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Interaction of *Lupinus angustifolius* L. α and γ conglutins with 13-hydroperoxide-11,9-octadecadienoic acid

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Abstract

Food proteins suffer losses of functional and nutritional value due to reaction with lipid peroxidation products. The seed globulins conglutin α and γ from *Lupinus angustifolius* L. have been incubated with 13-hydroperoxide-11,9-octadecadienoic acid at pH 9 as a model of the interactions between seed storage proteins and lipid peroxidation products during storage and processing of protein-based products. The incubation lead to fragmentation and/or polymerization of the conglutins as determined by chromatographic and electrophoretic analysis. Losses of tryptophan, methionine, cysteine, proline, valine and leucine were shown by amino acid analysis. In vitro digestibility assays did not show clear differences between the digestibilities of the native conglutins before and after incubation with the hydroperoxide. Nevertheless, it is concluded that reaction with 13-hydroperoxide-11,9-octadecadienoic acid lead to a decrease in the nutritional value of the conglutins because the losses of essential amino acids were substantial. In addition, fragmentation and polymerization reactions most likely have a detrimental effect in the functional properties of the conglutins. These results highlight the deleterious effects that lipid peroxidation products have on products such as protein concentrates, isolates and hydrolysates obtained from lipid-rich seeds.
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Keywords: *Lupinus angustifolius*; Conglutin; Lipid–protein interaction; Lipid oxidation; Protein digestibility

1. Introduction

Up to now only four of the 300 existing *Lupinus* species have been domesticated. Three of them (*Lupinus albus*, *L. luteus*, and *L. angustifolius*) have their origin in the Mediterranean region, whereas the fourth, *Lupinus mutabilis* is derived from South America. Lupin as a grain legume is recognised as an important alternative source of highly nutritive, protein rich seeds. It is also a source of vegetable oil that compares favorably with other major crops such as soybean. Lupin seed proteins are relatively rich in cysteine and lysine. A study with laboratory animals showed that the nutritional value of a lupin seed-based diet (*L. angustifolius*) was substantially improved by methionine supplementation (Lilley, 1986). These data indicate the importance of having a thorough knowledge of the nature and levels of the various protein components in lupin seed, particularly those rich in sulphur amino acids (Lilley, 1986). Blagrove and Gillespie (1975) resolved the globulins of *L. angustifolius* into conglutins α, β and γ. The minor globulin, conglutin γ, which is of interest because of its high-level of the sulphur amino acids methionine and cysteine, has been characterised in detail (Blagrove, Gillespie, Lilley, & Woods, 1980; Duranti, Restani, Poniatowska, & Cerletti, 1981). Conglutin γ has a profile of essential amino acids far better than the major lupin seed proteins (Duranti et al., 1981), but it is resistant to in vitro proteolysis in its native conformation (Duranti, Gius, Sessa, & Vecchio, 1995)

Food proteins are often exposed to oxidants or oxidizing conditions during processing and storage. Oxidation of polyunsaturated fatty acids leads to the formation of hydroperoxides and their secondary products such as *n*-alkanals, 2-alkanals, hydroxyalkenals and malondialdehyde (Esterbauer, Cheeseman, Dianzani, Poli, & Slat, 1982; Schanenstein, Esterbauer, & Zollner, 1977). Proteins and amino acids in foods are susceptible to reaction with lipid hydroperoxides and their secondary products (Finley, Wheeler, & Witt, 1981; Gardner, 1979).

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The capacity of oxidizing compounds to react with the functional groups of proteins contributes to the destruction or reduction of flavor, color, and nutritional value. It can also cause toxicity in biological systems and it can be a basic pathological process in vivo (Faunes, Weiss, & Karel, 1982; Finley et al., 1981; Kanner, German & Matsuo, 1962; Richardson, 1984; Kinsella, 1987; Tappel, 1973). Among the negative effects produced by peroxidizing lipids in foods, their chemical interaction with proteins have received considerable attention (Hidalgo & Kinsella, 1989; Pokorny et al., 1988).

The conglutins α and γ of *L. angustifolius* have been purified and characterized in order to study their interaction with the 13-hydroperoxide that results from the oxidation of linoleic acid. Linoleic acid is a major fatty acid in *L. angustifolius* (Aguilera & Trier, 1978). The objective of this work was to determine the changes induced in conglutin α (11S) and conglutin γ (10S) by incubation with 13-LOOH. This incubation was carried out as a model of the reaction between proteins and lipid peroxidation products that occur in protein isolates of lupin during storage and processing. To our knowledge, this is the first report of this kind of reaction in lupin storage proteins.

