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ORIGINAL ARTICLE

Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: a Fourier transform infrared (FT-IR) spectroscopic study

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Abstract The secondary structure of proteins in legumes, cereals, milk products and chicken meat was studied by diffuse reflectance infrared spectroscopy in the region of the amide I band. Major secondary structure components (β-sheets, random coil, α-helix, turns), together with the low- and high-frequency side contributions, were resolved and related to the in vitro digestibility behaviour of the different foods. A strong inverse correlation between the relative spectral weights of the β-sheet structures and in vitro protein digestibility values was measured. Structural modifications in legume proteins induced by autoclaving were monitored by the changes in the amide I spectra. The results indicate that the β-sheet structures of raw legume proteins and the intermolecular β-sheet aggregates, arising upon heating, are primary factors in adversely affecting the digestibility.

Keywords FT-IR · Diffuse reflectance · Food protein structure · Thermal aggregation · Legumes · Digestibility

Introduction

Low digestibility of plant proteins, such as those from legumes and cereals, together with a limiting content of essential amino acids (lysine, methionine, cysteine, tryptophan), represents a major issue for their low nutritional value compared with animal proteins. It has now been ascertained that protease inhibitors and lectins are inactivated by proper technological treatments of plant foods. In addition, heat-stable compounds (phytic acid, tannins), being present at low concentrations in domesticated species, have only a limited effect on the susceptibility to proteolysis of plant proteins (Carbonaro et al. 2000; Gilani et al. 2005). However, increasing evidences suggest that: (1) the structural properties of proteins in plant foods have a major role in the resistance to denaturation (Deshpande and Damodaran 1989; Carbonaro 2008), and (2) plant proteins manifest stability upon gastrointestinal digestion (Carbonaro et al. 2000; Shewry and Halford 2002).

Relationship among structural elements, susceptibility to proteolysis and availability of essential amino acids has not been clarified so far. Improvement in the susceptibility to proteolysis by sulfitolysis or S-carboxymethylation has been observed for both animal and plant proteins (Reddy et al. 1988; Clemente et al. 2000). In the latter case, besides disulphide involvement, structural features associated to the oligomeric structure and hydrophobicity of storage proteins may be responsible for their incomplete digestibility, even after heating (Carbonaro et al. 1997, 2000). Far-UV and circular dichroism spectroscopy studies have suggested that heating produces greater adverse changes in structure in vicilin (*Pisum sativum* L.) than in phaseolin (*Phaseolus vulgaris* L.) 7S globulins, thus reversing the susceptibility to proteolysis of the two proteins (Deshpande and Damodaran 1989). Studies on the in vivo gastrointestinal digestion of autoclaved 7S globulin have indicated that high amount of intermediate proteolytic fragments (MW 22–27 kDa) are present in the rats' small intestine, together with stable complexes of high molecular weight. Impairment in the in vivo digestibility of globulins upon heating has often been observed, although not

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systematically (Carbonaro et al. 2005; Montoya et al. 2008; Tang et al. 2009). In particular, vicilin-rich extracts from different *Phaseolus* species showed different aggregation and digestibility behaviours: heat-induced decrease in digestibility has been observed only in red and mung beans, but not in kidney bean (Tang et al. 2009). It has been suggested that the existence in *Phaseolus* seeds of several phaseolin types (such as T, I, and S) can be partially responsible for the different results.

A few studies have been performed on digestibility of proteins in whole cooked legumes and cereals. Adverse effect of heating on digestibility has been reported for faba bean and sorghum, unlike other species (common bean, pea, wheat), where the overall effect appeared to be positive (Carbonaro et al. 1997, 2000; Emmambux and Taylor 2009). It is worth considering that characterization of proteins in processed legumes and cereals suffers from their high propensity to aggregation (Carbonaro et al. 1993, 1997; Morel et al. 2002). This aspect makes currently employed biochemical techniques for protein characterization unsuitable in the case of denatured plant proteins, requiring more specific approaches.

Fourier-transform infrared (FT-IR) spectroscopy in the mid-infrared region is widely used in protein secondary structure determination through analysis of amide I band (stretching vibrations of C=O in the peptide bond) (Jackson and Mantsch 1995). The sensitivity of amide I to conformational changes makes it possible to study not only protein folding and unfolding, but also aggregation processes. Thus, FT-IR appears particularly suitable in controlling functional properties of food proteins during technological processes. FT-IR spectroscopy has been successfully applied to monitor structure of isolated soy, buckwheat, oat globulins and wheat gliadins under the effect of pH, salts, and thermal treatments (Choi and Ma 2005; Secundo and Guerrieri 2005). In addition, structural modifications underlying development of hard-to-cook phenomenon in common beans, have been explored by FT-IR analysis of extracted protein fractions (Maurer et al. 2004).

In the field of FT-IR spectroscopy, recent studies have been performed by diffuse reflectance (DR) FT-IR spectroscopy on food proteins (Liu and Yu 2010; Yu 2010; Yu and Nuez-Ortín 2010). Diffuse reflectance technique is particularly effective on high-absorbing powdered samples and it is not affected by artifacts due to the scattering of the radiation from sample particles (a problem commonly observed in the transmission of pellets).

