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TNO report

V9436

Literature study on the properties of Rubisco

Date	14 December 2011
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Number of pages	34

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1 Introduction

Replacement of animal protein by plant based proteins is seen as an important strategy to provide essential nutrients to the ever growing world population. In many cultures, animal products have been the primary source of proteins and other important nutrients. However, mass production of animals requires vast areas for the growing of feed and burdens the environment with waste streams, such as manure. The increasing demand for agricultural products and a limit availability of arable soil will render increased animal production unsustainable. Among the plant protein sources, legumes are likely the most important group. Soy beans, but also other seeds like peas, beans and lentils, have traditionally been important protein sources in China, India and other areas, and are still produced and processed into various products. However, the anti-nutritional factors in these protein sources make a harsh purification necessary. The heating steps and solvent purifications involved leave the protein highly insoluble and therefore less easily applicable in food.
A novel source, which is not being utilized commercially, is leaf protein. To the best of our knowledge, only one factory worldwide purifies proteins from plant leaves for feed applications [1]. Leaf protein consists of two major protein fractions:

- The membrane bound enzymes like the photosystem enzymes that contain the chlorophyll.
- The soluble proteins that contain enzymes like Rubisco

This literature search focuses on the properties of Rubisco, though in some cases due to lack of data on Rubisco the term leaf protein is mentioned. Leaf protein is mostly used to describe the soluble fraction of the leaf in which the membrane proteins together with the chlorophyll are largely removed. During the preparation of such leaf proteins several methods are used like pH precipitation, organic solvents etc. These procedures will strongly affect the functionality of proteins like Rubisco. It is therefore difficult to use data of leaf protein when searching for the properties of Rubisco. The data presented here are intended to give a general overview on the properties of Rubisco.

2 The enzyme Rubisco

Rubisco is the enzyme that catalyses the first step in photosynthesis, the conversion from ribulose-1,5-diphosphate to two molecules of 3-phosphoglyceric acid[2]. The enzyme is located in the soluble fraction (the stroma) of the chloroplasts [3,4]. Rubisco is activated by carbamylation, and a magnesium ion is essential for the activating CO_2 binding [2], after which a second CO_2 molecule serves as substrate. The enzyme Rubisco is present in all photosynthetic organisms, from bacteria to C3 and C4 plants, although its appearance is different between organisms (see below).
The enzyme is slow – the rate constant is about 5 s^{-1}, among the lowest for biological catalysts [5]. Furthermore, besides carbon fixation it also catalyses the reverse reaction at low vapor pressures of CO_2. The specificity of the enzyme for the carbon fixation reaction versus the oxidation varies between plant species. The sluggish performance and the schizophrenic character of the enzyme causes this first step in photosynthesis to be the rate limiting step. To compensate plants create large amounts of the enzyme in the leaves. Therefore, in C3 plants, up to 50% of the protein in the leafs is Rubisco [6,7]. In stems that percentage is reported to be lower [8] – not very surprisingly, since the stem needs to provide structure and not only catalytic activity. In algae and C4 plants the percentage of Rubisco is much lower – 16% for algae [6] , and 8-16% for C4 plants [9]. In C4 plants the Rubisco enzyme is located only in the bundle sheets, explaining the lower overall percentage[7].
The Rubisco content is highest in young leaves and decreases during the life time [3,10]. Therefore, the harvesting time is critical – a few days difference can make a large difference in yield [4].

2.1 Evolution

The evolution of Rubisco probably started in an atmosphere high in carbon dioxide and low in oxygen, and possibly in an aqueous environment. Rubisco developed as an enzyme consisting of two subunits of about 53 kDa (so-called form II). The form present in plants (form I) is thought to have developed from this dimeric form. Form I consists of 8 subunits similar to those encountered in the bacterial Rubisco, called the large subunits, coupled to 8 small subunits of 14 kDa. The specificity of form I Rubisco is higher than that of form II [5] . The large subunit structure of form I is remarkably similar to that of form II [2] , given that there is a homology of only 30% between them[2,11-13]. This homology is concentrated in two highly conserved regions[11].
The large subunit of Rubisco is responsible for the enzyme activity, and is coded in the chloroplast DNA.[14] The small subunit is encoded in the nucleus for Chlorophyll a/b plants and in the plastids (as is the large subunit) for all other organisms[15]. The small subunit affects the enzyme affinity for CO_2 by changing the large subunit conformation[13] . Although the enzyme is still active without the small subunits and its specificity is unaffected, its activity is hundred times less [2]. Another function of the small subunit may be to prevent inactivation of the enzyme [11] .
The hexadecameric form I Rubisco occurs in bacteria (including cyanobacteria) and in all green plants and algae. Its structure has been subdivided into four categories.

Form II is found in bacteria, some eubacteria and in dinoflagellates and is dimeric. It has a lower specificity for CO_2 than form I. There is no clear structural subdivision between species.
Two more forms of Rubisco have been defined – form III which is present in archae bacteria, and form IV which is present in bacteria but does not contain the catalytic site. Therefore, the latter is also called Rubisco-Like-Protein [16].
Between the Rubisco forms, there is a high variation in structure, catalytic properties and oxygen sensitivity [17] .
In a phylogenetic tree (Figure 1) the differences between species are visualized. The close relationship of the form I proteins is striking. The large variety in form IV is probably caused by the inactivity of the enzyme, which causes the structural demands to weaken. Table 1 shows the most important members of each class.

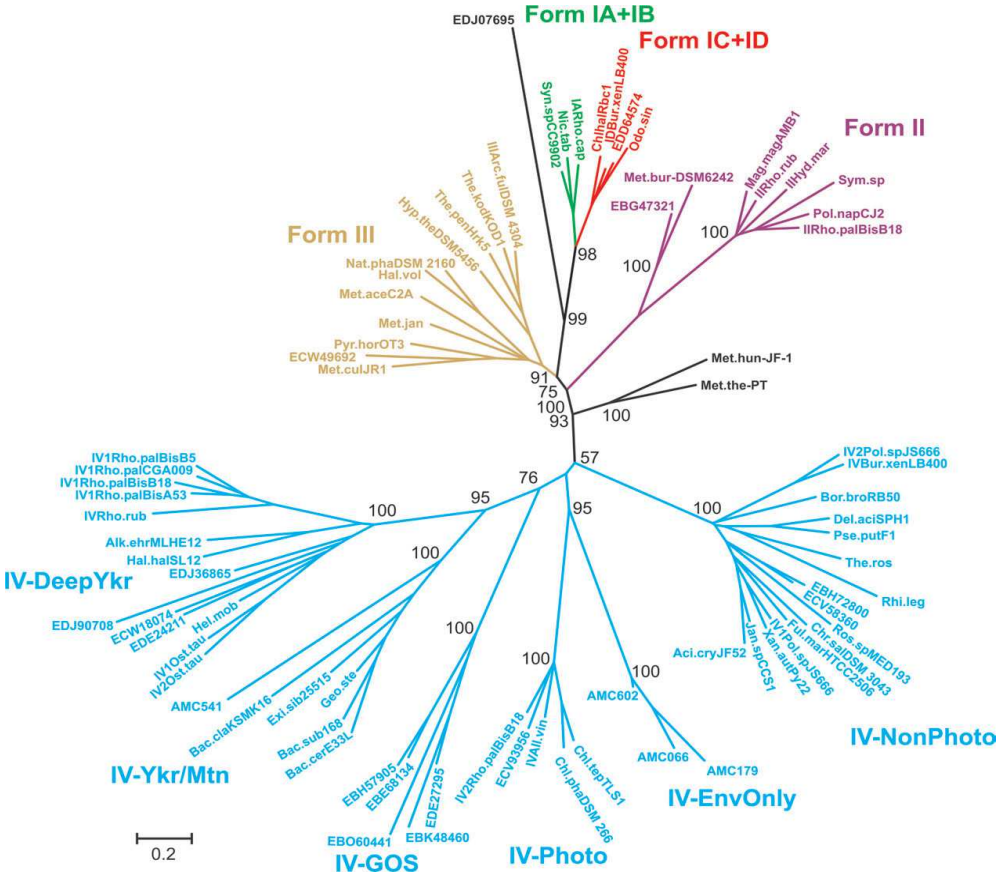


Figure 1: A phylogenetic tree which shows how far several species of Rubisco are removed genetically from each other. The similarity in the form I Rubisco is striking.[13]

Table 1: Diversity of several lineages of Rubisco.[5]

Lineage	Protein size (aa)	% identity		Phylogenetic distribution
		Avg	*Min*	
I-A	470-479	79	43	α, β and γ proteobacteria, cyanobacteria, prochlorales, sargasso sea metagenome, GOS metagenome
I-B	470-477	85	79	Cyanobacteria, prochlorales, eukaryotes – viridiplantae (streptophyta, chlorophyta), euglenozoa, sargasse sea metagenome
I-C	477-488	79	67	α and β-proteobacteria, chloroflexi
I-D	459-490	78	51	α, β and γ-proteobacteria, eukaryotes-stramenopiles, rhodophyta, haptophyceae
II	458-585	68	44	α, β and γ-proteobacteria, eukaryotes-Alveolata (Dinophyceae)
III-1	414-444	68	48	Methanogenic crenarchaeota
III-2	425-428	53	41	Methanogenic and thermophilic crenarchaeota, thermophilic and halophilic euryarchaeota.

