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Commercial γ-Oryzanol Inhibits the Formation of C-7 Oxidized Cholesterol Derivatives (OCDs) in an Aqueous Model System during Cholesterol Autoxidation

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수용성 모델시스템 내에서의 상업적 γ-Oryzanol의 C-7 산화 콜레스테롤 유도체 생성 저해효과

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ABSTRACT – The inhibition of cholesterol autoxidation by commercial γ-oryzanol (0, 50, 100, and 300 ppm) was studied in an aqueous model system for 20 h at pH 5.5 and 80℃. The inhibition effectiveness of the commercial γ-oryzanol was followed by the retention of cholesterol and the formation of C-7 oxidized cholesterol derivatives (OCDs). Changes in the amount of γ-oryzanol in the aqueous system were determined during cholesterol autoxidation. A method to detect the levels of 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol in an aqueous model system with γ-oryzanol was developed by using the hexane-ethyl acetate extraction system and high-performance liquid chromatography. Results showed that the levels of C-7 OCDs in an aqueous dispersion containing 300 ppm of γ-oryzanol were not significantly ($p>0.05$) increased, when compared to other treatments (0, 50, and 100 ppm), during the accelerated cholesterol oxidation.

Key words: Cholesterol, Autoxidation, γ-Oryzanol, OCD

Introduction

Crystalline cholesterol or aqueous cholesterol dispersion readily undergoes oxidation at relatively mild temperatures when exposed to air, with the oxidation reactions producing a variety of products[1-3]. Some cholesterol oxidation products (COPs) have been shown to be cytotoxic, atherogenic, mutagenic, and carcinogenic, when ingested by the laboratory animals[4-6]. The C-7 oxidized cholesterol derivatives (OCDs), 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol, are major COPs found at high concentration in certain foods such as muscle tissue[7]. These toxic sterols are potent inhibitors of the cholesterol biosynthesis, which is essential for cell function[8]. γ-Oryzanol, a rice bran extract, has a substantial commercial significance in Japan as a food and medical antioxidant. γ-Oryzanol, initially thought to be a single compound, is now known to be a mixture of ferulate esters with sterols and triterpene alcohols, predominantly campesterol, 24-methylenecycloartanol, and cycloartenol[9-11]. Compared with other cereals, rice bran has a high level of γ-oryzanol (3500 mg/kg bran)[10]. Current trend in the food industry is the use of natural antioxidant components rather than synthetic antioxidant[12]. An emulsion of fatty acids and aqueous buffer was used as a relatively simple model system for studying the lipid oxidation of the muscle-based foods[13]. For this reason, the incorporation of γ-oryzanol into a simple model system that represents the state of cholesterol in foods and human tissues may have utility in studying the effectiveness of the natural antioxidants in inhibiting the cholesterol autoxidation. In addition, only a limited number of the quantitative data exist on COPs in foods, mostly due to the difficulties associated with the isolation and detection of the oxidized cholesterol

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derivatives (OCDs). The objectives of this research were to develop a mild procedure for the quantification of C-7 OCDs, (7-ketocholesterol together with its reduced stereo-isomers, 7α-hydroxycholesterol and 7β-hydroxycholesterol) in a dispersion model system with γ-oryzanol as well as determine the efficacy of γ-oryzanol in suppressing the formation of C-7 OCDs during the cholesterol autoxidation in a dispersion model system.

Materials and Methods

Materials

Cholesterol and 7-ketocholsterol were purchased from Sigma Chemical Co. (St. Louis, MO). Other steroid standards (7α-hydroxycholesterol and 7β-hydroxycholesterol) were from Steraloid Inc. (Wilton, NH) and γ-oryzanol from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Solvents used were of high-performance liquid chromatography (HPLC) grade from various sources.

Cholesterol dispersion media preparation

The aqueous dispersion system was prepared using the method described by Kim et al., with slight modification[3]. The aqueous dispersion used to examine the cholesterol autoxidation consisted of 500 ppm cholesterol prepared by dissolving cholesterol and sodium dodecyl sulfate (SDS) in ethanol and adding 0.01 M histidine buffer prepared with deionized water. γ-Oryzanol in acetone (1 mL) at different concentrations (0, 50, 100, and 300 ppm) were added to the mixture of SDS and buffer solution. Because the typical pH of meat product is 5.5, the pH of the aqueous dispersion was adjusted to 5.5 using 2N HCL at room temperature and the pH was maintained to provide an internal condition similar to that of the cholesterol in the meat products during the cholesterol autoxidation.

