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Impact of trolox, quercetin, genistein and gallic acid on the oxidative damage to myofibrillar proteins: The carbonylation pathway

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ABSTRACT

The carbonylation pathway involves the oxidative deamination of lysine residues to yield a carbonyl compound (α-aminoadipic semialdehyde) that can be further oxidised to α-aminoadipic acid and form Schiff bases structures. The effect of trolox and other phenolic compounds (PhC) (namely genistein, quercetin and gallic acid) on the protein carbonylation pathway occurred during the oxidation of myofibrillar proteins (MP) catalysed by a Fe^{3+}/H_2O_2 system was studied. Trolox and PhC can exert either antioxidant or pro-oxidant capacities depending on their concentration, the oxidation conditions and the target in proteins. In general, quercetin and genistein showed an antioxidant activity towards lipid oxidation and the carbonylation pathway at different concentrations under the analysed conditions. Plausible mechanisms for the antioxidant and pro-oxidant effects of trolox and PhC on MP are discussed. Further research is needed to shed light on the effect of PhC mixtures on both lipid and protein oxidation.

1. Introduction

Lipid oxidation has been considered the one of the most important threats to food quality as it leads to formation of rancid odours, colour changes and potentially toxic compounds (Ventanas, Estévez, Tejeda, & Ruiz, 2006). Consequently, the mechanisms by which lipids oxidised have been studied in detail. In contrast, the analogous reactions by which proteins undergo oxidation are poorly characterised despite the fact that proteins have been identified as possible targets of reactive oxygen species (ROS). Due to their structure, certain amino acids residues, such as tryptophan, histidine, proline, lysine, cysteine, methionine, and tyrosine, are particularly prone to oxidation leading to various chemical modifications on proteins such as loss of sulfhydryl and amino groups and the formation of carbonyl compounds (Stadtman, 1993). Increasing interest has been focused on protein carbonylation since it has been highlighted as the most remarkable modification in oxidised proteins and the major outcome of direct oxidative attack to proteins (Estévez, 2011). As reviewed by Stadtman (1993) proteins undergo metal catalysed oxidation (MCO) in presence of transition metals, such as iron and copper, whereby proline, arginine and lysine are preferentially oxidised. Lysine residues are oxidised into the α-aminoadipic semialdehyde (AAS), while proline and arginine residues are degraded to the γ-glutamic semialdehyde (GGS) (Requena, Chao, Levine, & Stadtman, 2001). In food and biological systems, AAS and GGS have been found to account up to 60% of the total of the protein carbonyls and hence, they are considered suitable protein oxidation markers (Requena et al., 2001; Utrera, Morcuende, Rodríguez-Carpena, & Estévez, 2011).

Protein carbonyls may be involved in advanced oxidative reactions due to the highly reactive moieties found in their structure (Estévez, 2011). AAS, in particular, can be further oxidised into the α-aminoadipic acid (AAA) (Utrera & Estévez, 2012b; Utrera, Rodríguez-Carpena, Morcuende, & Estévez, 2012). In addition, proteins may cross-link by the reaction between a carbonyl moiety from a AAS residue and an ε-amino group from a neighbouring protein-bound amino acid or with another protein-bound AAS residue to form a covalent bond via Schiff base (SB) or aldol condensation structure, respectively (Estévez, 2011; Utrera et al., 2012). Hence, the complete oxidation route of lysine, recently described as the carbonylation route, involves the oxidative deamination of the original amino acid to yield a primary carbonyl product that may eventually be degraded into the corresponding carboxylic acid or be involved in the formation of Schiff bases (Utrera & Estévez, 2012b).

It is generally accepted that oxidative damage to proteins may be responsible for an impaired functionality (Estévez, 2011). In this regard, Utrera and Estévez (2012b) recently shed light on the potential impact of the chemical modification occurred during the carbonylation route on the functional properties of myofibrillar proteins (MP) oxidised by Cu^{2+}, Fe^{3+}, and Mb in combination with H_2O_2. According to their findings, intense protein carbonylation leads to fast and severe impaired water-holding, foaming and

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gelling capacities (Utrera & Estévez, 2012b). Likewise, the production of AAA and Schiff bases took place under stronger oxidation conditions, compromising to a larger extent the solubility of MP and worsening the aggregation and their gelling capacity (Utrera & Estévez, 2012b). These results reinforce the need of seeking and developing effective strategies for controlling protein oxidation. In addition, the growing concern among consumers over the safety of using synthetic antioxidants has resulted in a demand for natural antioxidant resources.

Among the natural antioxidant resources, α-tocopherol (vitamin E) has been proved to confer effective antioxidant protection mainly when used as a dietary supplement (Ventanas et al., 2006). 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox), a water-soluble analogue of α-tocopherol, has shown a higher antioxidant capacity than α-tocopherol (Cort et al., 1975) that may be attributed to the ability of trolox to interact with both water and lipid phases. Recently, phenolic compounds (PhC), a widely spread group of functional phytochemicals in fruits and vegetables, have been associated with health benefits such as antioxidant, antimicrobial, antimutagenic, as well as anti-inflammatory activities (Heinonen, 2007). Therefore, the antioxidant activities of PhC and phenolic-rich extracts from different sources have been studied in several foods and food model systems (Brettonnet, Hewavitarana, DeJong, & Lanari, 2010; Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011; Kerry & Abbey, 1998; Record, Dreosti, & McInerney, 1995; Shahidi, 2000; Shahidi, Amarowicz, He, & Wettasinghe, 1997; Utrera & Estévez, 2012a). Whereas PhC have been shown to be effective at reducing lipid oxidation in a large variety of muscle foods, their effectiveness against protein oxidation is a subject of debate (Estévez, 2011). In a previous study, Utrera and Estévez (2012a) reported that PhC could exert both antioxidant and pro-oxidant effects on tryptophan oxidation mainly depending on their chemical structure. According to their findings, phenolic acids such as gallic acid acted as pro-oxidants in general, while PhC with a catechol structure and other flavonoids (i.e. quercetin and genistein) acted as antioxidants (Utrera & Estévez, 2012a). Since the oxidative deterioration of tryptophan is just one of the multiple expression of the oxidative damage to food proteins, further investigation are needed to clarify the effect of these PhC on the oxidative stability of food proteins.