2.2 Form I in somewhat more detail

Almost all plants and algae have form I Rubisco. Form I Rubisco has been subdivided into classes A-D, where classes A and B are called the 'green' and C and D the 'red' form (Figure 1) [5,15,17] . The green group includes plants, algae, α, β and γ proteobacteria and cyanobacteria, while the red group consists of β purple bacteria, non-green algae and some other α and β proteobacteria [17,18]. Between the green and red groups there is only 55% homology, while within the red group a homology of 84% is observed[15]. Especially the small subunit is different in the two groups. Within the groups the small subunit shows more variation in the red group, but the large subunit is equally conserved within the two groups[18]. As can be seen in Figure 1, form I is rather conserved between species. In algae the non-variable sites are located at different positions compared to green plants, which may suggest a difference between land and sea based organisms[19] . The sea organisms have a higher specificity for CO_2. The green plant lineage consists of class B of the form I Rubisco. The enzyme is formed by 4 dimers of the large subunits, and each large subunit is associated with a small subunit. The large subunit of this form consists of 476 amino acid residues, folded into a beta barrel of eight alpha helices and eight beta sheets, with the sheets on the inside. Of these 476 residues, 76 are involved in the dimerization of the large subunits, 29 in dimer-dimer interactions and 54 in the interactions between the large and the small subunits. The subunit has been compared for 499 plants and is rather conserved.[19] Almost a quarter (105 residues) of the residues are completely the same, while an additional 110 residues only deviates in one species of Rubisco. Furthermore, 85% of the positions are used by less than 5 alternative residues. Though it was expected that the residues responsible for subunit interaction would be conserved in the protein, this turns out not to be the case (51% conservation versus 40% average) [19] . The active site is the only part of the protein that is

much more conserved. Because of the high overall conservation of the Rubisco enzyme[11,19] , it has been used to make evolutionary trees of plant species [20,21].

Rubisco is, as mentioned, present in all photosynthetic organisms. The most relevant sources are plants which are either a waste stream of another process, or which can be easily produced. In the first category sugar beet leaves, soy leaves or potato leaves are a possibility. Furthermore, grass and alfalfa are produced in large quantities for feed, and may get added value by purifying the protein for food applications. In the second category, algae should be mentioned. They can be easily grown on a large scale and can be tuned to produce several components. Proteins are always a large part of the dry material. To compare Rubisco of these species of interest, spinach has been used as a reference and the sequences are compared using the website uniprot.org [22]. The sequences were aligned and the similarity was calculated. The number of amino acids in the large subunit as given by genetics is shown, and the number of identical and similar subunits when comparing the sequence to that of spinach (Table 2). The website contains both reviewed and non-reviewed sequences, and in the table it is shown whether the used sequence is reviewed. The grass species available close to the laboratory facilities is perennial ryegrass, and is therefore the grass species included in the tables.

Table 2. For a selected number of species (plants and microalgae) the sequences of the large subunit of Rubisco are compared. The "**code**" is the code at the uniprot.org website, "**no AA"** is the number of amino acids of the large subunit of Rubisco of the selected species. The percentages are relatively to the spinach Rubisco, and both the percentage of identical as the number of identical and similar amino acids are given.

plant	code	reviewed sequence	No. AA	Identical (no.)	Similar (no.)	Identical (%)	Similar (%)
Spinach (spinacia oleracea)	P00875	yes	475	475	0	100.0	100.0
Sugar beet (beta vulgaris)	Q4PLI7	no	475	465	8	97.9	99.6
Soybean (glycine max)	P27066	yes	475	448	22	94.3	98.9
Alfalfa (medicago sativa)	P04991	yes	474	447	20	94.3	98.5
Potato (Solanum tuberosum)	P25079	Yes	477	444	24	93.5	98.5
Tobacco (nicotiana tabacum)	P00876	yes	477	441	26	92.8	98.3
Perennial ryegrass (lolium perenne)	A8Y9H8	Yes	477	440	24	92.6	97.7
Desmodesmus serratus	D2KAG0	no	290	261	21	90.0	97.2
Chlorella Vulgaris	QP12466	yes	475	419	39	88.2	96.4
Nannochloropsis gradinata	Q8MFM6	no	466	244	129	52.4	80.0
Nannochloropsis oculata	A8CCX4	no	458	243	122	53.1	79.7

As expected, the green plants had a higher similarity to spinach than the algae. However, for chlorella and desmodesmus the similarity was still very large –

although the given sequence of desmodesmus was only a fragment of the whole subunit and thus only half as large as that of spinach Rubisco. Of the investigated algae, only the nannochloropsis gave a low sequence similarity

The small subunit of Rubisco has been investigated less extensively than the large subunit. Fewer organisms have been sequenced. The similarity of the small subunit between species is much less than for the large subunit, though still the green plants are more similar to spinach than the only microalgae for which data were given in the database (Table 3). The larger variance can be explained by the later development of the small subunit. For perennial ryegrass only a fragment of the total sequence is known, explaining the low number of amino acids.

Table 3. Comparison of the small subunit sequence of several Rubisco proteins

plant	code	reviewed sequence	no AA	Identical (no.)	Similar (no.)	Identical (%)	Similar (%)
Spinach (spinacia oleracea)	Q43832	yes	180	180	0	100.0	100.0
Soybean (glycine max)	P00865	Yes	178	126	36	71.9	92.1
Tobacco (nicotiana tabacum)	P69249	yes	180	136	29	75.6	91.7
Potato (Solanum tuberosum)	P32764	Yes	181	138	30	76.7	93.3
Alfalfa (medicago sativa)	O65194	yes	180	122	46	67.8	93.3
Perennial ryegrass (lolium perenne)	Q66LL4	No	65	44	17	67.7	93.8
Chlorella Vulgaris	Q9AVB6	no	214	53	69	29.4	67.8

3 Protein functionality

Proteins are used in food for two major purposes, first of all for their nutritional value, but secondly also for their functionality. Clearly the nutritional value is determined within the primary structure and the digestibility of the protein. The functionality of a protein can be largely divided in four areas: solubility, foaming, emulsion and gelation. It is especially this functionality that is the target when proteins are purified for food purposes. For feed functionality is less relevant, with the exception of fish feed which requires large amount of lipids to be bound. Comparison of the functionality of Rubisco of different research groups is sometimes difficult because of difference in purity but also in processing methods. Every purification or drying step can have an influence on the final functionality of the Rubisco. The different functionalities are described below.

3.1 Solubility

Protein solubility is the major parameter that determines the other functionalities. A protein that is made fully insoluble for instance by heating has almost no functionality. The solubility of Rubisco is dependent on many factors: pH, temperature, ionic strength, the presence of divalent cations, the protein concentration and also the species from which the protein was isolated [23]. For example, treatment with divalent magnesium causes Rubisco from tobacco to completely precipitate at a pH below 7.5 (effect of pH and divalent cations), while the effect on spinach Rubisco was much smaller (effect of species). Alfalfa leaf protein (consisting mainly of Rubisco) is least soluble close to its iso-electric point (pH 5 to 6.5), soluble up to 20% in acid and in alkaline solution an increase in solubility between 10 and 90% is observed from pH 7 to 12[24]. Soy leaves gave soluble protein below pH 2 and above pH 6 after alkaline extraction[25]. Spinach Rubisco was reported to be soluble under food conditions (pH 6-8). The solubility of spinach Rubisco in shown in figure 2.

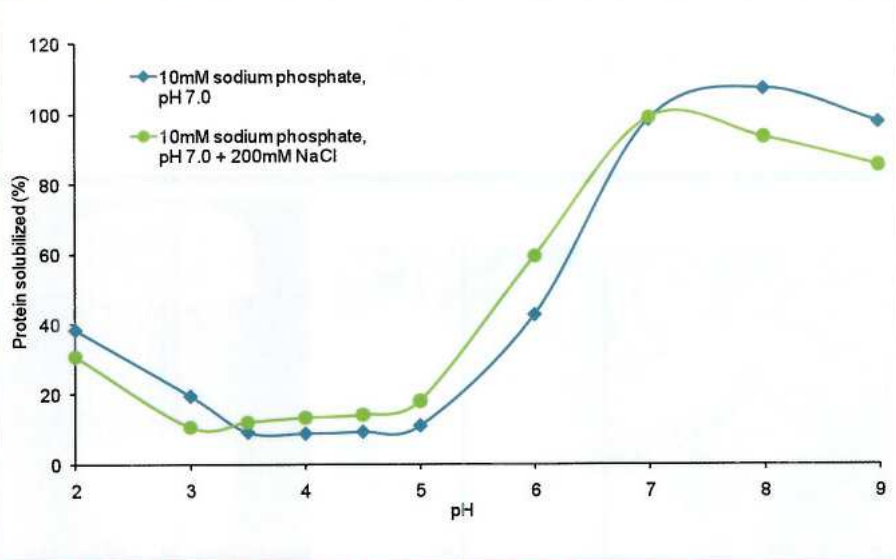


Figure 2 Solubility of spinach Rubisco as a function of ionic strength and pH. (copied from van de Velde *et al* [26]).

