Protein structural changes during processing of vegetable feed ingredients used in swine diets: implications for nutritional value

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Abstract

Protein structure influences the accessibility of enzymes for digestion. The proportion of intramolecular β-sheets in the secondary structure of native proteins has been related to a decrease in protein digestibility. Changes to proteins that can be considered positive (for example, denaturation and random coil formation) or negative (for example, aggregation and Maillard reactions) for protein digestibility can occur simultaneously during processing. The final result of these changes on digestibility seems to be a counterbalance of the occurrence of each phenomenon. Occurrence of each phenomenon depends on the conditions applied, but also on the source and type of the protein that is processed. The correlation between denaturation enthalpy after processing and protein digestibility seems to be dependent on the protein source. Heat seems to be the processing parameter with the largest influence on changes in the structure of proteins. The effect of moisture is usually limited to the simultaneous application of heat, but increasing level of moisture during processing usually increases structural changes in proteins. The effect of shear on protein structure is commonly studied using extrusion, although the multifactorial essence of this technology does not allow disentanglement of the separate effects of each processing parameter (for example, heat, shear, moisture). Although most of the available literature on the processing of feed ingredients reports effects on protein digestibility, the mechanisms that explain these effects are usually lacking. Clarifying these mechanisms could aid in the prediction of the nutritional consequences of processing conditions.

Key words: Processing: Protein structure: Protein digestibility: Secondary structure

Introduction

The increasing world population, along with a higher income, especially in developing countries, is predicted to increase the demand for animal protein. This increased demand and the limits to arable land increase the demand for feed ingredients and their price. As feed represents approximately 50 % of the total production costs in animal farming activities[1], a rise in ingredient price requires a higher efficiency in nutrient utilisation in order to be able to produce animal protein without increasing production costs.

Protein deposition in single-stomached production animals is directly linked to the digestibility of protein in the feed and the adequacy of the amino acid profile to match the animal's requirements[2]. This is evident from the vast amount of research conducted over the last decades to define the nutritional requirements of production animals (to maximise protein deposition and to minimise protein oxidation) and to evaluate the nutritional quality of the ingredients or feeds that are fed to meet these requirements[2–7].

Large variation exists in the nutritional quality of protein in feed ingredients, especially in those that received a thermal processing step, such as the most commonly used oilseed co-products (for example, soyabean (*Glycine max*) meal or rapeseed (*Brassica* spp.) meal)[8,9]. Heat used during production of ingredients facilitates the separation of the oil fraction from the full-fat oilseed, inactivates antinutritional factors present, and removes the residual organic solvents used for oil extraction[10,11]. However, depending on the severity of the conditions employed, thermal processing may negatively affect the digestibility and nutritional value of proteins[12]. As many of the ingredients used for compound feed production have already undergone a processing step, compound feed production is described here as secondary processing. Secondary processing can alter the protein quality of native as well as previously heat-processed protein sources[3,13]. Compound feeds are processed in order to control the physical properties of ingredients and improve nutrient availability, which leads to improvements in performance, and to the reduction of the pathogenic burden in feed[14]. It is usual yet unintentional, however, that during processing of ingredients and feed compounding, the factory throughput of material and the physical quality of the feed are of prime importance over

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the nutritional consequences of the processing parameters employed[15]. These processes could potentially decrease protein digestibility and amino acid bioavailability.

Most nutritional studies conducted on feed or feed ingredient processing focus on digestibility of nutrients as an end parameter, and fail to identify the underlying mechanisms for the differences found[13,16–18]. For example, extrusion of field peas (Pisum sativum) at 75, 115 and 155°C increased the standardised ileal digestibility of crude protein from 81 % in untreated field peas to 89, 94 and 92 % for the extrusion treatments, respectively[13]. These authors suggested that denaturation of either storage proteins or antinutritional factors explain the observed effects, but did not measure this. Furthermore, processing conditions in many studies are poorly described and when complex equipment (for example, pelleting or extrusion) was used, simultaneously involving several processing parameters, such as heat, pressure and shear, it is impossible to disentangle their effects[19–21]. It is important to understand the effects of each processing parameter individually, as well as interdependency of parameters, on the structural and chemical changes of the protein fraction, which could influence its nutritional value.

Processing, as described in the present review, is defined as any action that results in physical or chemical changes or disrupts the conformation of a native or previously processed ingredient or mixture of ingredients for compound feed production. Here, we address the effects of processing on the physico-chemical changes of proteins and the consequences of these changes for the nutritional quality of protein, linked to the end point of protein digestion. Selection of literature was limited to vegetable ingredients commonly used in swine diets, which reported effects of processing on both protein structure and digestibility. The review starts with a brief summary of the biochemistry of native proteins and its relationship with crude protein digestibility. Although subject to discussion, a protein with a structural conformation equal to its presence in the original source can be considered as native. We continue with an overview of the available literature on the effects of processing at various conditions on protein structure of ingredients used in swine feeds, and the consequences of these changes for protein digestibility in single-stomached production animals. When possible, correlations between studies are evaluated with the CORR procedure of Statistical Analysis System software version 9.3 (SAS Institute, Inc.)[22] using the information also included in the tables and online Supplementary tables. The correlations were used to identify trends on the mechanisms by which protein structure and structural modifications due to processing influence digestibility.

There are shortcomings to the different digestibility techniques reported in literature which might confound the correlations between structural properties of proteins and digestibility. For example, the effects of gut microbiota, antinutritional factors and dietary fibre on digestion are difficult to simulate using in vitro methods[23]. In addition, the standardised ileal digestibility technique does not take into account the specific endogenous losses originating from antinutritional factors and dietary fibre. Finally, the measurement of faecal protein digestibility includes the fermentation of proteins in the large intestine, which might overestimate protein digestibility at the level of the ileum. For this reason, correlations between structural properties and digestibility were performed for studies in which digestibility was analysed using similar techniques (for example, in vitro or faecal).

Biochemistry and bioavailability of protein in vegetable feed ingredients

Structure of native proteins

Proteins are polymers made up of different amino acids linked by peptide bonds. This linear chain of amino acids forms the primary structure of the protein and their sequence, specific for every protein, also determines the secondary, tertiary and quaternary structures of the native form of the protein[24]. The main configurations of the secondary structure of protein are the α-helices and β-sheets[25,26] (Fig. 1), although others exist, mostly resulting from variations of these configurations. The α-helices and β-sheets provide strength and rigidity to proteins[27]. Regions in the primary structure of a protein that lack structural elements are considered as random coils[28], which by definition are less stable[27]. The secondary structure of proteins can be determined by several techniques, such as Fourier transform IR (FTIR) spectroscopy and circular dichroism, the latter one used solely for soluble proteins. Circular dichroism uses the polarisation angle of polarised light in the far-UV region for the determination of the secondary structure of proteins and the near-UV region for the determination of changes in the tertiary structure. With FTIR spectroscopy, the amide I region (1600–1700/cm) is used to measure the secondary structure of proteins by detecting the vibrations of the carboxyl groups of amino acids[29,30]. This region of the spectra includes bands for the main secondary structures: α-helices (1650–1660/cm) and β-sheets (1630–1638/cm). Within the amide I region, also bands that indicate modifications to the native structural conformation can be distinguished, such as
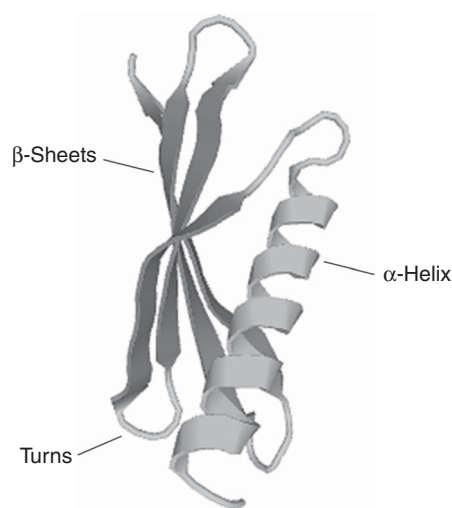
Fig. 1. Cartoon view of the main secondary structures of proteins. The protein in the cartoon is Protein Data Bank ID: 4R80[25,26].

