EDUCATIONAL PAPER

What makes protein indigestible from tissue-related, cellular, and molecular aspects?

Petra M. Becker[1] and Peiqiang Yu[2,3]*

[1] Wageningen UR Livestock Research, Lelystad, The Netherlands
[2] Department of Animal and Poultry Science, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, Canada
[3] Tianjin Agricultural University, Xiqing District, Tianjin, P. R. China

Received: September 6, 2012
Revised: February 20, 2013
Accepted: February 21, 2013

This paper gives an insight into key factors, which impair enzymatic protein digestion. By nature, some proteins in raw products are already poorly digestible because of structural peculiarities, or due to their occurrence in plant cytoplasmic organelles or in cell membranes. In plant-based protein, molecular and structural changes can be induced by genetic engineering, even if protein is not a target compound class of the genetic modification. Other proteins only become difficult to digest due to changes that occur during the processing of proteinaceous products, such as extruding, boiling, or acidic or alkaline treatment. The utilization of proteinaceous raw materials in industrial fermentations can also have negative impacts on protein digestibility, when reused as fermentation by-products for animal nutrition, such as brewers' grains. After consumption, protein digestion can be impeded in the intestine by the presence of antinutritional factors, which are ingested together with the food or feedstuff. It is concluded that the encircling matrix, but also molecular, chemical, and structural peculiarities or modifications to amino acids and proteins obstruct protein digestion by common proteolytic enzymes in humans and animals.

Keywords:
Antinutritional factors / Genetic modification / Matrix effects / Protein digestion / Protein structures

1 Proteins that have a low digestibility by nature

1.1 Animal structural proteins or scleroproteins

Scleroproteins are fibrous proteins that form supporting structures in the body. They are resistant to digestion. Scleroproteins comprise collagen, elastin, keratin, and silk fibroin, but are also present in insect cuticles and marine organism, where they might be halogenated to some extent [1, 2].

A scleroprotein's peptide sequence often consists of a limited selection of amino acids with a repeated order of appearance [3]. Scleroproteins can form unusual secondary structures, such as the collagen triple helix. The protein structures often feature stabilizing cross-links between peptide chains.

1.1.1 Collagen and gelatin

Collagen contains high percentages of alanine, glycine, and proline [4]. During posttranslational processing, prolines and lysines are hydroxylated by the enzymes prolyl hydroxylase and lysyl hydroxylase, resulting in hydroxyproline and hydroxylysine. The hydroxylation is important for the cross-linking of three peptides to a stable triple helix in collagen. This enzymatic step requires vitamin C as a cofactor [5]. Collagen is quite resistant to digestion [6], although the bovine and

Correspondence: Professor Peiqiang Yu, Department of Animal and Poultry Science, College of Agriculture and Bioresources, University of Saskatchewan, 51 Campus Drive, Saskatoon, S7N 5A8, Canada
E-mail: Peiqiang.Yu@usask.ca

Abbreviations: **BAL**, β-aspartyl-ε-lysine; **BSA**, bovine serum albumin; **CLA**, agglomerative hierarchical cluster analysis; **GGL**, γ-glutamyl-ε-lysine; **HAL**, histidinoalanine; **LAL**, lysinoalanine; **LAN**, lanthionine; **LDA**, linear discriminant analysis; **Me-HAL**, methyl-histidinoalanine; **Me-LAL**, methyl-lysinoalanine; **Me-OAL**, methyl-ornithinoalanine; **OAL**, ornithinoalanine; **PCA**, principal component analysis; **PCP**, pyrrolidone carboxylate peptidase; **ROS**, reactive oxygen species

*Additional corresponding author: Dr. Petra M. Becker
E-mail: Petra.Becker@wur.nl

porcine pancreas contains collagenase activity [7]. As protein source for humans, only digestible, denatured collagen plays a role [8]. Due to its lack of essential amino acids, collagen is inferior to, e.g., muscle protein as protein source [8]. On the other hand, as a nitrogen source, collagen could reinforce the body's own amino acid synthesis [8]. Gelatin is a hydrolyzed product from collagen [9].

1.1.2 Elastin

Ligaments, tendons, and arteries contain elastin. Although elastin resembles collagen in its structure, elastin cannot be broken down into gelatin. This is because elastin, in contrast to collagen, contains desmosine and isodesmosine. Desmosine and isodesmosine contain heteroaromatic derivatives of pyridinium, which are formed by cross-linking of lysine residues [10]. Elastin is largely indigestible [8].

1.1.3 Keratin

Keratins, proteins with a predominantly pleated sheet configuration, are poorly soluble in water and resistant to proteolytic enzymes [11, 12]. Keratin is the building material of robust external protein structures [13]. The strength and rigidity of keratin is based on permanent, thermally stable cross-linkings formed by intra- and intermolecular hydrogen bonds and disulfide bridges between cysteine molecules. Wool and hair contain about 14% cystine (cysteine dimer), and horn 12% [3]. Protein in hair is extensively cross-linked with disulfide bonds, which are not very susceptible to proteolytic enzymes as reported by Clark et al. [14].

The amino acid patterns of the keratins from mammals, namely sheep wool, pig hair, and cattle horn, resemble one another closely. However, avian keratin, such as in chicken feathers, differs from mammalian keratin in being lower in arginine, cystine, glutamate, histidine, lysine, and tyrosine and slightly higher in isoleucine and valine [3].

The production of feather meal from feathers is called rendering. For nutritional purposes, rendered feather protein is mainly used in ruminant nutrition as a high ruminal escape protein [15–17], and can be used in fish diets [18].

1.1.4 Silk fibroin and sericin

Insects of the Saturnidae and Bombycidae spin silk fiber [19]. The chemical structure of the silkworm (*Bombyx mori*) fibroin consists basically of an alternation of glycine residues with two thirds of alanine and one third of serine [20]. Sericin makes up about 20–30% of the total cocoon weight. It contains about 32% serine, and 46% hydroxy amino acids [21].

Both sericin and fibroin have low digestibilities [21, 22]. Water-soluble fibroin extracted from silk yarn, by contrast, had an in vitro digestibility of 58% after treatment with pepsin–trypsin–chymotrypsin. The in vivo true digestibility determined in rats was 65.7% [22].

1.2 Insect cuticle and marine scleroprotein

Insects as alternative sources of protein for use in food and feed are a current research focus to meet the world's growing food demand, although the idea is not new [23]. Especially the larval stages of several insect species and their muscle protein seem interesting in this regard [24]. While juvenile larvae have a soft exoskeleton or cuticle, during metamorphosis, the insect cuticle is hardened by sclerotization. Protein-containing, hardened cuticle structures in insects are, e.g., the firm exoskeleton of cockroaches or grasshoppers. In sclerotized cuticles, which were shown to contain protein and chitin among other extremely diverse materials, these two components are often cross-linked by aromatic groups [25]. According to St. Leger et al. [26], apart from proteases, chitinase seems essential for the biodegradation of insect cuticles. Although some human beings were shown to produce gastric endochitinase, in vitro studies revealed that fly wings incubated in gastric liquid with high chitinase activities were at most moderately digested [27].

In marine organisms, both chitin as well as proteins can be halogenated [2]. Moreover, chlorotyrosine has been detected not only in saltwater invertebrates, but also in land insects. However, the reason for the halogenation of this amino acid is still largely unknown [1].

1.3 Plant storage proteins

Plant proteins, particularly those in legume grains, generally have lower in situ digestibilities than those in animal tissue. This may result from their relative insolubility, their intracellular organization in discrete protein bodies [28], and the low permeability of plant cell walls as well as the protective covering of the seed by the seed coat. Hence, vegetable matter generally requires processing, e.g., cooking, to improve the protein digestibility. Boiling and subsequent freezing of legume seeds increased their digestibility to more than 95% [12, 29]. Digestion-resistant, stable peptides may provoke allergic reactions in susceptible individuals [30, 31].

