α-Aminoadipic and γ-Glutamic Semialdehydes as Indicators of Protein Oxidation in Myofibrillar Proteins

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Abstract

In the present study, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) were used as indicators of the oxidative deterioration of myofibrillar proteins (MP). MP were extracted from porcine *longissimus dorsi*, suspended (20 mg protein/mL) in 15mM piperazine-N,N bis(2-ethane sulphonic acid) buffer (pH 6.0) containing 0.6M NaCl and oxidized with an iron-catalyzed oxidizing system (10 μM $FeCl_3$, 0.1 mM ascorbic acid, 1 mM H_2O_2) while kept in a oven at 37ºC for 12 days. Sampling (200μL of MP suspension) was carried out at fixed times for HPLC analysis. The samples were derivatized with ABA and the proteins subsequently hydrolyzed in the presence of 6N HCl. Both semialdehydes were detected in oxidized MP by using HPLC with fluorometric detection. Identification of both compounds was confirmed by running the same samples together with synthesized AAS and GGS standards in LC-ESI-MS. AAS and GGS increased over time during the oxidation essay with the highest level being detected at the end of the storage. According to our findings, both AAS and GGS could be used as indicators of protein oxidation in meat systems.

Introduction

Protein oxidation is a key topic of increasing interest amongst food scientists. The oxidation of myofibrillar proteins involves the loss of essential amino acids and the generation of oxidation products such as cross links (disulphide bonds, dityrosines), amino acid oxidized derivatives and protein carbonyls. The oxidation of myofibrillar proteins during processing and storage of meat products is associated with the loss of quality, leading to texture and color changes (1-3). The methods applied so far for assessing protein oxidation do not provide specific information about particular protein oxidation products. Amongst those, the quantification of carbonyls through derivatisation with dinitrophenylhydrazine (DNPH) and subsequent spectrophotometric measurement at 370 nm (4) has been generally accepted and commonly used in a large variety of muscle foods. However, so far, there are no reliable and consistent protein oxidation markers. α-Aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) are considered the main carbonyl products of metal-catalyzed oxidized proteins and have been highlighted as protein oxidation biomarkers. However, there is no information about the presence of these compounds in oxidized meat proteins and hence, the suitability of using these compounds as protein oxidation markers is unknown. The purpose of the present study was to detect AAS and GGS using HPLC/MS and evaluate their suitability as indicators of protein oxidation.

Material and methods

All chemicals were supplied by J.T Baker (Deventer, Holland), Riedel de-häen, and Sigma Aldrich (Steinheim, Germany). Porcine *longissimus dorsi* muscle was purchased in a local supermarket in Helsinki.

Methods

Extraction and in vitro oxidation of muscle proteins

MP were extracted from porcine *longissimus dorsi* muscle according to the procedure used by Park et al. (5). After the extraction, the myofibrillar protein isolated was stored in a tightly capped bottle, at 0ºC and used within 48 hours. MP (20 mg/mL) were suspended in 15mM PIPES buffer (pH=6) containing 0.6N sodium chloride and oxidized (10 μM $FeCl_3$, 0.1 mM ascorbic acid, 1 mM H_2O_2) while kept in a oven at 37ºC for 12 days.

Synthesis of AAS-ABA and GGS-ABA

Standard AAS and GGS were synthesized from N-α-acetyl-L-lysine and N-α-acetyl-L-ornithine, respectively, using lysyl oxidase activity of egg shell membrane following the procedure described by Akagawa et al. (6). Both compounds were derivatized as explained below and analyzed using LC-ESI-MS in a positive mode. The protonated molecular ions $[M+H]^+$ and their fragment ions were recorded.

Preparation and HPLC analysis of AAS and GGS

An aliquot (200 µL) of the oxidized protein suspensions was dispensed in a 2 mL-Eppendorf tube and proteins precipitated with cold 10% TCA. Samples were derivatised with p-aminobenzoic acid (ABA) in MES buffer according to the procedure described by Akagawa et al. (6). After several cleaning steps with ethanol:dietil eter (1:1), proteins were precipitated again and hydrolyzed with 6N HCl. Hydrosylates were dried *in vacuo*, reconstituted with 200 µL milli-Q water and filtered through PVDF syringe filter before HPLC analysis. Samples (30 µL) were injected in a HPLC with a C-18 reversed phase column eluted with 2.5% acetic acid water and methanol. AAS-ABA and GGS-ABA were detected by fluorescence (FLD) (Ex: 280; Em: 350 nm). Both compounds were also tentatively identified by LC-ESI-MS according to their m/z values and fragmentation pattern

Experimental design and statistical analysis

A Full Factorial design (Unscrambler Software, v.9.0, Camo Process AS) was chosen for evaluating the impact of oxidation time (0, 6 and 12 days), hydrolysis time (12, 18 and 24 hours) and amount of derivatisation agent (ABA) (50, 100 and 200 mM) on the levels of AAS and GGS detected by LC-MS.

Results and discussion

Both, AAS and GGS (Figure 1) were detected by using HPLC coupled to a FLD detector (Figure 2). Identification of both compounds was confirmed by using LC-MS. Peaks corresponding to AAS and GGS in the samples had the same retention time and molecular weight (GGS m/z: 253; AAS m/z: 267) than standard compounds. Additionally, for identification purposes, a fragmentation procedure (MS_3) was applied to both compounds, and those detected in the oxidized samples showed the same fragmentation pattern than standard compounds (GGS: 253→235→190 ions, AAS: 267→249→204 ions).