the intermolecular β-sheets (1620–1630/cm) and A2 bands (1690–1695/cm), indicative for protein aggregation[30]. Inter-molecular β-sheet structures originate from intermolecular hydrogen bonds[31] and A2 regions from intermolecular aggregation of proteins or the vibration of the carboxyl groups of the amino acid side chains[30].

Intramolecular formation of non-covalent and disulfide bonds between side chains of amino acids holds the three-dimensional tertiary structure of protein together. The hydro-phobic amino acids are hidden in the interior of the structure. Non-covalent bonds between amino acids can be due to Van der Waals interactions between hydrophobic amino acids (for example, proline, tryptophan), to hydrogen bonding, or to electrostatic interactions between amino acids with opposite charges (for example, lysine and glutamate)[27]. Moreover, proteins in their tertiary structure can interact with each other and form non-covalent and covalent (disulfide) intermolecular bonds, which give rise to the quaternary structure of proteins. Most of the storage proteins in vegetable sources are present in a quaternary conformation, which can also be considered their native conformation. Examples of such proteins are napin (albumin) and cruciferin (globulin) in rapeseed, and the globulins β-conglycinin and glycinin in soyabean. Rupture of intermolecular bonds releases the individual proteins that compose the quaternary structure. For example, under reducing conditions, the acidic and basic polypeptides of soyabean glycinin and rapeseed cruciferin are separated as seen by electrophoresis (SDS-PAGE)[32,33]. The energy required to break disulfide covalent bonds is higher than the energy required to break non-covalent bonds (Table 1). Proteins with large numbers of disulfide bonds are regarded to have a higher resistance to enzymic activity during digestion and also a lower sensitivity to structural changes during processing than proteins with small numbers of disulfide bonds[34].

Proteins from cereals and legumes are usually stored as protein bodies. In general, approximately 80 % of the protein content of legumes can be classified as storage proteins and is stored in protein bodies[35]. It has been defined that any protein present at least in 5 % of the total protein content can be con-sidered as storage protein[36]. The remainder of the proteins, which comprise the proteins with biological activity (for example, enzymes and inhibitors), are not contained within protein bodies.

An overview of the structural properties of the major proteins that are present in important vegetable ingredients used for commercial swine compound feed production is shown in Table 2. The proteins are described based on the most abun-dant protein types for each ingredient. For example, although classified under the same name (i.e. zein), the prolamins from maize consist of four types of proteins with different secondary structures (i.e. α-, β-, γ- and δ-zein). The secondary structure of α-zein is mostly α-helical, whilst that of β-zein (which is also related to γ-zein) consists mostly of β-sheets and turns[37]. The difference in the secondary structure of these proteins originates from their different amino acid compositions[38].

Overall, globulins are the predominant protein types in oil (for example, rapeseed) and legume seeds (for example, soyabean), whilst prolamins and glutelins are the most

Table 1. Types, strength and amino acids involved in bonds found in proteins and solvents that can dissolve these bonds*

Type of bond	Description	Strength (kJ/mol)	Distance (nm)	Amino acids involved	Broken by
Non-covalent					
van der Waals	Induced or permanent interactions between dipoles	0·4–4	0·30–0·60	Tyrosine, tryptophan, phenylalanine, proline, methionine, leucine, isoleucine, valine, alanine, glycine	Ionic and non-ionic detergents (for example, SDS, thiourea, triton, CHAPS, Na salts of long-chain fatty acids)
Hydrophobic interactions	Interactions between non-polar molecules following localised water structuring	<40 NH→O: 8	1·00		Molecules with strong polarity (for example, urea, thiourea, SDS)
Hydrogen bonds	Between hydrogen and strong electronegative atom	NH→N: 13 OH→O: 21 OH→N: 29	0·30 0·31 0·27 0·28	Asparagine, glutamine, threonine, serine, cysteine	
Ionic/electrostatic	Between opposite charged groups	20	0·25	Aspartic acid, glutamic acid, lysine, arginine, histidine	Acids, alkali or salt solutions
Covalent					
Disulfide bonds	Two sulfur atoms linked together	215–251	0·21	Cysteine	Reducing reagents (for example, DTT, Na₂SO₃, 2-mercaptoethanol)
Peptide bonds	Amide bonds between amino acids	C–N: 308	0·13–0·15	All amino acids	Very strong forces (for example, combustion, hot acid hydrolysis)
Isopeptide bonds	Amide bonds between side chains of amino acids	305–308	0·13–0·15	Lysine, glutamic acid, aspartic acid, glycine	Very strong forces (for example, combustion, hot acid hydrolysis)

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol.
* Modified after Liu & Hsieh[81].

Table 2. Biochemical characteristics reported for major proteins of vegetable ingredients frequently used in pig feed

Ingredient	Protein	Osborne class (protein type)	Content (% of total protein)	MM (kDa)	pI (pH)	Secondary structure				Protein digestibility coefficient*	Reference
						α-Helices (%)	β-Sheets (%)	α:β Ratio	Random coils (%)		
Rapeseed	Cruciferin	Globulin	60	300	7.2	11	31	0.35	58	0.89 ± 0.003	35, 42, 115
	Napin	Albumin	15–45	12–14	9.0–12.0	25	38	0.66		0.87 ± 0.002	35, 42, 116
Soyabean	Glycinin	Globulin (legumin)	31	300–380	4.6–5.0	15	37	0.41	23	0.79 ± 0.008	33, 35, 42, 117, 118
	β-Conglycinin	Globulin (vicilin)	30–50	150–200	4.9–6.0	13	38	0.34	23	0.86 ± 0.011	35, 42, 43, 119
Pea	Legumin	Globulin	65–80	398	4.5–8.8	9	46	0.20		0.63 ± 0.02*	41, 120, 121
	Vicilin	Globulin		180	5.0–6.0	35–36	40–49	0.80	7–15	0.88 ± 0.02*	41, 120, 122
	Albumin	Albumin	20–35	26		3	49	0.06	31	0.41 ± 0.12*	41, 123
Kidney beans	Phaseolin	Globulin (vicilin)	36–46	163–600	4.2	16 ± 0.2	39 ± 0.4	0.41	26 ± 0.2	0.11 ± 0.02–0.27 ± 0.02*	35, 46, 124
Sunflower	Helianthinin	Globulin (legumin)	55–60	300–350	5.4–6.8	2	28	0.07	70		125, 126
Wheat	Gliadin	Prolamin	34	28–55	6.5–7.8	22–36	25–31	1.04	11–16	0.90 ± 0.007	42, 127, 128, 129, 130
	Glutenin	Glutelin	47	100–1000	7.0					0.91 ± 0.005	42, 127, 130
Maize	Zein	Prolamin	40–52	22–25	6.8	44–65	0–30	1.29–3.63	41–51	0.53–0.99 ± 0.001*	131, 132, 133, 134, 135
Barley	Hordein	Prolamin	80	36–44	6.0	18 ± 2.3–34 ± 1.6	14 ± 1.3–25 ± 2.4	1.40–2.00		0.54–0.70*	38, 136, 137, 138
Sorghum	Kafirin	Prolamin	68–73	13–27	6.0	40–60	36–43	1.27		0.76 ± 0.03*	135, 139