2 Processing as protein digestibility-affecting treatment

2.1 Heat treatment

2.1.1 Enhanced polymerization, and change in secondary structure

Apart from a digestibility-increasing effect of heat treatment, heat treatment can also decrease digestibility. Cooking

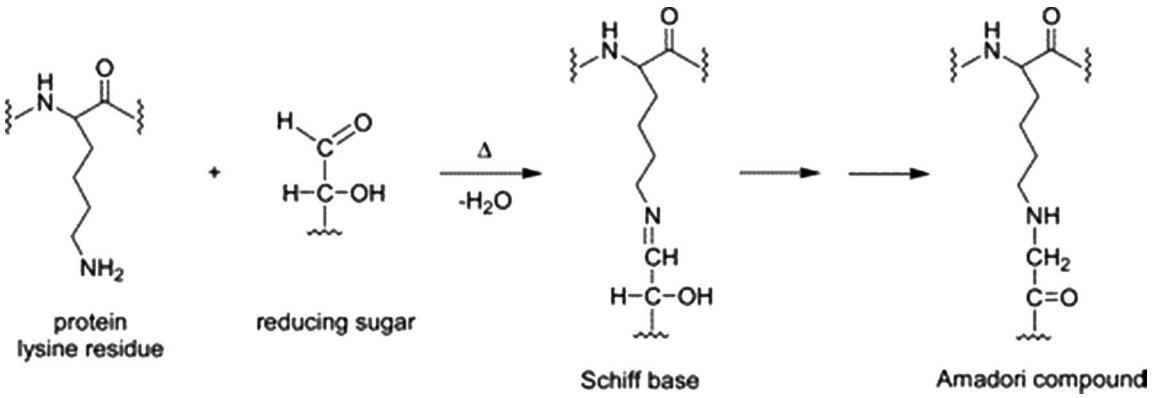

Figure 1. Maillard reaction of lysine residues in protein with reducing sugars. Reprinted with permission from Czerwenka et al. [110]. Copyright (2012) *American Chemical Society*.

negatively affects the enzymatic digestibility and solubility of sorghum protein, but not of maize protein in children [32–34]. Cooking was shown to enhance disulfide linkage formation and polymerization in sorghum [32, 35, 36].

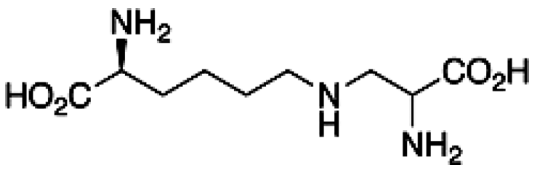

Figure 2. Lysinoalanine (LAL).

2.1.2 Chemical reaction with reducing sugars (Maillard reaction)

The Maillard reaction can cause a decrease of protein nutritional quality [37–40]. According to Finot et al. [41], biologically available ε-N substituted derivatives of lysine, such as ε-N-(α-amino acyl)-, α-N-ε-N-di-amino acyl-, and Schiff's bases were four to seven times less reactive than free lysine in the Maillard reaction. Hence, these reversible substitutions of lysine can possibly prevent digestibility loss of protein in food or feed, which has to be subjected to severe heat treatment.

2.1.2.1 Early Maillard reaction

The first step in the Maillard reaction is a simple condensation reaction between the carbonyl group of a reducing carbohydrate and the free amino groups of a protein, peptide, or amino acid (Fig. 1). Up to this point, the reaction is reversible. The first addition products of lysine with reducing sugars were found to be fully bioavailable in rats, by releasing lysine in the acidic environment of the stomach [42].

After the early Maillard reaction, an "Amadori" rearrangement takes place, in which the aldose (glucosylamine) is transformed into a ketose, namely a 1-amino-1-deoxy-2-ketose. This reaction is irreversible and the "Amadori" compound formed is relatively stable (Fig. 2). In food proteins, the main reactive amino group is the ε-amino group of lysine. When the sugar is glucose, the main "Amadori" compound in a heated protein is, therefore, ε-N-deoxyfructosyl-L-lysine in a bound form. In milk, the corresponding "Amadori" compound formed is ε-N-lactuloselysine. Under mild conditions (37°C, air, pH 7.4), ε-N-deoxyfructosyl-L-lysine can undergo oxidative decomposition to ε-N-carboxymethyllysine (see also Section 2.1.4.2.).

Tryptophan can also undergo an early Maillard reaction with its indole-NH group and reducing sugars, but only at acidic pH. Thus, Nα-acetyl-1-(β-D-glucopyranosyl)-DL-tryptophan amide was found to be generated through condensation reactions between Nα-acetyl-DL-tryptophan amide and D-glucose. This may be considered an N-substituted glycosylamine. Whether this reaction occurs in processed foods is not yet known. It could be of some importance in canned foodstuffs with low pH values. The sulfur amino acids cystine and methionine are not involved in the early Maillard reaction [43].

According to Finot [42], intestinal absorption of Amadori compounds of free amino acids occurs by passive diffusion, while Maillardized peptides are likely to be unabsorbed by the gut. Degradation of Amadori compounds by the intestinal microbiota to CO_2 seems to contribute to their low fecal and urinary excretion in rats (and in pigs), as was indicated by whole-body autoradiographies of rats after oral ingestion of ^{14}C-fructose-lysine.

2.1.2.2 Advanced Maillard reactions

Under more severe heating or storage conditions, the Maillard reaction proceeds further to different advanced steps. The reactions in question lead to the formation of literally thousands of compounds, which are responsible for the numerous flavors and odors of heated foods. Regarding essential amino

acids, a few remarks seem necessary, because they cannot be formed by the body but have to be taken up from protein sources. Lysine heated with glucose (6 h at 105°C) forms ε-(2-formyl 5-hydroxymethylpyrrol-1-yl)-L-norleucine (ε-pyrrol-lysine), an advanced Maillard product of lysine [41].

Tryptophan can react with its indole-NH group with active carbonyl intermediates formed during the advanced Maillard reaction. Reaction products between α-N-acetyl-tryptophan and 3-deoxyglucusone have been identified. Whether tryptophan residues in an intact food protein react in a similar way is not clear [41]. In contrast to cysteine, cystine seems not to be affected by the advanced Maillard reaction, whereas methionine is progressively destroyed by an unknown mechanism [41].

Some more reaction products have been identified, such as ε-N-carboxymethyllysine, hydroxymethyl-furfuraldehyde, and 3-deoxyglucosone, which are produced in the advanced Maillard reaction step and are considered as premelanoidins (cf. Section 2.1.3; [42]). Also, acrylamide, which gave cause for serious concern because of its carcinogenicity when discovered in fried, baked, or toasted starchy foods in 2002 [44], seems to be a by-product of the Maillard reaction. Mottram et al. [45] showed that acrylamide can be produced by reaction between asparagine and dicarbonyl compounds formed in the Maillard reaction, such as 2,3-butanedione, when heated above 120°C. Another objectionable compound is the highly toxic volatile acrolein. Acrolein can be formed by thermal decomposition of methionine, via the flavor compound of baked potatoes, methional. Acrolein can also be formed from threonine during heat treatment of food, or in vivo by threonine conversion with the enzyme myeloperoxidase under acute oxidative stress conditions, such as during myocardial infarction and stroke [46]. Part of the toxicity of acrolein has been ascribed to impairing protein function by its reaction with nucleophiles in amino acid residues, such as cysteine, histidine, arginine, and lysine. Further reactions of amino acid adducts of acrolein with nucleophilic sites in protein leads to cross-links between amino acid chains [46].

2.1.2.3 Final Maillard reaction

The final phase produces brown melanoidin pigments, which result from the polymerization of the many highly reactive compounds formed during the advanced Maillard reactions.

The course of the Maillard reaction is strongly influenced by temperature, duration, water activity, and pH. It is slowed down by very high and very low water activity in the food or feed matrix, and by lowering the pH [43].

2.2 Thermomechanical modification: irradiation

Fombang et al. [47] attributed the reduced digestibility caused by higher doses of irradiation to cross-linking and aggregation and to the formation of Maillard products, which inhibit enzymatic protein digestion.

2.3 pH Denaturation

2.3.1 Cross-linking of protein via lysinoalanine (LAL), histidinoalanine (HAL), lanthionine (LAN), or phenylethylaminoalanine

The cross-linked amino acid product LAL (Fig. 2) can be formed in protein by a reaction of the ε-amino group of lysine with dehydroalanine. Dehydroalanine, in turn, can be formed by β-elimination from, e.g., cystine, cysteine, or serine residues in the protein chain [48–50]. Alkaline processing of protein-containing foods promotes LAL production, such as a hot alkaline dipping prior to pretzel-baking, or isoelectric precipitation followed by neutralization at alkaline pH employed for casein isolation on an industrial scale, or alkaline extraction of deboning residues from poultry and red meats for the recovery of additional food-grade proteins [48].

Measurements revealed that the LAL content of raw and pasteurized milk, e.g., amounted to up to 15 mg/kg, and the one of calcium caseinate to up to 1560 mg/kg [48]. LAL formation in food proteins has been reviewed by Finot [51] and by Friedman [48].

While the formation and presence of LAL in food has been extensively studied and covered, much less is known about other, similar products such as HAL, LAN, or phenylethylamine (cf. Section 2.3.2). HAL was first detected in soybean protein treated with alkali [48], but also occurs in milk products and baby formulas, and in human tissues, such as connective tissue, bone, dentin, and eye cataracts [52, 53]. According to Friedman and Noma [54], the amine phenylethylamine, which can be formed by decarboxylation of free phenylalanine, reacts like lysine in a competitive reaction with dehydroalanine residues to form phenylethylaminoalanine in, e.g., casein and soybean.