Cholesterol Autoxidation

The dispersion was incubated at 80°C to increase the rate of the cholesterol autoxidation over time (0, 5, 10, 15, and 20 h). The reason for setting the incubation temperature at 80°C was because C-7 OCDs such as 7α-hydroxycholesterol and 7β-hydroxycholesterol, unlike 7-ketocholesterol, were relatively stable under mild conditions such as low temperature, short incubation time, and no prooxidant requirement. Therefore, with the limitation of extending the oxidation time in the *in vitro* experiment, the incubation temperature was increased up to 80°C to promote the cholesterol autoxidation followed by the addition of 25 μL of copper (II) sulfate (0.005 M) to promote the autoxidation. The aqueous model system used in this research was highly prooxidative (high temperature, copper-catalyzed, and oxygen-saturated). The concentrations of C-7 OCDs and cholesterol were determined to compare the degree of cholesterol oxidation over the 20-h experimental period.

Sample extraction and preparation

A 5-mL aliquot of the cholesterol dispersion was extracted three times using 10 mL of hexane: ethyl acetate (8:2) by liquid-liquid extraction to yield sterol compounds such as cholesterol, C-7 OCDs, and γ-oryzanol. The collected extracts were filtered through anhydrous sodium sulfate. These extracts were then evaporated completely under a stream of nitrogen and diluted in 1 mL mobile phase before HPLC analysis.

High-performance liquid chromatography (HPLC) analysis

HPLC was performed using a Waters (Milford, MA) system consisting of a model-510 pump, a model Rheodyne 7725 I injector, and a model 486 teunable absorbance detector. A Supelcosil™ (Supelco, Bellefonte, PA) LC-Si, 5 μm, 25 cm × 4.6 mm i.d. normal-phase column was used for C-7 OCDs, and the mobile phase was hexane/isopropyl alcohol (97:3), whereas a 5 μm C18 100A reverse-phase column was used for cholesterol with the mobile phase of methanol/acetonitrile (7:3). γ-Oryzanol was analyzed through the HPLC reverse-phase system as described by Xu and Gober[10]. The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50:44:3:3), and the flow rate was controlled at 1.3 mL/min. The detection wavelengths of the UV detector were 203 for cholesterol and 330 for γ-oryzanol. Quantification was accomplished using an Autochrowin chromatography workstation (Young-in Science Co. Ltd., Seoul, Korea) with the external standards.

Experimental design and statistical analysis

Three replication separate replications were performed in a randomized complete block design with treatments assigned in a 4 × 5 factorial arrangement. With the replications (n=3) blocked, the main treatment factors were the γ-oryzanol treatment at different levels (0, 50, 100, and 300 ppm) and the incubation time (0, 5, 10, 15, and 20 h). The General Linear Model (GLM) procedure was applied to the data at the level of $p<0.05$ for statistical analysis[14], and Least Significance Difference (LSD) was used to compare the mean differences among the treatment combinations.

Results and Discussion

Fig. 1 shows the trends of retention of cholesterol in the aqueous dispersion with different concentrations of γ-oryzanol during the cholesterol autoxidation. Cholesterol