2. Materials and methods

2.1. Material

Lupin seeds were a gift from Koipesol Semillas S.A. (Sevilla, Spain). Linoleic acid and lipoxygenase were purchased from Sigma (St. Louis, MO, USA). Blue dextran 2000, thyroglobulin, β-amilase, bovine serum albumin and ribonuclease were obtained from Amersham Pharmacia Biotechnology (Uppsala, Sweden). Diethyl ethoxymethylenemanolate was purchased from Fluka (Buchs, Switzerland).

2.2. Purification of globulins

In order to prepare the proteins for incubations, lupin seeds (100 g) were ground in a blender (particle size, 0.2–0.5 mm) and extracted under stirring using 1000 ml 0.1 M sodium borate buffer (pH 8.3) at room temperature for 1 h, according to the method of Singh, Rao, Singh, and Jambunathan (1988). The extract was centrifuged at 8000 $\times$ *g* for 15 min. The supernatant was dialyzed against 25 mM sodium citrate buffer (pH 4.6) at 4 °C for 15 h and centrifuged as before. The supernatant and pellet corresponded to the albumin and globulin fractions, respectively. The globulin precipitate was dissolved in 0.1 M sodium borate 0.2 M sodium chloride buffer (pH 8.3), dialyzed against 25 mM sodium citrate buffer (pH 4.6) at 4 °C for 15 h and centrifuged. The supernatant was discarded, and the precipitated legumin fraction was freeze-dried.

For further purification, the freeze-dried legumin fraction was dispersed by shaking in 0.1 M sodium borate and 0.2 M sodium chloride buffer (pH 8.3) (1:20 w/v), and insoluble materials were eliminated by centrifugation at 8000 $\times$ *g* for 15 min. The supernatant was eluted with 0.19 M sodium phosphate and 19 mM sodium citrate buffer (pH 7.0) in a PD-10 Sephadex G-25M column in order to remove non-protein compounds. The legumin solution was then loaded on a protein Pak DEAE 8 H 1000 A 8 μm exchange column (Waters, Milford, MA). Protein purification was carried out using a binary gradient system. The solvents used were as follows: buffer A, 0.19 M sodium phosphate, 19 mM sodium citrate buffer (pH 7.0); buffer B, 0.19 M sodium phosphate, 19 mM sodium citrate, 0.5 M sodium chloride buffer (pH 7.0). Solvents were delivered to the column as follows: time 0–10 min, elution with 100% buffer A; 10–25 min, linear gradient from 0 to 28% buffer B; 25–35 min, elution with 28% buffer B; 35–45 min, linear gradient from 28% to 100% buffer B; 45–55 min, elution with 100% buffer B; 55–60 min, linear gradient with 100% buffer A. Flow rate was 1 ml/min, and volume injection and sample concentration were 10 ml and 18 mg of protein/ml, respectively. Legumin enriched fractions were pooled for further purification by gel filtration chromatography.

Conglutin α and γ were obtained by gel filtration using a Superose 12 column. Elution was carried out using 25 mM sodium phosphate and 0.5 M sodium chloride buffer (pH 7.0) The flow rate was 0.5 ml/min. Volume injection and sample concentration were 500 μl and 8–10 mg of protein/ml, respectively. Conglutin α and γ were considered suitable for lipid-protein interaction studies as monitored by native PAGE and gel filtration chromatography.

2.3. Amino acid analysis

Samples (10 mg) were hydrolyzed with 4 ml of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC), according to the method of Alaiz, Navarro, Girón, and Vioque (1992), using D,L-α-aminobutyric acid as internal standard. The HPLC system consisted of a Model 600E multi-system with a 484 UV–vis detector (Waters) equipped with a 300 $\times$ 3.9 mm i.d. reversed-phase column (Novapack C_{18}, 4 μ; Waters). A binary gradient was used for elution with a flow of 0.9 ml/min. The solvents used were (A) sodium acetate (25 mM) containing sodium azide (0.02% w/v) pH 6.0 and (B) acetonitrile. Elution was as follows: time 0.0–3.0 min, linear gradient from A/B (91:9) to A/B (86/14); 3.0–13.0 min, elution with A/B (86/14); 13.0–30.0 min, linear gradient from A/B (86:14) to A/B (69/31);

30.0–35.0 min, elution with A/B (69:31). The column was maintained at 18 °C.