2.3.2 Racemization

The partial conversion of an L-amino acid to a D-amino acid, resulting in a mixture of both enantiomers (Fig. 3), is called racemization. Usually D-amino acids are poorly bioavailable and utilized in vivo [53]. Most eukaryotes, except plants, possess D-amino acid oxidases, which catalyze the oxidative deamination of D-amino acids to the corresponding α-oxoacids and ammonia [55]. In these reactions, O_2 functions as electron acceptor and is reduced to H_2O_2 [56]. Moreover, humans are unable to utilize D-lysine, D-leucine, D-threonine, D-tryptophan, D-isoleucine, or D-valine [52]. In addition, diets high in D-amino acids reduce the interconvertibility of D-amino acids, because they overload the amino acid oxidase system, and might also inhibit the bioavailability of essential D-amino acids [52].

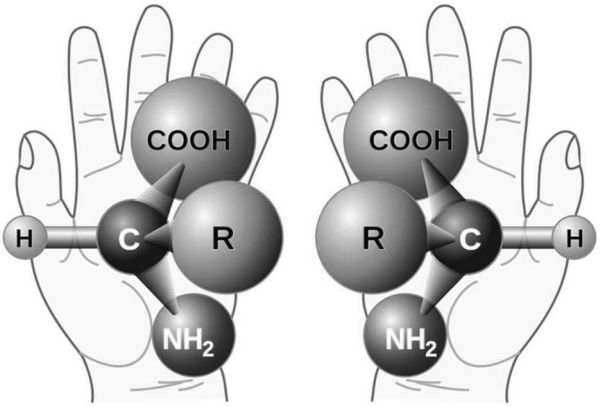

Figure 3. Two enantiomers of a generic amino acid (from http://www.newworldencyclopedia.org/entry/Chirality_(chemistry), original location: http://nai.nasa.gov/library/images/news_articles 159_1.jpg).

Possibly due to their negligible role in protein anabolism and catabolism, D-amino acids act as messenger molecules in the brain, such as the neuromodulator D-serine [57], or the hormone secretion regulator D-aspartate [58], with their concentrations being controlled by D-amino acid oxidase. In long-living tissues, levels of some D-amino acids appear to be correlated with human age or disorders (schizophrenia, epilepsy, Alzheimer [58]).

Racemization of L-amino acids occurs during alkaline treatments (pH 8–12) concurrently with LAL formation [48]. Severe heating alone can also result in racemization, but only of aspartate among the biogenic amino acids. Alkaline treatment (pH 12, 90°C, 2 h) of a *Vicia faba* protein isolate led to a significant racemization of phenylalanine (45%) and threonine/serine (79%) whereas leucine, isoleucine, and methionine were stable under these conditions [43].

Jenkins et al. [59] treated commercial zein, a hydrophobic protein with a very low lysine content, with either 0.1 N NaOH or $Ca(OH)_2$ at 85°C for 4 h, and then neutralized, washed, and freeze-dried the respective products. The alkaline treatments led to an increase of D-amino acids; the NaOH- and $Ca(OH)_2$-treated samples were shown to contain 20.3% and 15.3% D-amino acids versus 1.5% in the untreated zein. Racemization affected aspartate and serine > phenylalanine, glutamate, tyrosine, and threonine > methionine > alanine > valine, leucine, isoleucine, and proline. The last four amino acids are nonpolar ones. In addition, proline is a secondary amine, with its α-amine nitrogen being incorporated in a, probably stabilizing, ring structure. Young rats fed NaOH-treated zein as only protein source failed to grow and showed extreme diarrhea. The effects were less severe with $Ca(OH)_2$-treated zein.

Under careful processing conditions, racemization is considered negligible and to involve aspartate only. However, to be on the safe side, new alkaline treatments should always be checked for their possible effects in terms of racemization [43].

3 Antinutritional factors as protein digestibility-impeding factors

3.1 Tannins

In livestock diets, tannins can diminish weight gains, apparent digestibility, and feed utilization efficiency [60, 61]. Tannins are usually hold responsible for these antinutritional effects due to their inhibition of digestion of dietary proteins [62]. However, multifactorial effects of tannins can contribute to the observation of a reduced protein digestion. Tannins were, e.g., reported to have a negative impact on feed intake [63], reduce feed digestibility by formation of tannin-nutrient complexes [64–68], inhibit rumen enzymes [63], and inhibit the growth of rumen bacteria [61,69,70]. In addition, the quick absorption of low molecular weight polyphenols associated with tannins might inhibit postdigestive metabolism [62].

3.2 Protease inhibitors

Protease inhibitors inhibit the activity of the enzymes trypsin and chymotrypsin in the gut, thus preventing protein digestion. Trypsin inhibitors are found in many plant species. These species include a range of grain legumes, such as common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), Lima bean (*Phaseolus lunatus*), peanut (*Arachis hypogaea*), field or garden pea (*Pisum sativum*), soybean (*Glycine max*), and winged bean (*Psophocarpus tetragonolobus*). However, they are also found in cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), potatoes (*Solanum tuberosum*), and a number of species in the genus *Curcurbita*. Because of the extensive use of soybean meal in monogastric feeding, the trypsin inhibitors associated with this plant species have been studied most extensively [71].

In legume seeds, the two most important inhibitor families are the Kunitz trypsin inhibitor family and the Bowman–Birk inhibitor family [72].

The Kunitz inhibitor family was the first family to be isolated. The Kunitz-type trypsin inhibitor from soybean is a peptide comprising 181 amino acids with two disulfide bridges and a molecular weight of about 21 000 Da. As this inhibitor reacts stoichiometrically with trypsin to form a stable complex, it is known as a single-headed inhibitor. It primarily inhibits trypsin, but it can also weakly inhibit chymotrypsin. This inhibitor is highly stable to thermal and acid denaturation [30, 71].

The Bowman–Birk inhibitor family is widely distributed in legume seed. Bowman–Birk inhibitor from soybean is a smaller peptide molecule and consists of 71 amino acids. It contains a high level of cystine and has seven disulfide bridges. The molecular weight is about 8000 Da. It is a double-headed molecule and inhibits both trypsin and chymotrypsin at two different binding sites. Bowman–Birk inhibitors are resistant to digestion in gastric juices and to proteolytic

enzymes. There is also a suggestion that they may be resistant to breakdown by heat [71, 73].

3.3 Antigenic proteins

Many allergenic food proteins resist digestion, and are absorbed by the intestinal mucosa, where they act as antigens. The storage proteins glycinin and β-conglycinin in soybean and peanuts are glycoproteins that are associated with allergic reactions. The human body responds to them by producing antibodies in the Peyer's patches of the gut and their associated lymphoid tissue. These antibodies are then released into the gut and react with the antigens to prevent their absorption. Antigens that escape absorption cause inflammation of the intestinal mucosa [71]. In pecans, stable, digestion-resistant antigenic polypeptides from 2S albumin protein (Car i 1) may contribute to allergic sensitization in susceptible individuals [31].

The remarkable stability of the soybean Kunitz trypsin inhibitor to both thermal and acid denaturation may also be important for its role as a food allergen [30].

3.4 Phytate

The status of phytates as antinutritional factors is somewhat unclear. They may decrease protein solubility [74] and digestibility [75], e.g., of casein and BSA [12, 76].

4 Chemical structures as protein digestibility-impeding factor

4.1 Disulfide bonds

The presence of cross-linkers, such as disulfide bonds (Fig. 4), tends to stabilize the protein structure [77]. For the creation of a ruminal escape protein, Morrison et al. [78] designed a protein with a high content of selected essential amino acids, MB-1, which was stabilized by an intermolecular disulfide bridge, resulting in an MB-1-Cys dimer. This dimer was exposed to a Pronase E protease preparation as well as to proteases extracted from ruminal microbes. It was found that in both cases, the MB-1-Cys dimer had a better resistance to proteolytic degradation than MB-1 [78].

4.2 Cyclic peptides and cystine-knot structures

Naturally occurring small circular peptides have long been known to exist in the bacterial world. These cyclic bacterial peptides often contain uncommon amino acids, and they are biosynthetically produced by large peptide synthetases, rather than the standard ribosomal protein synthesis machinery. Cyclic peptides, and by extension circular proteins, have therefore remained a bit of an anomaly until the middle of the 1990s, when the discovery of several ribosomally synthesized macrocyclic proteins was reported. These proteins were all extracted from plant material, and in addition to having a cyclic backbone, they were also found to contain a cystine-knot structure. The first cyclic cystine-knot protein to be fully structurally characterized was the protein kalata B1 from the African plant *Oldenlandia affinis*. Its cystine-knot structure consists of three disulfide bonds, two of which form a ring structure in the peptide backbone, the third one passing in between these. This cross-linking pattern gives the protein backbone a high stability and forces it to adopt a β-sheet structure. Such protein cystine-knot structures were later reported to be rather common; e.g., they are also found in certain human growth hormones [79].

