

DEGRADATION CHANGES INDUCED BY HYDROXYL RADICALS IN BEAN (*PHASEOLUS VULGARIS*) AND PEA (*PISUM SATIVUM*) PROTEINS

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The oxidative modification of pea and bean proteins caused by hydroxyl radicals was investigated. The changes that occurred during the oxidation process were examined using fluorescence detection and electrophoretic methods. The modification of proteins resulted in a loss of Trp residues and a gain in its degradation product – formylkynurenine (FK); Tyr residues were oxidized to bityrosine (BT). The oxidative modification induced also the formation of protein carbonyl derivatives (PC). The degradation products were observed in minor amounts in pea proteins in comparison to bean proteins. The electrophoretic patterns of bean proteins showed the appearance of new fractions of high molecular weights corresponding to the polymers formation. On the contrary, hydroxyl radical-oxidized pea proteins exhibited a significant reduction in band surface.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen or peroxy radicals, are one of the major factors causing deterioration during the storage and processing of food. ROS preferentially oxidize lipids including polyunsaturated fatty acid, but they also attack other components, including proteins, saccharides and vitamins. In some circumstances, the degree of oxidation changes occurring in proteins may be greater than in lipids [Davies *et al.*, 1999].

Exposure of proteins to oxygen free radicals induces modifications of amino acid residues [Davies *et al.*, 1987; Gardner, 1979; Salvi *et al.*, 2001]. The effect of these disadvantageous interactions may also be the fragmentation of protein molecules or cross-links formation and aggregation of proteins, depending upon the nature of damage-sensitive components and the type of radical attacking [Gardner, 1979; Kamin-Belsky *et al.*, 1996]. Proteins are particularly sensitive to hydroxyl radical attack [Davies *et al.*, 1987].

The metabolites (intermediate products) arising in oxidative processes might be used as sensitive markers for the determination of the extent of protein damage. Amino acids such as Tyr, Trp, Phe and His easily undergo oxidation and some of their modification products may be used for the determination of the degree of protein changes. Products of Tyr, Trp and Phe degradation are well known, contrary to the compounds formed during His oxidation.

As an effect of the aromating ring cleft, oxidation of Trp causes formation of such compounds as kynurenine, FK, 5-hydroxytryptophan and several non-physiological components (dioxindolylalanine, oxindolylalanine) [Davies *et al.*, 1999; Simat & Steinhart, 1998]. Tyr oxidation product, BT, creates intra- or intermolecular (Protein₁-Tyr-Tyr-Protein₂)

protein bonds [Kato *et al.*, 2001]. Phe undergoes conversion to o-tyrosine residues. Many works (5 000 papers) have been devoted to the usage of carbonyl groups as protein oxidation markers [Levine, 2002]. Protein carbonyl derivatives are the effect of Lys-, Pro- and Arg-ended side chain oxidation [Stadtman *et al.*, 1992].

Oxidative modification of proteins may change their susceptibility to digestive enzymes action [Kamin-Belsky *et al.*, 1996; Liu & Xiong, 2000]. Metabolites formed from degraded proteins may also inhibit the activity of proteases [Stadtman *et al.*, 1992]. Products arising during protein oxidation are not utilized for protein synthesis [Nielsen *et al.*, 1985]. However, not all proteins are equally susceptible to the oxidation-caused damage. The studies describing food proteins degradation changes mostly concern proteins of animal origin [Kamin-Belsky *et al.*, 1996; Liu & Xiong, 2000; Martinaud *et al.*, 1997; Salvi *et al.*, 2001], and only very few regard vegetable proteins [Carbonaro *et al.*, 1996; Sanchez-Vioque *et al.*, 1999].

To determine the oxidative changes in proteins and the products formed in these reactions, the following methods were used: HPLC [Levine, 2002; Simat & Steinhart, 1998], GC-MS [Kell & Steinhart, 1990], SDS-PAGE [Martinaud *et al.*, 1997], and fluorimetric methods [Davies *et al.*, 1987; Rice-Evans *et al.*, 1991].

This work is a continuation of investigations into the usage of legume proteins as natural antioxidants. In the previous publications, it was stated that proteins derived from legume seeds possess good free radical-scavenging properties [Worobiej & Klepacka, 1999; Worobiej, 2001] and an ability to decrease the reaction rate of linoleic acid autooxidation [Wołoskiak & Worobiej, 1999].

In the present study, the degradation of bean and pea proteins caused by hydroxyl radicals generated in metal ion-

-catalysed oxidation systems (a Fenton-type reaction) were studied.