Also in the pH dependent solubility the pretreatment and species play a role. For soybean leaves, for example, there is almost no change in solubility between pH 8 and 11[25]. Furthermore purification conditions influence solubilityis shown by isolation of tobacco Rubisco after isolation from an acid, alkaline and neutral (suspension of crystals) solution, which shows large differences[27]. Also for alfalfa Rubisco variation in solubility was observed – depending on the way of precipitation and drying of the protein. After spray-drying at 85℃ the solubility was comparable to a freeze-dried samples. However, increasing spray-dry temperature to 95℃ or 140℃ dramatically decreased solubility[28]. Generally speaking, the solubility of Rubisco is good, considering its rather high hydrophobicity[27]. Tobacco Rubisco is even reported to stay soluble upon prolonged boiling[23], but alfalfa Rubisco has a denaturation temperature of 76.2℃ and also spinach Rubisco is heat-instable [27]. Under the right conditions, tobacco Rubisco was better soluble than soy protein isolate[29]. However, the purification steps may significantly decrease solubility. One approach of increasing solubility after protein isolation is enzymatic hydrolysis [24 30]. Although the hydrolysis doesn't change the nutritional value and solubilises the protein, an extensive hydrolysis decreases the other functional properties. All in all, it is difficult to generalize these data, as they are dependent on many parameters.

3.2 Water binding

Water binding is one of the functional parameters often used to describe the possible applications for a protein. In most food products, water binding is a important as it determines the texture of the food. With low water binding capacity, the product will become dry and/or will loose water during storage. Water binding is for instance a very critical parameter in meat products. the Water binding in meat is associated with the amount of water that can be complexed by the product. It can be calculated both for heated products and non heated products. For non heated meat products water binding is the binding of water to the native protein solution, measured on a freshly prepared extract. In this case water binding is the binding of water to the native proteins in a solution. Through the water binding, the meat extract becomes viscous. In contrast the water binding of a heated meat extract which produces a gel like structure is something else. Here in fact the water binding is caused by the gelled structure of the protein matrix which captures the water. The water binding of Rubisco in its native state seems very low. Douillard and Mathan [31] showed no clear indication of water binding with freeze dried or low temperature (85 ºC) spray dried leaf protein, which seems to be correlated with the excellent solubility of the Rubisco. The sample that was spray dried at 140 ºC had a water binding capacity of 428 % by volume compared to 612 % for soybean isolate. The increased water binding suggests that the protein is denatured and aggregated which can increase the water binding. All commercial soy bean protein isolates are partially denatured, which explains their water binding capacity before heating. Tobacco leaf protein also show water binding capacity in the order of soy protein isolate [29] but it depends on the isolation procedure and therefore most probably on the partial unfolding of the protein

3.3 Fat binding

The ability of proteins to bind fat is an important functional property for applications such as meat replacers and extenders, mainly because it enhances flavor retention and reputedly improves mouth feel. Fat absorption is usually measured by adding excess liquid fat to a protein powder, thoroughly mixing, centrifuging and determining the amount of bound or absorbed oil. Fat absorption of Rubisco seems to be higher for alfalfa leaf protein than for soy protein isolate, independent on the water solubility of the protein [28]. Once more, preparation procedure is very important: for two leaf protein samples it was higher, but for one lower than soybean protein isolate [29].

3.4 Emulsifying

Emulsifying properties, as many other functionalities, depend on the pretreatment. Rubisco emulsifies badly after heat coagulation, but good after pH precipitation followed by freeze-drying [32]. The emulsification of spinach Rubisco was worse than that of BSA and soy protein [10] However, for alfalfa leaf protein spray-dried at low temperature (85℃), it w as better than for soy protein[28]. Lamsal et al [33] reported that similar to other functionalities like foam stability and solubility, emulsifying properties depend very much on the purification procedure. They compared pH precipitated leaf protein versus ultrafiltrated leaf protein. From electrophoresis, an percentage of 80% Rubisco was estimated. The emulsion stability of the ultrafiltrated samples was almost twice as good as the precipitated leaf protein. Both were better than egg white protein. Emulsion stability and stability of the Rubisco samples were similar to that of the egg white protein.

3.5 Foaming

The foaming properties of Rubisco is dependent on work-up procedures but compared in general favorable to known foaming proteins such as egg white protein[10],[27] , though this is again dependent on work-up procedures[28]. Under favorable conditions, it gives the same foam volume as egg white, but with a longer stability. Furthermore, the behavior upon heating is similar. Therefore, Rubisco may be used as a replacement for egg white protein. It has been used already to make merengue pie, with a taste ‘quite acceptable to the 12-15 people who consumed the pies[34]. The foam is most stable with minimal repulsive forces, i.e. close to the iso-electric point or at high ionic strength[10]. Figure 3 shows a comparison of the foam stability of Rubisco versus whey and soy protein.

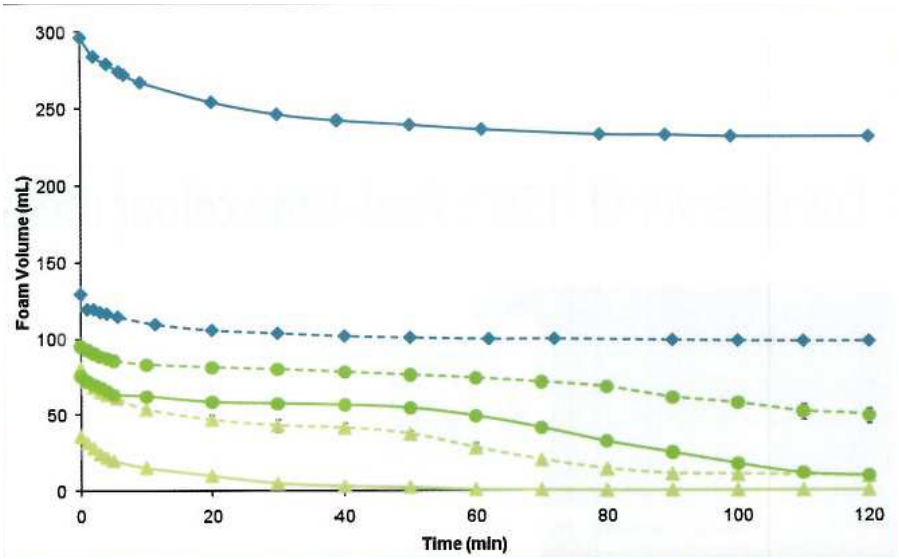


Figure 3 Foam volume of 2 hours of Rubisco (♦), soy (▲) and whey protein (●) isolates at two different pH's(4.5: dotted line and 7: sold line). (Copied from van de Velde *et al* [26]).

3.6 Gelation

Rubisco gels readily. The gelation is caused by denaturation followed by network formation. During the denaturation, ionic bridges are ruptured, aromatic groups get more exposed and ions can move in and out of the active site [35]. The combination of these processes determines the network formation. Once more, the exact conditions define the properties. Lu and Kinsella [36] investigated the effect of pH, temperature and calcium ions on the gelation of alfalfa Rubisco. Calcium decreases the coagulation temperature[37]. The strongest gel was obtained upon heating alfalfa Rubisco 30 min at 80℃ at pH 12 [36]. Also tobacco Rubisco and spinach Rubisco were reported to yield strong gels[10]. The gel formed upon heating spinach Rubisco had a higher strength than gels of soy glycinin after preparation of a >5% (w/v) concentration which was heated for 10 min at 90℃ [10]. This seems to be in contradiction with the lower heat stability of spinach Rubisco. However, the gelation may be better with decreasing solubility or denatured proteins. Again, because of the many different purification methods, combined with the many factors influencing the gelation, general conclusions cannot be drawn. However, the former examples give an indication of the possibilities of the Rubisco protein. Figure 4 shows a comparison of the gelation of pure Rubisco versus whey and soy protein.

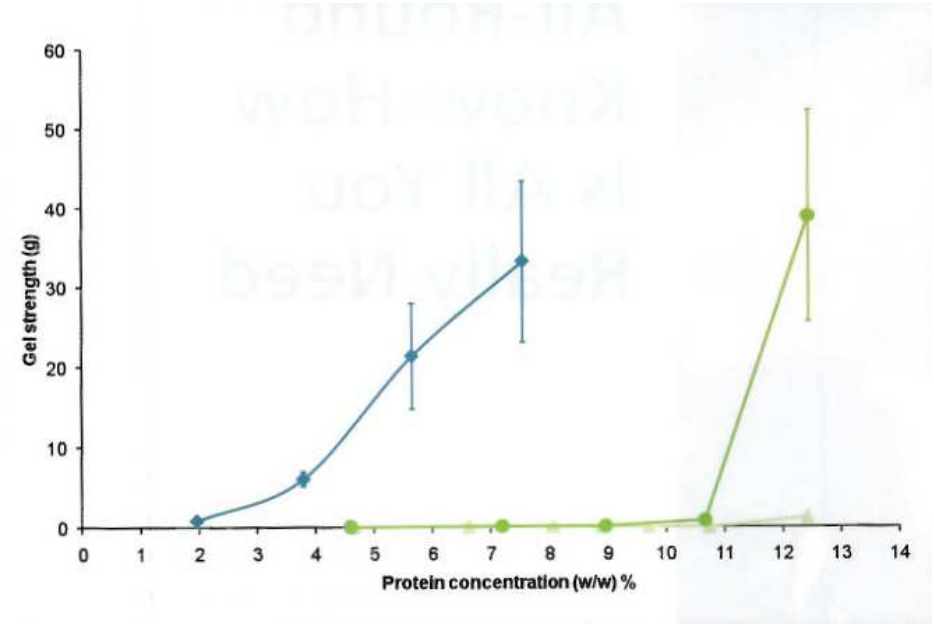


Figure 4 Gel strength as a function of protein concentration at pH 7. Rubisco (♦), soy (▲) and whey protein (●) isolates. (Copied from van de Velde *et al* [26].)