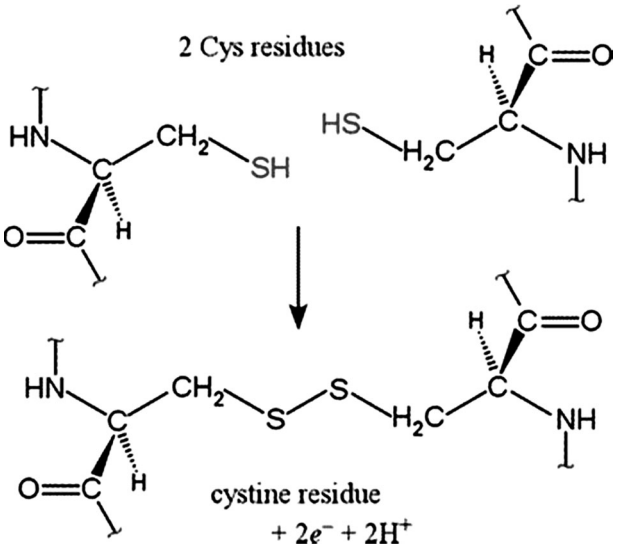

Figure 4. Disulfide bond: two cysteine residues react to a cystine dimer (source from Cronk [111]).

4.3 Blocking of the ε-amino group of lysine

Lysine with a blocked ε-amino group as such is not bioavailable for animals [5]. Lysine residues in protein can undergo a Maillard reaction with reducing sugars, react with alanine derivatives, with carboxyl groups of aspartic and glutamic acid residues, or with nitrosating agents.

4.3.1 Formation of isopeptide bonds between the lysine ε-NH_2 and β- and γ-carboxyl groups

According to Sikorski [5], low water activity in combination with high temperature can lead to the formation of isopeptide bonds between the ε-NH2 of lysine and the β- and γ-carboxyl groups of aspartic and glutamic acid residues. γ-Glutamyl-ε-lysine (GGL), and β-aspartyl-ε-lysine are also produced by catalytic action of enzymes of the transglutaminase family.

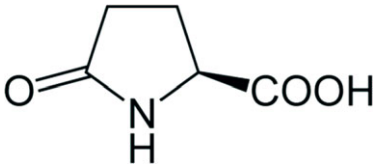

Figure 5. Pyroglutamic acid.

Transglutaminase, nicknamed "meat glue," is used in food processing to create imitation meat or fish products by enzymatically cross-linking smaller protein pieces [42]. In contrast to free β-aspartyl-ε-lysine, the lysine of which proved unavailable for rats, free GGL was 100% bioavailable to the rodents. Protein-bound GGL is expected to amount to maximally 3.5% of the initial lysine value in heat-treated proteins, and therefore nutritional consequences are expected to be low [42].

4.3.2 β-Elimination of nucleophiles leading to cross-links with free amino or thiol groups

At alkaline pH, a hydroxide-ion catalyzed β-elimination of H_2S, H_2O, phosphate, or sugar from, e.g., cystine, cysteine, or serine residues can occur in a protein chain, resulting in the formation of dehydroalanine [48–50]. Dehydroalanine in turn, can undergo a Michael addition with a nucleophilic group in a suitable position of the protein chain, such as the ε-amino group of lysine, leading to the formation of LAL (Nε-(2-amino-2-carboxyethyl)-lysine) [80]. Dehydroalanine can also react with other residues with nucleophiles, such as free amino or thiol groups, e.g., with ornithine to ornithinoalanine (OAL, Nδ-[2-amino-2-carboxyethyl]-ornithine), with cysteine to LAN, and with histidine to HAL [48]. In fact, both nitrogen atoms of histidine may react, giving rise to the region-isomers Nπ-HAL (α,α'-diamino-1H-imidazole-1,5-dipropanoic acid) and Nτ-HAL (α,α'-diamino-1H-imidazole-1,4-dipropanoic acid) [80].

An analog mechanism to the formation of dehydroalanine is the formation of dehydroaminobutyric acid (i.e., methyl-dehydroalanine) from threonine residues [48, 50]. Methyl-dehydroalanine can react with the same amino acids as dehydroalanine to give methyl-LAL (Me-LAL, Nε-[2-amino-2-carboxy-1-methylethy])-lysine), methyl-ornithinoalanine (Me-OAL, Nδ-[2-amino-2-carboxy-1-methylethyl]-ornithine), methyl-LAN (Me-LAN), and methyl-HAL (Me-HAL), respectively [80].

Under highly alkaline conditions, mainly LAL is being formed. At lower pH, lysine residues are protonated, and dehydroalanine preferably reacts with histidine to HAL, or with cysteine to LAN, which then dominate among the cross-links [53]. Neither Me-LAL nor Me-HAL was detected in milk products, probably because the reactivity of methyl-dehydroalanine toward nucleophiles is more than tenfold lower than the one of dehydroalanine. Hence, the formation of Me-LAL as well as of Me-HAL during food processing seems negligible [81].

4.4 Pyroglutamic acid

Pyroglutamic acid (also known as pyrrolidone carboxylic acid, and pyroglutamate or pyrrolidone carboxylate in its basic form) is an uncommon amino acid derivative, in which the free amino group of glutamic acid cyclizes to form a lactam (Fig. 5). "Pyroglutamate is formed by cyclization of N-terminal glutamine or glutamate residues" [82]. When containing an N-terminal pyroglutamate, the peptide chain cannot be prolonged because pyroglutamate does not have a free primary amino group for reaction with the carboxyl group of another amino acid. Upon hydrolyzation of protein, pyroglutamyl peptides can be formed by cyclization of N-terminal glutamine residues, which is stimulated by mild alkaline conditions [83,84]. The N-terminal pyroglutamate bond is not hydrolyzed by pepsin, trypsin, or pancreatin, but can be cleaved from peptides and proteins by the enzyme pyrrolidone carboxylate peptidase (PCP). PCP activity has been detected in tissues of man, pig, cow, rabbit, guinea pig, rat, mouse, pigeon, hen, and carp, plants, and bacteria. By contrast, no activity was found in human urine and serum [85]. Different synthetic L-pyroglutamyl compounds (basic form and amidified with alanine or proline), were shown to rupture lysosomal membranes in vitro, which suggests that these compounds can contribute to tissue damage in vivo [83]. However, Monsuur et al. [87] showed that the enzyme PCP is not involved in the etiology and pathology of celiac disease.

Indigestible pyroglutamyl peptides were, e.g., detected in an enzymatic hydrolyzate of wheat gluten [87,88], and found to convey a specific glutamate-like ("umami") taste [84].

4.5 Oxidative damage to proteins

Proteins are a major target for oxidation, because of their abundance in biological material and their reactivity. Oxidation can occur at the protein backbone and the amino acid side chains [89]. When proteins unfold due to oxidation, protein function can be impaired, leading to an increase of protein hydrophobicity, which often results in the formation of toxic aggregates [89,90]. In addition, oxidative changes result in the loss of sensory and nutritional quality.

Reactions with reactive oxygen species (ROS) play a role during processing and prolonged storage of food [5], and within living cells under oxidative stress conditions, which play a critical role in cell ageing and disorders such as Alzheimer's and Parkinson's [91–93]. The rate of oxidative changes in proteins is controlled by the activity of the oxidizing agents and inhibitors, the presence of sensitizers, such as chlorophyll, methylene blue, erythrosine, and riboflavin; different peroxidants and antioxidants, temperature, and the sensitivity of various amino acid residues [5]. In general, the most reactive radicals are the least selective and lead to both backbone and side-chain damage, and thus protein fragmentation.

As lysine and sulfur amino acids are often first limiting amino acids in food and feed protein, any loss of these amino acids, which are prone to oxidation, is critical for the nutritional quality of the protein [94]. Methionine, e.g., can be oxidized to methionine sulfoxide (Met-SO) and to methionine–sulfone (Met-SO_2). However, not only free, but also casein-bound Met-SO proved as utilizable by rats as free methionine in their diet [94]. While many studies indicate that Met-SO is bioavailable, free Met-SO_2 is not [42]. Rexroth et al. [93] recently showed that ROS as side products of aerobic metabolism in mitochondria selectively target only one specific tryptophan of ATP synthase, which stands out due to a metal binding site in its proximity, leading to irreversible oxidation of this tryptophan.