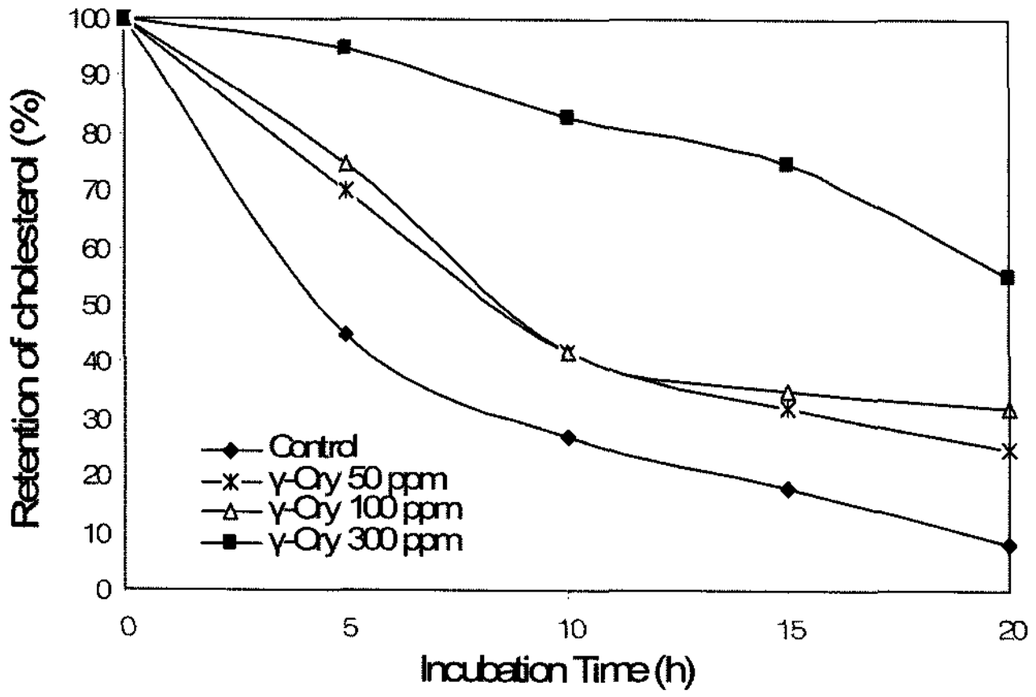

Fig. 1. Time course of autoxidation of cholesterol in an aqueous dispersion medium with different concentrations of commercial γ-oryzanol incubated for 20 h at pH 5.5 and 80°C.

was significantly autoxidized in all of the aqueous dispersion during the 20-h incubation period at pH 5.5 and 80°C. However, at a higher concentration (300 ppm) of γ-oryzanol, the retention of cholesterol remained relatively more stable. Fig. 2 shows the amounts of total C-7 OCDs formed in the aqueous dispersion during the 20-h incubation period at pH 5.5 and 80°C. The contents of C-7 OCDs significantly increased ($p<0.05$) up to 15 h in all treatments except for the aqueous dispersion with 300 ppm of γ-oryzanol, as the incubation time increased. At the 20-h of cholesterol incubation without γ-oryzanol treatment (Fig. 2), the amount of C-7 OCDs decreased when compared to that at the 15-h cholesterol oxidation. This showed the existence of an interaction between the incubation time and the γ-oryzanol treatment. In the dispersion with higher concentration (300 ppm) of γ-oryzanol, the amount of C-7 OCDs was not significantly increased ($p>0.05$) up to 15-h cholesterol oxidation, which could be due to the presence of a phenolic hydroxyl group in the ferulate esters of γ-oryzanol. The group trapped the peroxy radical to yield the hydroperoxide, which reacted to produce a radical and propagate a free radical chain reaction. Fig. 3 shows the decrease in the production of 7-ketoholesterol at 15-h incubation compared to that at 20-h cholesterol incubation. The decrease in 7-ketocholesterol might be due to the pH sensitivity of 7-ketocholesterol in the aqueous dispersion medium resulting from the accelerated cholesterol autoxidation at a high temperature. 7-Ketocholesterol is highly sensitive to alkaline pH and is subjected to thermal dehydration, yielding 3, 5-cholestadien-7-one[3]. The trend of the inhibition effect of γ-oryzanol on the production level of 7-hydroxycholesterol during the cholesterol oxidation was similar to that of 7-ketocholesterol (Fig. 4). When cholesterol in the aqueous dispersion medium without γ-oryzanol was incubated for a 20-h period at pH 5.5 and 80°C, the epimeric ratio of 7-hydroxycholesterol (7α-hydroxycholesterol:7β-hydroxycholesterol) was approximately α:β=1:2 after 5 h incubation (Fig. 4). In the aqueous dispersion without γ-oryzanol treatment, the production levels of 7-hydroxyoholesterols were lower at the 20-h incubation when compared to that at the 15-h cholesterol incubation (Fig. 4). The decrease of 7-hydroxycholesterol in the aqueous dispersion medium after 15-h incubation (Fig. 4) might be due to the inherent thermal instability of the cholesterol oxide products and pH change resulting from the extended cholesterol autoxidation at high temperature. The retention of γ-oryzanol in the aqueous dispersion with cholesterol during oxidation is shown in Fig.