MATERIALS AND METHODS

The protein preparations were obtained from the coloured bean seeds, *i.e.* red bean (Red Kidney, Florpak, Poland) brown bean (Nida, IHAR Radzików, Poland), white bean (Prosna, IHAR Radzików, Poland), and pea (Poa, Łagiewniki, Poland). The seeds were dehulled, ground and sieved (100 mesh). The protein preparations were obtained by isoelectrical precipitation from basic (pH 9.2) extracts of the flour and centrifugation. The precipitates were re-dispersed in distilled water adjusted to pH 7.0 and lyophilized.

The changes in the proteins in the oxidation process were determined by the measurement of Trp fluorescence and Trp and Tyr oxidative breakdown of products' fluorescence, as well as the fluorescence of PC [Giełauf *et al.*, 1995; Rice-Evans *et al.*, 1991].

The protein samples were incubated at 37°C for 1, 2, 3, 48 h and 3, 7 and 10 days in the mixture generating hydroxyl radicals, containing H₂O₂ (2.5 mM), CuSO₄ (0.1 mM) in a phosphate buffer (pH 7.2).

All fluorescence measurements were performed with a Shimadzu RF-1501 spectrofluorophotometer. The excitation and emission wavelengths were 282/331 nm for Trp, 360/454 nm for formylkynurenine, 325/415 nm for bityrosine, and 350/450 nm for PC.

Sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis (SDS-PAGE) was used to monitor the subunit structure changes in oxidized proteins [Laemmli, 1970]. The proteins before electrophoretic assay were incubated at 37°C for 24 h in a hydroxyl radical-generating solution. The oxidation reaction was stopped by the addition of a synthetic chelator – diethylaminopentacetic acid (DETAPAC, Sigma) [Carbonaro *et al.*, 1996].

The proteins were stained with Coomassie Brilliant Blue R-250. The molecular weight reference proteins were: bovine albumin (66.0 kDa), egg albumin (45.0 kDa), trypsinogen (24.0 kDa), beta-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

RESULTS AND DISCUSSION

Oxidation of the proteins induced by hydroxyl radicals caused a loss of Trp residues. The changes in Trp content are shown in Table 1. The rate of Trp destruction was affected by the protein nature. Proteins of pea seeds were more susceptible to oxidation by hydroxyl radicals than bean proteins. The rate of Trp destruction increased to 55% and 36–46% in pea and bean proteins, respectively, after 3 h of oxidation, whereas at the end of the incubation period (10 days), 96% Trp residues were degraded in pea and 84–93% in bean proteins.

The oxidative modification of proteins by hydroxyl radicals leads to the formation of compounds such as FK. The results presented in Table 2 show the effect of FK formation after different exposure periods of proteins to hydroxyl radicals. The content of FK formed from Trp residues of bean proteins increased over 48 h of oxidation, but it decreased after 3 days and increased again upon prolonged incubation (7 and 10 days).

TABLE 1. Loss of tryptophan residues (Trp) of bean and pea protein preparations during incubation at 37°C with hydroxyl radicals.

Incubation time min	Loss of Trp [%]			
	PWB	PBB	PRB	PP
60	27.6 (±0.9)	36.4 (±0.6)	30.4 (±1.2)	41.4 (±0.7)
120	35.9 (±1.1)	41.4 (±0.4)	35.0 (±1.5)	50.7 (±0.5)
180	35.7 (±1.4)	45.9 (±0.4)	39.3 (±0.5)	54.7 (±1.5)
days				
2	64.0 (±1.7)	77.1 (±1.1)	77.5 (±1.2)	91.7 (±1.1)
3	72.5 (±1.2)	83.3 (±0.8)	88.3 (±0.2)	94.4 (±0.4)
7	81.8 (±0.7)	86.3 (±0.8)	92.0 (±1.9)	95.8 (±0.2)
10	83.7 (±1.6)	88.3 (±1.1)	93.1 (±1.6)	95.9 (±0.7)

PWB – protein preparation from white bean; PBB – protein preparation from brown bean; PRB – protein preparation from red bean; PP – protein preparation from pea. The results are means (±SD) of three determinations.

TABLE 2. Changes of formylkynurenine (FK) content in bean and pea protein preparations during incubation at 37°C with hydroxyl radicals.

Incubation time min	Gain of FK [%]			
	PWB	PBB	PRB	PP
60	45.1 (±2.2)	94.6 (±1.5)	127.7 (±2.9)	n.d.*
120	60.8 (±3.6)	113.7 (±4.0)	149.1 (±2.0)	n.d.
180	76.0 (±6.0)	124.4 (±2.4)	158.6 (±3.7)	n.d.
days				
2	166.7 (±5.5)	319.4 (±9.6)	186.4 (±1.9)	55.1 (±2.0)
3	156.8 (±5.9)	200.5 (±1.9)	157.6 (±7.6)	51.4 (±1.6)
7	175.3 (±2.9)	233.5 (±5.5)	163.7 (±3.6)	76.8 (±3.7)
10	227.7 (±10.9)	278.4 (±13.9)	207.5 (±14.1)	78.7 (±3.9)

PWB – protein preparation from white bean; PBB – protein preparation from brown bean; PRB – protein preparation from red bean; PP – protein preparation from pea. The results are means (±SD) of three determinations; * not detected.