2.4. Preparation of 13-hydroperoxide-11, 9-octadecadienoic acid (13-LOOH)

13-LOOH was obtained according to Gardner (1975). The reaction mixture (480 ml) included 5.4 mM linoleic acid, 0.09% Tween 20, 5.0 mM borate, and 0.04 mg lipoxygenase/ml. The reaction solution was adjusted to pH 10 with KOH before lipoxygenase was added. In order to minimize by-product formation, care was taken to ensure good oxygenation by using a vessel with a sintered glass bottom through which pure oxygen was bubbled. The oxidation was allowed to proceed for 40 min at 21 °C, at which time pH was adjusted to 4.0. After extraction using diethyl ether, purification of 13-LOOH was carried out by silica gel thin-layer chromatography using diethyl ether–hexane–acetic acid (40:60:1) as the mobile phase. Fractions were collected by scrapping off the plates and elution with diethyl ether, and were monitored for 13-LOOH by measuring absorbance at 232 nm, characteristic of the conjugated diene. The resulting 13-LOOH was estimated to be 98% pure.

2.5. Incubation of conglutin α and γ protein with 13-LOOH

Conglutins (2 mg/ml) in 0.2 M sodium borate an 0.03% Triton X-100 buffer (pH 9.0) were mixed by sonication with 13-LOOH (ratio 1:2 w/w), bubbled with air for 15 min, and incubated at 37 °C for 48 h according to Millán and Vioque (1983).

2.6. In vitro protein digestibility

In vitro protein digestibility was determined according to the method of Hsu, Vavak, Satterlee, and Miller (1977). Samples containing 62.5 mg of protein were suspended in 10 ml of water and the pH was adjusted to 8.0. An enzymatic solution containing 1.6 mg trypsin (17.7 BAEE U mg^{-1}), 3.1 mg α-chymotrypsin (43 U mg^{-1}) and 1.3 mg peptidase (50 U g^{-1}) per ml was added to the protein suspension in a 1:10 v/v ratio. The pH of the mixture was measured after 10 min and the in vitro digestibility was calculated as a percentage of digestible protein using the equation:

$$(\%)\text{Digestible protein} = 210.464 - 18.103 \times \text{pH}$$

2.7. Electrophoresis

Native polyacrylamide gel electrophoresis (native PAGE) was performed using 0.75 mm thick gels and run at 20 mA in a Mini Protean unit (Bio-Rad, Richmond, CA). Gels were stained with 0.25% Coomassie Brilliant Blue G-250 in water/methanol/ acetic acid (5:5:1) for 2 h and destained first with water/methanol/ acetic acid (5:5:2) for 1 h and finally with 5% acetic acid. Gels were prepared as following: 4% (w/v) acrylamide/bis (97.3:2.7), 62 mM Tris (pH 6.8) for the stacking gel; and 8% (w/v) acrylamide/bis (97.3:2.7), 375 mM Tris (pH 8.8) for the separating gel. The runing buffer was 25 mM Tris and 190 mM glycine (pH 8.4). Protein samples (2 mg/ml) were mixed 1:1 (v/v) with a solubilization buffer containing 62 mM Tris, 10% glycerol, and 0.01% bromophenol blue (pH 6.8).

3. Results and discussion

3.1. Purification of L. angustifolius conglutin α and γ

L. angustifolius seed globulins were resolved by anion exchange chromatography into four main peaks, named: A, B, C, and D (Fig. 1). The composition of each peak was further analysed by gel filtration chromatography. Fraction A, which included the proteins not bound to the ion exchange column, corresponds to conglutin γ, with molecular weight of 97 kDa (Fig. 2a). Similar results were obtained by Blagrove and Gillespie (1975). Fraction B and C from ion exchange chromatography showed a similar gel filtration pattern with a main peak corresponding to α conglutin (11S) with 216 kDa apparent molecular weight (Fig. 2b, c). Higher molecular weight proteins in the eluate could be aggregates of conglutin α because this protein polymerizes easily (Sánchez-Vioque et al., 1999; Wolf & Nelson, 1996). Fraction D, eluted with 58% 1M NaCl (Fig. 1), is a minor fraction. It is composed of two main peaks (Fig. 2d), the first corresponding to conglutin α, and the