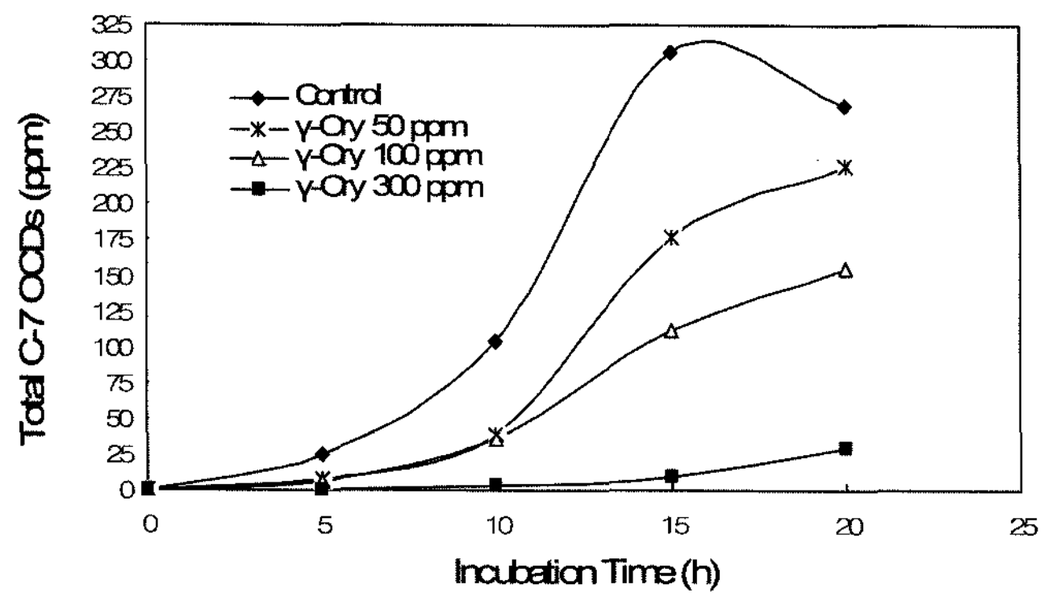

Fig. 2. Production of total C-7 oxidized cholesterol derivatives (OCDs) in an aqueous dispersion medium with different concentrations of commercial γ-oryzanol incubated for 20 h at pH 5.5 and 80°C.