In this work, to gain additional information at a molecular level about the relationship between structure of food proteins and their digestibility behaviour, DR spectroscopy in the solid state (powdered) was applied to examine the secondary structure of proteins. The amide I band from DR spectra of plant (legumes, cereals) and animal (meat, milk products) foods was analyzed. Besides amide I modes deriving from α-helix, β-sheet and turns, low- and high-frequency contributions in the amide I band, assigned to amino acid side-chains, β-sheet intermolecular aggregates and intramolecular antiparallel β-sheets were quantified. The in vitro protein digestibility behaviour of the various foods was examined and a structure–digestibility relationship was inferred. The modifications of secondary structure of proteins in whole legume (common bean, chickpea, lentil, soybean) flours upon mild (dry heating) and severe (autoclaving) thermal treatments were then analyzed. A relationship between structure and digestibility changes in these processed legumes was finally presented.

Materials and methods

Whole pasteurized fresh milk (Centrale del Latte, Rome), mozzarella cheese (from pasteurized cow milk), chicken breast meat, dry seeds of white common bean (*Phaseolus vulgaris* L. var. *cannellino*), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* L.), soybean (*Glycine max* L.), barley (*Hordeum vulgare* L.) and emmer wheat (*Triticum dicoccum* L.) were obtained from the local market. Milk, mozzarella cheese, and chicken meat were immediately freeze-dried and stored at −30°C until use. Dry seeds of legumes and cereals were ground in a Cyclotec 1093 Tecator ($\leq$50 μm). Legumes were autoclaved at 120°C for 20 min (1 atm) after soaking in water at room temperature for 2 h (1:4 w/v) and then freeze-dried. Dry heated legumes were prepared in a thermostatic oven at 120°C for 30 minutes, 24 and 48 h. Chickpea, soybean and mozzarella cheese powders were defatted according to AOAC (1999). Total nitrogen (N) content was determined by the Kjeldahl method (AOAC 1999).

DR measurements

Measurements were performed in the mid-infrared region (500–5,000) with a resolution of 2 cm^{-1} by using a Bruker IFS66V interferometer (Bruker Optics, Ettlingen, Germany) working under vacuum to avoid intense spectral components due to atmospheric CO_2 and H_2O, and equipped with accessories for the acquisition of the DR. Previous to FT-IR measurements, the grain size of all samples was reduced to about 2 μm by grinding and then mixed with KBr powder treated in the same conditions. A spectrum of the radiation diffused by KBr powder, $I_0(\omega)$ was first collected. This spectrum was considered as a reference provided that the absorption coefficient of KBr is negligible small. A further spectrum I_c was acquired when

a small concentration c of sample is dispersed in the KBr matrix. The ratio

$$R(\omega) = I_c(\omega)/I_0(\omega) \quad (1)$$

provides an estimate of the DR of the sample itself. The sample concentration c was kept around 5% in weight as control experiments indicated that higher concentration may cause distortions in the spectra due to saturated absorption lines. According to the Kubelka–Munk theory, the absorption coefficient for unity length $\alpha(\omega)$ can be obtained from the diffuse radiation through the relation:

$$\alpha(\omega) \approx \frac{(1 - R(\omega))^2}{2R(\omega)}. \quad (2)$$

Data analysis was performed by using Gaussian curve-fitting procedures, together with Fourier self-deconvolution (FSD) and second derivative (SD) methods for the search of the amide components. Both FSD and SD procedures were first applied to the amide I band in order to better resolve its components and to determine their peak frequencies (ω_i) and their widths (Γ_i). Best curve fitting, at the lowest possible χ^2 values, were performed on the measured spectra using the values of ω_i and of Γ_i previously obtained by FSD and SD. Gaussian lineshape is commonly used in multipeak deconvolution since it corresponds to the most likely distribution function of independent absorbing processes (Susi and Byler 1987; Yu 2005). Each Gaussian component is assigned to a specific secondary structure of the sample. The ratio between the integrated intensity of the i-th Gaussian component and that of the whole amide I band provides the relative spectral weight W_i of specific secondary structure of the protein, assuming for them identical values of the molar absorption coefficient.

It is worth noticing that, within a multipeak approach, FT-IR spectra of proteins can only provide relative estimation, and not exact determination, of the secondary structure components. Indeed, several variables may affect the mathematical modeling, such as number of the bands, their shape and difference in the molar absorptivity of secondary structure elements. However, we point out that the purpose of modeling the relative proportion of secondary structure was to detect the food differences, as well as the effects of processing on different legumes, in relation to their digestive behaviour.

The relative weights obtained by multipeak analysis may often be considered as estimates of the secondary structure percentages. Indeed, previous studies on well-characterized proteins showed a good agreement between the values of the relative spectral weights obtained by multipeak fitting and the percentages of secondary structure as reported from X-ray crystallographic data (Carbonaro et al. 2008).

In vitro protein digestibility

In vitro protein digestibility values were obtained by the multienzyme method of Bodwell et al. (1980), that provides values related to the in vivo digestibilities for both plant and animal proteins. Porcine pancreatic trypsin (type IX, 15,310 units/mg of protein), bovine pancreatic chymotrypsin (type II, 48 units/mg of solid), porcine intestinal peptidase (P-7500, 115 units/g of solid), and bacterial protease (type XIV, 4.4 units/mg of solid) (Sigma Chemical Co., St. Louis, MO) were used for the enzymatic digestion. For each sample, 10 mg N (on a Kjeldahl basis) in 10 mL of distilled water was equilibrated at 37°C and the pH was adjusted to 8.0. 1 mL of a three-enzyme solution in water (1.58 mg of trypsin, 3.65 mg of chymotrypsin, and 0.45 mg of peptidase) was added to the sample, and digestion was allowed to proceed for 10 min at 37°C. After addition of 1 mL (1.48 mg) of protease solution, the digestion was continued for 9 min at 55°C The pH value was measured after 1 min at 37°C and used to estimate the in vitro protein digestibility in the equation

$$D = 234.84 - 22.56(pH)_{20} \quad (3)$$

where D is the in vitro protein digestibility (%) and pH_{20} is the pH value of the suspension after 20 min digestion (Bodwell et al. 1980).