MM, molecular mass; pI, isoelectric point.
* Values were determined from *in vitro* measurements.

abundant cereal proteins. In addition, proteins with very different structural characteristics can be found within the same ingredient. For example, cruciferin in rapeseed has a higher molecular weight and α-helix:β-sheet ratio than napin. According to the 'Osborne'[39] classification of proteins, albumins are soluble in water, globulins in diluted salt solutions, prolamins in ethanol–water solutions and glutelins in diluted alkali solutions. Although many proteins are classified as being from the same 'Osborne' type[39], proteins from different ingredients usually differ in their amino acid composition. This is reflected in different conformations, thereby making it impossible to generalise on the types of bonds present and their secondary, tertiary or quaternary structures. Globulins and albumins seem to have a higher content of intramolecular β-sheet structures compared with prolamins. This is also reflected in a lower α-helix:β-sheet ratio of the globulins and albumins compared with the prolamins.

Carbonaro et al.[30] suggested that the secondary structure of the protein in a (processed) ingredient is a good predictor of digestibility. These authors reported that the proportion of intramolecular β-sheet structures in food sources from vegetable and animal origin is negatively correlated with *in vitro* crude protein digestibility (r –0.98) using porcine trypsin and peptidase, bovine chymotrypsin and a bacterial protease (the latter added in a subsequent incubation). A higher α-helix:β-sheet ratio has been described to negatively influence *in vitro* intestinal digestibility of rumen undegraded protein in ruminants[40], although the mechanism of digestion in ruminants differs from that in single-stomached animals. The relationship between the α-helix:β-sheet ratio and digestibility was also studied *in vitro*[41]. It was found that the *in vitro* digestibility of the albumin, globulin and vicilin fractions of peas (41, 63 and 88 %, respectively), using porcine pepsin and pancreatin, corresponded with the α-helix:β-sheet ratio of 0.06, 0.20 and 0.80, respectively. Variation in the secondary structure of proteins, however, does not seem to explain the variation in digestibility completely. Napin and cruciferin, which have very different secondary structures (for example, ratio α-helix: β-sheet is 0.66 in napin and 0.35 in cruciferin), do not differ much in their apparent faecal digestibility in rats[42]. Also, proteins with similar secondary structures, such as glycinin and β-conglycinin from soyabeans, differ in their apparent faecal digestion coefficients[42]. The larger number of disulfide bonds in glycinin compared with β-conglycinin has been postulated to provide a further explanation for the differences in the digestibility values between these proteins[33,43].

There was no correlation ($P > 0.05$) between the secondary structure of isolated native proteins and *in vitro* or faecal digestibility (Table 2). However, literature on food allergens[44,45] suggests that the native structure of some proteins (for example, Ara h 1 from groundnuts or β-conglycinin from soyabeans) influences the accessibility of gastric proteases, which might also limit the access of intestinal peptidases for proteolysis. For example, the main globulin from kidney beans (*Phaseolus vulgaris*), phaseolin, has high resistance to pepsin and pancreatin hydrolysis (19 % *in vitro* digestibility), which was attributed to its closed tertiary or quaternary structure that restricts enzyme accessibility[46].

Structural changes due to protein denaturation

Exposure of plant storage proteins to harsh conditions, like extreme pH values, heat or pressure, causes protein denaturation. For example, a pH higher than 11·5 or lower than 3·0 or pressure at 400 MPa for 10 min cause complete denaturation of soya glycinin[47,48]. Denaturation is the unfolding of proteins from their tertiary or secondary structures and will occur when the denaturing influence is sufficiently large to break the non-covalent or covalent bonds that hold the structure together. Conditions for denaturation depend on the type and structure of each native protein[49]. Refolded proteins with a structural conformation different from their native state can also be considered as denatured. Proteins refold in an attempt to minimise their free energy state, with simultaneous formation of new bonds or interactions[50]. It has been suggested[51] that the new bonds formed are not representative of the original conformation of the protein, as this is statistically unlikely to occur. These new bonds result from intramolecular electrostatic and hydrophobic interactions between amino acids, or from intermolecular non-covalent and/or disulfide bonds between two unfolded proteins. The formation of these bonds or interactions between amino acids may lead to protein aggregates, which can be soluble or insoluble depending on the molecular weight of the aggregate. As defined by Wang *et al.*[52], protein aggregates consist of proteins that have lost their native state and are at least twice the size of the native protein. The mechanisms that explain the aggregation of proteins were extensively reviewed by these authors. Protein aggregation can also occur due to crosslinking reactions between proteins, resulting from protein oxidation and the formation of covalent bonds between amino acids, which is favoured at alkaline pH[53]. An example of the latter is the formation of lysinoalanine, which is a crosslink between lysine and alanine and is mediated through the formation of dehydroalanine from β-substituted amino acids (for example, phosphoserine) and the subsequent nucleophilic addition of the ε-amino group of lysine[53].

During digestion, proteins are partially denatured by the acidic pH conditions in the stomach, which facilitates the accessibility of proteases to the peptide bonds. Pepsin is secreted in the stomach and cleaves peptide bonds between hydrophobic or neutral amino acids, except for proline. Proteins that are resistant to the acidic and proteolytic conditions in the stomach can reach the intestinal mucosa and cause allergic reactions[44]. Trypsin and chymotrypsin are secreted in the pancreatic juices in the duodenum. Trypsin is highly specific to lysine and arginine, whilst chymotrypsin has a preference for large hydrophobic amino acids, such as tryptophan, tyrosine and phenylalanine. This specificity becomes important when the cleavage sites for these enzymes are physically or chemically blocked by structural constrains, such as protein aggregation due to non-covalent or covalent bonds formation or by modification of the amino acid residues due to protein oxidation (for example, dityrosine bonds) or Maillard-type reactions.

Changes in the structure of proteins due to denaturing treatments, such as heat, pH and pressure, can lead to proteins that become either more susceptible or more resistant to proteolysis. Protein denaturation can result in the formation of random coils, which exposes groups that are not usually accessible in the native form, thus becoming more susceptible to enzymic hydrolysis[30,54]. The formation of random coils due to processing results from the breakdown of the tertiary structure and that of the secondary structure, such as α-helices and intramolecular β-sheets. Denaturation, especially at extreme conditions involving high temperatures or pH extremes, promotes protein aggregation[30,55–59] and crosslinking between amino acids[60]. Aggregation and crosslinking reactions could decrease the accessibility of digestive enzymes, thereby reducing protein digestibility[61,62].

