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Transformation of egg cholesterol during bacterial fermentation

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Microbial fermentation of separated egg yolks was carried out at 25°C for up to 9 days using a *Bacillus coagulans* strain B1. Yolk samples were analyzed for cholesterol prior to and following fermentation. Little change in cholesterol was observed during the first 2 days of fermentation although after 5 days and after 8 days it was reduced by 32·9 and 49·6%, respectively. During the last 2 days of fermentation, the reduction in cholesterol levelled off at 49·6%. Examination of the cholesterol products formed showed that 35% of cholesterol was oxidized with 12% of products identified. Of those identified, the major ones were 7β-hydroxycholesterol (32·5%), cholesterol triol (19·2%), 7α-hydroxycholesterol (19·1%) and 25-hydroxycholesterol (7·2%).

INTRODUCTION

Health concerns regarding cholesterol have led to a decreased consumption of eggs. This is particularly unfortunate as eggs are an excellent source of protein, vitamins and minerals. In order to reverse this trend considerable efforts have been made to reduce yolk cholesterol through diet, breeding, extraction with solvents and more recently with supercritical liquids (Turk & Barnett, 1971; Larsen & Froning, 1981; Hargis, 1988; Froning *et al.*, 1990).

Fioriti *et al.* (1978) proposed the use of vegetable oil as an extractant to reduce the cholesterol level in yolk. Another more 'natural' way of controlling the cholesterol content in eggs was by modification of feed (Klein, 1980) or by using additives such as long chain branched amines and derivatives of sterols (Cecil *et al.*, 1981). Using either of these compounds, cholesterol was almost totally removed, although egg production was reduced and a new sterol—desmosterol—was detected in eggs at elevated amounts.

Baker & Darfler (1977) proposed a partial substitution of yolk with egg white, when eggs are used as a bakery ingredient, to reduce cholesterol consumption. Several patents have been registered based on substituting egg yolk with vegetable oil, gum, and milk, and egg proteins (Seeley, 1974; Glasser & Matos, 1976; Anon., 1976).

Enzymic removal of cholesterol from egg yolk was also reported, using extracellular enzymes from *Rhodococcus equi* #23; however, these authors did not specify what happened to this egg component (Aihara *et al.*, 1988*a*,*b*). Cholesterol reductase, identified in certain parts of green plants and in several bacteria, reduces cholesterol to coprostanol (Beitz *et al.*, 1990). The transformation of the cholesterol into other derivatives is a way of eliminating this compound; however, their metabolic significance remains to be explored (Pollak & Kritchevsky 1981; Smith, 1987).

The presence of cholesterol in foods is undesirable because it is susceptible to oxidation in the presence of molecular oxygen, generating a wide array of oxidized products, some of which are known to be toxic and atherogenic (Smith, 1981; Peng *et al.*, 1982; Park & Addis, 1986; Nourooz-Zadeh & Appelqvist, 1989; Przybylski & Eskin, 1991; Kummerow *et al.*, 1992). This paper examines the potential of a *Bacillus coagulans* strain B1 bacteria culture for reducing cholesterol in egg yolk by fermentation.

MATERIALS AND METHODS

Materials

Cholesterol, cholesterol oxide standards and chemicals for the colorimetric assay of cholesterol were obtained from Sigma Chemicals (St. Louis, MO). Sylon BTZ (a mixture of bis(trimethylsilyl acetamide (BSA), trimethylchlorosilane (TMCS) and trimethylsilylimidazole

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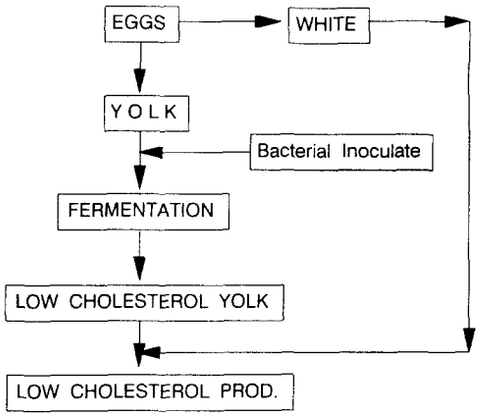

Fig. 1. Procedure for egg product preparation with reduced cholesterol by bacterial fermentation.

