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ESR studies on the thermal decomposition of trimethylamine oxide to formaldehyde and dimethylamine in jumbo squid (*Dosidicus gigas*) extract

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ABSTRACT

The effects of ferrous iron, heating temperature and different additives on the decomposition of trimethylamine oxide (TMAO) to formaldehyde (FA) and dimethylamine (DMA) and generation of free radicals in jumbo squid (*Dosidicus gigas*) extract during heating were evaluated by electron spin resonance (ESR). The thermal decomposition of TMAO to TMA, DMA and FA and free radical signals was observed in squid extract, whereas no DMA, FA and free radical signals were detected in cod extract or in aqueous TMAO solution *in vitro* at high temperatures. Significant increase in levels of DMA, FA and radicals intensity were observed in squid extract and TMAO solution in the presence of ferrous iron with increasing temperature. Hydrogen peroxide stimulated the production of DMA, FA and ESR signals in squid extract, while citric acid, trisodium citrate, calcium chloride, tea polyphenols and resveratrol had the opposite effect. Similar ESR spectra of six peaks regarded as amminium radical were detected in the squid extract and TMAO–iron(II) solution, suggesting that the amminium radical was involved in the decomposition of TMAO.

1. Introduction

Trimethylamine oxide (TMAO) is a natural osmolyte and an important non-protein nitrogen component in marine species. It is found in all marine fish species in quantities form 1% to 5% of the muscle tissue (dry weight) but is virtually absent from freshwater species (Chung & Chan, 2009; Hebard, Flick, & Martin, 1982). In marine products TMAO is one of the principal precursors of formaldehyde (FA) and dimethylamine (DMA). Extensive studies have shown that TMAO is decomposed to equimolar amounts of DMA and FA, catalysed by TMAO demethylase (TMAOase), in gadoid fish, crustaceans and squid during *post-mortem* frozen storage (Fu et al., 2006; Harada, 1975; Kimura, Seki, & Kimura, 2000; Phillippy & Hultin, 1993; Sotelo & Rehbein, 2000). The possibility of non-enzymic degradation of TMAO to DMA and FA by endogenous tissue catalysts, i.e. Fe^{2+}, Sn^{2+}, SO_2 and catabolites of cysteine (Spinelli & Koury, 1979, 1981), has been reported. Significant DMA and FA, accompanied by large amounts of TMA, were formed by non-enzymatic reactions in squid muscle (*Illex argentinus*) and extract (*Illex illecebrosus*) when heated (Kołodziejska, Niecikowska, & Sikorski, 1994; Nitisewojo & Hultin, 1986). Similarly, 90% of TMAO in five species of squid was converted to DMA and TMA after heating at 200 °C for 1 h (Lin & Hurng, 1985). FA, DMA, and TMA were continuously accumulated during the processing of squid, affecting its physicochemical properties (Chou, Chang, Lo, Lan, & Shiau, 2000). The difference in the thermal decomposition of TMAO between squid and gadoid was observed, indicating that FA, DMA, and TMA were gradually produced in squid, whereas TMA increased and FA decreased in gadoid (Kołodziejska et al., 1994; Zhu, Li, & Jia, 2012). Thus, TMAO in squid could form FA and DMA by at least two pathways, i.e. *via* enzymatic catalysis during frozen storage and *via* non-enzymatic breakdown during thermal processing (Zhu, Li, et al., 2012).

Jumbo squid (*Dosidicus gigas*) is one of the most abundant cephalopod species. Most of the undervalued squid species are processed into dried/seasoned squid or fried squid in many aquatic manufacturing industries owing to their acidic taste and ammonia-like smell. A large amount of squid products is consumed in east and south-east Asia, where many different squid products are available commercially. Recently, great attention has been paid to breakdown products of TMAO, especially FA, as important factors effecting the quality and safety of squid products. The high levels of DMA and FA in the various species of squid products is considered a serious toxicological problem (Lin, Lee, & Chang, 1983). In 2004, FA was classified in Group I as "carcinogenic to humans" by the International Agency for Research on Cancer (IARC). Because harmful FA and DMA are produced continuously during thermal processing of squid product, it is necessary to

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clarify the mechanism of non-enzymatic degradation of TMAO to improve the quality of squid products.

The application of electron spin resonance (ESR) spin-trapping techniques appears to be one promising approach for the detection of radicals produced and can serve as a valuable tool to increase understanding of the reaction mechanisms. Free radicals, *o*- and *p*-benzosemiquinone, in aqueous extracts of cigarette tar were detected by direct ESR (Zang, Stone, & Pryor, 1995).

Ferris, Gerwe, and Gapski (1968) reported that the non-enzymatic reaction of TMAO aqueous solution containing ferrous iron could proceed by a free radical path. The phenomenon of FA and DMA formation by non-enzymatic pathway in squid during thermal processing was reported in the 1980s (Lin, Chang, & Lin-Shiau, 1984). However, few reports have explored the formation mechanism of DMA and FA *via* non-enzymatic reactions in the squid during thermal processing.

The aim of the present study was to confirm the free radicals produced in jumbo squid extract at high temperatures by ESR and evaluate the role of the radical in the thermal decomposition of TMAO to DMA and FA in jumbo squid.