Data were subjected to analysis of variance. The significance of the differences between means was obtained by Duncan's multiple-range test ($P < 0.05$). Correlation between in vitro protein digestibility values and elements of secondary structure was checked by calculating the Pearson's correlation coefficient (r).

Results and discussion

Secondary structure of food proteins

The present DR data provided secondary structure of proteins in different foods: legumes (common bean, chickpea, lentil, and soybean), cereals (barley and emmer wheat), milk products (milk and mozzarella cheese) and chicken meat. The amide I lineshape in food samples appears in general much broader than the same band detected in purified proteins: this occurrence can be ascribed to the coexistence of different proteins in whole food which results in a convolution of spectral bands. To this respect, the detection and assignment of the finer spectral contributions is not straightforward, and the use of narrowing methods as FSD and SD is well established. In the case of food samples, both FSD and SD provided not more than seven spectral contributions, thus less than those generally observed in FT-IR experiment of purified proteins (Dong

et al. 1990; Dave et al 2000). However, some of these contributions appear as broad bands even after the application of narrowing procedures, suggesting an unresolved finer structure. It is also remarkable that the molar absorption coefficient may differ between the secondary structure components. However, the origin and amount of these differences are still controversial. Literature reports of relative absorption coefficient are scattered. Most of the IR papers assume identical extinction coefficients for the components, whereas few works claim for significant difference between them, even though the values provided for purified proteins are conflicting (de Jongh et al. 1996; Vedantham et al. 2000).

In Fig. 1 is reported the amide I spectrum of soybean (taken as an example) together with its Gaussian spectral deconvolution (panel a); in the same figure, SD and FSD spectra are reported in panels b and c, respectively. Seven Gaussian bands have been resolved for this sample, centred at 1,615, 1,636, 1,654, 1,663, 1,678, 1,685 and 1,694 cm^{-1}.

In Fig. 2 is shown the Gaussian deconvolution of the amide I band of common bean, chickpea and lentil, as obtained from the above described procedure. Small frequency shifts of homologous structural contributions are observed in the different food proteins: therefore, a general description of the amide I band in foods can be made by grouping the Gaussian contributions observed within proper frequency regions. In Table 1 are reported the values W_i of secondary structure relative spectral weights for any specific range here considered. In the range between 1,630 and 1,638 cm^{-1} legumes and cereals spectra show one of the most intense contribution, that can be safely assigned to the β-sheet secondary structure (hereafter indicated as β-band). According to several authors, there is no experimental evidence for a difference between the frequencies of parallel and antiparallel β-sheets in the 1,630–1,638 range (Susi and Byler 1987; Khurana and Fink 2000; Pelton and McLean 2000): thus the β-band is assumed to contain either structures. For soybean, lentil and barley seeds β-sheet arrangement accounts for at least 30% of the whole secondary structure, while in chickpea and common bean seeds the relative spectral weight of the β-band reaches significantly higher values (37.2 and 43.6%, respectively). Conversely, a small content of the β-sheet contribution (21.7%) has been found in the emmer wheat seed. The Gaussian bands centred in the frequency range 1,650–1,660 are assigned to α-helix structure (α-band). A significant amount of α-band is found in barley and emmer wheat (28 and 29%, respectively), while for most legumes this relative spectral weight is around 18–20%, even lower in the case of soybean (11.8%).

The Gaussian components centred within 1,660–1,670 cm^{-1}, 1,670–1,680 cm^{-1} and 1,680–1,688 cm^{-1} are less intense than the previously discussed α- and β-bands. Their assignment is not straightforward, since different origins have been proposed for them in the literature (Pelton and McLean 2000; Barth 2007). In globular proteins β-turn conformations are common structures providing spectral contribution at frequencies between 1,660 and 1,690 cm^{-1}. Moreover, a component around 1,682 cm^{-1} assigned to a high wavenumber antiparallel β-sheet

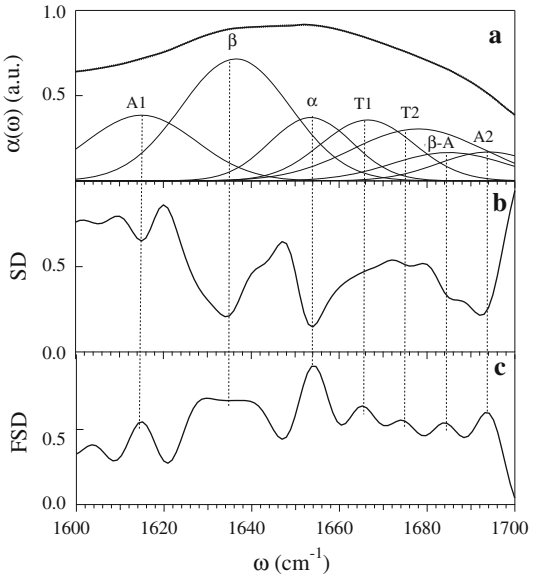

Fig. 1 The amide I spectrum of soybean as obtained from DR measurements (panel *a*). In panel *b* the second derivative of the spectrum is reported. In panel *c* the FSD spectrum is shown, as obtained with a bandwith = 5 cm^{-1} and a resolution enhancement factor = 2. *Thin dashed vertical lines* indicate the central frequencies of the Gaussian contributions