Polyunsaturated fatty acids readily react with oxygen to their peroxides, and then form secondary autoxidation products, e.g., 9-oxononanoic acid or 4-hydroxynonenal from linoleate. Incubation of casein with autoxidation products resulted in insolubility, and depending on incubation time and reactivity of secondary products, indigestibility, loss of amino acid residues, and polymerization of casein [95]. Reactions between casein and oxidizing lipids during incubations with (i) methyl linoleate (50°C, 80% relative humidity, 0–10 days) [96] and (ii) technical grade methyl linolenate (37–55°C, 33–84% relative humidity, 1–4 mol O_2/mol lipid, 4 weeks) [94] were shown to result in (i) losses of methionine, tryptophan, histidine, and lysine [96], and (ii) reduced bioavailabilities for rats of e.g., cyst(e)ine, lysine, tryptophan, and methionine [94].

4.6 Reaction with nitrite

Nitrous acid generated in food at low pH from endogenous or added nitrite decomposes readily to very reactive nitrosating agents. These nitrosating agents may react with amines (lysine) to form N-nitroso compounds or nitrosamines [5], which have been associated with causing cancer [97].

5 Overview of available detection methods for protein in complex products

5.1 Unique infrared band of protein structure

Protein secondary structure usually consists of large amounts of alpha-helix and beta sheet, and small amounts of beta-turn and random coil. Each fine structure has relatively unique infrared absorption bands. Under infrared radiation, a protein will produce amide I and II bands in the region of ca. 1710–1475 cm^{-1} by infrared spectroscopy (Fig. 6, adapted from Yu et al. [98] with the Journal's permission). Amide I bands depend very much on the protein secondary structure, and hence are usually used to quantify model-fitting protein alpha-helixes and beta sheets. Amide II bands depend on other chemical functional groups, and are less used for protein secondary structure prediction due to overlapping with other bands (e.g., lignin band 1510 cm^{-1}) [99, 100]. The detailed band assignments in spectroscopy, such as for antiparallel β-sheets (ca. 1685–1680 cm^{-1}), turns and coils (ca. 1680–1660 cm^{-1}), α-helices (ca. 1660–1648 cm^{-1}), unordered structures (ca. 1660–1648 cm^{-1}), β-sheets (ca. 1640–1625 cm^{-1}), 3_{10} helixes (ca. 1637 cm^{-1}), and aggregated strands (ca. 1625–1610 cm^{-1}), were summarized before by Marinkovic and Chance [101]. The functional group band assignments or band shifting may be affected by the type of samples and various treatments.

5.2 Univariate molecular spectral analysis of protein structure

For studying protein inherent structures or processing-induced structural changes of protein, two kinds of molecular spectral analyses can be applied. The first one is univariate molecular spectral analysis, which can be used for protein fine structure-associated band as well as integrated band analyses in terms of intensity, frequency, and ratio [100, 102–104]. For example, Yu et al. [98] used univariate molecular spectral analysis to study the gene-transformation-induced changes on protein in transgenic plants. Liu et al. [105] used univariate molecular spectral analysis to study bioethanol processing effect on the metabolic characteristics of protein in dairy cattle, in which truly absorbed protein supply or indigestible protein could be predicted. Zhang and Yu [106] used this method to indicate protein structure characteristics of bioethanol products after dry fractionation process.

5.3 Multivariate molecular spectral analysis of protein structure

The secondary method for protein spectral analysis is multivariate molecular spectral analyses (e.g., CLA, PCA, LDA analysis), in which the whole spectral region is taken into account, i.e., not only one band, but a multiband region [107]. The big advantage of these analyses, as reported by Yu [100], is that spectral assignments do not have to be assigned to specific structures, but that samples can be compared with each other according to their similarity. These methods could be used to detect protein structural make-up or conformation between feed varieties, feed processing, feed treatments, and gene transformation.

5.4 Multicomponent peak modeling for protein structure analysis

To analyze protein fine structures by means of infrared spectra, multipeaking modeling methods can be used. There are several different methods for peak fitting, such as Gaussian function, Lorentzian function, or combined Gaussian–Lorentzian function. Using these methods, protein amide I component peak shape, center, offset, wide, and areas can

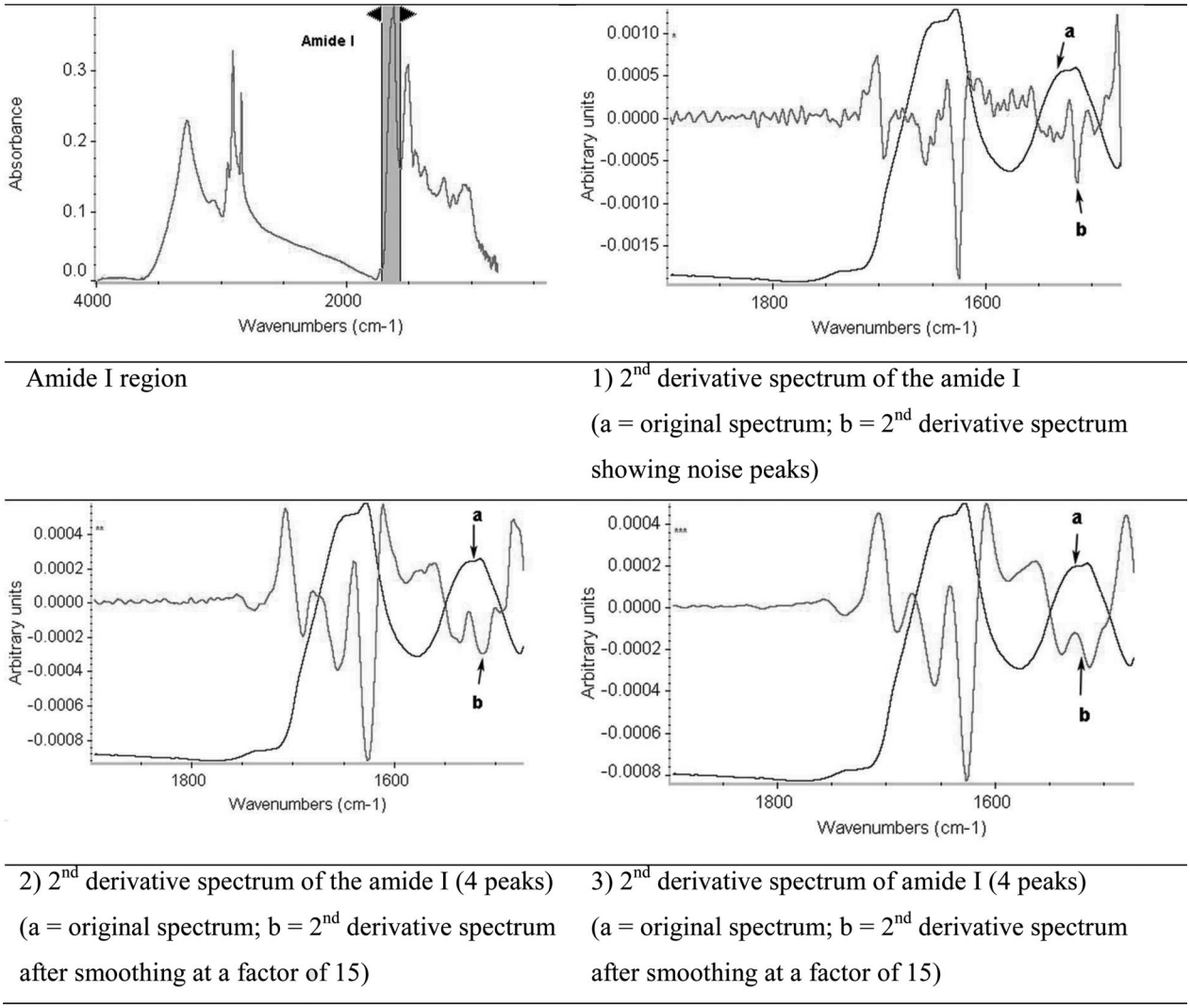

Figure 6. A spectra from Synchrotron-based infrared microspectroscopy (SR-IMS), and its second derivative and Fourier Self-Deconvolution (FSD) spectrum for plant alfalfa tissues in the amide I and II regions (ca. 1710–1475 cm^{-1}) (adapted from Yu et al. [98] with permission from Elsevier).

be determined using various program (e.g., Origin, OMINIC) [107]. After obtaining model-fitted protein fine structures, the protein availability and utilization could be linked or associated to the protein fine structures.

6 Induced changes of protein molecular structure affecting degradation and digestibility detected with synchrotron analytical techniques

6.1 Adverse effect of biological component matrix on protein digestion

In a study by Yu et al. [108], it was reported that matrix effects in cereal grains affected protein degradation and digestion. In comparison with Harrington barley, Valier barley had a lower ratio of starch to protein IR absorbance intensity. The starch granules in Valier are more closely associated with the protein matrix. This closer association prevented the starch granules from being rapidly degraded in the rumen.