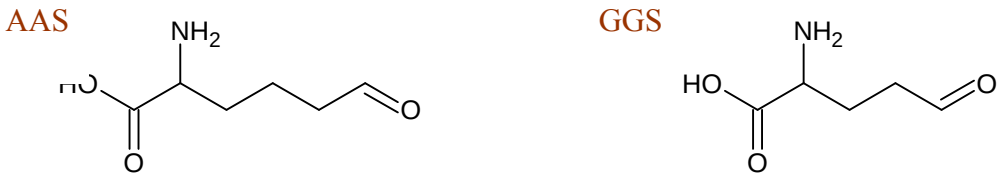

Figure 1. Chemical structure of AAS and GGS.

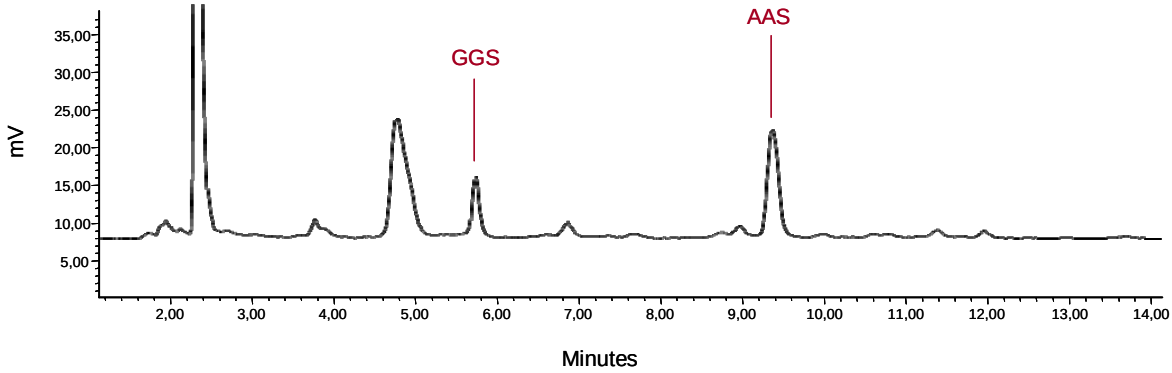

Figure 2. HPLC-FLD chromatogram of oxidized MP (Ex: 280; Em: 350 nm).

The oxidation time had a significant effect on AAS and GGS as long as the areas of both compounds increased during the oxidation essay (Figure 3, Table 1). AAS derives from lysine while GGS derives from arginine and proline (7). Both compounds are thought to comprise around 60% of total protein carbonyls derived from metal catalyzed protein oxidation (7). The same authors used both compounds as indicators of aging and diseases linked to protein oxidation. On the other hand, the amount of reagent had no impact on AAS and GGS. Increases in the amount of reagent (ABA) led to an increase of such compound in chromatograms while no significant increase of AAS or GGS was observed (Table 1). Therefore, the reagent at a concentration of 50 mM was not a limiting factor for the derivatisation of myofibrillar proteins under the conditions of the present experiment. The hydrolysis time had no effect on the compounds of interest (Figure 3). MP were fully

hydrolyzed after 12 hours in the oven. This finding is of great interest since the procedure for preparing samples is considerably time-consuming and by performing the shortest hydrolysis time (12 hours) the whole process will be shortened without affecting the detection of the two protein carbonyls. In conclusion, the method applied is effective for detecting AAS and GGS in oxidized meat proteins. The present results suggest that both oxidation products could be used as oxidation indicators in muscle proteins.

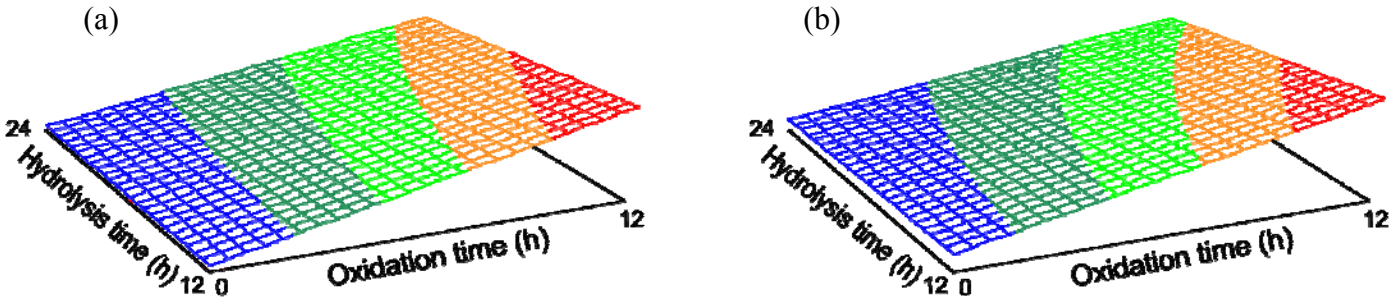

Figure 3. Effect of hydrolysis and oxidation time on mass detection of AAS-ABA (a) and GGS-ABA (b).

Table 1. Effect of studied factors on the detection of ABA, AAS and GGS by LC-MS.

	ABA	AAS	GGS
Oxidation Time (h)	ns	+++	++
Reagent (nM)	++	ns	ns
Hydrolysis Time (h)	ns	ns	ns

++: $p < 0.01$; +++: $p < 0.001$; Ns: Non-significant effect

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