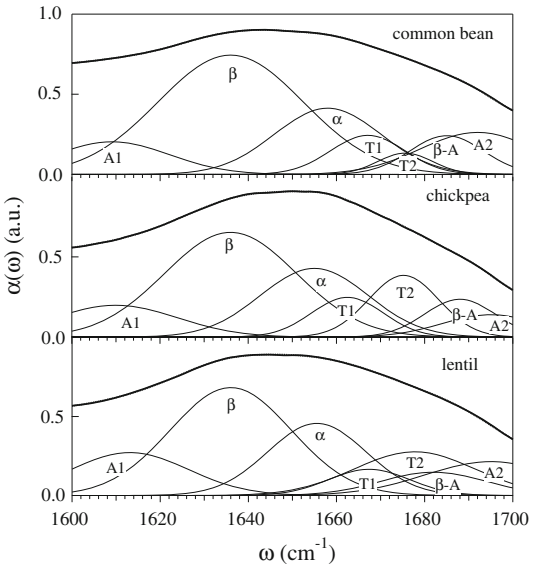

Fig. 2 The amide I band and its spectral deconvolution with Gaussian contribution of common bean, chickpea and lentil

Table 1 Relative spectral weights (W_i) of the secondary structure components of various food proteins as obtained from the spectral deconvolution of the amide I band

	W_{A1} (1,610–15)	$W_{\beta-1}$ (1,620–30)	W_{β} (1,630–38)	W_{RC} (1,640–48)	W_{α} (1,650–60)	W_{T1} (1,660–70)	W_{T2} (1,670–80)	$W_{\beta-A}$ (1,680–88)	W_{A2} (1,690–95)
Common bean	9.4		43.6		17.7	7.2	2.9	7.1	12.1
Chickpea	10.5		37.2		19.9	7.7	11.7	7.2	5.8
Lentil	12.7		33.0		18.0	5.4	13.4	6.2	11.3
Soybean	16.2		30.2		11.8	12.8	15.4	7.1	6.5
Barley	9.9		31.0		28.0	6.6	2.4	7.4	14.7
Emmer wheat	10.8		21.7		29.0	13.2	5.5	3.0	16.8
Milk	14.1	7.0	10.7	7.6	15.3	6.5	12.7	5.5	20.6
Mozzarella cheese	15.4	11.8	4.0	23.0	4.1	4.2	14.2	3.0	20.3
Chicken meat	16.3		6.9	8.6	26.2	8.2	8.3	2.2	23.3

The frequency intervals reported for each component are expressed in cm^{-1}

contribution, has been detected in several plant food proteins, including wheat gliadins, red bean globulins and lentil lectin (Chehin et al. 1999; Meng and Ma 2001; Secundo and Guerrieri 2005). Thus, we assume that the bands centred in the ranges 1,660–1,670 cm^{-1} and 1,670–1,680 cm^{-1} are due to turns' conformations (T1 and T2 bands, respectively), while those between 1,680–1,688 cm^{-1} originate from antiparallel β-sheet structures (β − A). In legumes and cereals, the percentange of T1 and T2 contributions has not a constant value, ranging from 9% in barley to 28.2% in soybean. Conversely, the relative spectral weight of the β − A structure is quite constant in most of proteins in the foods plant examined (around 7%), with the only exclusion of the emmer wheat where this relative spectral weight is definitely small (3%). The spectral contributions detected in the 1,610–1,615 cm^{-1} (A1) and 1,690–1,695 cm^{-1} (A2) ranges may be assigned to protein aggregates as well as to absorption of amino acid side-chains (Murayama and Tomida 2004; Barth 2007). A band at 1,690 cm^{-1} has been observed in the FSD spectrum of red bean globulin and lentil lectin (Chehin et al. 1999; Meng and Ma 2001). Moreover, high frequency contributions around 1,690 cm^{-1} have been found to increase in gliadins subjected to heat treatments and unambiguously associated to intermolecular protein aggregation (Secundo and Guerrieri 2005). The presence of a low-frequency band at 1,610–1,620 cm^{-1} has been addressed as a marker for intermolecolar protein complexes (Murayama and Tomida 2004; Carbonaro et al. 2008). However, several authors report that 10–30% of the amide I absorption of purified proteins derives from the amino-acid side chains (Barth 2000). Absorption from the carboxyl stretching of the amino acid side chains in proteins is predicted in the range 1,670–1,720 cm^{-1}, while contributions at the lowest frequencies (1,600–1,620 cm^{-1}) are commonly assigned to C–C and N–H molecular modes of the side chains. In particular, the 1,600–1,614 cm^{-1} band is partially originated from the NH_2 scissoring of the glutamine side chain, to arginyl asymmetric stretching and to protonated histidine (Venyaminov and Kalnin 1990; Barth 2000). We cannot unambiguously distinguish between the two possible origins of the A1 and A2 contributions at this stage, even if according to previous findings, high amount of protein aggregates in raw cereals and legumes are not expected (Carbonaro et al. 1997, 1999; Emmambux and Taylor 2009). With the only exception of soybean (A1 = 16.2%), the average relative spectral weight of the A1 component is around 10% for both cereal and legume proteins. As far as the A2 contribution is concerned, its relative spectral weight is found to vary between 5.8% (chickpea) and 16.8% (emmer wheat). A different proportion of structural components is found for the animal foods: pasteurized milk, mozzarella cheese and chicken meat, also reported in Table 1. In these foods a low content of the β-band is measured: 10.7, 4.0 and 6.9% for milk, mozzarella cheese and chicken meat, respectively. Small amount (2.2–5.5%) of the β-A band (1,680–1,688 cm^{-1}) likely representing antiparallel β-sheet, are observed in animal foods. On the contrary, A1 and A2 contributions in animal foods (14.1–16.3% and 20.3–23.3%, respectively) are more intense than the corresponding bands in legume and cereal proteins. In chicken meat the major contribution is represented by the α-band (26.2%): this latter holds 15.3 and 4.1% in milk and in mozzarella cheese, respectively. Besides these spectral contributions, a further band in the amide I spectrum characterises milk products and chicken meat: this band, centred at 1,640–1,648 cm^{-1}

