Enzymatic Degradation of Egg Yolk Cholesterol

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ABSTRACT

The capacity of four cholesterol oxidases from Nocardiia erythropolis (Ne), Streptomyces species (Ss), Brevibacterium species (Bs) and Pseudomonas fluorescens (Pf) to degrade cholesterol in egg yolk at 4, 25 and 37°C has been determined. The effects of enzyme substrate concentration and incubation temperature on the enzymatic activity of these cholesterol oxidases have also been investigated. The cholesterol degrading capacity of these enzymes follows the sequence Pf>Ne>Ss>Bs. Up to 93.4% cholesterol was degraded by cholesterol oxidases from P. fluorescens and Streptomyces species after 72 h of incubation at 37°C. At 4°C, P. fluorescens cholesterol oxidase could degrade up to 64.9% cholesterol after 48 h.

Key Words: Enzymatic degradation, eggs, cholesterol.

High cholesterol content in egg is a factor contributing to declining egg consumption (27). A suspected causative association between dietary cholesterol intake and the development of coronary heart disease is still a topic for debate (30). Several studies have also indicated no or insignificant association of cholesterol intake from egg with serum cholesterol (11,17,32). Nevertheless, to address this cholesterol concern, the egg industry has attempted to produce low cholesterol eggs as well as egg products by various approaches.

Genetic selection to develop a chicken line which could produce low cholesterol eggs has only yielded moderate results (2.4% cholesterol reduction) (13,22). Modification of the hen’s feed by dietary lipid treatment or by adding garlic oil did not influence cholesterol concentration in the egg (26,36). Consequently, cholesterol reduced egg products are prepared by rather complex processes (for example, by partially replacing egg yolk with vegetable oil or water then combining with egg white to meet the market demand) (4,8). The recombined egg does not retain the desirable egg flavor. Therefore, there is a need for a simple technique to produce cholesterol-free or cholesterol reduced egg yolk without losing egg flavor, and as a food ingredient for various egg based products.

Extraction of egg cholesterol using organic solvents has met with limited success due to a large amount of cholesterol remaining in the extracted egg as well as some undesirable effects on the functional properties of the egg yolk (20). Recently, β-cyclodextrin has been used to remove egg cholesterol by the formation of an insoluble complex with cholesterol or its ester in an aqueous solution. However, even with an additional enzymatic treatment it is still difficult to completely remove all residues of β-cyclodextrin from the yolk (9). This is a major impediment since β-cyclodextrin is not an approved food ingredient in some countries.

Supercritical fluid extraction could provide another means to remove egg cholesterol. In spite of the high capital investment required, this process already has several commercial applications in the food industry. Although up to 80% of egg cholesterol can be removed by supercritical fluid extraction using carbon-dioxide as the fluid, an economical technique to recover the cholesterol from the significant amount of co-extracted egg lipid is required as a prerequisite for commercial feasibility (12,14).

Microbiological degradation of cholesterol by some strains of Nocardiia, Rhodococcus, Brevibacterium, Corynebacterium or Streptomyces has been reported (1,2). However, some of these organisms may accumulate steroid intermediates with consequent adverse health effects (3). Some strains which could degrade cholesterol without accumulating steroid intermediates have also been isolated (3). When egg yolk was inoculated with Rhodococcus equi, up to 70% of cholesterol was degraded with almost no accumulation of steroid intermediates (2).

Johnson and Somkuti also found Rhodococcus strains could reduce up to 80% of egg cholesterol (16). Although these findings indicate that enzymatic treatment could be used for egg cholesterol degradation, for any commercial application it would be necessary to shorten the degradation time of 3 days reported in these studies.

In a previous study, we reported that cholesterol oxidase could degrade all cholesterol in a buffer system and up to 65% in milk at low temperature (3 to 7°C), in less than 24 h (36).

This paper reports cholesterol degradation activities of cholesterol oxidases originating from four different microorganism genera, Brevibacterium, Nocardiia, Pseudomonas and Streptomyces, in an egg yolk system under different environments in order to define the optimal conditions for each enzyme.
DEGRADATION OF CHOLESTEROL

MATERIALS AND METHODS

Egg yolk.

Yolks and whites from 6L eggs (weighing 55 g each) were carefully separated. The yolks were combined and mixed until homogenous. A 0.2 g/ml yolk suspension in 0.1 M potassium phosphate buffer (KPB) pH 7 containing 0.02% thimerosal, as an anti-bacterial agent, was portioned into 20 ml lots and stored at -4°C until required.

Cholesterol oxidases.

Cholesterol oxidases (COD) derived from Ss, Bs, Ne and Pf were purchased from Sigma Chemical Company, St. Louis, MO as lyophilized powders. Cholesterol oxidases from Ne was reconstituted in 0.1 M KPB, pH 5 and the others in the same buffer but at pH 7. These pH's have been reported as being the most stable environment to store these enzymes to prevent appreciable loss of activity (28).