(TSIM) in proportion of 3:3:2 v/v) and pyridine were supplied by Supelco Canada (Oakville, Ont.). Sep-Pak aminopropyl cartridges were obtained from Waters Canada (Missassagua, Ont.). All chemicals were of reagent grade.

Fermentation

Whole eggs were separated into white and yolk fractions as shown in Fig. 1. The yolk fraction was mixed with water in a 1:1 ratio and 1% (v/v) of the bacterial inoculum of *B. coagulans* strain B1 was added. The mixture was allowed to ferment at 25°C for up to 9 days. Samples were removed initially and daily over the 9-day period. After fermentation both yolk and white fractions were mixed to form a product with reduced cholesterol.

A detailed description of the fermentation performed is given by D. R. Collimore (1993).

Lipid extraction

Lipids were extracted using the method of Bligh & Dyer (1959). Lipid extracts were evaporated to dryness, the residues were dissolved in chloroform, and 10 mg of total lipids was loaded onto an aminopropyl solid phase extraction (SPE) cartridge. Neutral lipids, including cholesterol and its esters, were eluted with 8 ml of chloroform–2-propanol (2 : 1, v/v). The eluted lipids were then dissolved in hexane and applied to a second SPE cartridge for further separation. Cholesterol esters were eluted with 8 ml of hexane, and cholesterol with 12 ml of 5% ethyl acetate in hexane (v/v).

For colorimetric measurement of cholesterol, lipids were extracted from egg yolk with ethyl acetate.

The control for all analyses performed was raw unfermented egg.

Cholesterol assay

Cholesterol was monitored colorimetrically as well as by gas chromatography.

Colorimetric method

The cholesterol content was analyzed colorimetrically with *p*-toluenesulfonic acid, as described by Pearson *et al.* (1953). The colorimetric assay, faster and simpler, was used to monitor multiple fermentations.

Gas chromatography—saponification

The eluates from SPE separations were evaporated to dryness and to the dry residues 5 ml of 2 N KOH in methanol and 1 ml of internal standard solution (containing 40 μm of 5α-cholestane per ml of methanol) were added. The mixtures were blanketed with nitrogen, and vigorously shaken until a monophasic system was obtained. Saponification was carried out overnight in the dark at room temperature. The saponified mixture was diluted with 10 ml of glass distilled water, and the non-saponifiables were extracted five times with 10 ml of diethyl ether. The extracts were washed twice with distilled water and evaporated to dryness. The residue of water was removed by coevaporation with 1 ml of benzene and 2 ml of 2-propanol.

Gas chromatrography—derivatization and separation

The dried ether extract residues were redissolved in 200 μl pyridine and 100 μl of Sylon BTZ was added to derivatize the cholesterol and its oxides. The reaction was conducted at room temperature for a minimum of 1 h with doubler sonication for 2 min each. The derivatized cholesterols were separated on a fused silica capillary column DB-17 (30 m × 0·25 mm i.d.; J&W, Folsom, CA) with 0·25 μm phase film. The column temperature was programmed from 120 to 195°C at 30°C/min, then to 235°C at 10°C/min and finally to 285°C at 6°C/min. The upper temperature was held for 15 min. Approximately 0·5 to 1·0 μl of the sample was injected, with a split ratio of 1:100, into the column.

A Hewlett-Packard 5890 gas chromatography (Avondale, PA) equipped with a 5970 mass selective detector, was used. GC–MS analyses were performed, on the same capillary column as described above, in the splitless injection mode. Compounds were identified by comparison of mass spectra of analyzed compounds with standards and library spectra. Also, spectra of cholesterol oxides published by Park & Addis (1986) were used for conformational purposes. Data on oxidized cholesterols represent the total amount of derivatives present in fermented medium.