mainly originates from random coil (RC), the unordered protein structure. Random coil component accounts for 7.6 and 23% in milk and mozzarella cheese, respectively. Random coil conformation has been claimed to be typical of caseins, the major class of milk and mozzarella cheese proteins. It has been found to represent 48 and 40% of the whole amide I intensity in purified α- and β-/κ-caseins, respectively (Carbonaro, personal communication). In chicken meat the RC relative spectral weight is around 9%. In milk products a Gaussian contribution centred between 1620–1630 cm^{-1} has been observed and assigned to intermolecular β-sheets (β-I band): its importance will be discussed in the following sections for the thermally treated foods.

The data reported in Table 1 suggest some preliminary comments. First, we note that legume seeds are characterized by the highest content of β and β-A band. The higher amount in β-sheet structure of common bean compared with the other legume species can be ascribed to its content in 7S globulin (Carbonaro et al. 2008). This globulin has been shown by X-ray analysis to contain a higher β-sheet percentage than the 11S protein, the other major globulin of legumes (Lawrence et al. 1994). As a further statement, we remark that the relative spectral weight of the α-helix structure does not exceed 20% in all legume species. The secondary structure of the cereal proteins of the two species here analyzed is quite dissimilar to that of legumes, being characterized by higher α-helix contents.

Relationship between secondary structure and digestibility

In vitro protein digestibilities D of the various foods are presented in Table 2. The highest digestibility values are measured for animal foods: chicken meat (92.0%), mozzarella cheese (87.0%) and pasteurized milk (84.0%). Among plant foods, emmer wheat presents the highest protein digestibility value (82.7%), slightly lower than that of milk. Soybean is the legume species with the highest digestibility (79.9%), followed by lentil (79.3%), chickpea (77.0%) and common bean (73.5%). Barley shows a digestibility (77.5%) comparable to that of chickpea.

In Fig. 3 are shown the relative spectral weights of β-sheet structures of the various foods as a function of their digestibilities D. For the milk products, the β-sheet contribution also includes $W_{\beta-1}$, the intermolecular β-sheet. From the data in Fig. 3, it appears that the digestibility process does not involve protein regions whose amino acids are arranged in β-sheet structures. Indeed, a high negative linear correlation coefficient ($r = -0.980$) is found between the β-sheet contents of all proteins and the food digestibility values. An inverse linear correlation is also observed between $W_{\beta-A}$, the minor antiparallel β-sheet contribution, and protein digestibility ($r = -0.881$), although with a rate about 7 times lower with respect to the β-band. The decrease in protein digestibility as a function of the amount of β conformations can be explained by the high hydrophobic character of these structures, which involves aromatic residues (tyrosine, phenylalanine and tryptophan) and β-branched amino acids (threonine, valine and isoleucine). Hydrophobic interactions are believed to make a major contribution in stabilizing native protein structure. These forces have been suggested to adversely affect the solubility of legume proteins by promoting protein-protein interaction and aggregate formation, especially upon thermal treatment, likely reducing accessibility of susceptible sites to proteases (Carbonaro et al. 1993, 1997).

In Fig. 4 the relative spectral weight W_{A1} and W_{A2} of the lowest and the highest frequency components are shown as function of D. For these two spectral components is

Table 2 In vitro protein digestibilities (%) of food samples (D) and autoclaved legumes (D_a)

	D (%)	D_a (%)
Common bean	73.5 ± 0.3 aA	80.2 ± 0.5 aB
Chickpea	77.0 ± 0.4 bA	82.0 ± 0.5 bB
Lentil	79.3 ± 0.5 cdA	80.8 ± 0.4 aB
Soybean	79.9 ± 0.3 dA	84.1 ± 0.5 cB
Barley	77.5 ± 0.5 b	
Emmer wheat	82.7 ± 0.5 e	
Milk	84.0 ± 0.3 f	
Mozzarella cheese	87.0 ± 0.5 g	
Chicken meat	92.0 ± 0.5 h	

The values represent means ± standard deviations of at least four determinations. Within a same column, values followed by different lowercase letters are significantly different at $P < 0.05$. Within a same row, values followed by a different capital letter are significantly different at $P < 0.05$

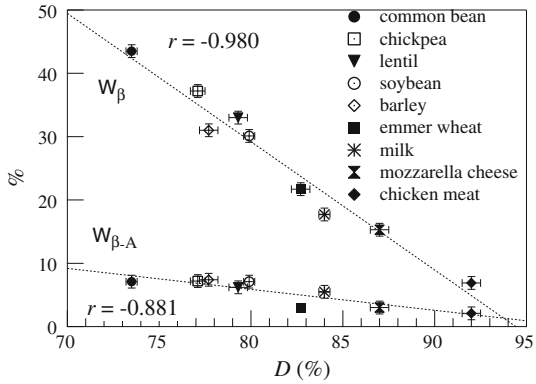

Fig. 3 Relative spectral weights of the β-band and of the β-A band as a function of the food digestibilities

