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Protein–lipid interactions during the incubation of whey proteins with autoxidizing lipids

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ABSTRACT

The changes induced in whey proteins due to incubation with oils containing different amounts of polyunsaturated fatty acids (fish, sunflower, soybean and olive oil) and with different initial oxidation status were studied under autoxidizing conditions. Changes upon incubation with fresh vegetable oils were very limited. Only upon incubation with fish or highly oxidized soybean oil, most significant changes with respect to lysine, histidine, phenylalanine and arginine residues occurred. Remarkably, the content of methionine and tryptophan remained unchanged under the experimental conditions. The most prominent impact of incubation of whey proteins with lipids was severe protein aggregation as shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

1. Introduction

It is well recognized that polyunsaturated fatty acids (PUFAs) can provide extensive nutritional and health benefits. Thus, ω-3 PUFAs have been considered to contribute to the prevention of several diseases such as coronary heart disease, hypertension, type 2 diabetes, rheumatoid arthritis, Crohn's disease and obstructive pulmonary disease (Simopoulos, 1999). Recognition of the potential benefits of ω-3 fatty acids has stimulated increased interest towards the fortification of foods with oils rich in these particular fatty acids (Betti et al., 2009; Iafelice et al., 2008; Ye, Cui, Taneja, Zhu, & Singh, 2009). However, enrichment of food products with such unsaturated fatty acids should be carefully evaluated since they are highly susceptible to oxidation. Nevertheless, oxidation of lipids in complex food matrices should not be considered as a separate series of oxidation reactions.

Refsgaard, Tsai, and Stadtman (2000) showed that fatty acid induced protein modifications such as generation of carbonyl derivatives and loss of reactive lysine residues are dependent on the degree of unsaturation of the fatty acids. They also reported that polyunsaturated fatty acid alkoxyl radicals formed during breakdown of lipid peroxides are likely to be involved in the formation of protein carbonyls. On the other hand, saturated and monounsaturated fatty acids are able to induce less or no protein oxidation at all (Refsgaard et al., 2000). Thus, exposure of proteins to peroxidizing lipids or their secondary breakdown products may induce severe changes in proteins, including polymerization, insolubilization and formation of lipid–protein complexes (Hidalgo & Kinsella, 1989). Several amino acids, mainly cysteine, methionine, histidine, tyrosine and lysine are affected by the secondary lipid oxidation products, therefore leading to reduced availability (Gardner, 1979).

Most of the studies of interactions between oxidizing lipids and proteins have been carried out between a model lipid, mostly linoleic acid or its peroxides, and model proteins, usually lysozyme, casein or bovine serum albumin (Hidalgo & Kinsella, 1989; Kanner & Karel, 1976; Matoba, Yoshida, & Yonezawa, 1982; Zamora & Hidalgo, 2003). The objective of this study was to characterize changes induced in a more complex whey protein isolate (a mixture of primarily α-lactalbumin and β-lactoglobulin) through interaction with autoxidizing lipids. Whey protein was chosen as a reaction system because whey protein isolates are widely used as low-cost protein-rich ingredients in foodstuffs due to their high content of essential amino acids and versatile functionality (Liu, Xiong, & Butterfield, 2000). The impact of the oxidative susceptibility and the oxidation status of the oils on the interaction between the proteins and the autoxidizing lipids were investigated.

2. Materials and methods

2.1. Reagents and standards

Whey protein isolate (Lacprodan® DI-9224) was kindly provided by Acatris Food Belgium (Londerzeel, Belgium). Food grade oils

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were purchased from a local store. Chemicals and standards of analytical grade were obtained from Sigma–Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium). Analytical grade solvents were purchased from Chem-Lab (Zedelgem, Belgium) except for hexane which was of technical grade. Gel and standards for SDS-PAGE were from Bio-Rad (Nazareth, Belgium) while the Krypton protein stain was provided by Thermo Scientific (Aalst, Belgium).

2.2. Oxidation of the oils used in the reaction systems

Soybean oil was oxidized under UV light (235 nm) at 35 °C for up to 48 h. Oxidation status of the oils used in the reaction systems was followed by iodometric determination of the peroxide value (AOCS, 1989b). The amount of secondary oxidation products in the oils was determined by measuring their *p*-anisidine values (p-AV) (AOCS, 1989a).

2.3. Preparation of the reaction systems

The reaction systems were prepared in 50 mM 3-morpholinopropanesulfonic acid (MOPS) pH 7.4 by mixing 1% (w/v) oil with 2% (w/v) whey protein isolate in a vortex mixer for 2 min. The reaction systems in sealed falcon tubes were incubated at 70 °C in the presence of 10 μM copper sulphate solution to stimulate the oxidation and 0.2 g L^{-1} sodium azide to prevent microbial growth. A reaction system without oils was used as control. At different periods of time, subsamples were removed for analytical measurements. First, samples were defatted 4 times with technical hexane to remove free lipids. Next, the defatted fractions were collected and precipitated with trichloroacetic acid (TCA, 10% final concentration) on ice. After centrifugation, the precipitated proteins were finally redissolved in 2% sodium dodecyl sulphate (SDS) and the pH was adjusted to approximately 10 with 10 M NaOH to facilitate redissolving the pellet. Any undissolved particles were removed by centrifugation at 10,000 × *g* for 10 min. The clear supernatant was immediately used for the determination of the protein content, free and total thiol groups, reactive lysine residues and protein bound carbonyls. Aliquots for amino acid composition determination and SDS-PAGE were stored at −20 °C until further use.