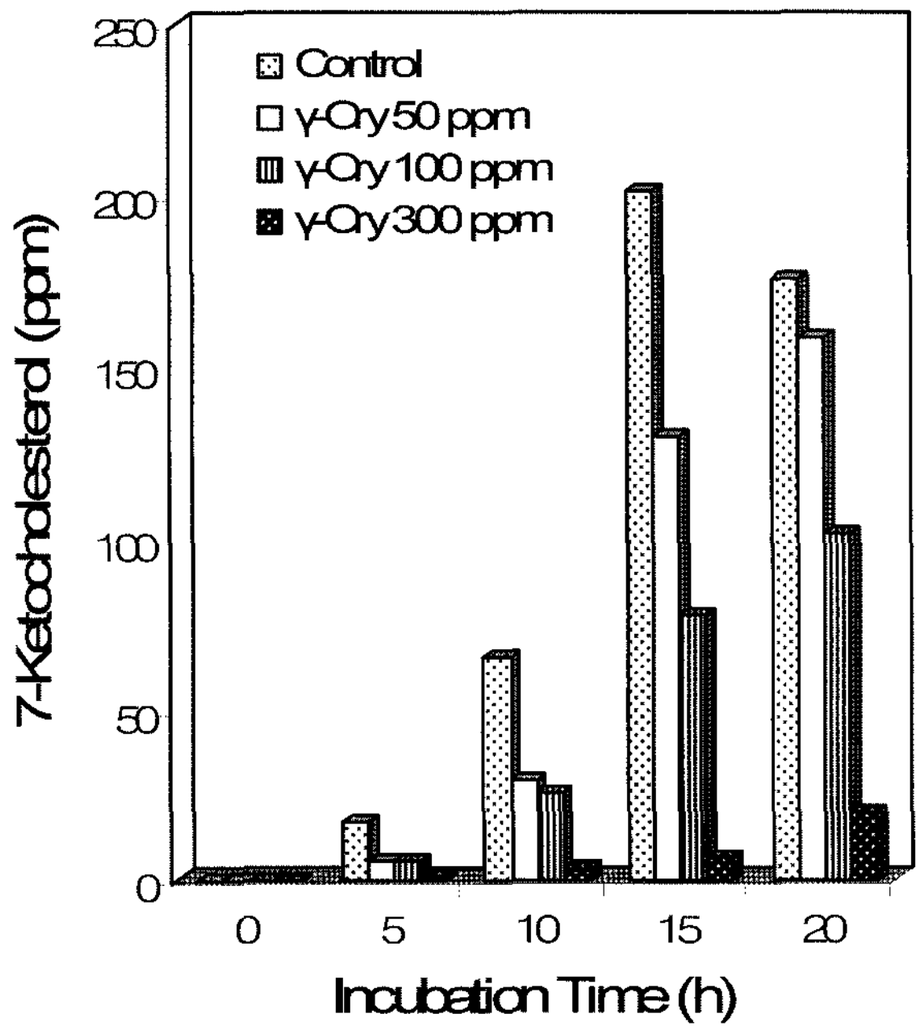

Fig. 3. Inhibition effect of commercial γ-oryzanol on the production of 7-ketocholesterol in an aqueous dispersion medium incubated for 20 h at pH 5.5 and 80°C.