RESULTS AND DISCUSSION

Change in cholesterol during the 9 days of yolk fermentation with *B. coagulans* strain B1 is shown in Fig. 2. Each point on the graph is an average of six replications of fermentation performed under the optimum conditions where the coefficient of standard deviation was 6 ± 2%. It is evident that very little

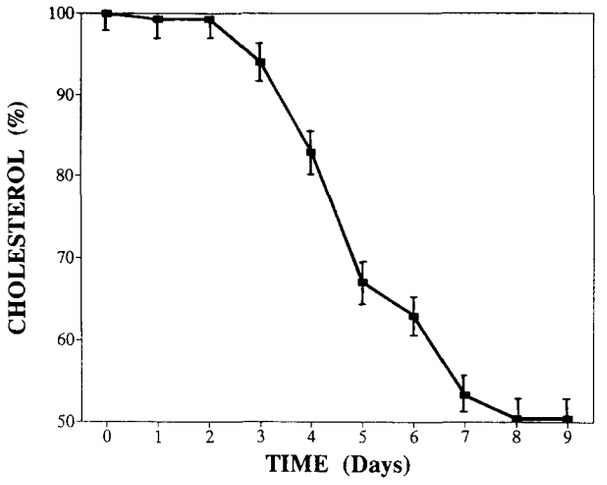

Fig. 2. Effect of fermentation time on cholesterol content in yolk.

reduction in cholesterol occurred during the first 2 days of fermentation, but was followed by a decrease in cholesterol content from 97·8 to 62·9% over the next 5 days. After a further 2 days of fermentation an additional 10% of cholesterol was removed.

The amount of cholesterol remaining leveled off at around 50·4% of the initial value at the conclusion of 8 and 9 days of the fermentation. The reduction in cholesterol by 50% resulting from fermentation can be directly related to baterial enzymic activity and bacterial growth, although this still remains to be fully confirmed. One consistent phenomenon noticed during fermentation was the complete lack of contaminant bacterial spoilage in the fermented material. This is particularly noteworthy since the eggs were not subjected to any cleaning or pasteurization prior to fracturing for the production of fermentation medium–egg yolk.

It could be concluded from these data that the *B. coagulans* bacteria has considerable potential for reducing cholesterol in egg yolk products. Also, it would appear that the bacteria were capable of 'out competing' the contaminant bacteria that are normally present in the medium.

The changes in cholesterol content were measured both colorimetrically and by gas chromatography. The chromatographic method measured cholesterol as a separated single component, and the results showed about 11% less cholesterol removed compared to colorimetry (49·6 versus 38·6% cholesterol removal). These differences have to be related to the poor specificity of the colorimetric method where other components can interfere (Naito, 1985).

Removal of cholesterol itself was important, although further evidence using gas chromatography and mass spectrometry indicated that over one-third of this reduction could be attributed to the transformation of cholesterol into oxidation products. As shown in Fig. 3(a), 34% of the products formed during bacterial fermentation of cholesterol appeared to be oxides (OXIDES) of which 12% were identified (IDENTIFIED OXIDES), while the remaining 66% were not identified (OTHERS). Tentative identification of oxides was based on similarity of mass spectrum fragments of detected compounds to identified components. Translating these percentages into actual concentrations, based on the average amount of cholesterol in eggs being 550 mg/100 g, about 277·2 mg/100 g of this component was transformed into oxidation products. Of these, one-third were tentatively identified as oxidation compounds and accounted for about 92 mg/100 g (920 ppm) of total cholesterol. Only 12% of these were identified as specific compounds, as discussed later, which account for about 32·6 mg/100 g (326 ppm) of total cholesterol. Based on these results it is clear that fermented egg products will have significantly reduced levels of cholesterol due largely to its transformation into a wide variety of oxidised products.