2.4. Fatty acid composition

The determination of the fatty acid composition was done by means of gas chromatography (AOCS, 1989c). The triacylglycerols were saponified with a methanolic NaOH solution. Subsequently, the fatty acids were esterified with BF_3/MeOH – reagent in the presence of sodium hydroxide. The methyl esters were further separated using the gas chromatographic equipment 6890N (Agilent Technologies, Diegem, Belgium) equipped with a flame ionization detector. The capillary column was a CP-Sil 88 from Varian (Sint-Katelijne-Waver, Belgium). The cold on column injection was used and the detector was operated at 300 °C. The oven was programmed as follows: 50 °C for 4 min, which was raised to 120 °C (10 °C min^{-1}) and further to 163 °C (5 °C min^{-1}) and held at 163 °C for 19 min. Finally the temperature was raised to 225 °C (10 °C min^{-1}) and held for another 16 min. Helium was used as carrier and make up gas. Fatty acid composition of the oils was calculated from the peak areas using nonadecanoic acid as internal standard.

2.5. Protein bound carbonyls

Protein oxidation was measured by estimation of the amount of protein bound carbonyl groups after converting them to the corresponding 2,4-dinitrophenylhydrazones. Briefly, 300 μL defatted protein solution was 1 h incubated with 400 μL 2.4-dinitrophenylhydrazine (DNPH) 10 mM in 2 M HCl. Next, the proteins were precipitated with 10% trichloroacetic acid (final concentration). Because carbonyl group containing compounds derived from lipid oxidation products may increase the protein carbonyl values in the DNPH assay, the proteins were thoroughly washed with 1 mL ethanol/ethyl acetate (1:1) after derivatization. Hence, any unreacted DNPH or DNPH bound to any other components than protein was removed. Finally the pellet was redissolved in 6 M urea and absorbance was measured at 370 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer. The protein bound carbonyl content was calculated using a molar absorption coefficient of 22,000 m^{-1} cm^{-1} and results were expressed as nmol mg^{-1} protein.

2.6. Free and total thiol group content

The oxidation of the sulphur containing amino acids was monitored by their derivatization with 5.5′-dithiobis-2-nitrobenzoate (DTNB) as previously described (Beveridge, Toma, & Nakai, 1974). Total thiol groups were determined in a similar manner after a previous reduction of the disulphide bridges with β-mercaptoethanol. Free and total thiol groups were calculated using a molar absorption coefficient of 13,600 m^{-1} cm^{-1}.

2.7. Loss of reactive lysine

The loss of reactive lysine was monitored using derivatization with *o*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol, which yields a fluorescent product with a maximum excitation wavelength of 340 nm and emission of 450 nm (Ferrer et al., 2003) using a Spectramax Gemini XPS fluorimeter (Molecular Devices, Brussels, Belgium). A standard of β-casein was used to prepare a calibration curve and samples were pre-treated and analyzed using a fluorimeter as described previously by Morales, Romero, and Jimenez-Perez (1996).

2.8. Protein determination

Protein content was determined according to the Kjeldahl procedure (AOAC, 1981). A factor of 6.38 was used to convert nitrogen to whey protein. The non protein nitrogen (NPN) was determined in the supernatant after a previous protein precipitation with 15% TCA (final concentration).

2.9. Amino acid analysis

Amino acid analysis of the protein samples was carried out after acid or basic hydrolysis. A treatment with 34 mM $NaBH_4$ for 4 h at 37 °C was performed to stabilize the protein–lipid adducts and to make them resistant to acid hydrolysis (Sanchez-Vioque et al., 1999). Further, the samples were hydrolyzed with 6 M HCl for 24 h at 110 °C. For tryptophan determination alkaline hydrolysis was performed with 4.2 M NaOH for 24 h at 110 °C. Hydrolysates were neutralized with NaOH or HCl to bring the pH to 2.2 and 4.25, respectively. The high performance liquid chromatography (HPLC) system employed consisted of an Agilent 1100 model (Agilent Technologies, Diegem, Belgium). The chromatographic column was ZORBAX Eclipse AAA Rapid Resolution column 4.6 × 150 mm, 3.5 micron (Agilent Technologies) operated at 40 °C at a flow rate of 2 mL min^{-1}. The chromatographic separation was achieved by injecting 0.5 μL sample and using a gradient elution of mobile phase A (40 mM NaH_2PO_4 with 0.2 g L^{-1} NaN_3 pH 7.8) and mobile phase B (acetonitrile/methanol/water in a 45/45/10 ratio) and allowed