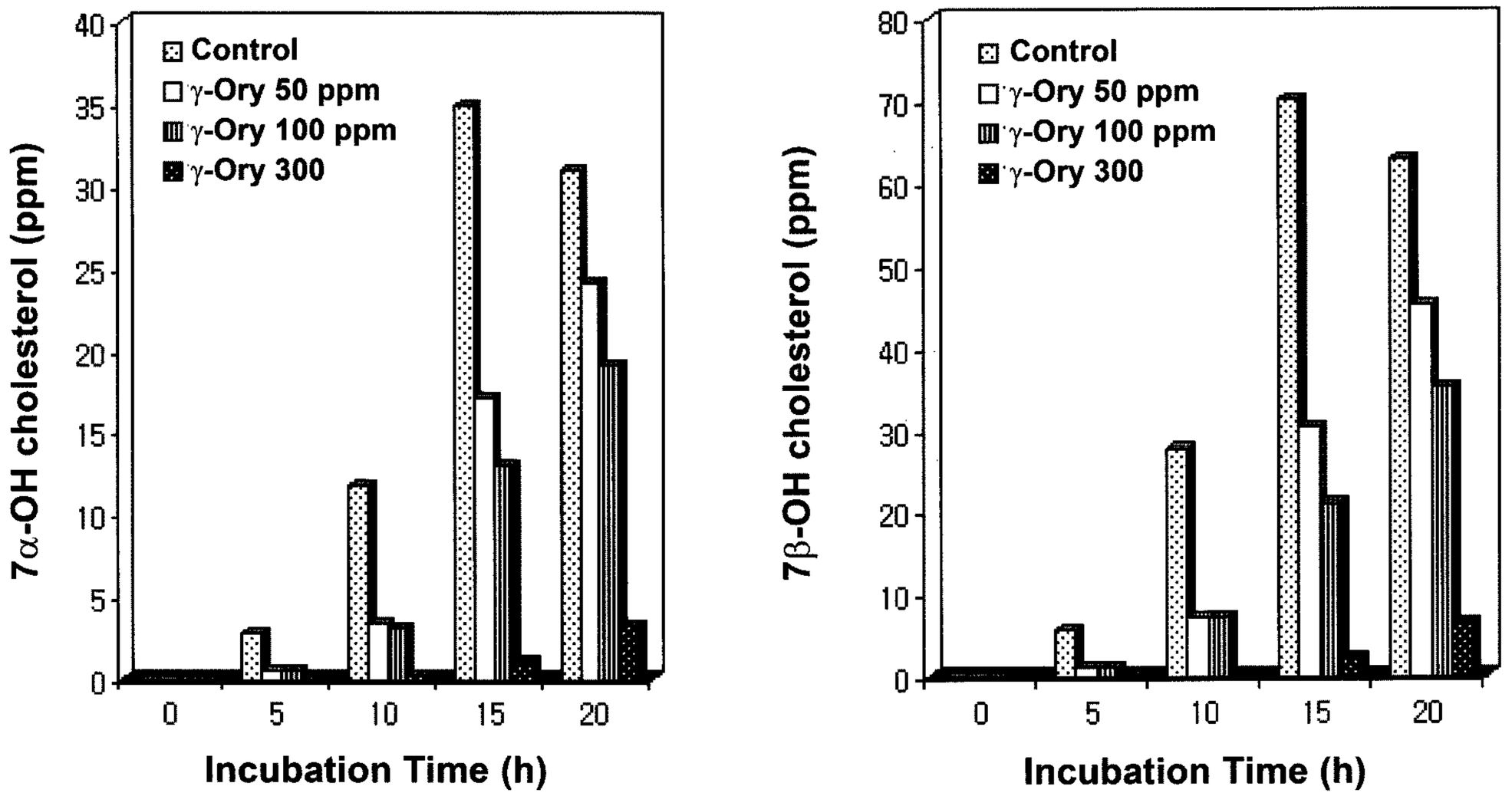

Fig. 4. Inhibition effect of commercial γ-oryzanol on the production levels of 7-hydroxycholesterols in an aqueous dispersion medium incubated for 20 h at pH 5.5 and 80°C.

5. Increased incubation times reduced the level of γ-oryzanol in the aqueous dispersion medium because γ-oryzanol, which, in itself, played a role as an antioxidant to inhibit the cholesterol autoxidation in the aqueous dispersion medium, should be oxidized during the incubation period. The order of the loss of γ-oryzanol in the dispersion medium from 0 to 20-h incubation period was 50 ppm (79.98%) > 100 ppm (64.22%) > 300 ppm (29.91%). This implied a relatively lower loss of γ-oryzanol in the aqueous dispersion medium at the higher concentration of 300 ppm. In light of the biological activities of OCDs, these data raise concerns over the safety of storage and cooking of cholesterol-containing foods, particularly the muscle-based products. According to several animal studies, dietary OCDs could play a significant role as strong initiators of the atherosclerotic lesions in the blood vessels[15-18]. Jacobson et al.[17] have shown that trace amounts of OCDs could lead to the coronary stenosis in the White Carneau pigeons. Lyons and Brown[19] have reported that 7-ketocholesterol could be cytotoxic as well as induce apoptosis in the vascular cells. In addition, Kosykh et al.[20] have shown that feeding rabbits with cholesterol containing 5% (w/w) OCDs significantly modified the very low density lipoprotein secretion in the hepatocytes. However, whether these observations are applicable to human is still questionable; nevertheless, the importance of understanding the inherent potency of the health risk of cholesterol-containing muscle-based food product, which might result in the development of OCDs during food processing, cannot be overstated.