2. Materials and methods

2.1. Sample preparation

Frozen jumbo squid (*D. gigas*) and Pacific cod (*Gadus macrocephalus*) stored at −20 °C, were provided by Zhoushan Xingye Industrial Company (Zhejiang, China). TMAO was purchased from Sigma–Aldrich (St. Louis, MO). Jumbo squid and Pacific cod were extracted according to the method of Zhu, Li, et al. (2012). Briefly, minced jumbo squid and Pacific cod were homogenised with cold Tris–acetic acid buffer (20 mM, pH 7.0) (1:4 v/v) at 9500 rpm for 30 s (Ultra-Turrax T25; IKA, Staufen, Germany), then centrifuged (Biofuge Stratos; Thermo Fisher Scientific Inc., Waltham, MA) at 10000*g* for 15 min at 4 °C. The water-soluble fraction of squid or cod was obtained as the supernatant.

2.2. Heat treatment

A 200 mM TMAO aqueous solution containing 10 mM Tris–acetic acid (pH 7.0) was prepared. The squid extract, cod extract and TMAO solution with or without 2 mM ferrous iron were heated at 100 °C for 15 and 30 min in a water bath. Additionally, the supernatant of squid and TMAO–iron(II) aqueous solution were heated in a water bath at temperatures between 40 °C and 100 °C for 15 min. The heated extract and TMAO aqueous solution were cooled by running water.

Heated samples (2 ml) of squid and cod extract, and TMAO aqueous solution, with or without ferrous iron, were mixed with 1 ml of 7.5% trichloroacetic acid (TCA) solution. After centrifugation (6000 *g*, 15 min) at room temperature, the supernatant of the mixture for squid and cod extract was transferred to vials. The pH values of all treated supernatants and TMAO solutions were adjusted to 6.0 by 1.0 M NaOH. These samples were then analysed for their contents of TMAO, TMA, DMA and FA.

2.3. Different additives treatments

Different additives, including hydrogen peroxide (H_2O_2), calcium chloride, citric acid, trisodium citrate were obtained from Huadong Medicine Inc. (Hangzhou, China). Tea polyphenols (purity $\geqslant$ 60%) was purchased from Zhejiang University Institute of Tea (Hangzhou, China). Resveratrol (purity $\geqslant$ 98%) was purchased from Shanghai Sea Biotech Company (Shanghai, China). Three millilitres of each substance were added to a tube containing 3 ml of the supernatant of jumbo squid. The final concentrations of additives were 0.1% H_2O_2, 5 mM calcium chloride, 5 mM citric acid, 5 mM trisodium citrate, 0.1% tea polyphenols, 0.1% resveratrol. The mixture was boiled for 15 min at 100 °C. After cooling the treated sample by running water, 1 ml of 7.5% TCA was added. The squid extract with distilled water was used as a control. After centrifuging (6000*g*, 15 min) at room temperature, the content of FA and DMA in the supernatant of squid was measured.

2.4. Determination of TMAO, TMA and DMA

The amounts of TMAO, TMA and DMA were measured by ICS-2000 (Dionex, Sunnyvale, CA) with a Dionex IonPac CS17 analytical column (250 mm × 4 mm i.d.) and a Dionex IonPac CG17 guard column (50 mm × 4 mm i.d.) as decribed by Li et al. (2009). Deionised water was obtained from a water purification system (Millipore, Billerica, MA).

2.5. FA determination

FA value was measured by HPLC according to the method of Li, Zhu, and Ye (2007), with little modification. For the derivatisation of FA, 0.5 ml sample and 0.5 ml of 1 mg/ml DNPH solution were heated for 30 min at 60 °C in a water bath. After filtering through a 0.45-μm syringe filter (Bonna-Agela Technologies, Wilmington, DE), these solutions were used to assess FA concentration by HPLC (Agilent Technologies Inc., Santa Clara, CA). Twenty microlitres of supernatant were injected. Separations were achieved on a 15 cm × 4.6 mm C18 Hypersil ODS 5 μM reverse-phase column (Agilent Technologies Inc.). The mobile phase for this separation was an aqueous solution of 70% methanol at a flow rate of 1 ml/min. The eluant was monitored at 355 nm using an ultraviolet detector (Agilent Technologies Inc.).

2.6. Reducing power

The reducing power was determined according to the method of Ferreira, Baptista, Vilas-Boas, and Barros (2007). A 1-ml aliquot of each sample solutions was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.0), and 1 ml of 1% potassium ferric cyanide. After incubation at 50 °C for 20 min, 1 ml of 10% TCA was added and then centrifuged for 15 min at 6000*g*. Then, 2 ml of treated solution were extracted and mixed with 2 ml of distilled water and 2 ml of 0.1% $FeCl_3$. Absorbance was measured at 700 nm with a UV-2550 ultraviolet spectrophotometer.

2.7. ESR measurements

The spin traps 5,5-dimethy-1-pyrroline-*N*-oxide (DMPO) and *N*-tert-butyl-α-phenyl-nitrone (PBN) were purchased from Sigma. One millilitre of the squid extract, cod extract and TMAO aqueous solution with or without 2 mM ferrous iron were boiled at 100 °C for 15 min in a water bath. After adding 0.37 M PBN or 0.2 M DMPO, the mixture was heated at 60 °C for 60 min and then transferred to a quartz capillary. The ESR spectrum of the mixture was recorded at room temperature (298 K) on a Bruker ESP A300 spectrometer. The squid extract in the presence of 2 mM ferrous iron, and 20 mM TMAO–2 mM iron(II) were both heated at 40, 60, 80 and 100 °C for 15 min in a water bath. The squid extract in the presence of various additives, including 0.1% H_2O_2, 5 mM calcium chloride, citric acid, 0.1% TP and resveratrol, was heated at 100 °C for 15 min. In all heated samples the radical signals were determined by ESR. The operating parameters of the ESR spectrometer were as follows: centre field 3512 G, sweep width 100 G, modulation frequency 100 kHz, modulation amplitude 2.00 G, microwave