4 Taste and Flavor perception

A pure protein does not have an off flavor or off taste. Off flavors are caused by other compounds. This means that off flavors have to come from other sources. Heating of proteins is known to cause the formation of sulphurous compounds that have a bad smell and a bad taste. Whey proteins are notorious for this source of off flavor. Another source of off flavor are the bound polyphenols. A third even better known source are the oxidation product of fatty acids. Oxidation off fatty acids yield small, volatile compounds which, as the polyphenols, can be bound to proteins by hydrophobic interactions. These molecules are responsible for the beany flavor of soybean products, since they are released upon preparation or consumption of food. The oxidation can be caused by enzymes such as lipoxygenase, as is reported for both soy and whey protein. Another oxidation mechanism is via metal ions. Metallo proteins such as hemoglobin, myoglobin, catalase or peroxidase cause oxidation, and even after protein denaturation the active-site metal ion can still oxidize lipids. Furthermore heat-treatment [38] and hydrogenation [39] can lead to off-flavours by small compounds, for example a musty taste because of decarboxylation reactions. Since these oxidized lipid off-flavors limit the applicability of the proteins, and are notorious in soy, much research has been performed on soy beans to remove the off-flavors or prevent their formation. By selection of the plants and the right harvesting time, the amount of off-flavors can be decreased, though only by a small amount [40]. Inspired by this, soy beans have been genetically modified so that the lipid oxidases, one of the main oxidation sources, were not expressed[41]. Though small differences were observed between soy species, this approach was not yet successful.
The activity of lipoxygenases can be diminished using the same approaches (heat, pH, inhibitors etc.) as employed for polyphenoloxidases. Removal of the precursors and the small-compound off-flavors requires different strategies than employed for the polyphenols. Precursors of the off-flavors can be removed by extraction with alcohol, hexane [38], or dichloromethane, though a complete removal has not yet been achieved. However the proteins denature severely, resulting in solubility loss [42]. In another approach, a CO_2 extraction was used to remove off-flavors [43], where supercritical CO_2 was more effective than liquid CO_2. The CO_2 treatment caused a pH drop, but not so severe as to cause functionality loss of the proteins. Because of the difference in hydrophobicity, hydrophobically bound butanone was easier to extract than hexanone [43]. Also in removal of off-flavors from anchovies, critical CO_2 is effective, and in comparison with solvent extraction a smaller amount of compounds of interest is removed [44]. However, extraction with supercritical CO_2 is reported not always to be efficient [45].
The previous section is very much focused on the off flavor problems for soy protein. This is because a lot of effort has been put in the research of those off flavors. The off flavors in leaf extracts and therefore also in Rubisco arise from a similar origin and seem to involve the same of off-flavor compounds. The off flavor formation starts whenever the plant material is disrupted to extract the soluble proteins. During the extraction lipoxygenase comes into contact with the membrane lipids of the chloroplasts. As a consequence purified Rubisco has a grassy smell even when it is isolated from algae. There are two possible approaches to solve the off-flavor problem:

1) prevention of the formation of off-flavors
2) removal of the off flavors

The easiest way of removing the chlorophyll containing fraction is by heating the extract for half an hour at 50 ºC. At this temperature, the membrane fraction starts to aggregate which makes them easier to remove by centrifugation. In this process the off flavors are already formed To prevent formation of the off flavors, which is preferable to removing them this process has to be changed, which is undesirable. The second approach seems more realistic but requires special columns which have a higher affinity for the off flavors than for Rubisco.

5 Allergenicity

Allergenicity is a major problem in food industry. From the major food ingredients, only proteins are be able to produce allergenic reactions. Carbohydrates and lipids do no induce allergenicity, although the carbohydrates that are bound to glycosylated proteins can have a strong influence on the potency of a protein to become an allergen. It is very difficult to define what the exact criteria that determine the possible allergenic potential of a protein. In literature the most mentioned aspect of protein allergenicity is the susceptibility towards the enzymes in the human digestive system. The general idea is that proteins that digest easily will not cause an allergic reaction. However, a report by Fu et al [46] compared the *in vitro* digestibility of allergenic protein (alpha-lactalbumin) with that of proteins with unproven allergenicity (spinach-leaf Rubisco). They observed that degradation of the proteins in a simulated gastric fluid neither predict their allergenicity, nor the prevalence of allergies against the tested allergenic proteins. This indicates that not only digestibility is relevant but also formation of protease resistant peptides and the ease in which protein fragments are taken up by the intestine cells. Protein abundance also partially determines allergenicity, although the most abundant protein on earth, Rubisco is not allergenic [47].

Although there might not be a one to one relation between allergenicity and digestibility of proteins, the important food allergens are fairly stable to digestion in the gastric model (simulated gastric fluid). For example, soybean ß-conglycinin was stable for 60 min, while non-allergenic food proteins, such as spinach Rubisco, were digested within 15 sec. In 2008 there was a report on a group of Rubisco allergic people [48]. Since this is to the best of our knowledge the only article describing Rubisco as an allergenic protein and is about only four people, for the time being we still assume Rubisco to have a low allergenicity. Rubisco consumed by humans up to now are proteins that are still in the leaf of the vegetables. The proteins did not undergo modification as happens during extraction and purification. The Rubisco samples used in several research articles have been purified on small scale which makes it possible to easily remove polyphenols and carbohydrates. It is not clear whether purification on a large scale works the same. Reactions of polyphenol or carbohydrates with Rubisco during large scale processing might be able to trigger allergenicity, although at this moment here is no evidence that this occurs. Based on the evidence present, Rubisco can be regarded as being non allergenic.

6 Bioactivity

For the last decade, many bioactive peptides derived from various food proteins were characterized. Main biological activities reported were immunomodulating, opioid like, mineral-carrying , diazepam like, antimicrobial and antihypertensive activities. These bioactive peptides are latent in the primary sequences of proteins and may be released during gastro-intestinal like proteolysis or food processing such as cheese ripening and fermentation. Hydrolysates containing bioactive peptides may also be generated by controlled hydrolysis carried out in enzymatic reactor, intending to produce nutraceuticals or ingredients suitable for functional foods [49].

6.1 Angiotensin I converting enzyme (ACE) inhibitory peptides

ACE inhibitors or angiotensin-converting enzyme inhibitors are a group of drugs used primarily for the treatment of hypertension (high blood pressure) and congestive heart failure. Antihypertensive peptides have been derived from milk, corn and fish protein sources [50]. Research has shown that such peptides can also be formed during hydrolysis of Rubisco [50-52].

Four inhibitory peptides for angiotensin I-converting enzyme (ACE), namely, MRWRD, MRW, LRIPVA, and IAYKPAG, were isolated from the pepsin−pancreatin digest of spinach Rubisco with using HPLC. IC50 values of individual peptides were 2.1, 0.6, 0.38, and 4.2 μM, respectively. MRW and MRWRD had an antihypertensive effect after oral administration to spontaneously hypertensive rats. Maximal reduction occurred 2 hours after oral administration of MRW, whereas MRWRD showed maximal decrease 4 hours after oral administration at doses of 20 and 30 mg/kg, respectively [51].

6.2 Opioid peptides

Opioid peptides are short sequences of amino acids that bind to opioid receptors in the brain; opiates and opioids mimic the effect of these peptides. Some opioid peptides for example endorphins are produced by the body itself. The effects of these peptides vary, but they all resemble opiates. Brain opioid peptide systems are known to play an important role in motivation, emotion, attachment behavior, the response to stress and pain, and the control of food intake [53].

Opioid-like peptides may also be absorbed from partially digested food (casomorphins, exorphins, and rubiscolins), but have limited physiological activity compared to opiates. The opioid food peptides have lengths of typically 4-8 amino acids. The body's own opioids are generally much longer.

The sequences YPLDL and YPLDLF in the large subunit of spinach D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) have opioid activity. The IC50 of these peptides in mouse vas deferens assay were 51.0 WM and 24.4

WM, respectively, and those in N receptor binding assay using [3H]deltorphin II as radioligand were 2.09 WM and 0.93 WM, respectively. Both peptides were selective for N receptor. We named them rubiscolin-5 and -6, respectively. The enzymatic conditions to release rubiscolin were investigated using both spinach Rubisco and synthetic fragment peptides [54].