Since the incubation pH may have a strong effect on the enzymatic activity, the degradation capacity of each enzyme was measured at pH 7, a pH at or close to their optimum physiological pH based on available literature. Cheillan et al. (6) observed activity by Pf COD remaining constant as long as the pH was maintained between 6.5 and 8.5. Uwajima et al. (33) have shown that the enzyme isolated from Brevibacterium had a broad pH optimum in the range of pH 6.0 to 8.5 and Nomura and Nakayama (23) observed pH optimum of 7.0 for Ne and Bs COD, and pH 7.5 for enzyme from Streptomyces.

For enzyme activity, one enzyme unit (1 U) is defined as the amount of COD that will promote the conversion of 1 μM of cholesterol to cholest-4-en-3-1 per min under the prescribed test conditions (6).

Cholesterol determination.

Cholesterol was determined by gas chromatography, following a method of Kovacs (18) with some modifications (7), with the principal change being that samples containing 1 to 2 mg cholesterol were converted to trimethylsilyl (TMS) derivatives prior to gas chromatography, by heating with 100 μl MSTFA (Sigma) at 100°C for 15 min. Octacosan-1-ol or dihydrocholesterol were used as internal standards. Coefficient of variation for the reproducibility and the mean recovery of the method used ranged from 2.2 to 2.9% and 90.2 to 95%, respectively.

A 25QC2/BPI0-0.25 capillary column was used in a Hewlett Packard 5890 gas chromatograph. Column temperature was set at 285°C and injection temperature at 300°C. A flame ionisation detector was used, and this was set at 320°C. A split injection (1 μl) was made with a split ratio of 30:1. Flow rate of the carrier gas (He) was 0.8 ml/min and the head pressure set at 24 psi. The run time was 17 min, with the retention times of the TMS derivatives of cholesterol and dihydrocholesterol being typically 10 and 10.3 min, respectively.

Sterols were identified by co-chromatography using TMS derivatives of commercially purchased standards (Sigma).

Cholesterol degradation products.

In order to detect COD products, thin-layer chromatography (TLC) was performed with pre-coated silica Gel 60 F254 sheets (Merck). Incubation products were separated with a mixed solvent system (chloroform: diethyl ether (60:40)) at room temperature and spots were made visible by spraying with 2,7'-dichlorofluorescein and viewed under ultra-violet (UV) light (254 nm). Standards of cholesterol and possible oxidation by-products (5-cholesten-3-one, 4-cholesten-3-one, 25-hydroxycholesterol and 7-ketocholesterol) were purchased from Sigma.

The identification of by-products was confirmed using gas chromatographic retention times in conjunction with mass spectra obtained. Incubation products were extracted with diethyl ether (2 ml + 1 ml), the extracts evaporated to dryness and the residues dissolved in chloroform (500 μl). Samples (1 μl) were then taken for GC-MS using a Hewlett Packard 5890 gas chromatograph fitted with a Hewlett Packard 5971A Mass Selective Detector (MSD). The column and chromatographic conditions were the same as for cholesterol determination.

Enzymatic treatment of egg yolk cholesterol.

Effect of time and temperature.

The effect of incubation time on the cholesterol degrading capacity of each enzyme was initially investigated. Yolk suspensions containing 0.1 g yolk (approximately 3.9 μM cholesterol) were added to tightly capped (teflon-lined) 10 ml test tubes and incubated, in triplicate, with 3.9 U of each enzyme at 4, 25 and 37°C. Residual cholesterol was determined at 3, 6, 9, 12, 24, 48 and 72 h by gas chromatography. The experiment was not replicated. Results of this investigation were used to select 12 h as a suitable time for a more detailed study of incubation temperature.

To four of seven test-tubes containing 0.1 g yolk in buffer suspension, 3.9 U of COD enzyme from either one of four types (Ne, Ss, Bs and Pf) was added. The other three test-tubes contained no enzyme and were treated as controls. These treatments were randomly positioned into test-tube racks, and these racks were randomly assigned to incubators at each of the temperatures, 4, 15, 25, 37, 45 and 60°C. Incubation time was 12 h.

The experiment was replicated on three different days and as a split-plot type design (29). Days were considered as blocks, incubators as plots and test-tubes containing yolk as the sub-plots. Temperature was the main plot treatment and the enzymes (including controls) were the sub-plot treatments. The main temperature effect, and the temperature by enzyme type interaction, were divided into orthogonal polynomial components (29) up to cubic order to determine if there were linear and quadratic trends. The data was analyzed as a temperature by enzyme type factorial with the control being considered in the analysis as an enzyme type. Pairs of means were compared using Fisher's least significant difference (LSD) at the 5% level.

Effect of concentration.

