischen Proteinstabilität und der Festigkeit des Isolats wurde gezeigt: Die Festigkeit stieg mit sinkender thermischer Stabilität der Proteine. Aufgrund des besonders milden Herstellungsverfahrens zeichnete sich das MLP durch den geringsten Grad der Proteindenaturierung aus und wies den Charakter einer viskoelastischen Flüssigkeit (G'' > G') sowie eine ausgezeichnete Emulgierkapazität auf. Im Gegensatz dazu zeigte ILP den höchsten Denaturierierungsgrad und ein viskoelastisch feststoffähnliches Verhalten (G'' < G'), während keine Emulgierkapazität mehr nachweisbar bar.

Neben der Eignung als „natürlicher“ Fettaustauschstoff wies MLP hervorragende Eigenschaften für die Nutzung als grenzflächenaktive Substanz in Emulsionen auf.

CURRICULUM VITÆ

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Titel der Doktorarbeit: Properties of protein isolates from lupin (*Lupinus angustifolius* L.) as affected by the isolation method.
Lehrstuhl für Lebensmittelchemie, apl. Prof. Dr. P. Köhler

Abschluss: **Doktor der Naturwissenschaften (Dr. rer. nat.)**

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2006 – 2008 **Masterstudium der Biomedizin**, Technische Universität München

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2003 – 2006 **Bachelorstudium der Ernährungswissenschaften**, Technische Universität München

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