Although loss of Trp residues in pea proteins was higher than in bean proteins, no significant changes in formation of FK from pea proteins occurred. During 3 h of oxidation, formation of FK in pea proteins was not detected and after 48 h the amounts of FK were significantly minor comparing to those in bean proteins. These data are likely due to differences of Trp amounts in globulin species, pea globulins contained less Trp than bean globulins [Bhatty, 1982].

An interesting observation is that decreased FK formation after 3 days of protein oxidation took place. One interpretation of these data is based on the fact that FK is formed in two phases [Giełauf *et al.*, 1995]. The initial phase coincided with converting Trp residues to Trp-peroxy radicals and Trp-hydroperoxides. During the second phase, Trp radicals are decomposed to FK and prolonged oxidation caused its degradation and formation of other compounds (dioxindolylalanine, oxindolylalanine) [Simat & Steinhart, 1998]. The re-increase of FK content after 7 days might be caused by decomposition of Trp radicals to FK in this period.

Among the products of oxidized protein decomposition there is also BT, a product of Tyr modification. The results presented in Table 3 demonstrate that the level of BT

TABLE 3. Changes of bityrosine (BT) content in bean and pea protein preparations during incubation at 37°C with hydroxyl radicals.

Incubation time	Gain of BT [%]			
	PWB	PBB	PRB	PP
min				
60	68.4 (± 3.1)	117.7 (± 1.7)	94.6 (± 2.6)	4.5 (± 1.0)
120	106.6 (± 4.1)	168.1 (± 2.6)	142.9 (± 4.6)	4.0 (± 1.5)
180	129.1 (± 2.5)	198.3 (± 4.3)	156.1 (± 2.3)	7.3 (± 1.6)
days				
2	194.4 (± 5.8)	298.3 (± 6.4)	148.8 (± 5.9)	158.5 (± 6.3)
3	36.3 (± 3.4)	n.d.*	62.2 (± 3.8)	n.d.
7	227.8 (± 4.0)	324.8 (± 11.1)	272.1 (± 5.8)	219.0 (± 5.5)
10	303.5 (± 3.4)	349.9 (± 8.1)	316.5 (± 9.2)	184.3 (± 7.9)

PWB – protein preparation from white bean; PBB – protein preparation from brown bean; PRB – protein preparation from red bean; PP – protein preparation from pea. The results are means (\pm SD) of three determinations; * not detected.

TABLE 4. Changes of protein carbonyls (PC) content in bean and pea protein preparations during incubation at 37°C with hydroxyl radicals.

Incubation time	Gain of PC [%]			
	PWB	PBB	PRB	PP
min				
60	34.9 (± 2.0)	67.5 (± 1.4)	69.6 (± 1.3)	n.d.*
120	47.9 (± 3.0)	88.8 (± 2.0)	86.7 (± 1.6)	n.d.
180	60.4 (± 5.0)	99.7 (± 1.4)	103.1 (± 4.2)	n.d.
days				
2	161.9 (± 2.7)	239.7 (± 7.6)	190.9 (± 3.8)	55.58 (± 2.0)
3	145.4 (± 6.7)	148.3 (± 6.5)	172.4 (± 6.6)	58.32 (± 3.9)
7	158.7 (± 6.8)	212.2 (± 6.8)	161.2 (± 5.2)	69.55 (± 5.0)
10	209.1 (± 10.5)	273.6 (± 7.0)	254.3 (± 9.0)	85.18 (± 6.3)

PWB – protein preparation from white bean; PBB – protein preparation from brown bean; PRB – protein preparation from red bean; PP – protein preparation from pea. The results are means (\pm SD) of three determinations; * not detected.

increased during 48 h of oxidation but a significant drop occurred during the following 3 days of incubation with hydroxyl radicals. However, after 7 and 10 days, BT content increased again. More BT was detected from the decomposition of Tyr than compounds formed from Trp residues; suggesting that Tyr is more susceptible to oxidation by hydroxyl radicals.

The hydroxyl radicals can modify some amino acid residues and convert them to PC. As shown in Table 4, PC content increased during 48 h-incubation of bean proteins with hydroxyl radicals, but at the same time PC from pea proteins were not observed. PC were not formed before 48 h-oxidation of pea proteins. These differences observed in the proteins of individual legume could result from small amounts of amino acid residues susceptible to oxidation, which can be converted to PC.