The extent of structural changes in proteins depends on the conditions employed during processing, and also on the types of proteins present. Upon thermal processing of rapeseed protein isolates, the globulin fraction (cruciferin) was more affected and formed a larger amount of aggregates than the albumin fraction (napin)[63]. Similar results have been reported for soyabean proteins, in which β-conglycinin is more heat-sensitive than glycinin due to the disulfide bonds present in the latter protein type[51]. Disulfide bonds can be broken during processing, but depending on the severity of the conditions, the free thiol residues can also react to form new bonds via sulfydryl-disulfide interchange reactions and increase aggregation[59]. Differences in amino acid composition and structure between different types of the same protein (for example, α-, β- and γ-kafirins) might also influence the response of the proteins to denaturing conditions and digestion[64].

Furthermore, it has been suggested that the net charge of the protein influences the structural response to thermal processing[59]. Increasing NaCl concentrations from 0 to 1·5 M at pH 8 increases the denaturation temperature of soya glycinin from 79·1 to 98·5°C[47]. This is an indication of a protective effect of the salt on the native structure of glycinin from protein denaturation at increasing temperatures. When similar processing conditions were used, a higher content of hydrophobic and uncharged polar amino acids and higher acidic:basic amino acid ratio were linked to a decrease in aggregation[59]. It has been proposed[55] that heat-induced aggregation promoted by basic amino acids (lysine, arginine or histidine) could have detrimental effects on protein digestibility.

In addition, proteins are highly susceptible to react with reducing sugars upon heating to produce Maillard reaction products. Some essential amino acid residues, such as lysine and arginine, are more susceptible than other amino acids to these type of reactions. Lysine susceptibility is due to the presence of the additional amino group, whilst in arginine it is due to the additional guanidinium group[65]. The nature of these reactions and the wide range of compounds formed have been reviewed elsewhere[65]. It must be considered that structural changes *per se* (for example, protein aggregation) and chemical changes due to Maillard reactions may occur simultaneously[66] and that the effects of both on proteolysis might be confounded.

Protein digestion is a multifactorial process that is influenced not only by the physico-chemical properties of the proteins, but also by the matrix in which the protein is embedded. Microstructural components of the feed are not often described in the literature and this aspect should be taken into account in

future research that links modifications of proteins due to processing to protein digestibility.

Processing, protein structure and protein digestibility

Extrusion and pelleting, which are frequently used agglomeration processes in feed production, involve the simultaneous utilisation of heat, moisture, shear and pressure. These agglomerating technologies can be considered as complex processes, in which process parameters (for example, temperature, moisture, screw configuration) and system parameters (for example, mechanical energy, throughput, residence time) are interrelated and can influence each other[67–69]. Both process and system parameters influence the final structural conformation of proteins and enzyme accessibility for proteolysis, and consequently affect protein digestion. Hence, it would be important for further research efforts to disentangle the effects of individual processing factors on protein, which will allow maximisation of dietary protein utilisation in animal production.

Heat-induced changes

Thermal treatments are involved in most common processing technologies to achieve the desired physical state of the ingredients and ingredient mixtures. Other reasons are to improve the digestibility of starch and proteins, to eliminate residual organic solvents after oil extraction and antinutritional factors, and to maintain hygienic levels of the processed feeds[15,70–72].

The maximum temperature achieved during commercial oil extraction of soyabeans and rapeseeds ranges between 100 and 110°C[10,11,73,74], a temperature which is held constant for 60–90 min[12]. It must be emphasised that these temperatures are achieved by toasting, which comprises the use of steam, which is a source of heat that involves large amounts of moisture. Large differences with respect to the formation of Maillard reaction products in soyabean meal proteins have been detected between dry (for example, oven-drying) and wet (for example, autoclaving) sources of heat[75], making it difficult to disentangle the individual effects of heat and moisture.

The temperature applied during compound feed production (secondary processing) depends on the technology employed. Common pelleting temperatures for swine feed production range between 60 and 100°C, whilst higher temperatures are achieved with expander processing (90–130°C) and extrusion (60–160°C)[76].

Changes in the secondary structural conformation of proteins due to thermal processing have been reported (Table 3), although only a limited number of studies have linked these changes to protein digestibility. After autoclaving at 120°C for 20 min, an increase in the relative amount of random coil conformation in different whole ingredients was reported[30]. Heating at 100°C for 15 min also increased the proportion of random coils in rapeseed protein isolate from 23 % in the unheated material to 28 % after heating, whilst increasing simultaneously the proportion of β-sheets from 10 to 33 %[63]. Formation of random coils is an indication of successful protein denaturation, also linked to a positive correlation with *in vitro* digestibility (r 0·91)[30]. The increase of the *in vitro* digestibility reported in this study after processing ranges from 2 to 6 %, which can be considered as low. In addition, inactivation of protease inhibitors was not considered in this study. The effect of the formation of random coils on the increase in digestibility after the thermal treatment might be confounded with the inactivation of protease inhibitors. These authors also reported that autoclaving induced the disappearance of the intramolecular β-sheet structures and the appearance of intermolecular β-sheet structures and A2 bands in FTIR spectra. These two bands in the spectra (intermolecular β-sheets and A2 bands) have been related to protein aggregation and the proportion of intermolecular β-sheets was highly predictive (r –0·99) for *in vitro* protein digestibility. As formation of aggregates and random coils occur simultaneously during processing, the net effect on digestibility is related to the relative frequency of each phenomenon. When taken together (Fig. 2), the total content of intramolecular β-sheet structures from native proteins and the intermolecular β-sheet structures from proteins after thermal treatment has a high negative correlation (r –0·95; $P < 0·001$; n 8) with *in vitro* crude protein digestibility. The total content of intramolecular and intermolecular β-sheets can be used as a fast

Table 3. Relative spectral weights of the secondary structure of proteins and *in vitro* crude protein digestibility coefficient after processing under different conditions
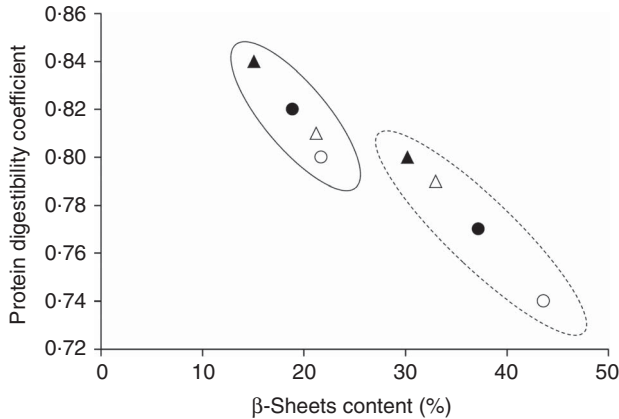
| Feedstuff/ material | Treatment | Secondary structure (relative spectral weights) | | | | Protein digestibility coefficient | | Reference |
		α-Helices (%)	β-Sheets (%)	α-Helix:β-sheet ratio	Random coils (%)	Reported average	SE	
Kidney bean	Control	11–18	32–44	0·34–0·41	0	0·74	0·003	30, 95
	Dry heated 120°C, 30 min	0	13		18			
	Autoclaved 120°C, 20 min	0–13	0–22	0·58	18–33	0·80	0·005	
Lentil	Control	8–18	33–47	0·17–0·54	0	0·79	0·005	30, 95
	Dry heated 120°C, 30 min	8	46	0·17	0			
	Autoclaved 120°C, 20 min	0–22	7–21	1·02	9–23	0·81	0·004	
Chickpea	Control	20	37	0·53	0	0·77	0·004	30
	Autoclaved 120°C, 20 min	19	19	1·02	15	0·82	0·005	
Soyabean	Control	12	30	0·39	0	0·80	0·003	30
	Autoclaved 120°C, 20 min	12	15	0·80	17	0·84	0·005	