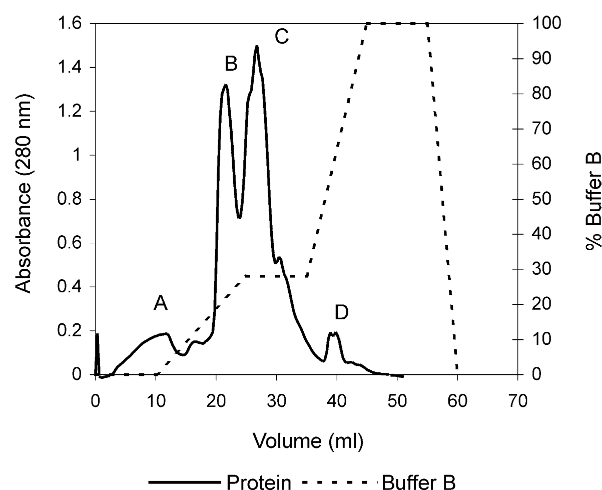

Fig. 1. Ion exchange chromatography of the globulins of *Lupinus angustifolius*. Fraction A (conglutin γ); fraction B and C (conglutin α soluble) and fraction D (conglutin α insoluble).

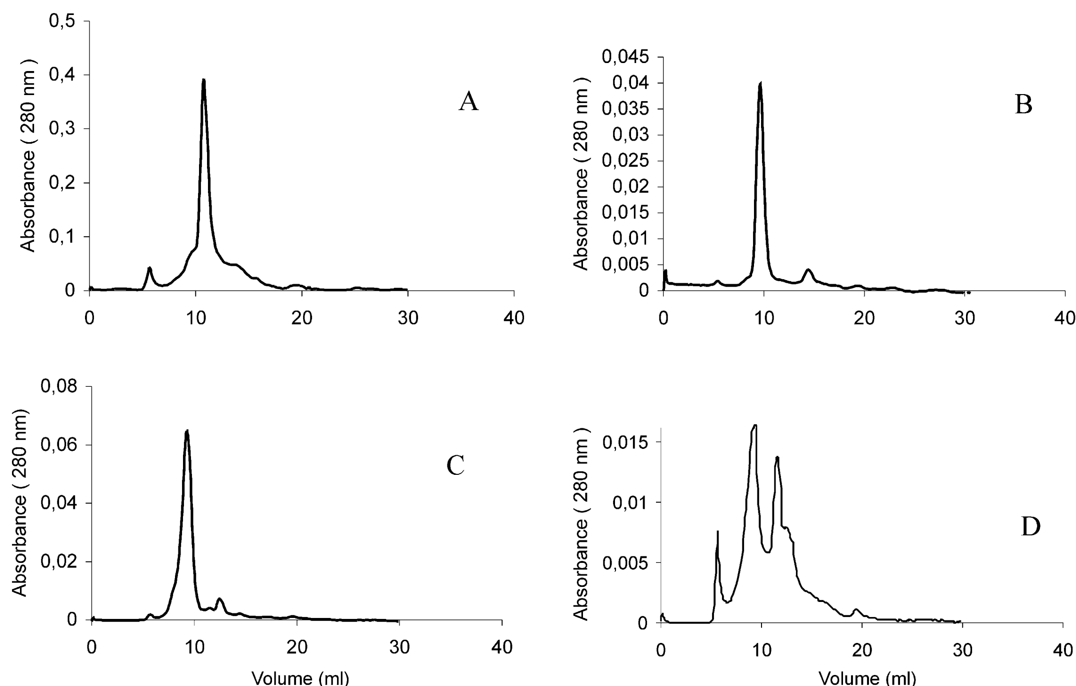

Fig. 2. Gel filtration chromatography of the fractions A to D obtained by ion exchange chromatography. Conglutin γ (A); Soluble conglutin α (B and C); insoluble conglutin α (D).

second peak probably corresponding to the αβ subunits of conglutin α with an apparent molecular weight of 60 kDa (Sánchez-Vioque et al., 1999). According to Blagrove and Gillespie, (1975), peaks B and C in Fig. 1 correspond to soluble conglutin α and peak D corresponds to insoluble conglutin α.

3.2. Amino acid composition of conglutins

Conglutins α and γ have different amino acid profiles (Table 1). Conglutin α is rich in glutamic acid, while conglutin γ has less arginine and glutamic acid and more sulphur-containing amino acids (methionine and cysteine). The content of essential amino acids in conglutin γ is similar to the FAO/WHO reference protein requirement (1991) except for lysine. From a nutritional point of view, it is better than conglutin α, because of its higher content of cysteine, methionine, threonine and valine.

