Bioconversion of Egg Cholesterol to Pro-vitamin D Sterols with *Tetrahymena Thermophila*

G. VALCARCE, A. NUSBLAT, J FLORIN- CHRISTENSEN, AND B.C. NUDEL

ABSTRACT: The potential use of the non-pathogenic ciliate *Tetrahymena thermophila* for converting cholesterol into pro-vitamin D sterols in egg suspensions was investigated. Conditions screened for efficient conversion included inoculum size and age, egg and egg yolk dilution, initial pH, and aeration. By optimizing these parameters, 55% ± 6 cholesterol reduction was obtained in 24 hours incubation. *Tetrahymena* converted cholesterol into three unsaturated sterols, D7, 22 bis-dehydrocholesterol, an ergosterol analog (27%), D7-dehydrocholesterol, also known as pro-vitamin D3 (4%), and Δ22- dehydrocholesterol (8%). Despite significant proteolysis, culturing with *Tetrahymena* did not result in net consumption of total amino acids content, which was in fact increased by 16% ± 2. These findings emphasize the potential use of *Tetrahymena* in the food industry.

Keywords: cholesterol bioconversion, pro-vitamin D formation, *Tetrahymena thermophila*, egg, egg yolk suspensions

Introduction

EGGS ARE ONE OF THE MOST COMPLETE, NATURALLY PRODUCED foods, supplying nutritionally important substances needed for a balanced diet (Belitz and Grosch 1992). Egg components are also important food ingredients used to confer desirable properties on a large variety of food products.

Major drawbacks of egg are its high content of cholesterol and hypersensitivity reactions. These concerns have led to innovations to improve egg acceptability; particularly, decreasing cholesterol content has been the aim of many attempts. Different methods have been proposed for the removal of cholesterol, such as adsorption with b-cyclodextrins (Courregelongue and others 1987) or digitonin immobilized on a carrier (Richardson and Jimenez-Flores 1992). Another approach has used extraction procedures with solvents, such as ethanol (Fallis and others 1977) or supercritical carbon dioxide (Ogasahara and others 1992). For biochemical treatments, isolated enzymes (Beitz and others 1990) or combination of enzymes (Saito and Ohuchi 1998) have been employed.

A different approach has been culturing egg yolk with live microorganisms that metabolize cholesterol, including members of the genus Rhodococcus (Aihara and others 1988; Watanabe and others 1987) and Nocardia (Chosson and others 1988).

The ciliate *Tetrahymena* is a non-pathogenic, generally considered as safe protozoan (Wheatley and others 1994). It has several peculiarities with promising potential in food processing applications, including the ability to desaturate cholesterol into D7-dehydrocholesterol (pro-vitamin Δ_3) and D7,22- bis-dehydrocholesterol, a close analog of ergosterol (pro-vitamin Δ_2) (Mallory and Conner 1970). *Tetrahymena* can also desaturate exogenous fatty acids taken up from the medium (Holz and Conner 1973). Thus, it can convert stearic acid (18:0) into γ-linolenic acid (18:3, *n*-6), a process entailing sequential desaturation of 18:0, including a step that involves Δ12-desaturase activity (Holz and Conner 1973). Moreover, *Tetrahymena* releases large quantities of hydrolases to the extracellular medium, which serve to predigest complex components of various food sources (Florin-Christensen and others 1989, 1990). Among the secreted hydrolases, a set of lipolytic enzymes (Florin-Christensen and others 1985), including phospholipase C (Florin-Christensen and others 1986 a), phospholipase A_1, and triacylglycerol lipase (Florin-Christensen and others 1986 b). This is especially relevant to cholesterol bioconversion, since such enzymes can aid cholesterol-removing processes, as found previously by other authors (Ohuchi and Saito 1992).

Tetrahymena enzymatic build-up is therefore potentially useful for the treatment of eggs to decrease cholesterol while simultaneously enhancing pro-vitamin D sterols. It could also increase essential fatty acids content, and predigest proteins. In this work we investigated the effects of treating whole egg and egg yolk with live *Tetrahymena* cells, particularly regarding cholesterol conversion to pro-vitamin D. Critical conditions for optimal bioconversion were assessed, including the influence of cell inoculum, dilution, pH, aeration, and agitation rate.