6.2 Effect of protein inherent structure on protein digestion

Liu and Yu [103, 104] studied various varieties of barley structural make-up and found that different protein molecular structure conformations among different varieties. These differences were highly associated with protein degradation. The protein conformation analysis was done based on both uni- and multivariate molecular analysis results [103, 104].

6.3 Genetic modification of protein source affecting protein degradability

The protein inherent structure in plants can be changed by foreign gene transformation, as was shown by Yu et al. [98]. Also, gene-induced changes of protein structure affect protein degradation and digestion. Yu et al. [98] used synchrotron-based infrared microspectroscopy to detect the relationship between fine structure of protein in *Lc*-transgenic alfalfa (in which anthocyanidin was enhanced) and nutritive value. Yu et al. [98] found that the transgenic alfalfa contained a lower percentage of the model-fitted α-helices and β-sheets and a higher percentage of other model-fitted protein fine structures. These results indicated that transgenic *Lc* alfalfa contained similar proteins to nontransgenic alfalfa, but protein molecular structures were different. These structures in combination with the anthocyanidin enhancement significantly affected protein degradation and utilization of alfalfa.

6.4 Biofuel or bio-oil processing affecting protein availability of coproduct

Yu and Nuez-Ortin [109] found that the protein-inherent structure in coproduct was different from parent feedstock gain and indicated that the structure was altered by biofuel processing (e.g., commonly available bioethanol processing). Such changes also altered the biological properties of protein sources for animals, such as change in solubility of protein, change in protein subfractions, change in digestibility of protein, change in rumen undegraded protein and degraded balance of protein in ruminants [109]. The alteration of protein structure is mainly associated with biofuel and bio-oil processing procedures such as fermentation.

7 Conclusions

It is concluded that some proteins in raw products are already poorly digestible by nature, due to structural peculiarities such as modifications to amino acids or cross-linking of amino acid chains. In the biological matrix the proteins are embedded in such as membranes or storage bodies in cells or tissues, which can also contribute to protein indigestibility. Other proteins only become difficult to digest due to changes, which occur during biotechnological processes to protein-containing by-products, or the processing of proteinaceous products for food or feed production. Last but not least, protein digestion can be impeded in the intestinal tract by antinutritional components. All of these factors can impair protein digestion by common proteolytic enzymes in humans and animals.

P. M. B. gratefully acknowledges financial support for this study from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR, and the Dutch Ministry of Economic Affairs, Agriculture & Innovation. P. Y. gratefully acknowledges financial support for his feeds research programs from Ministry of Agriculture Strategic Research Chair research programs, Natural Sciences and Engineering Research Council of Canada (NSERC), Saskatchewan Agricultural Development Fund (ADF), Beef Cattle Research Council (BCRC), and SaskCanola Development Commission as well as synchrotron beamtime support from the National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, New York, USA) and Canadian Light Source Inc. (Saskatoon, Canada). Figure 1 has been reproduced with permission of the Journal of Agricultural and Food Chemistry, Fig. 4 with permission of Dr. Jeff D. Cronk (Gonzaga University, Spokane, WA, USA), and Fig. 6 has been adapted with permission of the Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. All the other figures shown are not protected by copyright. The sources of the respective figures are given in their legends.

The authors have declared no conflict of interest.

8 References

[1] Andersen, S. O., Chlorinated tyrosine derivatives in insect cuticle. *Insect. Biochem. Mol. Biol.* 2004, *34*, 1079–1087.

[2] Ehrlich, H., *Biological Materials of Marine Origin: Invertebrates*, Springer, Heidelberg 2010.

[3] Graham, C. E., Waitkoff, H. K., Hier, S. W., The amino acid content of some scleroproteins. *J. Biol. Chem.* 1949, *177*, 529–532.

[4] Szpak, P., Fish bone chemistry and ultrastructure: implications for taphonomy and stable isotope analysis. *J. Archaeol. Sci.* 2011, *38*, 3358–3372.

[5] Sikorski, Z. E., in: Sikorski, Z. E. (Ed.), *Chemical and Functional Properties of Food Components*, Third Edition, CRC Press, Boca Raton, Florida, USA 2006, pp. 129–176.

[6] Straumfjord, J. V., Hummel, J. P., Collagen digestion by dog pancreatic juice. *Proc. Soc. Exp. Biol. Med.* 1957, *95*, 141–144.

[7] Houck, J. C., Patel, Y. M., Pancreatic collagenase, a new enzyme. *Proc. Soc. Exp. Biol. Med.* 1959, *102*, 421–423.

[8] Loewit, K., Über die ernährungsphysiologische Bedeutung des Bindegewebes. *Zeitschr. Lebens. Untersuch. Forsch. A* 1970, *143*, 5–10.

[9] Schrieber, R., Gareis, H. (Eds.), *Gelatine Handbook: Theory and Industrial Practice*, Wiley VCH Publishers, Weinheim 2007.

[10] Anwar, R. A., Oda, G., The biosynthesis of desmosine and isodesmosine. *J. Biol. Chem.* 1966, *241*, 4638–4641.

[11] Block, R. J., Bolling, D., The amino acid composition of keratins. *J. Biol. Chem.* 1939, *127*, 685–693.

[12] Lentle, R. G., Janssen, P. W. M., in: Lentle, R. G., Janssen, P. W. M. (Eds.), *The Physical Processes of Digestion*, Springer, New York 2011, pp. 47–61.

[13] Weiss, I. M., Kirchner, H. O. K., Plasticity of two structural proteins: alpha-collagen and beta-keratin. *J. Mech. Behav. Biomed. Mater.* 2011, *4*, 733–743.

[14] Clark, J. H., Murphy, M. R., Crooker, B. A., Supplying the protein needs of dairy cattle from by-product feeds. *J. Dairy Sci.* 1987, *70*, 1092–1109.

[15] Blasi, D. A., Klopfenstein, T. J., Drouillard, J. S., Sindt, M. H., Hydrolysis time as a factor affecting the nutritive value of feather meal and feather meal-blood meal combinations for growing calves. *J. Anim. Sci.* 1991, *69*, 1272–1278.

[16] Church, D. C., Daugherty, D. A., Kennick, W. H., Nutritional evaluation of feather and hair meals as protein sources for ruminants. *J. Anim. Sci.* 1982, *54*, 337–344.

[17] Encinias, A. M., Lardy, G. P., Leupp, J. L., Encinias, H. B. et al., Efficacy of using a combination of rendered protein products as an undegradable intake protein supplement for lactating, winter-calving, beef cows fed bromegrass hay. *J. Anim. Sci.* 2005, *83*, 187–195.

[18] Bureau, D. P., Harris, A. M., Bevan, D. J., Simmons, L. A. et al., Feather meals and meat and bone meals from different origins as protein sources in rainbow trout (*Oncorhynchus mykiss*) diets. *Aquaculture* 2000, *181*, 281–291.

[19] Mondal, M., Trivedy, K., Nirmal Kumar, S., The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* Linn.: a review. *Caspian J. Env. Sci.* 2007, *5*, 63–76.

[20] Lotz, B., Colonna Cesari, F., The chemical structure and the crystalline structures of *Bombyx mori* silk fibroin. *Biochimie* 1979, *61*, 205–214.

[21] Padamwar, M. N., Pawar, A. P., Silk sericin and its applications: a review. *J. Sci. Ind. Res.* 2004, *63*, 323–329.

[22] Minagawa, H., Maekawa, A., Yamamoto, Y., Tadakoro, T., Digestibility of water-soluble fibroin prepared from silk yarn. *J. Jpn. Soc. Nutr. Food Sci.* 2004, *57*, 215–220.

[23] DeFoliart, G. R., Insects as a source of protein. *Bull. ESA* 1975, *21*, 161–164.

[24] Hooper, S. L., Thuma, J. B., Invertebrate muscles: Muscle specific genes and proteins. *Phys. Rev.* 2005, *85*, 1001–1060.

[25] Andersen, S. O., Insect cuticular sclerotization: a review. *Insect Biochem. Mol.* 2010, *40*, 166–178.

[26] St. Leger, R. J., Charnley, A. K., Cooper, R. M., Cuticle-degrading enzymes of entomopathogenic fungi: mechanisms of interaction between pathogen enzymes and insect cuticle. *J. Invertebr. Pathol.* 1986, *47*, 295–302.

[27] Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N. et al., Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: a tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohyd. Polym.* 2012, *87*, 995–1012.

[28] Herman, E. M., Larkins, B. A., Protein storage bodies and vacuoles. *Plant Cell* 1999, *11*, 601–613.

[29] Khattab, R. Y., Arntfield, S. D., Nyachoti, C. M., Nutritional quality of legume seeds as affected by some physical treatments, part 1: protein quality evaluation. *LWT-Food Sci. Technol.* 2009, *42*, 1107–1112.