3.3. Amino acid composition of conglutins after incubation with 13-LOOH

Considering that lupin conglutins are relatively poor in sulphur-containing amino acids, any decrease in the bioavailablity of these amino acids may decrease the nutritional value of the protein. Reaction of conglutin α with 13-LOOH caused a significant loss in the content of the amino acids tryptophan, methionine, cysteine, proline and valine (Table 1). A smaller loss was observed in leucine. After incubation of conglutin α with 13-LOOH the methionine content was reduced to less than half the original value. This loss is probably due to the conversion of methionine to its sulfoxide, because the latter is readily formed by reaction of proteins with oxidizing lipids (Cuq, Besancon, Chartier, & Cheftel 1978; Nielson, Loliger, & Hurrel, 1985). Losses of methionine residues are especially significant since conglutin α is relatively poor in this amino acid (FAO/WHO, 1991). Cysteine residues were also affected by incubation with 13-LOOH, and decreased to about half the original value. This loss of cysteine is probably due to the transformation of cysteine to cysteinyl sulfoxide (Gardner & Jursinic, 1985).

The amino acid composition of conglutin γ was less afected by the incubation with 13-LOOH. Thus, only tryptophan residues were clearly lost by incubation with the oxidized lipid. This is consistent with other reports showing that tryptophan residues are very susceptible to degradation by reaction with peroxidizing lipids (Gardner, 1979; Hidalgo et al., 1989; Kanazawa, Danno, & Natake, 1975).

3.4. Electrophoretic and column chromatography analysis of conglutins after reaction with 13-LOOH

Native PAGE showed that α and γ conglutin were highly modified after incubation with 13-LOOH. The band corresponding to native conglutin α in the control disappeared after incubation with 13-LOOH and was sustituted by a smear of proteins along a wide molecular weight range (Fig. 3). Similar results were observed with

Table 1
Amino acid composition of native conglutin α and conglutin γ and before and after incubation (24 h) with 13-hydroperoxide (data, expressed as % Molar of amino acids are the mean±SD of two analyses)

	FAO[a]	Native α-conglutin	LOOH modified α-conglutin	Native γ-conglutin	LOOH modified γ-conglutin
Aspartic acid[b]		8.4±0.9	9.9±1.7	8.7±1.3	8.5±1.1
Glutamic acid[c]		20.2±1.4	23.9±1.1	9.0±1.2	10.7±2.9
Serine		7.8±0.0	7.5±0.0	9.1±1.3	8.4±0.6
Histidine	1.9	1.6±0.2	1.8±0.1	3.2±0.7	2.9±0.4
Glycine		7.2±0.1	7.5±0.6	9.8±0.9	9.7±0.9
Threonine	3.4	4.6±0.2	4.6±0.4	7.9±0.4	7.4±0.1
Arginine		8.9±1.0	8.9±0.1	3.6±0.1	4.6±1.0
Alanine		4.4±0.6	4.9±0.1	6.1±0.3	6.5±0.7
Proline		3.3±0.1	1.5±0.1	1.3±1.2	0.8±0.7
Tyrosine	6.3[d]	4.3±0.2	4.1±0.1	3.5±0.1	3.2±0.3
Valine	3.5	5.1±1.9	2.1±1.4	12.1±6.1	12.3±5.8
Methionine	2.5[e]	1.3±0.0	0.5±0.1	3.0±0.0	2.2±0.9
Cysteine		0.9±0.3	0.5±0.1	1.5±0.0	1.0±0.6
Isoleucine	2.8	4.4±0.4	4.8±0.5	3.9±0.2	4.7±0.6
Leucine	6.6	10.8±1.6	8.8±0.1	9.0±0.1	9.1±0.2
Phenylalanine		3.9±0.4	3.9±0.3	4.1±0.1	4.4±0.4
Lysine	5.8	3.4±0.0	3.2±0.2	5.6±0.1	5.0±0.5
Tryptophan		0.5±0.0	0.0	0.5±0.0	0.1±0.0

[a] FAO/WHO Energy and protein requirement (1991).
[b] Aspartic acid + asparagine.
[c] Glutamic acid + glutamine.
[d] Tyrosine + phenylalanine.
[e] Methionine + cysteine.

conglutin γ. This is consistent with reports showing that reactions between oxidized lipids and proteins cause fragmentation of proteins (Matoba, Kurita, & Yonezawa, 1984), cross-linking of protein chains (Sánchez-Vioque et al., 1999) and chemical modification by reaction with secondary products of lipid oxidation (Chio & Tappel, 1969).