Materials and Methods

Microorganism and culture conditions

Tetrahymena thermophila strain CU 399 was used throughout the study. Cells were grown in a proteose-peptone medium as described previously (Valcarce and others 2000). It consisted of (w/v) 1% proteose-peptone, 0.1% yeast extract, 1% glucose and 0.003% iron citrate. Cultures were inoculated daily by a 1:10 dilution of a 24 h culture. Cultivation was carried out with rotary shaking (100 rpm) at 29 °C.

Inoculation of cells into whole egg and egg yolk suspensions

Tetrahymena cells were harvested by centrifugation of cultures (1500 x g for 10 min at 4 °C) and the cell pellets were washed once in water. Washed cells were re-suspended directly

Table 1–The influence of the inoculum and the initial pH on cholesterol conversion in egg suspensions

Condition		Cholesterol conversion (%)
Inoculum [a]	12 h (exponential)	48 ± 3
	24 h (early stationary)	45 ± 5
	48 h (late stationary)	31 ± 6
Initial pH	7.0	22 ± 3
	8.0	27 ± 2
	8.5	30 ± 3

[a] The initial cell concentration was 7 x 10 ^{5} cells/mL in all tested conditions. The values of cholesterol conversion correspond to the average of three experiments ± Standard Deviation of the mean (S.D.). Results were analyzed by ANOVA for statistical significance, see text.

in 50 mL of whole egg or egg yolk suspensions prepared in distilled water. The initial cell concentrations tested were 3.5 x10 ^{5} cells/mL, 7 x10 ^{5} cells/mL, and 1.4 x10 ^{6} cells/mL.

The whole egg and egg yolk suspensions were prepared at various dilutions, from 1:5 to 1:50, to select the best conditions for simultaneous growth and cholesterol conversion. Ampicillin (50 μg/mL), streptomycin (10 μg/mL) and 0.003% iron citrate were added. Cultures were incubated with shaking for up to 72 h at 29 °C.

Other conditions tested in this study were: the effect of aeration (by bubbling sterile air at 1 vol / min), the variation in rotary agitation (100 rpm or 250 rpm), the initial pH (7.0, 8.0, and 8.5), and the age of the cell inoculum (12, 24, or 48 h cultures).

Materials

Whole egg suspensions were prepared from fresh egg by dilution with distilled water. For egg yolk suspensions, the yolks were carefully separated from the egg white in aseptic conditions, and diluted with sterile distilled water. In both cases, suspensions were homogenized by thorough hand shaking. Proteose-peptone, yeast extract, iron citrate, and solvents were purchased from Merck (Darmstadt, FRG). C-18 Ultrasphere (4.6 x 250 mm) HPLC columns were from Beckman (Palo Alto, Calif., U.S.A.). Sterol standards were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Radiolabeled cholesterol (1α,2α [^{3}H]-cholesterol, 48.0 Ci/mmole) and rainbow molecular mass protein marker were purchased from Amersham (Buckinghamshire, U.K.).

Analysis of sterols

Lipids were extracted by a modification of the Bligh and Dyer method (Bligh and Dyer 1959). For sterol analysis, 1 mL samples from egg cultures were saponified by addition of 1 volume of 2 M sodium hydroxide prepared in methanol/water (1:1 v/v) and heated at 60 °C during 1 h. After cooling and mixing, 5.6 mL of chloroform/ methanol (3/2) was added. The sterols partitioning in the lower phase were concentrated under nitrogen and separated by high performance liquid chromatography (HPLC) on a C-18 Ultrasphere column using methanol/water (98:2, v/v) as mobile phase at 41 °C. The absorbancy of the eluates was monitored at 205 and 285 nm. An increase in A_{285} is indicative of the cholesterol desaturation at position 7, with formation of conjugated Δ 5,7-dienes. For quantitation, stigmasterol (100 μg/mL) was added to the egg suspensions prior to saponification and used as internal standard. This sterol separates cleanly from cholesterol and its derivatives under the conditions employed. The identity of each compound was established by gas chromatography-mass spectrometry (Valcarce and others 2000).