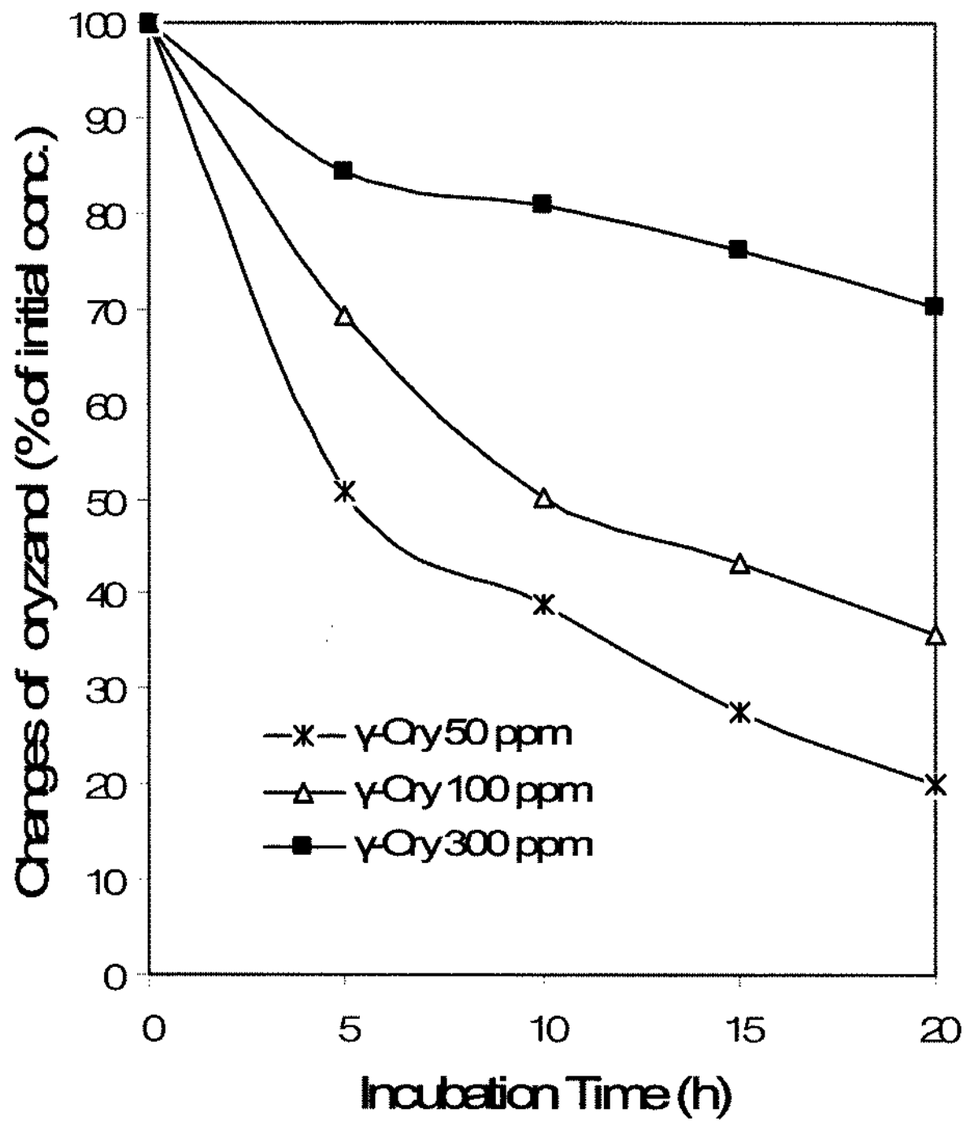

Fig. 5. Changes of retention of commercial γ-oryzanol in an aqueous dispersion medium incubated for 20 h at pH 5.5 and 80°C.

This study showed that the commercial natural γ-oryzanol extracted from the rice bran could effectively inhibit the formation of C-7 OCDs in a muscle-based model system during the accelerated cholesterol autoxidation. However, this investigation is limited in scope in understanding the effect of γ-oryzanol on the inhibitory mechanism of the

general cholesterol oxidation including both the side-chain oxidation and the epoxidation except C-7 oxidation. Further investigation is necessary to provide a more comprehensive understanding of the suppression effects of other COP formation by γ-oryzanol. In addition, determining the potency of γ-oryzanol through comparison of its inhibitory effect on the cholesterol autoxidation with the other natural phenolic compounds at the same concentration levels would also be useful.

요 약

미강 추출 상업용 유통 감마오리자놀의 콜레스테롤 자동산화에 의한 C-7 산화 콜레스테롤 유도체 생성 저해 효과가 수용성 모델 시스템을 이용하여 검토되었다. C-7 콜레스테롤 산화 유도체 (C-7 oxidized cholesterol derivatives: C-7 OCDs) 생성을 위해 콜레스테롤과 감마오리자놀이 분산된 수용성 모델시스템은 구리이온을 촉매로 pH 5.5와 80℃의 가혹 조건에서 20시간 동안 반응되었다. 산화 유도 기간에 따른 C-7콜레스테롤 산화 유도체 (7-ketocholesterol, 7α-hydroxy-cholesterol 과 7b-hydroxycholesterol)의 생성 정도와 감마오리자놀 및 콜레스테롤 변화 추이 정도가 핵산과 에틸아세테이트를 이용한 용매 추출법과 고속 액체크로마토그래프 (high-performance liquid chromatography) 테크닉을 이용 정량적으로 분석되었다. 분석 결과 콜레스테롤 산화 유도 기간에 따른7-ketocholesterol 생성 비율은 7-hydroxycholesterol 이성체 (α-형:β-형) 대비 약 2:1의 비율로 생성되었으며, 7-hydroxycholesterol 이성체에 있어서는 α-형 대비 β-형의 생성 정도가 약 1:2의 비율로 나타났고, 총 C-7 산화콜레스테롤의 생성은 상대적인 고농도(300 ppm) 감마오리자놀 처리 모델 시스템에서 효과적으로 저해되었다.

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