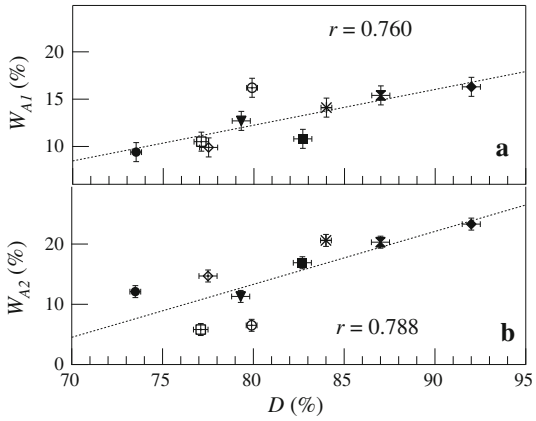

Fig. 4 Relative spectral weights of the A1-band (panel *a*) and of the A2-band (*b*) as a function of the food digestibilities. Symbols used are the same as in the legend of Fig. 3

possible to resolve a direct correlation with the digestibility, more pronounced for the A2 band. The linear increase of W_{A1} and W_{A2} with digestibility contrasts with the assignment of these contributions to protein aggregates, since resistance to proteolysis and, therefore, inverse correlation would be expected in this case. Our finding supports the hypothesis that absorption from charged amino acids side chains—lysine, arginine, histidine, aspartic and glutamic acids—exposed on the protein surface, largely influences the A1 and A2 components. This assumption is also consistent with a low extent of protein aggregation previously found in raw foods (Carbonaro et al. 1993, 1997).

No correlation among W_{α}, W_{T1}, W_{T2} and protein digestibilities could be established within the accuracy of the present data. The absence of an evident relationship with digestibility confers to these secondary structures a minor role, either adverse or favorable, in the proteolysis process. However, we observe that the RC structure is detected only in animal food proteins that show the highest digestibilites, thus suggesting a good amino acid availability from the unordered secondary structure.

Effect of thermal treatment and relationship with the digestibility of legume proteins

The effect of mild (dry heating) and severe (autoclaving) thermal treatments on the secondary structure of legume proteins was investigated: the secondary structure relative spectral weights W_i of the thermally treated legumes are reported in Table 3.

Table 3 Relative spectral weights (W_i) of the secondary structure components of legumes proteins after various thermal treatments as obtained from the spectral deconvolution of the amide I band

	W_{A1}	$W_{\beta-1}$	W_{β}	W_{RC}	W_{α}	W_{T1}	W_{T2}	$W_{\beta-A}$	W_{A2}
Common bean	9.4		43.6		17.7	7.3	2.9	7.1	12.1
30 min	11.4		43.4		15.6	7.5	3.0	7.3	11.8
24 h	11.0		42.3		15.3	5.7	3.5	8.1	14.1
48 h	10.7		43.9		11.6	10.3	2.7	4.0	16.8
Autoclaved	9.1	21.7		18.1	12.5	10.2	6.5	9.0	12.9
Chickpea	10.5		37.2		19.9	7.7	11.7	7.2	5.8
30 min	10.3		37.7		20.6	4.9	14.4	6.1	6.0
24 h	10.0		33.4		18.6	8.8	11.7	5.7	6.8
48 h	12.0		32.7		17.0	7.9	11.4	5.6	7.4
Autoclaved	11.6	18.9		14.7	19.3	9.8	6.7	7.1	11.9
Lentil	12.7		33.1		17.9	5.4	13.4	6.2	11.3
30 min	12.3		33.2		18.5	8.8	6.4	6.0	14.8
24 h	11.9		33.9		20.4	11.4	3.3	5.2	13.9
48 h	8.3		33.7		21.3	10.5	4.2	8.8	13.2
Autoclaved	9.0	21.2		8.5	21.6	6.1	7.8	4.9	20.9
Soybean	16.1		30.1		11.8	12.8	15.4	7.3	6.5
30 min	9.8		31.8		18.9	12.9	7.4	9.2	10.0
24 h	12.9		32.0		14.3	13.5	6.4	10.7	10.2
48 h	11.2		31.2		14.9	10.8	9.3	12.0	10.6
Autoclaved	11.6	15.1		17.1	12.1	10.7	9.6	10.0	13.8

For sake of comparison, the relative spectral weights of the raw legumes are also reported. 30 min: 120°C for 30 min, 24 (48) h: 120°C for 24 (48) hours

Dry heating at 120°C for 30 minutes, 24 and 48 hours caused only small structural changes in legumes. Major results are a decrease of W_α in common bean (from 17.7 to 11.6%) and chickpea proteins (from 19.9 to 17.0%), whereas the relative spectral weight of the β-band is slightly reduced only in the chickpea proteins (−4.5%). A small average increase (+3%) of the A2 band relative spectral weight is observed in all legumes, that might suggest a slightly higher accessibility of amino acid side chains in the thermally treated proteins. On the contrary, not a regular behaviour in the case of the A1 band is evident, as W_{A1} slightly decreases in lentil and soybean (about −4%), but not in common bean and chickpea (+1–2%). A reduction ot the relative spectral weights of the T1 and T2 bands is also observed in lentil and soybean proteins. These data indicate a high thermal stability of legume proteins, irrespective of the species considered. As expected, changes on the secondary structure of legume proteins induced by autoclaving were more severe than those detected upon dry heating. In Fig. 5 are reported the amide I spectra of autoclaved legumes together with their Gaussian deconvolution. Most evident modifications consisted in the disappearance of the β-band in all legume species, and in the