[30] Roychaudhuri, R., Sarath, G., Zeece, M., Markwell, J., Stability of the allergenic soybean Kunitz trypsin inhibitor. *Biochim. Biophys. Acta: Proteins Proteom.* 2004, *1699*, 207–212.

[31] Spiric, J., *Identification and Characterization of Putative Allergens in Pecan Species*. MSc thesis. University of Nebraska-Lincoln, USA 2011. (http://digitalcommons.unl.edu/foodscidiss/20/; accessed in Feb. 2013).

[32] Emmambux, M. N., Taylor, J. R. N., Properties of heat-treated sorghum and maize meal and their prolamin proteins. *J. Agric. Food Chem.* 2009, *57*, 1045–1050.

[33] Hamaker, B. R., Kirleis, A. W., Butler, L. G., Axtell, J. D. et al., Improving the in vitro protein digestibility of sorghum with reducing agents. *Proc. Natl. Acad. Sci. USA* 1987, *84*, 626–628.

[34] Hamaker, B. R., Kirleis, A. W., Mertz, E. T., Axtell, J. D., Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. *J. Agric. Food Chem.* 1986, *34*, 647–649.

[35] Oria, M. P., Hamaker, B. R., Shull, J. M., Resistance of sorghum alpha-, beta-, and gamma-kafirins to pepsin digestion. *J. Agric. Food Chem.* 1995, *43*, 2148–2153.

[36] Rom, D. L., Shull, J. M., Chandrashekar, A., Kirleis, A. W., Effects of cooking and treatment with sodium bisulfite on *in vitro* protein digestibility and microstructure of sorghum flour. *Cereal Chem.* 1992, *69*, 178–181.

[37] AlKanhal, H. A., Abu-Lehia, I. H., Al-Saleh, A. A., Changes in the quality of fresh and recombined ultra-high temperature treated milk during storage. *Int. Dairy J.* 1994, *4*, 221–236.

[38] Finot, P. A., *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*, Birkhäuser Verlag, Basel, Switzerland 1990.

[39] Moughan, P. J., Gall, M. P. J., Rutherfurd, S. M., Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. *J. Agric. Food Chem.* 1996, *44*, 1520–1525.

[40] Seiquer, I., Díaz-Alguacil, J., Delgado-Andrade, C., López-Frías, M. et al., Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11–14 y. *Am. J. Clin. Nutr.* 2006, *83*, 1082–1088.

[41] Finot, P. A., Mottu, F., Bujard, E., Mauron, J., N-Substituted lysines as sources of lysine in nutrition. *Adv. Exp. Med. Biol.* 1978, *105*, 549–570.

[42] Finot, P. A., The absorption and metabolism of modified amino acids in processed foods. *J. AOAC Int.* 2005, *88*, 894–903.

[43] Mauron, J., Influence of processing on protein quality. *J. Nutr. Sci. Vitaminol.* 1990, *36*, S57–S69.

[44] Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. et al., Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* 2002, *50*, 4998–5006.

[45] Mottram, D. S., Wedzicha, B. L., Dodson, A. T., Food chemistry: acrylamide is formed in the Maillard reaction. *Nature* 2002, *419*, 448–449.

[46] Stevens, J. F., Maier, C. S., Acrolein, Sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol. Nutr. Food Res.* 2008, *52*, 7–25.

[47] Fombang, E. N., Taylor, J. R. N., Mbofung, C. M. F., Minnaar, A., Use of γ-irradiation to alleviate the poor protein digestibility of sorghum porridge. *Food Chem. Toxicol.* 2005, *91*, 695–703.

[48] Friedman, M., Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. *J. Agric. Food Chem.* 1999, *47*, 1295–1319.

[49] Friedman, M., in: Stadler, R. H., Lineback, D. R. (Eds.), *Process-Induced Food Toxicants: Occurrence, Formation, Mitigation, and Health Risks*, John Wiley & Sons, Inc., Hoboken, NJ, USA 2008, pp. 473–508.

[50] Rombouts, I., Lagrain, B., Brijs, K., Delcour, J., Cross-linking of wheat gluten proteins during production of hard pretzels. *Amino Acids* 2012, *42*, 2429–2438.

[51] Finot, P. A., Lysinoalanine in food proteins. *Nutr. Abstr. Rev. Clin. Nutr.* 1983, *A53*, 67–80.

[52] Meade, S. J., Reid, E. A., Gerrard, J. A., The impact of processing on the nutritional quality of food proteins. *J. AOAC Int.* 2005, *88*, 904–922.

[53] Taylor, C. M., Wang, W., Histidinoalanine: a crosslinking amino acid. *Tetrahedron* 2007, *63*, 9033–9047.

[54] Friedman, M., Noma, A. T., Formation and analysis of [(phenylethyl)amino]alanine in food proteins. *J. Agric. Food Chem.* 1986, *34*, 497–502.

[55] Pollegioni, L., Piubelli, L., Sacchi, S., Pilone, M. S. et al., Physiological functions of D-amino acid oxidases: from yeast to humans. A review. *Cell. Mol. Life Sci.* 2007, *64*, 1373–1394.

[56] Tedeschi, G., Pollegioni, L., Negri, A., in: Pollegioni, L.; Servi, S. (Eds.), *Unnatural Amino Acids - Methods and Protocols*, Humana Press, New York 2012, pp. 381–395.

[57] Pollegioni, L., Sacchi, S., Metabolism of the neuromodulator D-serine. A review. *Cell. Mol. Life Sci.* 2010, *67*, 2387–2404.

[58] Khoronenkova, S., Tishkov, V., D-Amino acid oxidase: physiological role and applications. A review. *Biochemistry* 2008, *73*, 1511–1518.

[59] Jenkins, W. L., Tovar, L. R., Schwass, D. E., Liardon, R., Carpenter, K. J., Nutritional characteristics of alkali-treated zein. *J. Agric. Food Chem.* 1984, *32*, 1035–1041.

[60] Jonker, A., Gruber, M. Y., Wang, Y., Coulman, B. et al., Modeling degradation ratios and nutrient availability of anthocyanidin-accumulating Lc-alfalfa populations in dairy cows. *J. Dairy Sci.* 2011, *94*, 1430–1444.

[61] Jonker, A., Gruber, M. Y., Wang, Y., Narvaez, N. et al., Fermentation, degradation and microbial nitrogen partitioning for three forage colour phenotypes within anthocyanidin-accumulating Lc-alfalfa progeny. *J. Sci. Food Agric.* 2012, *92*, 2265–2273.

[62] Butler, L. G., Rogler, J. C., in: Ho, C.-T., Lee, C. Y., Huang, M.-T. (Eds.), *Phenolic Compounds in Food and Their Effects on Health I*, ACS Symposium Series 506, American Chemical Society, Washington D.C. 1992, pp. 298–304.

[63] Kumar, R., Singh, M., Tannins: their adverse role in ruminant nutrition. *J. Agr. Food Chem.* 1984, *32*, 447–453.

[64] Beauchemin, K. A., McGinn, S. M., Martinez, T. F., McAllister, T. A., Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *J. Anim Sci.* 2007, *85*, 1990–1996.

[65] de Oliveira, S. G., Berchielli, T. T., Pedreira, M. d. S., Primavesi, O. et al., Effect of tannin levels in sorghum silage and concentrate supplementation on apparent digestibility and methane emission in beef cattle. *Anim. Feed Sci. Technol.* 2007, *135*, 236–248.

[66] Hagerman, A. E., Robbins, C. T., Weerasuriya, Y., Wilson, T. C. et al., Tannin chemistry in relation to digestion. *J. Range Manage.* 1992, *45*, 57–62.

[67] Smith, A., Zoetendal, E., Mackie, R., Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microb. Ecol.* 2005, *50*, 197–205.

[68] Wang, Y., Waghorn, G. C., McNabb, W. C., Barry, T. N. et al., Effect of condensed tannins in *Lotus corniculatus* upon the digestion of methionine and cysteine in the small intestine of sheep. *J. Agr. Sci.* 1996, *127*, 413–421.

[69] Min, B. R., Attwood, G. T., McNabb, W. C., Molan, A. L. et al., The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen bacteria. *Anim. Feed Sci. Technol.* 2005, *121*, 45–58.

[70] Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N. et al., Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol.* 2005, *123–124*, 403–419.

[71] Hill, G. D., in: Caballero, B. (Ed.), *Encyclopedia of Food Sciences and Nutrition* (2nd Edn.), Academic Press, Oxford 2003, pp. 4578–4587.

[72] Tan-Wilson, A. L., Hartl, P. M., Delfel, N. E., Wilson, K. A., Differential expression of Kunitz and Bowman-Birk soybean proteinase inhibitors in plant and callus tissue. *Plant Physiol.* 1985, *78*, 310–314.