predictor for protein digestibility, at least for legume protein sources. As both β-sheet structures within the protein and between proteins correlate well with *in vitro* crude protein digestibility, it appears that the structure itself (i.e. β-sheets), but not its location, limits proteolysis. The correlation between the α-helix:β-sheet ratio and *in vitro* crude protein digestibility was not significant, but exhibits a trend (r 0·65; $P = 0·08$). Nevertheless, native legume ingredients tend to have a lower ratio and digestibility compared with thermally treated ingredients. It should be noted, however, that these correlations originate from a single study[30] and that the ingredients used

Fig. 2. Correlation between the β-sheets content and *in vitro* crude protein digestibility coefficient of native (- - -) and thermally treated (—) whole legume seeds: ○, *Phaseolus vulgaris*; ●, *Cicer arietinum*; △, *Lens culinaris*; ▲, *Glycine max*. Adapted from Carbonaro *et al.*[30].

probably contained protease inhibitors. It is likely that these protease inhibitors were inactivated during thermal treatment and that the increase in digestibility can be partly attributed to the inactivation of protease inhibitors and partly to the modifications of the secondary structure.

Although the *in vitro* digestibility of the most frequent protein fraction present in kidney beans (phaseolin) was improved after thermal processing (Table 4), the digestibility of other protein fractions from the same source (total albumins, protease inhibitor and lectins) was largely reduced[77]. This could explain why minor or no changes in digestibility were reported after thermal treatment of whole kidney beans or protein isolates thereof [30,55,59]. The overall effect on total protein in the feedstuff seems to be a combination of the positive and negative effects on each individual protein fraction. This means that for a complete understanding of the underlying mechanisms that explain the effects of processing on protein digestibility in feed ingredients, the effects on each individual protein type needs to be studied. Positive effects of the thermal treatment of kidney beans on apparent ileal crude protein digestibility in pigs have been reported[78]. It was discussed that these effects might be due to inactivation of antinutritional factors and conformational changes of proteins during 'high temperature short time' treatments (136°C; 1·5 min), which is in contrast to the lower temperature and longer times (ranging from 95 to 121°C; 15 to 30 min) applied in other studies[30,55,59,77].

Changes in the solubility of proteins, due to the heat-induced formation of insoluble aggregates, have been reported for a wide range of experimental conditions and were linked to changes in the types of bonds within and between proteins

Table 4. Change in *in vitro* and *in vivo* protein digestibility compared with a control of feed ingredients as affected by various processing treatments

Feedstuff/material	Treatment	Δ Digestibility (%)*	Reference
In vitro digestibility			
Red bean isolate	Cooked 95°C, 30 min	−9	59
Mung bean isolate	Cooked 95°C, 30 min	5	59
Kidney bean isolate	Cooked 95°C, 30 min	2	59
Kidney bean	Autoclaved 120°C, 20 min	7	30, 55
Faba bean	Autoclaved 120°C, 20 min	−4	55
Lentil	Autoclaved 120°C, 20 min	−1 to 2	30, 55
Chickpea	Autoclaved 120°C, 20 min	5	30, 55
Kidney bean isolated proteins			
Phaseolin	Cooked 99°C, 30 min	> 100	77
Total albumins	Cooked 99°C, 30 min	−33	
	Autoclaved 121°C, 15 min	−47	
Protease inhibitor – lectins	Cooked 99°C, 30 min	−11	
	Autoclaved 121°C, 15 min	−44	
Glutelins	Cooked 99°C, 30 min	3	
	Autoclaved 121°C, 15 min	−9	
Soyabean meal†	Extruded 115°C, 26 % moisture, 80 rpm	27	104
	Extruded 115°C, 35 % moisture, 80 rpm	22	
	Extruded 115°C, 26 % moisture, 140 rpm	32	
	Extruded 115°C, 35 % moisture, 140 rpm	22	
In vivo digestibility			
Faba bean‡	Autoclaved 120°C, 20 min	−30	140
Soyabean meal§	Extruded 116°C, 23 kWh/t	1	141
Soyabean white flakes§	Extruded 116°C, 23 kWh/t	18	141

* Change (%) within studies with respect to control.
† Data extrapolated from graphs.
‡ Standardised ileal digestibility, rats.
§ Faecal digestibility, mink.

(see online Supplementary Table S1). There is a parallel increase in the amount of insoluble protein aggregates with the severity of the thermal treatments applied. Already at 80°C, 31 % of the protein content in rapeseed protein isolate formed insoluble aggregates, compared with 0 % in the unheated material[63]. In the isolate, cruciferin (globulin) was more susceptible to aggregate formation than napin (albumin)[63]. Most studies agree on a decreased solubility of a protein after a thermal treatment, compared with that of the native protein[57,79–81]. The reduction in protein solubility is an indication of the formation of insoluble protein aggregates. Many native proteins are soluble as their hydrophobic groups are located on the inside of the molecule. After denaturation, involving reorientation of the hydrophobic groups and refolding, the initial protein aggregates formed are soluble, but they become insoluble when their size exceeds the solubility limit[31].

Protein solubility studies with agents capable of cleaving different types of bonds (for example, urea, SDS and dithiothreitol (DTT)) have been used in order to study the main causes for protein insolubility (see online Supplementary Table S1). Urea and SDS are good agents to cleave non-covalent bonds, whilst DTT is adequate for the reduction of disulfide bonds into thiols. The solubility of unheated proteins in buffer solutions is similar to the solubility of aggregated proteins with solutions that contain both non-covalent and covalent cleaving agents. Combining these two types of cleaving agents has a synergistic effect on the amount of solubilised protein, compared with the amount of protein solubilised by using them separately[57,79–81]. This indicates that non-covalent and covalent bonds are mutually important in maintaining the structure of aggregated proteins.