6.3 Anxic peptides

An anxiolytic (also antipanic or anti-anxiety agent) is a drug used for the treatment of anxiety, and its related to psychological and physical symptoms. Anxiolytics have been shown to be useful in the treatment of anxiety disorders. Beta-receptor blockers such as propranolol and oxprenolol, although not anxiolytics, can be used to combat the somatic symptoms of anxiety. Anxiolytics are also known as minor tranquilizers. This term is less common in modern texts, and was originally derived from a dichotomy with major tranquilizers, also known as neuroleptics or antipsychotics [53].

In their study, Zhao et al [55] found that rubimetide (Met-Arg-Trp), which was isolated from a pepsin–pancreatin digest of Rubisco, has anxiolytic-like activity in the elevated plus-maze test at a dose of 0.1 mg/kg (intraperitoneal.) or 1.0 mg/kg (orally.) in mice. They found that rubimetide lowered blood pressure via prostaglandin (PG) D2- dependent vasorelaxation in spontaneously hypertensive rats (SHR).

6.4 Miscellaneous

Hydrolysis of the small Rubisco subunit was performed for 20 h at 37 ℃ using pepsin in ammonia/formic acid buffer at pH 3. The hydrolysate was fractionated on a Sephadex G25 column. Biological activities were found in two fractions. The first fraction showed slight bacteriostatic properties against two pathogenic bacteria, *Salmonella arizonae* and *Shigella sonnei*. The second fraction, tested by radio immunoassay, presented a secretagogue activity comparable to that of gastrin [56]. A secretagogue is a substance that causes another substance to be secreted. One example is gastrin, which stimulates the H/K ATPase in the parental cells and so causes increased gastric acid production by the stomach. Pentagastrin, a synthetic gastrin, histamine, and acetylcholine are also gastric secretagogues.

6.5 Bioactivity in relation to the sequence

The bioactivity of peptides is related to the sequence and length of the peptides. This shows that some bioactive peptides found are not necessarily present in all Rubisco samples. As indicated in the sequences below, Rubiscolin-6 (red) and rubimetide (green) can be found in the sequence of the large Rubisco subunit from Spinach. In contrast, Rubiscolin could not be detected in the sequence of the large Rubisco subunit from the algae *Chlamydomonas reinhardtii*, whereas rubimetide (green) was detected. The presence of the peptide YPIDLF (blue) in the sequence is different from Rubiscolin, however, no information is available on bioactivity of this peptide.

Amino acid sequence large subunit spinach Rubisco

MSPQTETKASVEFKAGVKDYKLTYYTPEYETLDTDILAAFRVSPQPGVPPEEAGAA
VAAESSTGTWTTVWTDGLTNLDRYKGRCYHIEPVGEENQYICYVAYPLDLFEEGS
VTNMFTSIVGNVFGFKALRALRLEDLRIPVAYVKTFQGPPHGIQVERDKLNKYGRP
LLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLFCAE
ALYKAQATGEIKGHYLNATAGTCEDMMKRAVFARELGVPIVMHDYLTGGFTANTT
LSHYCRDNGLLLHIHRAMHAVIDRQKNHGMHFRVLAKALRLSGGDHIHSGTVVGK
LEGERDITLGFVDLLRDDYTEKDRSRGIYFTQSWVSTPGVLPVASGGIHVWHMPA
LTEIFGDDSVLQFGGGTLGHPWGNAPGAVANRVALEACVQARNEGRDLAREGNT
IIREATK WSPELAAACEVWKEIKFEFPAMDTV

Amino acid sequence large subunit *Chlamydomonas reinhardtii* Rubisco

MVPQTETKAGAGFKAGVKDYRLTYYTPDYVVRDTDILAAFRMTPQLGVPPEECGA
AVAAESSTGTWTTVWTDGLTSLDRYKGRCYDIEPVPGEDNQYIAYVAYPIDLFEE
GSVTNMFTSIVGNVFGFKALRALRLEDLRIPPAYVKTFVGPPHGIQVERDKLNKYG
RGLLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLF
VAEAIYKAQAETGEVKGHYLNATAGTCEEMMKRAVCAKELGVPIIMHDYLTGGFT
ANTSLAIYCRDNGLLLHIHRAMHAVIDRQRNHGIHFRVLAKALRMSGGDHLHSGTV
VGKLEGEREVTLGFVDLMRDDYVEKDRSRGIYFTQDWCSMPGVMPVASGGIHV
WHMPALVEIFGDDACLQFGGGTLGHPWGNAPGAAANRVALEACTQARNEGRDL
AREGGDVIRSACKWSPELAAACEVWKEIKFEFDTIDKL

7 Nutritional value

The nutritional value of a protein is dependent on the amino acid composition, and on the availability of the amino acids. The latter is determined by the folding of the protein, but also by the composition of the meal [57]. The nutritional value is often lower than expected from the amino acid composition, both due to functionality loss during drying and because of oxidation [58]. Polyphenol compounds, such as tannins or melanins, decrease the nutritional value of proteins by reaction with lysine or cysteine [45]. The binding of phenolic compounds such as rutin or chlorogenic acid to Rubisco have even been reported to cause digestion problems in humans [45], although in other studies small amounts of polyphenols had no adverse effects on digestibility[61]. Rubisco is reported to digest readily before heating. After heating digestion is worse[10]. For now we will focus on the 'potential nutritional value' of Rubisco, determined solely by the amino acid composition.
At the uniprot.org website[22], amino acid sequences of several forms of Rubisco have been compared and the amino acid score has been calculated by comparing the amount of the essential amino acids with the amino acid requirement pattern based on the requirements of preschool-age children (Table 4)[59]. The amino acid score (the percentage of the required amount present) is shown in

Table 5. The two essential amino acids in which Rubisco of most species is lacking are isoleucine and lysine. However, the differences between species are relatively large. For both spinach and alfalfa the small subunit compensates for the lysine deficiency of the large subunits. Tobacco does not have any deficient amino acids. Remarkably, for Chlorella the deficiencies in both isoleucine and lysine are worse in the small subunit than in the large subunit, which is the opposite trend to that in plants. The two other microalgae (*Desmodesmus* and *Nannochloropsis*) have no deficiencies in their large subunits (no data are available for the small subunits).

Table 4. Amino acid requirement pattern based on amino acid requirements of preschool-age children.

essential amino acids	ile	leu	lys	cys+ met	tyr+ phe	thr	trp	val
recommended (mg/g)	28	66	58	25	63	34	11	35

Table 5. Amino acid composition of Rubisco. L = large subunit, S = small subunit, T = total protein (1:1 L:S). Amino acids present in smaller quantities are shown in red, the limiting amino acid in bold type.

Essential amino acids		ile	leu	lys	cys+met	tyr+phe	thr	trp	val
Spinach (spinacia oleracea)	L	**91.8**	133.8	96.8	160.6	182.7	192.5	257.8	183.4
	S	**69.3**	117.0	105.5	255.8	272.3	142.0	462.0	135.3
	T	**86.9**	130.2	98.7	181.4	202.2	181.5	302.4	172.9
Tobacco (nicotiana tabacum)	L	**101.2**	133.4	104.9	140.2	181.6	163.7	257.1	198.9
	S	104.6	117.8	136.6	193.1	258.1	**102.1**	581.3	155.6
	T	**102.0**	130.0	111.7	151.7	198.2	150.4	327.3	189.6
Sugar beet (beta vulgaris)	L (1)	**96.7**	133.8	96.8	160.6	182.7	186.9	257.9	183.4
	L (2)	102.6	142.0	**84.8**	149.2	179.1	180.2	273.6	177.4
Alfalfa (medicago sativa)	L	101.7	127.6	**97.0**	163.1	186.5	175.9	258.4	178.4
	S	122.1	129.6	136.6	157.1	254.7	**102.1**	465.1	136.2
	T	106.2	128.0	**105.6**	161.8	201.4	159.9	303.3	169.2
Soybean (glycine max)	L	**92.1**	137.4	101.2	153.1	182.1	176.0	258.5	178.4
	S	105.0	141.8	137.1	193.8	271.7	**82.0**	466.7	136.7
	T	**94.9**	138.4	109.0	162.0	201.6	155.6	303.6	169.4
chlorella vulgaris	L	106.7	134.3	**92.9**	161.1	178.8	181.8	258.7	151.5
	S	**44.2**	119.4	48.1	234.9	141.0	168.2	221.0	147.9
	T	87.6	129.7	**79.2**	183.7	167.3	177.6	247.2	150.4
desmodesmus serratus	L	**102.2**	148.7	102.7	183.5	183.9	193.4	157.3	157.9
Nannochloropsis oculata	L	146.2	139.6	**109.7**	186.8	186.8	171.2	235.2	168.7
Nannochloropsis gradinata	L	138.4	133.5	**107.5**	209.4	192.7	167.8	263.6	170.9

In some species the protein is not limiting in any amino acid. Therefore, it can be compared nutritionally with meat protein[60], such as egg protein and beef muscle.

7.1 Digestibility

The word digestibility has several meanings that do not always correspond. It can be gastric digestibility used for allergy research. The major spinach protein, Rubisco, has been shown to be so easily digested by pepsin that it degrades completely after only 30 s in an a simulated gastric digestion experiment [62].This means that after 30 seconds the bands of the monomer subunits have disappeared. It does not say anything about the further digestion by pepsin, but the results also show that the protein is broken down further very fast by pepsin[63]. Figure 5 shows the digestion curves of several proteins with pepsin at pH 1.2 and pH 2 again sterssing the very fast digestion of rubisco. At both pH's the monomer band of the large subunit is already fully dissapeared at the first meassuring point after 30 seconds. From the 6 tested proteins, Rubisco is digested the fasted.