The present paper aims to examine the overall effect of trolox and selected PhC namely genistein, quercetin and gallic acid, on the carbonylation pathway during the in vitro oxidation of miofibrillar proteins (MP) by iron (Fe^{3+}) and hydrogen peroxide. Genistein, quercetin and gallic acid were chosen since they are PhC with different chemical structures and are ubiquitously found in nature, while trolox was used as positive antioxidant control.

2. Materials and methods

2.1. Chemicals and meat supply

All chemicals and reagents used for the present work were purchased from Merck (Darmstadt, Germany) or Sigma Chemicals (Sigma–Aldrich, Steinheim, Germany). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA). Porcine meat (muscle longissimus dorsi) was obtained from a local slaughterhouse. Solutions were freshly prepared prior to use. All concentrations refer to final concentrations if not otherwise stated.

2.2. Extraction of MP

MP were extracted from porcine *longissimus dorsi* muscle according to the procedure used by Estévez, Kylli, Puolanne, Kivikari, and Heinonen (2008), with minor modifications. Minced muscle was homogenised for 30 s with 4 volumes (v/w) of a cold isolation buffer (10 mM potassium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM Ethylene glycol-bis (2-amino ethyl ether)-N,N,N′,N′-tetraacetic acid at pH 7). Samples were centrifuged (429*g* for 15 min at 4 °C), and the supernatant was discarded; the pellet was washed twice with 4 volumes (v/w) of the same buffer. Then, the myofibrillar pellet was washed three times with 4 volumes of 0.1 M NaCl. Before the third centrifugation, the myofibrillar suspension was filtered through gauze and the pH was adjusted to 6.0 with 0.1 M HCl. MP suspensions (5 mg/mL) were prepared in 15 mM piperazine-N,N′-bis(2-ethanesulphonic acid) (PIPES) (pH 6) buffer containing 0.6 M NaCl.

2.3. Oxidation systems

MP suspensions (5 mg/mL) were oxidised in vitro by the addition of iron (25 μM $FeCl_3$) in combination with hydrogen peroxide (2.5 mM) as oxidation promoters. To evaluate the effect of trolox and selected PhC namely genistein, quercetin and gallic acid, on the oxidative stability of MP, such compounds were assayed at three concentrations (10, 50 and 200 μM). The effect of the oxidation system on MP was also analysed in control samples (no added trolox or PhC). Three experimental units (replicates) were prepared for each of the oxidation systems. Suspensions were oxidised in the dark at 37 °C for 12 days with constant stirring. According to preliminary studies, the chosen conditions of time and temperature guarantee the oxidation of myofibrillar proteins. Sampling was carried out at days 0, 3, 6, 9, and 12 for analyses.

2.4. Lipid oxidation

Lipid oxidation was assessed by measuring the levels of thiobarbituric acid-reactive substances (TBARS) according to the method described by Utrera and Estévez (2012b). One millilitre of MP suspension was incubated with 1% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.50 mL) and 2.8% (w/v) trichloroacetic acid (0.50 mL) in a boiling water bath for 10 min. After cooling at room temperature for 20 min, the pink chromogen was extracted with n-butanol (2 mL) and its absorbance measured at 535 nm against a blank of n-butanol. TBARS concentrations were calculated using malondialdehyde (MDA) as standard which was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane. Results were expressed as milligrams of MDA equivalents per mg of protein.

2.5. Protein oxidation

2.5.1. HPLC-FLD analysis of AAS

Samples were derivatised with 50 mM aminobenzoic acid (ABA) and subsequently hydrolysed with 6 M HCl according to the procedure described by Utrera et al. (2011). An aliquot of MP suspensions (200 μL) was dispensed in 2 mL screw-capped eppendorf tubes. Proteins were precipitated with 2 mL of cold 10% TCA and subsequent centrifugation at 429*g* for 30 min. The resulting pellets were treated again with 2 mL of cold 5% TCA and proteins precipitated after centrifugation at 2683*g* for 5 min. Pellets were then treated with 0.5 mL of 250 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer (pH 6.0) containing 1% sodium dodecyl sulphate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL of 50 mM ABA in 250 mM MES buffer (pH 6.0), and 0.25 mL of 100 mM $NaBH_3CN$ in 250 mM MES buffer (pH 6.0). The derivatisation was completed by allowing the mixture to react for 90 min while tubes were immersed in a water bath at 37 °C and stirred regularly. The derivatisation reaction was stopped by adding 0.5 mL of cold 50% TCA followed by a centrifugation at 2683*g* for 5 min. Pellets were then washed twice with 1 mL of 10% TCA