The cholesterol oxides (eight compounds identified) are summarized in Fig. 3(b). Of these, 7β-hydroxycholesterol accounted for 32·5%, followed by cholesterol triol (5α-cholestane-3β,5,6β-triol) with 19·2%, followed closely by 7α-hydroxycholesterol (19·1%). These three compounds accounted for over 70% of all the components identified, and their concentration in fermented egg products would be 230·8 ppm, based on the average cholesterol content in eggs. This compared with dried eggs reported to have the same cholesterol oxidation products, ranging from 21·9 to 64·9 ppm (Bovenkamp

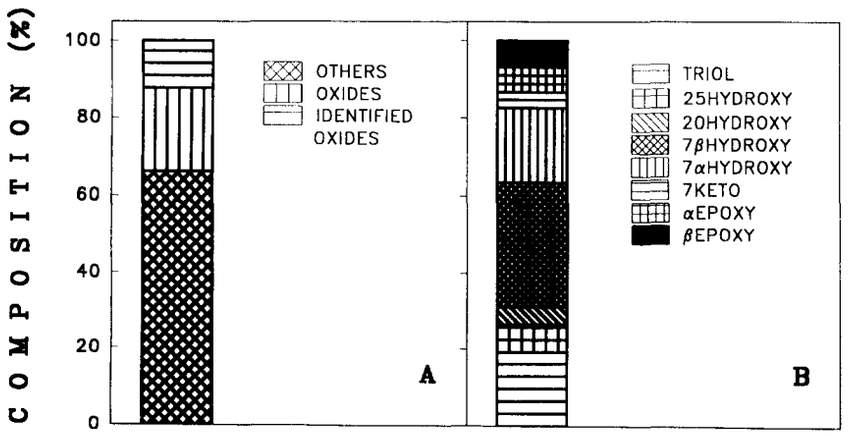

Fig. 3. Degradation products formed from cholesterol during fermentation: (A) general groups of products; (B) identified cholesterol oxides.

et al., 1988). Of these three compounds, triol was reported to exert the most adverse effects on living cells by having the highest toxicological impact at a concentration of a few ppm (Baranowski *et al.*, 1982; Sevanian *et al.*, 1986).

The next three compounds, 25-hydroxycholesterol (5α-cholestene-3β,25α-diol), 5,6α-epoxycholesterol and 5,6β-epoxycholesterol, accounted in total for 20·5% (67 ppm) of all identified compounds. The presence of these oxidized products was reported in dried eggs at about half the value reported in this study (Bovenkamp *et al.*, 1988). Of these, 25-hydoxycholesterol was shown to have a toxic effect on cells if exposed for an extended period of time and to cause defects in endothelium and smooth muscle cells of the aorta (Imai *et al.*, 1980; Peng *et al.*, 1985). All these components can initiate atherogenic changes in the human arteries that are directly related to formation of atherosclerotic plaques (Imai *et al.*, 1980; Smith, 1981, 1987; Sevanian & Peterson, 1986; Kummerow *et al.*, 1992).

The last two components identified were 7-ketocholesterol (3β-hydroxycholest-5-en-7-one) and 20-hydroxycholesterol (cholest-5-ene-3β,20α-diol) accounting for 4·1 and 4·6%, respectively. Both these compounds are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase activity (Erikson *et al.*, 1978; Kandutsch, 1980). The relative concentration calculated for both of these components was about 28 ppm, which is within the range of these cholesterol oxidation products reported in thermally treated eggs and foods (Nourooz-Zadeh, 1988).

The presence of these oxidized products suggests that an active oxidase system in *B. coagulans* is probably responsible, in part, for the transformation of cholesterol. In addition, aerobic fermentation, as used in this study, may also play a significant role in cholesterol oxidation and the formation of these undesirable compounds. Aihara *et al.* (1988*b*) analyzed products formed during the removal of cholesterol from egg yolk, using extracellular enzymes extracted from bacteria *R. equi* no.23. These authors found 3-ketocholesterol among the products formed, although they used TLC for separation in which the detection technique applied is not particularly sensitive. The separation of cholesterol oxidation products by TLC is probably incomplete when compared to capillary gas chromatography as it is unable to separate its derivative isomers (Maeker, 1987).

The results from this study demonstrate the importance of exercising extreme caution when attempting to reduce cholesterol by fermentation. During these processes cholesterol can be transformed into a wide range of oxidized derivatives, some of which may pose a far greater health hazard than cholesterol itself.

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