Bioconversion of radiolabeled cholesterol

Egg suspensions were supplemented with [1,2 ^{3}H] cholesterol (0.2 μCi/ mL, final concentration) prior to the inoculation with *Tetrahymena* cells. Radiolabeled cholesterol was added from a 1 mCi/mL stock solution in ethanol, by dilution in 50 mL of the egg suspension, followed by a 2 h incubation with shaking at 30 °C to help partition of cholesterol into egg fat globules. The desaturation of radiolabeled cholesterol by *Tetrahymena* cells was examined by HPLC coupled to a Flo-one Beta Radio chromatography detector (Radiomatic, Canberra Company).

Protein electrophoresis

Aliquots of whole egg or egg yolk suspensions cultured with *Tetrahymena* were removed at the indicated time points (0, 10, and 24 h), mixed with Laemmli sample buffer (Laemmli 1970) at a protein concentration of 19 mg/mL and analyzed using a Phast gel system (Pharmacia, Upsala, S.V.) according to the manufacturer instructions.

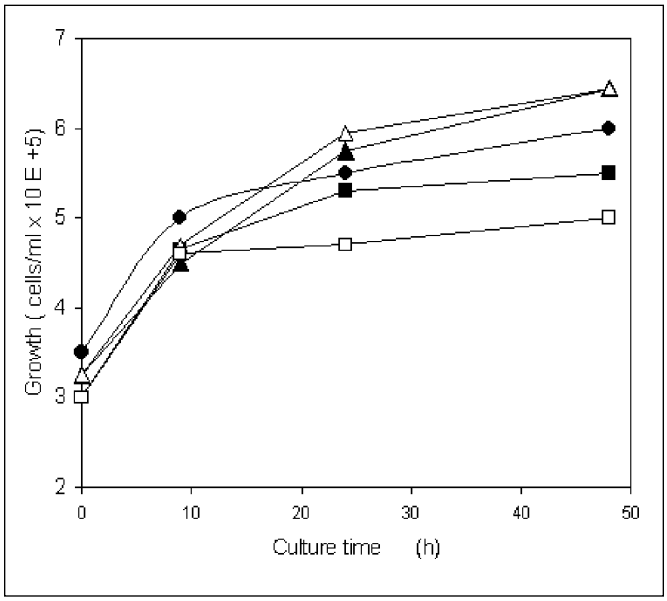

Figure 1–Growth of *Tetrahymena* in egg suspensions. Symbols: -▲- egg-yolk 1:10; -■- egg-yolk 1:50; -△- egg 1:10; -□- egg 1:50; -●- egg 1:5

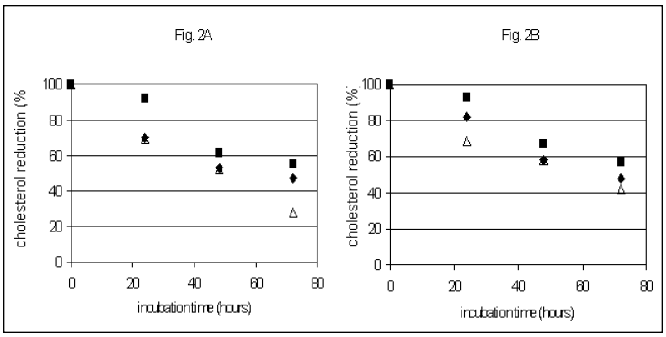

Figure 2–Residual cholesterol in egg (2A) and egg yolk (2B) suspensions after inoculation with different cell densities of *Tetrahymena thermophila*.. Symbols: -■- 3.5 x 10 ^{5} cells/mL; -◆- 7 x 10^5 cells/mL; -△- 1.4 x 10 ^{6} cells/mL.

Table 2–Changes in cholesterol and cholesterol unsaturated derivatives upon culture of egg suspensions with *Tetrahymena thermophila*.

	Sterol metabolites (μg /mL)				Radioactivity distribution (%)			
Time [a]	CH	D7-CH	D7,22-CH	D22-CH	CH	D7-CH	D7,22- CH	D22-CH
0 h	550 ± 12	0	30 ± 2	0	100 ± 2	0	0	0
10 h	430 ± 21	20 ± 3	94 ± 8	30 ± 3	78 ± 5	4 ± 1	17 ± 2	6 ± 1
24 h	250 ± 15	19 ± 2	151 ± 13	44 ± 3	45 ± 6	4 ± 1	27 ± 4	8 ± 2

[a] Egg suspensions (1:5) were supplemented with 20 ul of [1,2 ^{3}H] -cholesterol (1 mCi /mL) and inoculated with 1.4 x 10 ^{6} cells/mL. Immediately after inoculation (0 h) and after 10 and 24 h incubation with aeration, samples were withdrawn and analyzed for mass sterol composition (μg /mL) and radioactivity recovered in each of the cholesterol derivatives. The latter are expressed as percentage of the initial radioactive cholesterol added to the suspension (%). The values correspond to means ± Standard Deviation of three separate experiments. CH: cholesterol; D7-CH, D7- dehydrocholesterol; D7,22-CH: D7,22 bis-dehydrocholesterol; D22-CH, 22 dehydrocholesterol.