Protein damages resulting from the incubation with 13-LOOH were also studied by gel filtration chromatograhpy (Fig. 4). The profile in Fig. 4 shows that conglutins α and γ were highly modified by incubation with 13-LOOH, as observed also by native PAGE. The original peaks corresponding to α and γ conglutins shown in Fig. 2, dissappeared and were substituted by others of higher or lower molecular weight. Similar results were obtained by Millán and Vioque (1985) studing the reaction between BSA and hydroperoxide. These modifications were most likely due to cleavage and cross-linking of the proteins.

3.5. In vitro digestibility of modified conglutins

Protein digestibility may be altered by reaction with lipid peroxidation products, causing a reduction in nutritional value (Sánchez-Vioque et al., 1999). Nevertheless, incubation with 13-LOOH did not affect substantially the in vitro digestibility of conglutin α and γ as determined by incubation with digestive enzymes. After 24 h incubation with 13-LOOH, the in vitro protein digestibility of conglutin α and γ were similar to control values (Table 2). Similar proteins undergo a large decreased of in vitro digestibility when incubated with hydroperoxides. This is the case of chickpea legumin, which after incubation with 13-LOOH reduces it digestibility from 84.1 to 69.2% (Sanchez-Vioque et al., 1999).

4. Concluding remarks

Studies on the negative effects of oxidized lipids on protein digestibility are scarce, although changes in digestibility have been related to formation of protein polymers (Kamin-belsky, Brillon, Arav, & Shaklai, 1996; Millán & Vioque, 1983). Incubation with 13-LOOH caused extensive alterations in α and γ conglutins as shown by chromatographic and electrophoretic analysis. It is possible that the loss of digestibility caused by cross-linking and polimerization reactions may be com-

Table 2
In vitro protein digestibility of native conglutin α and γ and after incubation with 13-LOOH. Data expressed as g of protein digested/100 g of protein are the mean±SD of two analyses

	% in vitro digestibility
Native conglutin α	69.17±0.09
LOOH-modified conglutin α	66.18±0.18
Native conglutin γ	68.90±0.72
LOOH-modified conglutin γ	66.99±0.27

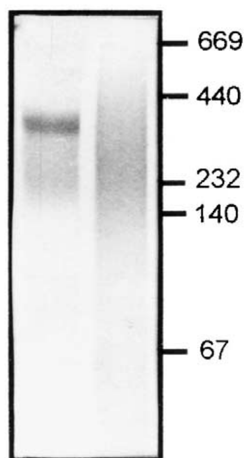

Fig. 3. Native-PAGE of conglutin α before (left) and after (right) incubation with 13-LOOH.

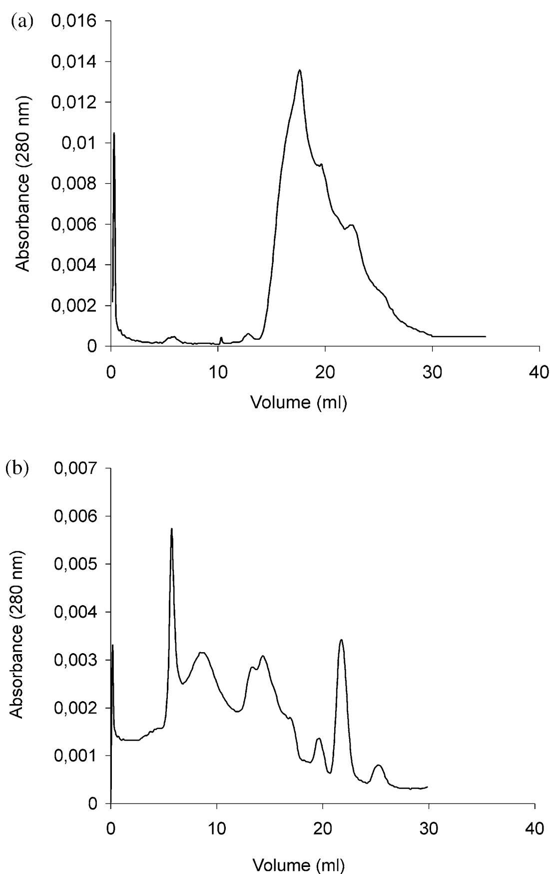

Fig. 4. Gel filtration chromatography of conglutin α (a) and γ (b) after incubation with 13-hydroperoxide.

pensated by fragmentation of the proteins, so that the overall digestibility is similar to controls. In any case, losses of essential aminoacids by incubation with 13-LOOH were very substantial, so that a decrease in nutritional value ocurred even though in vitro digestibility was not affected.

Acknowledgements

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