After thermal processing, there is an increase in the disulfide bond content with a simultaneous reduction in the content of thiols (Table 5). For example, thiol content was reduced from 6·6 μmol/g protein in the albumin and the protein inhibitors plus lectins fractions isolated from native kidney beans to 0·2 and 0·6 μmol/g protein, respectively, after heating at 121°C for 15 min[77]. At the same time, the disulfide bond content was increased from 24·8 to 28 μmol/g protein in the albumin fraction and from 22·5 to 25·5 μmol/g protein in the protein inhibitors plus lectins fraction. These changes were more evident with autoclaving at 121°C for 15 min than with cooking at 99°C for 30 min. This could be an indication that the effect of

Table 5. Free thiol and disulfide bonds content, and protein digestibility coefficient after processing under different conditions*

Feedstuff/material	Treatment	Disulfide bonds (μmol/g protein)	Free thiol groups (μmol/g protein)	Protein digestibility coefficient	Reference
Kidney bean isolate	Control		4·5 ± 0·20	0·64 ± 0·02	59
	Cooked 95°C, 30 min		1·3 ± 0·02	0·65 ± 0·02	
Mung bean isolate	Control		8·7 ± 0·07	0·56 ± 0·01	59
	Cooked 95°C, 30 min		2·2 ± 0·04	0·61 ± 0·01	
Red bean isolate	Control		10·3 ± 0·21	0·53 ± 0·01	59
	Cooked 95°C, 30 min		1·8 ± 0·01	0·48 ± 0·01	
Rapeseed protein isolate	Control		25		63
	Cooked 60°C, 15 min		24		
	Cooked 80°C, 15 min		17		
	Cooked 100°C, 15 min		13		
Soyabean meal	Control	29·5 ± 0·5	9·5 ± 0·4	0·85 ± 0·006†	141
	Extruded 122°C, 27 kWh/t	31·4 ± 0·3	7·8 ± 0·2	0·86 ± 0·002†	
Soyabean white flakes	Control	23·3 ± 0·8	10·6 ± 0·4	0·71 ± 0·020†	141
	Extruded 119°C, 24 kWh/t	32·7 ± 0·4	7·2 ± 0·1	0·84 ± 0·003†	
Kidney bean isolated proteins					
Albumins	Control	24·8 ± 0·3	6·6 ± 0·1–19·2 ± 0·2	0·29 ± 0·009–0·32 ± 0·004	77, 87
	Heated 60°C, 30 min		14	0·28 ± 0·002	
	Heated 80°C, 30 min		13	0·25 ± 0·003	
	Heated 100°C, 30 min		4·8	0·25 ± 0·006	
	Heated 121°C, 30 min		2	0·21 ± 0·010	
	Autoclaved 121°C, 15 min	28 ± 0·3	0·2 ± 0·0	0·15 ± 0·008	
	Heated 135°C, 30 min		0·1	0·18 ± 0·008	
Protease inhibitor lectin	Control	22·5 ± 0·1	6·6 ± 0·1	0·28 ± 0·001	77
	Cooked 99°C, 30 min	24·9 ± 0·1	1·9 ± 0·0	0·25 ± 0·015	
	Autoclaved 121°C, 15 min	25·5 ± 0·1	0·6 ± 0·1	0·16 ± 0·010	
Glutelins	Native	ND	ND		77
Red sorghum	Control	30·6–34·0 ± 0·16	0·9–1·5 ± 0·03	0·72 ± 0·001–0·79 ± 0·001	82, 83
	Pelleted 65°C	28·5	0·8	0·72 ± 0·001	
	Pelleted 80°C	29·7	0·9	0·72 ± 0·001	
	Pelleted 90°C	35·9 ± 0·16	1·3 ± 0·03	0·74 ± 0·001	
	Pelleted 95°C	29·0	0·8	0·76 ± 0·001	
White sorghum	Control	33·9 ± 0·16	1·2 ± 0·03	0·75 ± 0·001	82
	Pelleted 90°C	34·4 ± 0·16	1·1 ± 0·03	0·75 ± 0·001	
Yellow sorghum	Control	33·8 ± 0·16	1·5 ± 0·03	0·71 ± 0·001	
	Pelleted 90°C	34·7 ± 0·16	1·4 ± 0·03	0·73 ± 0·001	

ND, not detected.
* Reported average ± standard error.
† Values correspond to faecal crude protein digestibility in minks.

temperature on protein denaturation is more relevant than the effect of time, as reported elsewhere[78] for the storage protein phaseolin. There was a positive correlation between the in vitro crude protein digestibility and the content of thiol groups in thermally treated isolated albumins from kidney beans (r 0.80; $P < 0.003$; n 11) (Fig. 3). Correlations for the rest of the protein sources analysed were not significant. Some of these sources (Phaseolus beans and soyabeans) probably contained protease inhibitors and lectins. An increase in digestibility that resulted from heat inactivation of protease inhibitors and lectins could mask the decrease in digestibility resulting from heat-induced disulfide bonding. It has been suggested[82,83] that the formation of disulfide bonds after heat treatment of sorghum is responsible for the decreased digestibility of kafirin. However, correlations between disulfide bond content of sorghum after thermal treatments and standardised ileal digestibility were not significant ($P > 0.05$). Oddly, the disulfide bond content in the study of Selle et al.[83] did not increase along with the decrease in the thiol content. With increasing temperatures, there is also an increase in the surface hydrophobicity of the proteins in rapeseed protein isolate, which is a reflection of the exposure to the surface of the hydrophobic groups buried inside the molecules[63]. Surface hydrophobicity (S_O) increased from 600 in the unheated rapeseed protein isolate to 650, 1500 and 1100 after thermal treatments at 60, 80 and 100°C for 15 min, respectively[63].

Overall changes in the structure of protein can be estimated by comparing the denaturation enthalpy of native protein with that of the protein after processing (see online Supplementary Table S2). Denaturation enthalpy is a measure of the amount of energy required to denature a protein. Hence, denatured proteins exhibit a lower or a lack of denaturation enthalpy compared with the native ones. Protein denaturation usually increases with the intensity of the heat treatment and has been suggested as the main reason for improved protein digestibility in thermally heat-treated ingredients[70,84]. According to these authors, unfolding of the native secondary/tertiary structure of the protein could facilitate enzymic attack and their suggestion was based on several proteins of vegetable origin (for example, wheat and soya). The conditions applied during processing probably determine whether proteins become more susceptible (for example, unfolding and random coil formation) or more resistant (for example, protein aggregation and chemical changes to amino acids) to enzymic attack. However, enthalpy of denaturation can also increase after long thermal treatments as reported[85] for soyabean protein isolates prepared from heat-treated soyabeans at 40 and 80°C for 4, 8, 12 and 16 h. The denaturation enthalpy of this material decreased after 4 and 8 h of thermal treatment, but increased again with longer heating times. It is possible that protein refolding occurred at longer heating times, causing the denaturation enthalpy to increase. Most of the denaturation enthalpy observed, ranging from 36 to 70 % of the enthalpy in the original material (2.85 J/g), originated from glycinin, which was less denatured than β-conglycinin under these conditions.