[73] Wu, Y. V., Sessa, D. J., Conformation of Bowman-Birk inhibitor. *J. Agr. Food Chem.* 1994, *42*, 2136–2138.

[74] Bourdillon, J., A crystalline bean seed protein in combination with phytic acid. *J. Biol. Chem.* 1951, *189*, 65–72.

[75] Kratzer, F. H., Soybean protein-mineral interrelationships. *Fed. Proc.* 1965, *24*, 1498–1500.

[76] Knuckles, B. E., Kuzmicky, D. D., Betschart, A. A., Effect of phytate and partially hydrolyzed phytate on *in vitro* protein digestibility. *J. Food Sci.* 1985, *50*, 1080–1082.

[77] Mangino, M. *Protein Denaturation. Food science lecture*. The Ohio State University, Columbus, Ohio, USA 2007. (http://class.fst.ohio-state.edu/FST822/ lectures/Denat.htm, accessed in Feb. 2013).

[78] Morrison, J., McNally, J., Navidzadeh, A., Beauregard, M., Development of an optimized feeding technology for dairy cows: improvement in resistance to ruminal proteases in the de novo-designed protein MB-1. *Appl. Biochem. Biotechnol.* 2000, *87*, 247–264.

[79] Vogel, H. J., Chan, D. I., Circular proteins: ring around with NOESY. *Structure* 2005, *13*, 688–690.

[80] Boschin, G., D'Agostina, A., Arnoldi, A., A convenient synthesis of some cross-linked amino acids and their diastereoisomeric characterization by nuclear magnetic resonance. *Food Chem.* 2002, *78*, 325–331.

[81] Walter, A. W., Henle, T., Haeßner, R., Klostermeyer, H., Studies on the formation of lysinomethylalanine and histidinomethylalanine in milk products. *Z. Lebensm. Unters. Forsch. A* 1994, *199*, 243–247.

[82] Twardzik, D. R., Peterkofsky, A., Glutamic acid as a precursor to N-terminal pyroglutamic acid in mouse

plasmacytoma protein. *Proc. Nat. Acad. Sci.* 1972, *69*, 274–277.

[83] Caldwell, K. A., In vitro digestion of gliadin by gastrointestinal enzymes and by pyrrolidonecarboxylate peptidase. *Am. J. Clin. Nutr.* 1980, *33*, 293–302.

[84] Schlichtherle-Cerny, H., Amadò, R., Analysis of taste-active compounds in an enzymatic hydrolysate of deamidated wheat gluten. *J. Agric. Food Chem.* 2002, *50*, 1515–1522.

[85] Szewczuk, A., Kwiatkowska, J., Pyrrolidonyl peptidase in animal, plant and human tissues. *Eur. J. Biochem.* 1970, *15*, 92–96.

[86] Monsuur, A. J., Stepniak, D., Diosdado, B., Wapenaar, M. C. et al., Genetic and functional analysis of pyroglutamyl-peptidase I in coeliac disease. *Eur. J. Gastroenterol. Hepatol.* 2006, *18*, 637–644.

[87] Higaki-Sato, N., Sato, K., Esumi, Y., Okumura, T. et al., Isolation and identification of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. *J. Agric. Food Chem.* 2002, *51*, 8–13.

[88] Sato, K., Nisimura, R., Suzuki, Y., Motoi, H. et al., Occurrence of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. *J. Agric. Food Chem.* 1998, *46*, 3403–3405.

[89] Davies, M. J., The oxidative environment and protein damage. *Biochim. Biophys. Acta* 2005, *1703*, 93–109.

[90] Costa, V., Quintanilha, A., Moradas-Ferreira, P., Protein oxidation, repair mechanisms and proteolysis in *Saccharomyces cerevisiae*. *IUBMB Life* 2007, *59*, 293–298.

[91] Cuervo, A. M., Dice, J. F., When lysosomes get old. *Exp. Gerontol.* 2000, *35*, 119–131.

[92] Terman, A., Brunk, U. T., Oxidative stress, accumulation of biological 'garbage', and aging. *Antioxid. Redox Signal.* 2006, *8*, 197–204.

[93] Rexroth, S., Poetsch, A., Rögner, M., Hamann, A. et al., Reactive oxygen species target specific tryptophan site in the mitochondrial ATP synthase. *Biochim. Biophys. Acta: Bioenergetics* 2012, *1817*, 381–387.

[94] Nielsen, H. K., Finot, P. A., Hurrell, R. F., Reactions of proteins with oxidizing lipids. 2. Influence on protein quality and on the bioavailability of lysine, methionine, cyst(e)ine and tryptophan as measured in rat assays. *Br. J. Nutr.* 1985, *53*, 75–86.

[95] Kanazawa, K., Ashida, H., Natake, M., Autoxidizing process interaction of linoleic acid with casein. *J. Food Sci.* 1987, *52*, 475–479.

[96] Matoba, T., Yonezawa, D., Nair, B. M., Kito, M., Damage of amino acid residues of proteins after reaction with oxidizing lipids: estimation by proteolytic enzymes. *J. Food Sci.* 1984, *49*, 1082–1084.

[97] Bartsch, H., Montesano, R., Relevance of nitrosamines to human cancer. *Carcinogenesis* 1984, *5*, 1381–1393.

[98] Yu, P., Jonker, A., Gruber, M., Molecular basis of protein structure in proanthocyanidin and anthocyanin-enhanced Lc-transgenic alfalfa in relation to nutritive value using synchrotron-radiation FTIR microspectroscopy: a novel approach. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 2009, *73*, 846–853.

[99] Jackson, M., Mantsch, H. H., The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* 1995, *30*, 95–120.

[100] Yu, P., Plant-based food and feed protein structure changes induced by gene-transformation, heating and bio-ethanol processing: a synchrotron-based molecular structure and nutrition research program. *Mol. Nutr. Food Res.* 2010, *54*, 1535–1545.

[101] Marinkovic, N. S., Chance, M. R., in: Meyers, R. A. (Ed.), *Encyclopedia of Molecular Cell Biology and Molecular Medicine*, 2nd edition., Vol. *13*, Wiley-VCH, Weinheim 2006, pp. 671–708.

[102] Miller, L. M., Dumas, P., From structure to cellular mechanism with infrared microspectroscopy. *Curr. Opin. Struct. Biol.* 2010, *20*, 649–656.

[103] Liu, N., Yu, P., Using DRIFT molecular spectroscopy with uni- and multi-variate molecular spectral techniques to detect plant protein molecular structure difference among different genotypes of barley. *J. Agric. Food Chem.* 2010, *58*, 6264–6269.

[104] Liu, N., Yu, P., Characterize microchemical structure of seed endosperm within a cellular dimension among six barley varieties with distinct degradation kinetics, using ultraspatially resolved synchrotron-based infrared microspectroscopy. *J. Agric. Food Chem.* 2010b, *58*, 7801–7810.

[105] Liu, B., McKinnon, J. J., Thacker, P., Yu, P., Molecular structure and metabolic characteristics of the proteins and energy in triticale grains and dried distillers grains with solubles for dairy cattle. *J. Agric. Food Chem.* 2012, *60*, 10064–10074.

[106] Zhang, X., Beltranena, E., Christensen, C., Yu, P., Use of a dry fractionation process to manipulate the chemical profile and nutrient supply of a coproduct from bioethanol processing. *J. Agric. Food Chem.* 2012, *60*, 6846–6854.

[107] Yu, P., Multicomponent peak modeling of protein secondary structures: comparison of Gaussian with Lorentzian analytical methods for plant feed and seed molecular biology and chemistry research. *Appl. Spectrosc.* 2005, *59*, 1372–1380.

[108] Yu, P., Christensen, D. A., Christensen, C. R., Drew, M. D. et al., Use of synchrotron FTIR microspectroscopy to identify chemical differences in barley endosperm tissue in relation to rumen degradation characteristics. *Can. J. Anim. Sci.* 2004, *84*, 523–527.

[109] Yu, P., Nuez-Ortín, W. G., Relationship of protein molecular structure to metabolisable proteins in different types of dried distillers grains with solubles: a novel approach. *Br. J. Nutr.* 2010, *104*, 1429–1437.

[110] Czerwenka, C., Maier, I., Pittner, F., Lindner, W., Investigation of the lactosylation of whey proteins by liquid chromatography–mass spectrometry. *J. Agric. Food Chem.* 2006, *54*, 8874–8882.

[111] Cronk, J. D., *Biochemistry Dictionary*. Disulfide bond. 2011, http://guweb2.gonzaga.edu/faculty/cronk/biochem/D-index.cfm?definition=disulfide_bond; accessed in: April 2013.