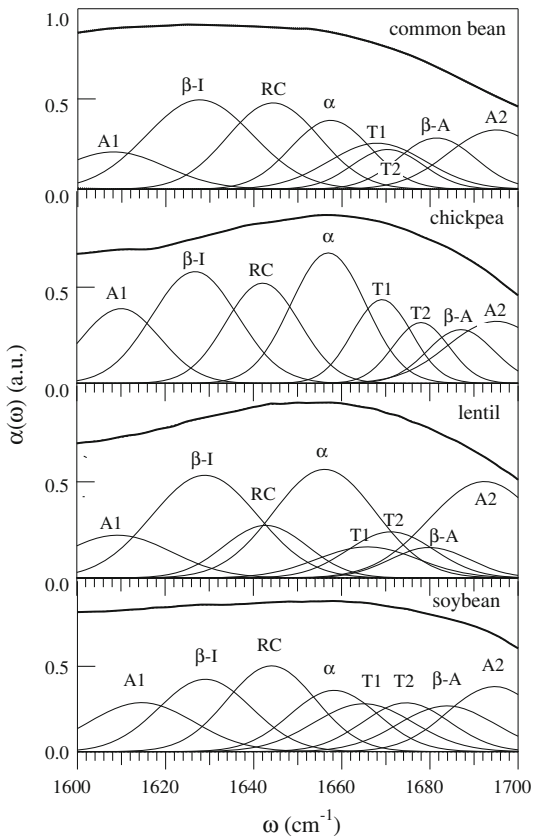

Fig. 5 The amide I band of the autoclaved common bean, chickpea, lentil and soybean and its spectral deconvolution with Gaussian contributions

appearance of the intermolecular β-sheets band (β-I band in Fig. 5) in the range 1,620–1,630 cm^{-1} This band, previously introduced for milk products, indicates the formation of protein aggregates (Jackson and Mantsch 1995; Carpenter et al. 1998), and accounts for 15.1–21.7% of the secondary structure. Our assignment is in agreement with previous FT-IR analysis of isolated thermally treated glycinin, the 11S soybean protein, that suggested the formation of intermolecular β-sheet aggregates, monitored by an increase of a band at 1,625 cm^{-1} (Mills et al. 2003). We notice that the RC contribution can be detected in all autoclaved legumes, ranging from 8.5 (lentil) to 18.1% (common bean). These data indicated that loss of β-sheet structures in autoclaved legumes is paralleled by an increase in both unordered conformations (RC) and intermolecular β-sheet aggregates (β-I band). This latter, in particular, have been demonstrated to represent protein complexes with a very stable conformation (Jackson and Mantsch 1995; Doglia et al. 2008).

The value of W_α decreases in autoclaved common bean, whereas it moderately increases in lentil. Moreover, a shift of the α-helix band toward a higher frequency (from 1,653 to 1,660 cm^{-1}) is clearly observed in the autoclaved soybean (Fig. 5). Studies on purified proteins, such as haemoglobin, suggest that bands at 1,653 and at 1,660 cm^{-1} belong to different species of α-helical protein domains, the second one corresponding to more flexible α-helix structures (Liu et al. 1998; Dave et al. 2000). As far as the relative spectral weight of the A1 and A2 bands is concerned, we observe for the former a moderately decrease in lentil and soybean, that may be attributed to a partial shielding by the intermolecular β-sheet aggregate band. On the contrary, A2 increases in chickpea (+6%), lentil (+10%) and soybean (+7%). No relevant changes of the A1 and A2 band intensities are detected in common bean. It is worth noticing that an increase of the highest frequency contributions has been observed in several heat-denatured proteins, together with the appearance of a band at 1,625 cm^{-1} both bands being assigned to intermolecular β-sheets (van de Weert et al. 2001). Moreover, changes in the secondary structure similar to those here reported, have been detected for thermally treated cereal proteins, such as gliadins, and have been ascribed to protein aggregate formation (Secundo and Guerrieri 2005). In vitro protein digestibility D_a of autoclaved legumes is reported in Table 2. The highest digestibility is found for autoclaved soybean, followed by chickpea, lentil and common bean. Digestibility of all legumes is significantly ($P < 0.05$) increased upon autoclaving, common bean showing the highest improvement (+6.7%), whereas lentil presenting the lowest increment (+1.5%) in digestibility. In Fig. 6 is reported the relative spectral weight of the β − I band in autoclaved legumes, as a function of D_a An inverse

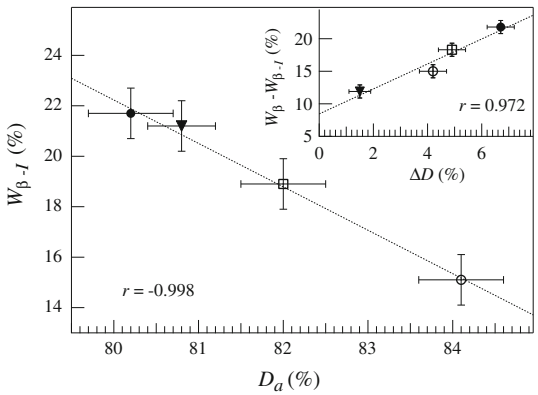

Fig. 6 Relative spectral weight of the $\beta - \mathrm{I}$ band of autoclaved legumes as a fuction of their digestibilities. Symbols used are the same as in the legend of Fig. 3. In the inset, the loss of β structure as a function of the digestibility increment is reported