Only a limited number of studies has been performed on the link between denaturation enthalpy and digestibility of processed vegetable protein sources[85–88]. The positive correlation between the relative degree of denaturation (i.e. decrease of enthalpy after thermal treatment with respect to the enthalpy of the native material) and in vitro crude protein digestibility for protein-containing ingredients (i.e. sweet potato protein, cowpeas and soyabeans) was significant (r 0.49; $P = 0.003$; n 34) (Fig. 4). However, these ingredients probably contained protease inhibitors. In that case, the relationship between the degree of protein denaturation and the in vitro crude protein digestibility would be somewhat confounded with increased apparent digestibility due to the heat inactivation of protease inhibitors. The amino acid sequence of sporamin (sweet potato protein) resembles that of Kunitz-type trypsin inhibitors, possibly explaining the increase in digestibility after denaturation[88]. Proteins from cowpeas and soyabeans can be classified under the same 'Osborne' type. However, their amino acid compositions are different, which possibly leads to different structural conformations (for example, secondary and tertiary) and explains the differences in the correlations between the relative
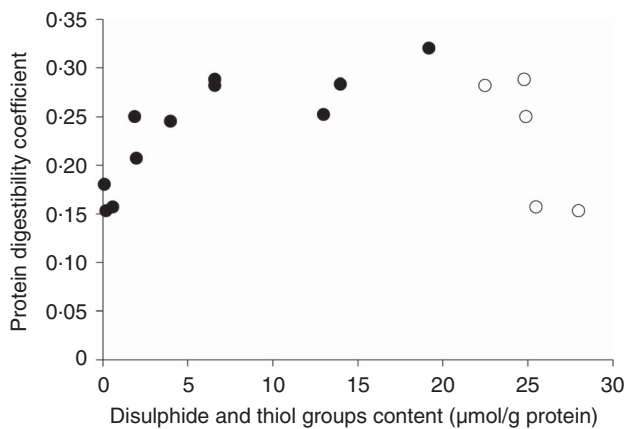
Fig. 3. Correlation between the content of disulfide (○) or thiol (●) groups and in vitro protein digestibility coefficient for thermally treated albumins isolated from kidney beans.
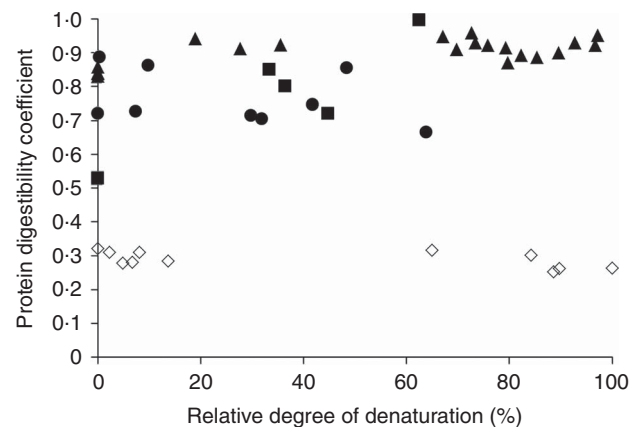
Fig. 4. Correlation between the relative degree of denaturation and in vitro crude protein digestibility coefficient of thermally treated isolated albumins from kidney beans (◇) and other protein sources (■, Ipomoea batatus; ▲, Vigna unguiculata; ●, Glycine max).

degree of denaturation and *in vitro* digestibility (Fig. 4). In addition, whilst the *in vitro* digestibility of sweet potato protein and cowpeas was determined using multiple enzymes (for example, porcine pepsin and pancreatin), the digestibility of soyabean proteins was determined after incubation with only pepsin. Incubation with a single enzyme might lead to an underestimation of protein digestibility. For isolated albumins from *P. vulgaris* there is a trend for a negative correlation between the relative degree of denaturation and the *in vitro* digestibility (r –0·53; $P = 0·09$; n 11) (Fig. 4). The degree of purity of the isolated albumin fraction is not described in that study[87]; thus this fraction might still contain trypsin inhibitors. The limited number of studies available, the inclusion of sources that contain protease inhibitors and the inconsistency in the results do not allow us to draw firm conclusions on the effects of protein denaturation on protein digestibility. The effect of protein denaturation on protein digestibility could be linked to the nature of the protein.

Effects of moisture

Water or steam is usually added as cofactors during processing in order to manage the moisture content and the physical properties of the processed material. The moisture content ranges between 12 and 18 % (w/w) for pelleting[15,76], whereas extrusion processing tolerates higher moisture inclusion ranging from 20 to 35 % (w/w)[76].

Proteins in dry conditions have high glass transition temperatures (T_g), which can be close to their degradation temperature[89,90]. The T_g can be reduced by the addition of water, which acts as a protein plasticiser, and decreases T_g approximately 10°C for every 1 % increase in moisture content[90]. Due to the small size of its molecules, water can move freely through the small openings of the structure of proteins. Water decreases the T_g by disrupting hydrogen bonds, van der Waals and ionic interactions that hold the structure of protein together, thereby increasing its flexibility[89]. Increased flexibility and rupture of the bonds also decrease the denaturation temperature of proteins. However, the flexibility of proteins is limited below water contents of 5–9 %. Exposure of hydrophobic amino acid residues due to changes in the conformation of proteins after the addition of water can lead to irreversible protein aggregation[91]. The denaturation temperature of sunflower (*Helianthus annuus*) oil cake globulins decreased from 189·5°C to 154·4, 133·4 and 119·9°C with increasing moisture contents of 0, 10, 20 and 30 %, respectively[92]. The T_g of these proteins showed a similar pattern, decreasing from 180·8°C with no water to 5·3°C at 26 % moisture content. Similar results were also reported for β-conglycinin[93] and glycinin[93,94] from soyabean meal. The denaturation temperature of β-conglycinin and glycinin were 76·5°C and 93·3°C, respectively, with a moisture content of 94 %. However, when the moisture content was decreased to 29 %, the denaturation temperature of β-conglycinin was increased to 180°C and no denaturation temperature could be found for glycinin. The addition of water decreases the T_g of both native and denatured glycinin, with a faster rate for the native proteins[94].

Thus, water addition increases the flexibility of protein structure and in combination with heat, shear or pressure,

changes the effects of these factors on the secondary protein structure (Table 3), *in vitro* crude protein digestibility (Table 4) and protein solubility (see online Supplementary Table S1). Increasing the moisture content during extrusion processing, whilst keeping a constant temperature, increases protein denaturation and refolding, with the formation of new bonds. For example, the percentage of protein solubilised by a solution of 50 mM-DTT in a phosphate buffer after extrusion at 170°C of a soya protein, wheat gluten and wheat starch mix (weight ratio 60:40:5) increased from 10 to 30 when moisture content (w/w) was increased from 60 to 72 %[81]. This indicates that during processing the extents of both covalent and non-covalent interactions increase with increasing moisture contents, possibly related to unfolding, which is the rate-limiting step. The increased interaction between proteins could explain the reduction of the *in vitro* digestibility of soyabean meal with increasing moisture contents at constant temperature and extrusion screw speed (Table 4). Alternatively, inactivation of trypsin inhibitors could have been higher at lower moisture contents, leading to higher *in vitro* crude protein digestibility.

Changes in the secondary structure of proteins have been reported when moisture-including sources of heat were used (for example, using autoclaving, desolventisation/toasting) in contrast with dry sources of heat (for example, roasting, IR radiation) at similar temperatures (Table 3). This was the case for lentils (*Lens culinaris*), in which no effects were reported for dry heating at 120°C for 30 min, whilst autoclaving at similar temperatures (120°C for 20 min) increases the α-helices and random coil contributions. The latter treatment also reduces the contribution of the intramolecular β-sheets conformation in exchange for a larger amount of intermolecular β-sheets[30]. However, the response of the secondary structure of proteins to changes in moisture could be source-dependent, as not all the ingredients analysed follow the same pattern. Whilst a dry source of heat at 120°C for 30 min induces the appearance of random coil structures in kidney beans, the same thermal treatment does not induce the formation of these structures in lentils[30,95]. These differences could be related to the proportion of the different types of proteins in each ingredient (for example, globulins *v.* albumins), or to the inherent structural characteristics of the proteins of each source. In these studies[30,95] the seeds were autoclaved, along with the water that was used for soaking them (1:4, w/v) during 2 h.