It is of particular interest, that brown bean proteins generated higher amounts of derivatives from Trp, Tyr and PC during oxidation than other varieties. The results of earlier studies showed that brown bean proteins were a more effective antioxidant towards hydroxyl radicals than other legume proteins [Worobiej & Klepacka, 1999]. Thus, the antiradical activity of brown bean proteins might explain

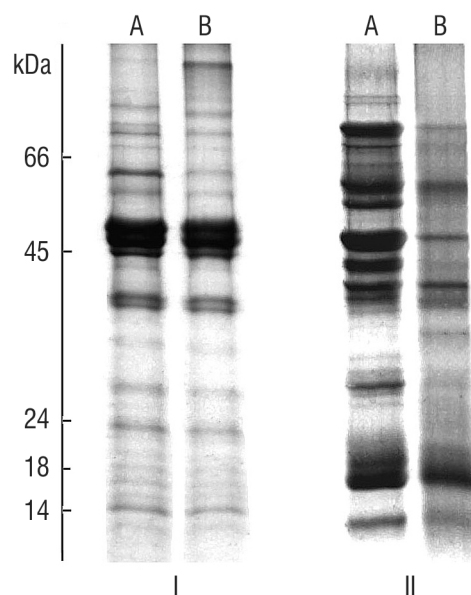


FIGURE 1. Gel electrophoresis patterns of bean (I) and pea (II) protein preparations (A – sample not incubated with hydroxyl radicals; B – sample incubated with hydroxyl radicals).

in the considerable destruction of this protein during the oxidative process.

Electrophoretic patterns of the protein preparations showed different degrees of degradation of individual variety-derived proteins (Figure 1). The bean-derived proteins showed insignificant changes upon hydroxyl radical action. There was a decrease in the band surface of about 66 kDa and about 22 kDa. Simultaneously, new fractions with lower electrophoretic mobility were formed. These bands appeared as the fraction of molecular weight above 66 kDa and dark stains and smears at the top of the separating gel. The electrophoretic patterns showed that oxidation did not induce fragmentation of bean proteins, but caused formation of polymers due to Tyr cross-linkages. On the contrary, SDS-PAGE results of hydroxyl radical-oxidized pea proteins exhibited much larger reduction in band surface than those of bean proteins. Thus, oxidation of pea proteins not only caused decreased band intensity, but also degradation of some high molecular weight bands and formation of new protein fragment with molecular weight of about 35 kDa. No polymers of pea proteins which could be formed by oxidation were detected by SDS-PAGE, but the formation of highly polymerized bands which could not pass the stacking gel is possible. Previous studies using SE-HPLC showed that incubation of pea proteins with hydroxyl radicals caused a significantly increased area of high molecular weight protein peak [Worobiej, 2000].

It is noteworthy that although the modification of pea proteins by hydroxyl radicals caused dramatic degradation of Trp and significant alteration in the structure of proteins, minor changes in the formation of Tyr and Trp modification products occurred.

CONCLUSION

To conclude, oxidative processes contribute to the subunit structure changes in legume proteins and protein oxidation also leads to the formation of degradation products derived

from Tyr and Trp residues. Pea proteins were more sensitive to oxidation than bean proteins.

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ZMIANY DEGRADACYJNE W BIAŁKACH NASION FASOLI (*PHASEOLUS VULGARIS*) I GROCHU (*PISUM SATIVUM*) POD WPLYWEM RODNIKÓW WODOROTLENOWYCH

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Badano modyfikację oksydacyjną białek fasoli i grochu pod wpływem działania rodników wodorotlenowych. Zmiany występujące podczas utleniania monitorowano poprzez pomiary fluorescencji i przy użyciu SDS-PAGE. Modyfikacja białek prowadziła do obniżenia zawartości tryptofanu (Trp, tab. 1) i formowania produktu jego degradacji – N-formylokinureiny (FK, tab. 2); reszty tyrozyny utleniane były do bityrozyny (BT, tab. 3). W wyniku reakcji białek z rodnikami wodorotlenowymi powstawały także pochodne karbonylowe (PC, tab. 4). Produkty degradacji generowane były w mniejszej ilości z białek grochu niż obserwowano to w przypadku białek fasoli. Elektroforetyczne rozdziały białek fasoli (rys. 1) wykazały pojawienie się nowych frakcji o wysokiej masie cząsteczkowej, które świadczą o tworzeniu się polimerów. W przeciwieństwie do tych zmian, w zmodyfikowanych przez rodniki wodorotlenowe białkach grochu stwierdzono znaczną redukcję powierzchni frakcji.