Yolk suspensions containing 0.1 g yolk cholesterol were incubated with 0, 0.5, 1, 2, 4 and 8 U of enzyme per μM egg cholesterol for 2 h at 45°C and residual cholesterol was determined. The analysis was replicated on 3 days, with each day considered as a block (29). Within the block there was one test-tube sample of each enzyme type by concentration combination, plus two control samples (without enzyme). The arrangement of the test-tubes placed in the incubator was fully randomized. The data was analyzed as an enzyme type by concentration factorial plus an external non-enzyme control treatment, with blocking for time. Within the factorial, the concentration effects and concentration by enzyme type interaction were divided into orthogonal polynomial components (29) up to cubic order to determine if there were linear and quadratic trends. Pairs of means were compared using LSD at the 5% level.

RESULTS AND DISCUSSION

Cholesterol content of treated and untreated egg yolks were compared and results expressed as either percent of cholesterol reduction or mg/g residual cholesterol.

Effect of time and temperature.

The time course of cholesterol degradation of four COD in egg yolk at 37, 25 and 4°C are shown in Fig. 1, 2 and 3 when the yolk was incubated with the enzymes for 72 h.

The cholesterol degradation activity of each enzyme at 37°C is presented in Fig. 1. At 37°C, the Bs COD could...
The performance of the Pf COD at 37°C (Fig. 2) was similar to that at 37°C in terms of reaction rate and cholesterol degradation capacity. The reaction of both Ne and Ss CODs occurred in two stages. In the first stage, the reaction rate became slower, and after 24 h remained almost unchanged.

The 12-h incubation chosen to investigate the effect of temperature on cholesterol degrading ability by all four enzymes was based on data presented in initial incubation studies (Fig. 1-3), which showed the majority of cholesterol being degraded in the first 12 h (Fig. 4). The effect of temperature on the enzyme activity after 12-h incubation differed greatly between enzyme types (interaction significant at p<0.001, Fig. 4). An attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, p<0.001).

The most effective temperature for Bs COD and that of the other CODs was around 37 and 45°C, respectively. The enzymes isolated from Pf, Ne and Ss showed good heat stability with substantial activity retained even at 60°C. The Pf COD has an effective temperature range from 5 to 60°C, and from 5 to 37°C it is clearly superior to the other three enzymes.
The effect of enzyme concentration on the degradation of egg yolk cholesterol after 2 h differed significantly (interaction significant as p<0.01, Fig. 5). As in the previous study on temperature effect, an attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, p<0.01).

The most effective concentration of Pf COD and Ne COD was around 0.5 U/μM cholesterol and 1 U/μM cholesterol, respectively. Increasing the concentration of either of these enzymes further did not produce significantly different reductions in cholesterol (LSD = 0.47 mg cholesterol/g). Streptomycetes species COD showed significant changes in activity up to the maximum concentration of 2 U/μM cholesterol used in this investigation (LSD = 0.47 mg cholesterol/g). Surprisingly, even at the low concentration of 0.125 U/μM cholesterol, COD from Pf degraded more than one-third (up to 34.6%) of the egg cholesterol in 2 h. This was the only significantly different reduction between treatments seen at this concentration (LSD = 0.47 mg cholesterol/g). Increasing the concentration of Pf COD further to 1 and 2 U/μM did not produce a reduction in cholesterol that was significantly different (LSD = 0.47 mg cholesterol/g).

Cholesterol degradation was significantly different to that of the control at all concentrations and enzyme types (LSD = 0.41 mg cholesterol/g). The most effective enzyme type/concentration combination was that of Pf COD at 0.5 U/μM.

**Cholesterol degradation products.**

A number of COD products have been detected in foods including spray-dried egg yolk powder (19). The adverse health effects of these oxidation products are still a topic for debate.

In this study, the degradation products of egg cholesterol were examined by TLC and confirmed by MSD. The only steroid-like component detected was 4-cholesten-3-one, for each of the four enzymes. The 4-cholesten-3-one has been described as the first degradation product of cholesterol oxidase catalysis in species of Nocardia, Brevibacterium, Pseudomonas and Streptomyces by many investigators (2,5,10,28,31,33). Aihara et al. (2) also found this compound in egg yolk inoculated with R. equi No. 23, a COD producing microorganism, but it was converted rapidly into non-steroid components.

**CONCLUSION**

The cholesterol degrading capacity of four COD enzymes in egg yolk, a complex food system, has been investigated. All enzymes catalyzed the transformation of cholesterol to 4-cholesten-3-one. More than 50% of egg cholesterol could be reduced by three COD's (Ne, Pf and Ss), within 24 h. The Pf COD was effective over a wide temperature range including at 4°C. The low concentration of COD from Pf required to achieve significant reduction in yolk cholesterol coupled with the ability of this enzyme to degrade cholesterol at 4°C may make this enzyme, com-
pared to the other three investigated, an economical and practical method for lowering the cholesterol level in egg yolk.

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