Shear effects

Multiple definitions have been provided for shear. As defined for extrusion processing[96], shear is the dissipation of the mechanical energy input by friction of the particles inside the extruder barrel. The estimation of shear is based on the torque produced by the engine and on the calculation of specific mechanical energy (SME) input. Different levels of shear are involved in the equipment, which is commonly used for compound feed production. Pelleting consumes a low level of SME, ranging between 23 and 45 kJ/kg, and the levels are highly dependent on the fat level inclusion in the diet[97,98] and the size of the die. The technology that is considered to cause the highest levels of shear is extrusion, with SME ranging from 839

to 1277 kJ/kg[68]. As a system parameter, torque is the result of the combination of several process parameters, such as temperature, moisture content, feeder-screw speed, extruder-screw speed and screw configuration. Thus, any change in the process parameters during extrusion can lead to an alteration of the SME input. Separating the effects of SME input from other process parameters used during processing in complex systems such as extrusion is difficult, and literature describing changes in protein structure due to shear only is scarce.

The effects of simple shear flow on protein structure and function have been reviewed before[99]. However, most of these research has been performed on model systems of proteins in solution[100,101], which do not resemble the conditions used during feed production. Simple shear of proteins can induce conformational changes to the structure of proteins by disrupting the secondary/tertiary structure, even at relatively low temperature, as illustrated by the work of Dunstan and colleagues[100,101].

Protein aggregation in soya isolates has been reported as a consequence of extrusion[68]. Vital factors during extrusion, such as a high temperature and pressure, were suggested to be responsible for protein aggregation[68]. However, with increasing SME levels, the molecular weight of the protein aggregates was reduced. The molecular weight of the aggregates was reduced by 5, 9 and 17 % at SME inputs of 1050, 1093 and 1277 kJ/kg, respectively, compared with the molecular weight at the 839 kJ/kg extruder setting[68]. It was suggested by Meade et al.[54] that the mechanical forces during extrusion could break the peptide bonds in the amino acid chain (effects on primary protein structure), thus producing a similar effect as proteolysis.

Increased levels of non-covalent and covalent bonds, which lead to larger amounts of aggregation, were reported[102] in soya proteins with increasing SME. In this experiment, shear contrasts were produced by adaptations to the screw configuration of the extruder. With the screw configurations that produced increased levels of shear, there was also a decrease in the solubility after the extraction of proteins with urea or DTT and the successive extractions with solutions of urea/DTT or DTT/urea. This could indicate that after extrusion at high mechanical energy inputs, proteins may interact by mechanisms other than the formation of disulfide or non-covalent bonds. These mechanisms could be covalent bonds related to crosslinking between amino acids[102].

The formation of Maillard reaction products (and possibly also protein structural modifications) is affected by the interaction between temperature and mechanical energy[103]. High SME inputs produced large amounts of Maillard reaction products when extrusion of a rice (Oryza spp.)–glucose–lysine model mixture was performed at barrel temperatures of 100 and 130°C, but not at 70°C[103]. Increasing the screw speed from 80 to 140 rpm had limited effect on the in vitro crude protein digestibility of commercial soyabean meal after extrusion[104].

Although there is little evidence that crude protein digestibility might be largely affected after shear-processing, the formation of crosslinked or Maillard-modified amino acids has a large impact on the nutritional value of the feed. The crosslinked and modified amino acids cannot be utilised after absorption[105], and therefore animal performance is decreased.

Particle size reduction processing

The amount of mechanical energy involved in particle size reduction is related to the type of equipment employed. SME ranges from negligible in roller milling to 104·4–126 kJ/kg for hammer milling[106] and 6·5–10·1 kJ/kg for the multicracker system[107]. Hammer and roller milling are the most frequently used technologies for particle size reduction of compound feed ingredients, although new technologies, such as the multicracker, have been developed[107].

The effects of particle size reduction on digestibility has been analysed before[21,108–110]. During particle size reduction, rupture of protein bodies from grains and legume seeds that contain storage proteins increases protein solubility[111], which in turn could facilitate enzyme accessibility. However, only few studies report improved enzyme accessibility due to a larger area of exposure of the substrate[112]. Few studies have been performed on the effects of particle size reduction on protein structure. This is probably related to the fact that particle size reduction is regarded as only a physical change and, as such, it is assumed not to have major effects on the structural characteristics of proteins.

Some of the technologies that are used to reduce particle size of ingredients involve the use of moisture, whilst others are performed under dry conditions. Wet-milling of maize dried distillers grains with solubles increased in vitro crude protein digestibility[113]. It is possible, as suggested by these authors, that the disruption of the cell wall structure leads to increased enzyme accessibility and that the improved in vitro digestion is not linked directly to changes in protein structure, although the latter point was not measured by the authors. Supporting this suggestion, is the observation that there were no changes in the secondary protein structure after wet-milling of silk from Philosomia cynthia ricini and Bombyx mori silkworms[114]. In contrast, when dry milling was used in the same study, the intramolecular β-sheet structures in these proteins completely disappeared, probably due to the processes involved in dry milling, including shear. The silk in that study was wet (i.e. addition of water) or dry milled using a ball mill followed by air jet milling. In the case of wet milling, water could act as a lubricant, thereby decreasing the amount of shear. It is possible that the disappearance of the intramolecular β-sheets after dry milling could increase protein digestibility as previously reported by Carbonaro et al.[30]. Furthermore, dry milling could have the additional benefit of the disruption of cell wall structures (as mentioned before for wet-milling), which can increase the access of enzymes for protein hydrolysis.

Conclusions

The structure of proteins influences enzyme accessibility for protein digestion. Some commonly used feed ingredients in swine diets exhibit structural constraints for digestion in their native proteins. Both primary processing of ingredients and compound feed production as a secondary treatment can affect the structure of native and partially denatured proteins. However, the link between the conformational changes and protein digestibility is not clear and further research should help

to elucidate these underlying mechanisms. Most literature available that links protein structure to digestibility describes protein sources or ingredients which still contain protease inhibitors. It is necessary for future research to take this aspect into account and study the relationship between the structure of proteins (native or processed) and digestibility in isolated proteins, excluding the effect of protease inhibitors on digestibility. Correlations between structural properties of proteins (for example, free thiol content or degree of denaturation) with protein digestibility after processing seem to be dependent on the protein nature. Heat-induced modifications in the structure of proteins during primary processing render bonds (intra- and intermolecular) and structures, which become irresponsive to secondary processing and allow limited access to proteolytic enzymes. When proteins did not undergo complete denaturation during primary processing, secondary processing might change protein structure, with positive or negative effects on digestibility. Heat seems to be the parameter during secondary processing with the largest influence on structural changes of proteins. Nevertheless, other factors involved during processing (for example, moisture and shear) could also have a decisive role. However, the multifactorial essence of the complex processing technologies used in compound feed production does not allow yet us to disentangle and explain the effects of each separate processing factor. In this sense, model systems could aid in separating the processing factors and explaining their effects on the structural conformation and nutritional value of proteins.

Acknowledgements

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0954422416000056

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