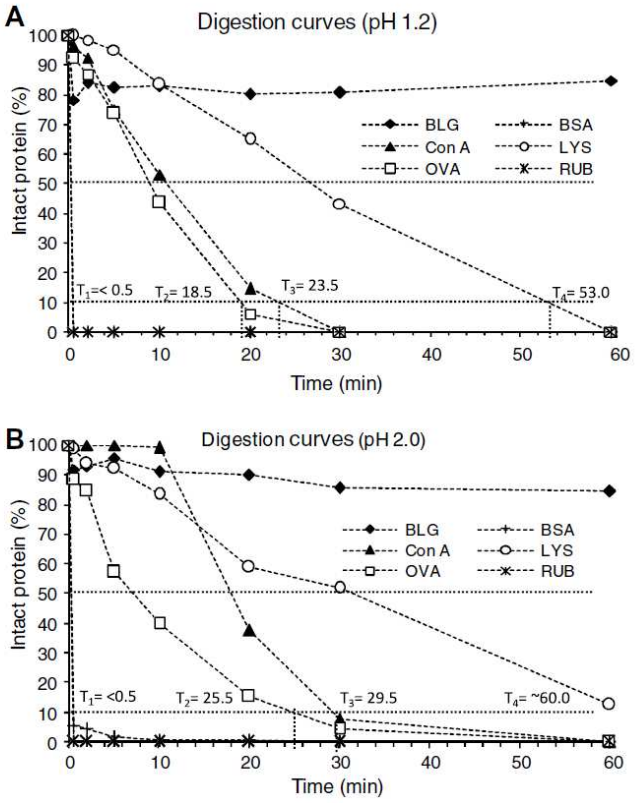


Figure 5 Pepsin digestion curves of 6 different proteins as a function of the pH. A) p 1.2. B) pH 2.0. Copied from van de Lahiri *et al* [63].

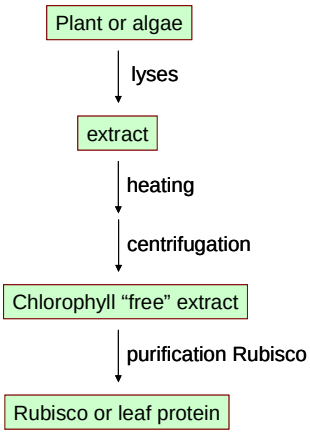
However to determine the nutritional value, the overall digestibility is of importance. This is in fact the protein breakdown in the stomach and in the intestine, followed by the uptake of amino acids by the small intestine. This is called true digestibility. In the past true digestibility was expressed by the measurement of the fecal digestibility, but nowadays it seems more realistic to measure the ileal digestibility. The ileal digestibility is determined by the amount of peptides that leave the ileum to enter the colon. It is beyond the scope of this study to exactly determine what the best method is. The true digestibility is determined by several factors. The susceptibility of the protein towards the digestive proteases is very important, but also other parameters like anti-nutritional factors and processing conditions are of interest. The fast gastric digestion of purified Rubisco can give the impression that the digestibility is very good, but we have to keep in mind that these Rubisco samples are very pure. This means that the amount of for instance polyphenols is very low. These polyphenols are one of the major reasons that digestibility of leaf protein is less compared to soy protein. For example bound chlorogenic acid creates off color problems in foods containing leaf protein as an ingredient and also lowers the digestibility of leaf protein by inhibiting the rate of trypsin attack on the protein [64]. There are two ways in which polyphenols inhibit digestion. One is by simple non covalent binding to the protein which inhibits the activity of proteases, the other is by covalent attachment through the action of polyphenol oxidase. The amino acids involved in the covalent reactions are lysine, cystein and tryptophan. These are all essential amino acids. Covalent attachment of

polyphenols to these amino acids makes them unsuitable to use for the human body and decrease digestion speed. So the nutritional value of the protein is lowered. No data were found on the true digestibility of pure Rubisco, only some results were found on the digestibility of leaf protein. These seem to vary a lot. As an example the true protein digestibility was measured for water-coagulated and steam-coagulated Lucerne and compared to that of lactalbumin. The values were 97,6% for lactalbumin, 80,9% for water-coagulated leaf protein and 76,7% for steam-coagulated leaf protein. The problem of the digestibility studies is the variability in processing methods, source of protein, solvents used, purity of the final samples. It is therefore very difficult to predict the true digestibility. In conclusion it can be said that the digestibility of pure Rubisco seems very good, but that the digestibility and thereby also the nutritional value of Rubisco samples is strongly influenced by several parameters.

8 Isolation of Rubisco

8.1 Introduction

There are many ways of purifying Rubisco. The majority of the methods described in literature have a part of the procedure that seems to overlap (scheme 1).



Scheme 1 isolation procedure of Rubisco

These four steps, lyses, heating and centrifugation and purification will be discussed below.

8.2 Lyses

The first step in the isolation of Rubisco is the production of an extract. This can be done in several ways, for instance by using a simple kitchen homogenizer or a small press. These procedures only seem to work with plants and not with algae. Algae have a strong cell wall and are in addition to small to be disrupted by the above mentioned methods. Therfore alternative methods have to be used for algae. The most common methods for opening algae are the Dyno-mill procedure (www.Eskens.com) or homogenizers. In the Dyno-mill equipment small inert balls are mixed with the algae apparatus which destroys the algae by friction. The machines were originally intended to decrease the size of paint pigments. Homogenizers are used for instance in milk industry where they decrease the average diameter of fat globules in milk and an increase in number and surface area, of the fat globules. The net result, from a practical view, is a much reduced tendency for creaming of fat globules. A problem in using a Dyno-mill or homogenizer is the relative high amount of energy required to open the algae and the heat that is generated during the processing which requires cooling of the extract. The high energy cost will affect the final price of the Rubisco and other products from the algae.
An important problem for the isolation of Rubisco from plants is the presence of polyphenols. Polyphenols tend to bind to proteins. This is accelerated by the presence of polyphenol oxidases. Unfortunately the breakdown of the plant cell structure results in mixing the proteins with the polyphenols and polyphenol

oxidase. From the moment the plant cells are disrupted, these reactions will start. To slow down the oxidation reactions, on small scale polyvinylpolypyrrolidone (PVPP) is added. PVPP binds polyphenols, but unfortunately not all polyphenols. Furthermore addition of PVPP complicates use of the insoluble compounds of plants. Therefore use of PVPP is not practical in a large scale biorefinery process. Sodium metabisulphite can be added to the plant material prior to the disruption of the plants. Sodium metabisulphite is an inhibitor of the polyphenol oxidase reaction. Without this inhibitor, the plant extracts will oxidize and become brown very quickly and part of the protein will have covalent bound polyphenols. Extracts of algae do not need the addition of sodium metabisulphite as they do not have polyphenols or only in a very low concentration. Experiments at TNO have shown that extracts of algae without addition of sodium metabisulphite do not turn brown during purification of Rubisco. In contrast extracts of grass and alfalfa discolor despite the presence of sodium metabisulphite. These brown colored polyphenols can be removed from the protein by means of chromatography as long as they are not covalently attached, but not by simple precipitation or filtration techniques. Spinach contains less polyphenols which explains why this source is often chosen for research purposes.

8.3 Heating followed by centrifugation

One of the most important components that make it difficult to purify Rubisco is the in soluble part that contains the chlorophyll. The chlorophyll is complexed with the photosystem proteins (photosystem I and II) that lie within the membranes of the thylakoids. These insoluble protein parts can be largely removed by centrifugation, but this requires a very high centrifugation force. The most common practice to circumvent this is by heating the extracts. This heating steps results in aggregation of the non soluble protein parts which makes it easier to centrifuge or filtrate. Alternative procedures, like the addition of flocculants can be used but they introduce an extra chemical that has to be removed later on. The heating step normally is within the range of 40 to 60 ºC. It should be sufficiently low to prevent the Rubisco from denaturation. Though the largest part of the chlorophyll can be removed by this method, there is still a small part of the insoluble chlorophyll containing protein fraction present in the soluble fraction in which the Rubisco is present. This minor chlorophyll contamination should be removed to avoid problems during the isolation of Rubisco. Methods like for instance activated carbon could remove the last part of the non soluble protein[65].

8.4 Purification of Rubisco

After the removal of the last part of the non soluble protein, the isolation of white Rubisco is possible. Several approaches such as pH precipitation[51], column chromatography[3,66], crystallization[29], ultrafiltration[67] have been used in literature. The purification procedures determine the functionality and the purity of the protein. For instance when using ultrafiltration, the final preparation will contain other proteins beside Rubisco. The purity of Rubisco is enhanced by using a pH precipitation method, but this lowers the solubility of the Rubisco.