Amino-acid analysis

Egg samples were extracted with hexane to remove most of the lipids. The protein (in the lower phase) was hydrolyzed in concentrated HCl and the amino-acid content of the samples was analyzed with an Applied Biosystems 477 automatic analyzer, as previously described (Valcarce and others 2001).

Statistic analysis

The data were analyzed by One way ANOVA test (Stastistix 7, Analytical Software).

Results and Discussion

THE ABILITY OF EGGS TO SUPPORT *TETRAHYMENA* CELL multiplication is a key step for the biotransformation process; therefore, growth of the ciliate on egg suspensions was first assessed. As shown in Figure 1, after a 24 h incubation period, the total cell counts approximately doubled the initial cell concentration, both in 1:10 and 1:5 whole egg or egg yolk dilutions, displaying similar growth yields. Lower biomass yield was attained in diluted suspensions (1:50).

Growth of *Tetrahymena* in egg suspensions takes place without formation of precipitates or other noticeable alteration in the sensory properties. In addition, as cultivation with *Tetrahymena* progressed, considerable decrease in the cholesterol initially present takes place. The cholesterol content decreases with the incubation time; the rate for this decrease depends on the amount of cells inoculated. By using 7 x10 ^{5} or 1.4 x10 ^{6} cells/mL as inoculum, 40% to 50% cholesterol reduction is obtained in 48 h incubation in 1:10 whole egg or egg-yolk suspensions (Figure 2A and B).

Cholesterol is converted into the unsaturated derivatives, Δ7-dehydrocholesterol (pro-vitamin D3), Δ7,22 bis-dehydrocholesterol (pro-vitamin D2 analog) and Δ22-dehydrocholesterol. Figure3 illustrates the kinetics of the process and compares a typical chromatogram obtained at the time of inoculation, showing cholesterol as the sole peak (Figure 3A) and a chromatogram obtained at 24 h culture (Figure 3B), in which cholesterol is reduced and the unsaturated derivatives are present. Under these suboptimal conditions, approximately 30% of cholesterol initially present in the egg suspension was metabolized in 24 h by a cell culture inoculated with 7 x 10 ^{5} cells/ mL, and more than 80% of the metabolized cholesterol was recovered in the form of pro-vitamin D sterols, mainly Δ7,22 bis-dehydrocholesterol. The decrease in cholesterol content and the simultaneous increase in the total amount of pro-vitamin D sterols formed (Δ7-dehydrocholesterol plus Δ7,22 bis-dehydrocholesterol) at 24, 48, and 72 h culture, is presented in Figure 4. The values shown correspond to a 1:10 egg suspension inoculated with 7 x 10 ^{5} cells/ mL .

A number of cultivation conditions, including the age of inoculum and the initial pH of the egg suspensions, were investigated in relation to cholesterol conversion. As shown in Table 1, the use of exponential or early stationary phase cultures significantly