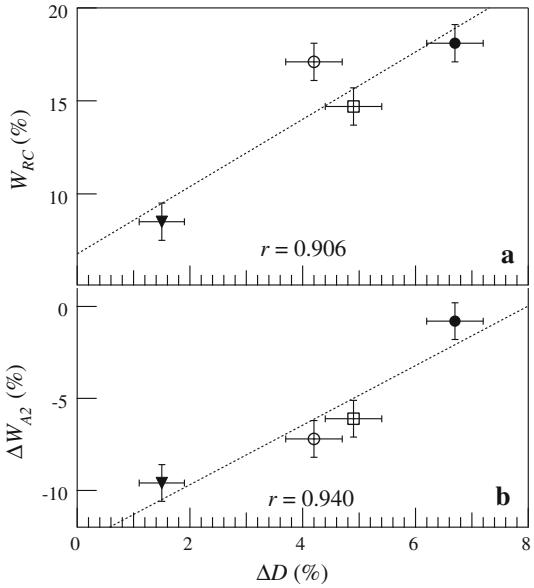

Fig. 7 Panel *a* relative spectral weight of the RC band of legumes as a fuction of the digestibility increment upon autoclaving. Panel *b* increment of the A2 band versus the digestibility increment. Symbols used are the same as in the legend of Fig. 3

correlation between these two parameters is evidenced ($r = -0.998$), even in such a short range of digestibility values, with a linear trend similar to that observed in the case of the β and the $\beta - \mathrm{A}$ bands. The tendency of the β-sheet structures to form complexes adverse to proteolysis is thus confirmed also by the present evidence on autoclaved legumes. It is worth noticing that the linear trend of W_β of foods discussed in the previous section and that of $W_{\beta-\mathrm{I}}$ of the autoclaved legumes do not entail a crossing point. Thus identical relative spectral weights of the $\beta - \mathrm{I}$ band and of the β-band provide systematically lower digestibility values for the former, suggesting a higher stability for the $\beta - \mathrm{I}$ band. This assumption is further exploited in the inset of Fig. 6, where the correlation between the digestibility increment $\Delta D = D_a - D$ and the loss in β-sheet structure of autoclaved proteins ($W_\beta - W_{\beta-\mathrm{I}}$) is reported. We can thus interpretate the disapperance of the β-band and the concomitant appearance of the $\beta - \mathrm{I}$ one as a partial conversion between similar structures upon thermal treatment, the latter structure showing a major role in the limited protein availability.

On the contrary, the formation of a less stable structure upon thermal treatment may be recognized in the appearance of the RC band (Table 3; Fig. 5). Actually, incremental digestibility ΔD is directly correlated to W_{RC} as shown in panel a of Fig. 7. In the panel b of the same figure is reported the increment of the A2 component, upon autoclaving, as a function of ΔD. An adverse effect towards proteolysis of the A2 increment is evident. In raw foods, this band was previously assigned to amino acid side chain absorptions. In autoclaved legumes, a different origin for the observed increment of the A2 band, likely related to the absorption of β-intermolecular aggregates in this high-frequency region, is inferred. Diffuse reflectance spectra of autoclaved legumes indicate that three out of seven structural modes are related with the digestibility, namely the β-intermolecular aggregate band , the random coil and the A2 bands. Adverse impact of aggregates (β-I and A2 bands) on protein digestibility is in agreement with our previous evidence, indicating that high molecular weight and low charge density characterize aggregates of legume proteins that are built up upon heating (Carbonaro et al. 1997, 2000). Such aggregates have been found to involve not only hydrophobic residues, such as phenylalanine, tryptophan, tyrosine, leucine, isoleucine, typically involved in the β-structure, but also significant amount of charged basic amino acids (lysine and arginine) (Carbonaro et al. 1993, 1999). Because of the restricted specificity of trypsin and chymotrypsin, impaired accessibility of lysine, arginine, phenylalanine, tryptophan and tyrosine in the intermolecular β-sheet aggregates is likely to exert a major impact on the small intestinal digestion of legume proteins.

Conclusions

Data analysis of the DR spectra of food proteins provided several markers to monitor changes in protein secondary structure upon thermal treatment and their effect on the nutritional properties. From the present data, besides major spectral amide I modes (α-helix, β-sheet, and turns), estimation of low and high frequency minor spectral contributions was provided. These latter were assigned to amino acid side-chains, intermolecular β-sheet aggregates and antiparallel intramolecular β-sheets.

β sheets were shown to be the main components in legume proteins, followed by cereal, milk product and chicken meat proteins. These secondary structure elements were found to play a major role in decreasing protein digestibility. Our analysis also disclosed a positive relationship between A1 and A2 bands and the digestibility process, thus supporting a major involvement of amino acid side-chains in these absorption bands. Gross changes in the secondary structure were shown in autoclaved legumes, consisting in the loss of the β-sheet contribution. Concomitantly, heat-induced intermolecular β-aggregates, that are recognized as very stable structures, were detected. Their adverse effect on the digestibility increment was demonstrated in this study. An adverse role was also envisaged for high frequency additional contributions, whereas the presence of random coil structures was related to the increment in digestibility.

These results are of relevance in the case of proteins in all plant foods, because aggregate formation is expected to be promoted by most of the technological procedures preceding human consumption. It is believed to highly affect not only nutritional value and functional properties, but also nutraceutical potential and safety of processed protein-rich plant foods.

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