Column chromatography is the best method as it is able not only to produce pure Rubisco, but also can remove polyphenols. This is however a very costly method for large scale purification. Currently ultrafiltration seems to be the best method for producing of a Rubisco samples on large scale. In this procedure there is a large difference in the source of Rubisco. As mentioned before algae do not contain large amounts of polyphenols and therefore the Rubisco sample is easily prepared by ultrafiltration. For plant derived extracts, polyphenols are a problem. The polyphenols partially stick to the protein, so that with ultrafiltration the polyphenols are concentrated in the protein fraction as well, despite the fact that they are low molecular weight molecules. This means that an additional step is necessary to remove polyphenols. This has to be done with the aid of a column with low affinity for Rubisco and high affinity for the polyphenols. Several column options are available but the outcome depends on the plant source, because every plant has its own polyphenol composition.

8.5 Functionality in relation to purification

The functionality of the protein is largely determined by the solubility of the protein, the purity of the protein samples and the bound polyphenols and off flavors. It is therefore difficult to predict the functionality of Rubisco from different sources. The measurements as performed with purified Rubisco show that this proteins has very promising functionalities for use in human food products. However large scale purification procedures are not yet available to provide pure, colorless and tasteless Rubisco. Therefore these impure samples have changed protein functionality in respect to pure Rubisco, because the hydrophobicity of the Rubisco changes as a result of the binding of the off flavors and polyphenols. Removal of these compounds will help to produce Rubisco samples with better defined functionality. In addition it has to be mentioned that apart from the change in functionality, the bound polyphenols and off flavors decrease customer acceptance. This may be a much bigger obstacle than the functionality differences as a result of impurities in the Rubisco samples.

9 Conclusion

Rubisco is the enzyme that catalyses the first step in photosynthesis, the conversion from ribulose-1,5-diphosphate to 2 molecules of 3-phosphoglyceric acid The name Rubisco is used for a very large group of enzymes that differ depending on their place in the phylogenetic tree. We like to refer to Rubisco as the enzyme found in plants and green algae that have 8 large and 8 small subunits. This Rubisco is a very attractive protein for human consumption because of a good nutritional value, functionality and digestibility. There are however drawbacks that hinder the introduction in food. These are mainly related to the extraction, purification and drying of the Rubisco. The problems in extraction and purification are related to the presence of chlorophyll, polyphenols and off-flavor molecules. In relation to the different sources it is of interest to know that the problems that arise as a result of polyphenols is not seen in Rubisco samples from algae. Algae have no or only a limited amount of polyphenols. In case of isolation of Rubisco from plants, metabisulphite is added to inhibit polyphenol oxidases and polyvinylpolypyrrolidone is added to capture part of the polyphenols. Despite these additions, the leaf extracts are turning brown as a result of polyphenol oxidation. This is not observed with extracts of algae. When making extracts of algae, sodium metabisulphite can be left out without any discoloration of the extracts. However it looks like the Rubisco content is less in algae compared to various plants. So there are differences in the way Rubisco is purified from algae or from plants and there will also be differences in the digestibility, functionality and amino acid composition.
The major conclusion is that Rubisco is an interesting protein for food products but there are still challenges in the purification on large scale.

10 Reference list

1. *www. france-luzerne. fr* **2011**.

2. Hartman, F. C.; Harpel, M. R. Structure, Function, Regulation, and Assembly of D-Ribulose-1,5-Bisphosphatecarboxylase Oxygenase. *Annual Review of Biochemistry* **1994,** *63*, 197-234.

3. Salvucci, M. E.; Portis, A. R.; Ogren, W. L. Purification of Ribulose-1,5-Bisphosphate Carboxylase Oxygenase with High Specific Activity by Fast Protein Liquid-Chromatography. *Analytical Biochemistry* **1986,** *153* (1), 97-101.

4. Fiorentini, R.; Galoppini, C. The Proteins from Leaves. *Qualitas Plantarum-Plant Foods for Human Nutrition* **1983,** *32* (3-4), 335-350.

5. Tabita, F. R.; Hanson, T. E.; Li, H. Y.; Satagopan, S.; Singh, J.; Chan, S. Function, structure, and evolution of the RubisCO-like proteins and their RubisCO homologs. *Microbiology and Molecular Biology Reviews* **2007,** *71* (4), 576-599.

6. Hilditch, C. M.; Jones, P. B.; Balding, P.; Smith, A. J.; Rogers, L. J. Ribulose Bisphosphate Carboxylases from Macroalgae - Proteolysis During Extraction and Properties of the Enzyme from Porphyra-Umbilicalis. *Phytochemistry* **1991,** *30* (3), 745-750.

7. Patel, M.; Berry, J. O. Rubisco gene expression in C-4 plants. *Journal of Experimental Botany* **2008,** *59* (7), 1625-1634.

8. Wang, J.; Kinsella, J. E. Composition of Alfalfa Leaf Protein Isolates. *Journal of Food Science* **1975,** *40* (6), 1156-1161.

9. Ku, M. S. B.; Schmitt, M. R.; Edwards, G. E. Quantitative-Determination of Rubp Carboxylase-Oxygenase Protein in Leaves of Several C-3 and C-4 Plants. *Journal of Experimental Botany* **1979,** *30* (114), 89-98.

10. Barbeau, W. E.; Kinsella, J. E. Ribulose bisphosphate carboxylase/oxygenase (rubisco) from green leaves - potential as food protein. *Food Rev. Int.* **1988,** *4* (1), 93-127.

11. Miziorko, H. M.; Lorimer, G. H. Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase. *Annual Review of Biochemistry* **1983,** *52*, 507-535.

12. Saito, Y.; Ashida, H.; Sakiyama, T.; de Marsac, N. T.; Danchin, A.; Sekowska, A.; Yokota, A. Structural and Functional Similarities between a Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RuBisCO)-like Protein from Bacillus subtilis and Photosynthetic RuBisCO. *Journal of Biological Chemistry* **2009,** *284* (19), 13256-13264.

13. Tabita, F. R.; Satagopan, S.; Hanson, T. E.; Kreel, N. E.; Scott, S. S. Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *Journal of Experimental Botany* **2008,** *59* (7), 1515-1524.

14. Schneider, G.; Lindqvist, Y.; Branden, C. I. Rubisco - Structure and Mechanism. *Annual Review of Biophysics and Biomolecular Structure* **1992,** *21*, 119-143.

15. Valentin, K.; Zetsche, K. Rubisco Genes Indicate A Close Phylogenetic Relation Between the Plastids of Chromophyta and Rhodophyta. *Plant Molecular Biology* **1990,** *15* (4), 575-584.

16. Portis, A. R.; Parry, M. A. J. Discoveries in Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. *Photosynthesis Research* **2007,** *94* (1), 121-143.

17. Selesi, D.; Schmid, M.; Hartmann, A. Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (cbbL) in differently managed agricultural soils. *Applied and Environmental Microbiology* **2005,** *71* (1), 175-184.

18. Assali, N. E.; Martin, W. F.; Sommerville, C. C.; Loiseauxdegoer, S. Evolution of the Rubisco Operon from Prokaryotes to Algae - Structure and Analysis of the Rbcs Gene of the Brown Alga Pylaiella-Littoralis. *Plant Molecular Biology* **1991,** *17* (4), 853-863.

19. Kellogg, E. A.; Juliano, N. D. The structure and function of RuBisCO and their implications for systematic studies. *American Journal of Botany* **1997,** *84* (3), 413-428.

20. Mummenhoff, K. Subunit Polypeptide Composition of Rubisco As A Taxonomic Tool in Separating Critical Lepidium Species. *Phytochemistry* **1994,** *35* (3), 683-684.

21. Newman, S. M.; Cattolico, R. A. Ribulose Bisphosphate Carboxylase in Algae - Synthesis, Enzymology and Evolution. *Photosynthesis Research* **1990,** *26* (2), 69-85.

22. *www. uniprot. org* **2011**.

23. Sheen, S. J. Comparison of Chemical and Functional-Properties of Soluble Leaf Proteins from 4 Plant-Species. *Journal of Agricultural and Food Chemistry* **1991,** *39* (4), 681-685.

24. Prévot-D'Alvise, N.; Lesueur-Lambert, C.; Fertin-Bazus, A.; Fertin, B.; Dhulster, P.; Guillochon, D. Continuous enzymatic solubilization of alfalfa proteins in an ultrafiltration reactor
11. *Enzyme and Microbial Technology* **2004,** *34* (5), 380-391.

25. Betschar, A.; Kinsella, J. E. Extractability and Solubility of Leaf Protein. *Journal of Agricultural and Food Chemistry* **1973,** *21* (1), 60-65.

26. van de Velde F; Alting, A.; Pouvreau, L. from wasteproduct to food ingredient: the extraction of the abundant plant protein Rubisco. *New Food,* **2011,** pp 10-14.

27. Barbeau, W. E. Functional properties of leaf proteins: criteria required in food applications. *Italian journal of food science* **1990,** *4*, 213-225.

28. Knuckles, B. E.; Kohler, G. O. Functional-Properties of Edible Protein-Concentrates from Alfalfa. *Journal of Agricultural and Food Chemistry* **1982,** *30* (4), 748-752.

29. Sheen, S. J.; Sheen, V. L. Functional-Properties of Fraction-1 Protein from Tobacco Leaf. *Journal of Agricultural and Food Chemistry* **1985,** *33* (1), 79-83.

30. Prévot-D'Alvise, N.; Lesueur-Lambert, C.; Fertin-Bazus, A.; Fertin, B.; Dhulster, P. Development of a pilot process for the production of alfalfa peptide isolate. *J. Chem. Technol. Biotechnol.* **2003,** *78* (5), 518-528.

31. Douillard, R.; de mathan, O. Leaf protein for food use: potential of Rubisco. In *New and developing sources of food protein*, 1994; pp 307-342.

32. Rault, M.; Giudiciorticoni, M. T.; Gontero, B.; Ricard, J. Structural and Functional-Properties of A Multienzyme Complex from Spinach-Chloroplasts .1. Stoichiometry of the Polypeptide-Chains. *European Journal of Biochemistry* **1993,** *217* (3), 1065-1073.

33. Lamsal, B. P.; Koegel, R. G.; Gunasekaran, S. Some physicochemical and functional properties of alfalfa soluble leaf proteins. *LWT - Food Science and Technology* **2007,** *40* (9), 1520-1526.

34. Knuckles, B. E.; Edwards, R. H.; Miller, R. E.; Kohler, G. O. Pilot Scale Ultrafiltration of Clarified Alfalfa Juice. *Journal of Food Science* **1980,** *45* (3), 730-734.

35. Libouga, D. G.; AguieBeghin, V.; Douillard, R. Thermal denaturation and gelation of rubisco: Effects of pH and ions. *International Journal of Biological Macromolecules* **1996,** *19* (4), 271-277.

36. Lu, P. S.; Kinsella, J. E. Extractability and Properties of Protein from Alfalfa Leaf Meal. *Journal of Food Science* **1972,** *37* (1), 94-99.

37. Dijkstra, D. S.; Linnemann, A. R.; Van Boekel, T. A. J. S. Towards Sustainable Production of Protein-Rich Foods: Appraisal of Eight Crops for Western Europe. PART II: Analysis of the Technological Aspects of the Production Chain. *Critical Reviews in Food Science and Nutrition* **2003,** *43* (5), 481-506.

38. Rackis, J. J.; Sessa, D. J.; Honig, D. H. Flavor Problems of Vegetable Food Proteins. *Journal of the American Oil Chemists Society* **1979,** *56* (3), 262-271.

39. Intarapichet, K. O. Off-flavors in foods: 3. Chemical changes. *Suranee J. Sci. Technol* **1996,** *3* (1), 21-29.

40. Fiorentini, R.; Galoppini, C. Pilot-Plant Production of An Edible Alfalfa Protein-Concentrate. *Journal of Food Science* **1981,** *46* (5), 1514-1517

41. Mellor, N.; Bligh, F.; Chandler, I.; Hodgman, C. Reduction of Off-Flavor Generation in Soybean Homogenates: A Mathematical Model. *Journal of Food Science* **2010,** *75* (7), R131-R138.

42. Arora, A.; Damodaran, S. ß-Cyclodextrin-Mediated Removal of Soy Phospholipids from the Air-Water Interface. *JAOCS J Am Oil Chem Soc* **2011,** *88* (2), 213-222.

43. Maheshwari, P.; Ooi, E. T.; Nikolov, Z. L. Off-Flavor Removal from Soy-Protein Isolate by Using Liquid and Supercritical Carbon-Dioxide. *Journal of the American Oil Chemists Society* **1995,** *72* (10), 1107-1115.

44. Park, J. Y.; Lee, M. K.; Uddin, M.; Chun, B. S. Removal of off-flavors and isolation of fatty acids from boiled anchovies using supercritical carbon dioxide. *Biotechnol. Bioprocess Eng.* **2008,** *13* (3), 298-303.

45. Pedone, S.; Selvaggini, R.; Fantozzi, P. Leaf protein availability in food: Significance of the binding of phenolic compounds to ribulose-1,5-diphosphate carboxylase. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie* **1995,** *28* (6), 625-634.

46. Fu, T. J.; Abbott, U. R.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - A comparative study. *J. Agric. Food Chem.* **2002,** *50* (24), 7154-7160.

47. Jeurink, P. V.; Savelkoul, H. F. J. Induction and regulation of allergen-specific IgE. In ***Volume 10 Allergy Matters: New Approaches to Allergy Prevention and Management***, Gilissen, L. J. E. J., Wichers, H. J., Savelkoul, H. F. J., Bogers, R. J., Eds.; 2006.

48. Genkov, T.; Spreitzer, R. J. Highly Conserved Small Subunit Residues Influence Rubisco Large Subunit Catalysis. *Journal of Biological Chemistry* **2009,** *284* (44), 30105-30112.

49. Kapel, R.; Rahhou, E.; Lecouturier, D.; Guillochon, D.; Dhulster, P. Characterization of an antihypertensive peptide from an Alfalfa white protein hydrolysate produced by a continuous enzymatic membrane reactor. *Process Biochem.* **2006,** *41* (9), 1961-1966.

50. Kitts, D. D.; Weiler, K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003,** *9* (16), 1309-1323.

51. Yang, Y.; Marczak, E. D.; Usui, H.; Kawamura, Y.; Yoshikawa, M. Antihypertensive Properties of Spinach Leaf Protein Digests. *J. Agric. Food Chem.* **2004,** *52* (8), 2223-2225.

52. Makino, A.; Sakashita, H.; Hidema, J.; Mae, T.; Ojima, K.; Osmond, B. Distinctive Responses of Ribulose-1,5-Bisphosphate Carboxylase and Carbonic-Anhydrase in Wheat Leaves to Nitrogen Nutrition and

Their Possible Relationships to Co2-Transfer Resistance. *Plant Physiology* **1992,** *100* (4), 1737-1743.

53. Wikipedia, Internet Communication 2011.

54. Yang, S.; Yunden, J.; Sonoda, S.; Doyama, N.; Lipkowski, A. W.; Kawamura, Y.; Yoshikawa, M. Rubiscolin-6, a δ opioid peptide derived from spinach Rubisco, has anxiolytic effect via activating σ1 and dopamine D1 receptors. *FEBS Lett.* **2001,** *509* (2), 213-217.

55. Zhao, H.; Ohinata, K.; Yoshikawa, M. Rubimetide (Met-Arg-Trp) derived from Rubisco exhibits anxiolytic-like activity via the DP1 receptor in male ddY mice. *Peptides* **2008,** *29* (4), 629-632.

56. Trovaslet, M.; Kapel, R.; Ravallec-Pl+¬, R.; Mouni, F.; Clarisse, M.; Faille, C.; Dhulster, P.; Guillochon, D.; Vercaigne-Marko, D. Secretagogue and bacteriostatic active fractions derived from a peptic hydrolysate of alfalfa RuBisCO small purified subunit. *J. Sci. Food Agric.* **2007,** *87* (3), 534-540.

57. Friedman, M. Nutritional value of proteins from different food sources. A review. *Journal of Agricultural and Food Chemistry* **1996,** *44* (1), 6-29.

58. Saunders, R. M.; Connor, M. A.; Booth, A. N.; Bickoff, E. M.; Kohler, G. O. Measurement of digestibility of alfalfa protein concentrates by in vivo and in vitro methods. *Journal of Nutrition* **1973,** *103* (4), 530-535.

59. Schaafsma, G. The Protein Digestibility-Corrected Amino Acid Score. *The Journal of Nutrition* **2000,** *130* (7), 1865S-1867S.

60. Loomis, W. D. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods in enzymology* **1974,** *31*, 528-544.

61. Hernandez, T.; Martinez, C.; Hernandez, A.; Urbano, G. Protein quality of alfalfa protein concentrates obtained by freezing. *Journal of Agricultural and Food Chemistry* **1997,** *45* (3), 797-802.

62. Bowman, C. C.; Selgrade, M. K. Utility of rodent models for evaluating protein allergenicity. *Regul. Toxicol. Pharmacol.* **2009,** *54* (3 SUPPL.), S58-S61.

63. Ofori-Anti, A. O.; Ariyarathna, H.; Chen, L.; Lee, H. L.; Pramod, S. N.; Goodman, R. E. Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. *Regul. Toxicol. Pharmacol.* **2008,** *52* (2), 94-103.

64. LAHIRY, N. L.; SATTERLEE, L. D.; HSU, H. W.; WALLACE, G. W. characterization of the chlorogenic acid binding fraction in leaf protein concentration. *Journal of Food Science* **1977,** *42* (1), 83-85.

65. van de Velde F; Alting, A.; Pouvreau, L. Process for the isolation dechlorophyllized rubisco preparation from a plant material
. WO 2011078671, 2011.

66. Sarkar, S. K.; Howarth, R. E.; Hikichi, M.; Mcarthur, J. Soluble-Proteins of Alfalfa (Medicago-Sativa) Herbage - Fractionation by Ammonium-Sulfate and Gel Chromatography. *Journal of Agricultural and Food Chemistry* **1975,** *23* (4), 626-630.

67. Knuckles, B. E.; De Fremery, D.; Bickoff, E. M.; Kohler, G. O. Soluble protein from alfalfa juice by membrane filtration. *Journal of Agricultural and Food Chemistry* **1975,** *23* (2), 209-212.