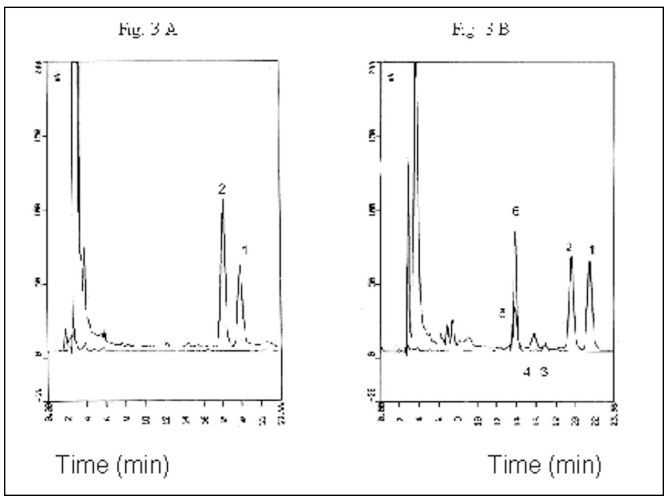

Figure 3–HPLC chromatograms showing the reduction of cholesterol and the appearance of dehydrocholesterol derivatives. A_{210} (upper tracing): Stigmasterol (1), Cholesterol (2), Δ22-dehydrocholesterol (4), Δ7,22 bis-dehydrocholesterol (5). A_{285} (lower tracing): Δ7-dehydrocholesterol (3), Δ7,22 bis-dehydrocholesterol (6).

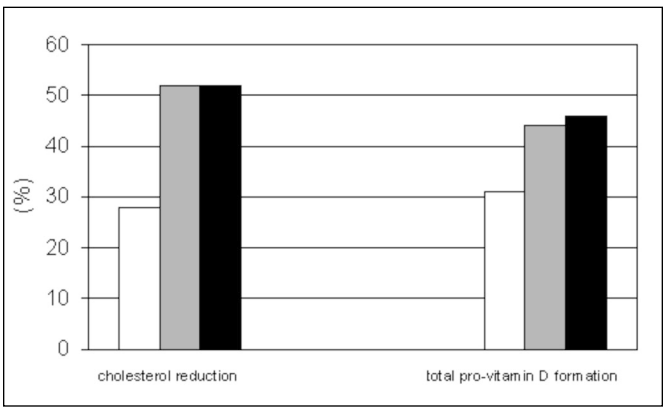

Figure 4–Cholesterol reduction and total pro-vitamin D formation at 24- h □, 48 h (▒), and 72 h ■ culture. Total pro-vitamin D was calculated as (r7,22 + r7) dehydrocholesterol.

($p < 0.05$) improved cholesterol conversion, compared to late stationary phase inoculum. Likewise, an initial pH between 8.0 and 8.5 yielded significantly better results than a lower starting pH.

The effects of agitation, aeration, and temperature were also examined. Figure 5 shows that aeration of the egg suspensions strongly improved cholesterol conversion by the cells, lowering the time of the process. Agitation (or the use of baffles) was either irrelevant or deleterious, probably due to shear stress imposed on the cells. Indeed, increasing the agitation speed from 150 to 250 rpm resulted both in lower growth yield and lower cholesterol conversion.

The combination of all the above mentioned factors (aeration, pH and the use of a fast growing culture) optimized the process. Under the selected conditions, cholesterol is reduced from an initial concentration of 550 ± 12 μg/mL to a final of 250 ± 15 μg/mL, implying a 55% ± 6 cholesterol reduction is obtained in a 24 h incubation period. Most of the converted cholesterol appears as dehydrocholesterol derivatives (see below).

The changes in egg- sterol composition upon cultivation for 0, 10, and 24 hours with live *Tetrahymena* cells in egg suspensions, with aeration, are summarized in Table 2. The amounts of cholesterol and the unsaturated derivatives, Δ7-dehydrocholesterol, Δ7,22 bis-dehydrocholesterol, and Δ22-dehydrocholesterol, were measured by absorbance (at 280_{nm} and 285_{nm}) for mass estimation, and by radioactivity determination using exogenous radiolabeled cholesterol as precursor (see Materials and Methods). An increase in total amino-acids content (16% ± 2) upon 24 h culture with *Tetrahymena* in whole-egg suspensions accompanied this process. However, significant proteolysis was also evident, as found by electrophoretic analysis on SDS-PAGE (Figure 6). Indeed, the protein content corresponding to lower molecular mass fractions increased during the incubation period. A similar proteolytic breakdown has been observed during the incubation of milk with cells of this strain (Valcarce and others 2001). A likely consequence of proteolysis is a better tolerance of egg by individuals with egg protein hypersensitivity.

Conclusions

A VARIETY OF METHODS HAVE BEEN PROPOSED TO DECREASE cholesterol content in foodstuffs, but none has yet attained commercial use. The physical and chemical techniques have the disadvantage that, in addition to cholesterol, fats, proteins, pigments, and flavor components are extracted, causing deterioration of quality. The enzymatic methods, although appropriate with regard to the resulting food quality, are difficult to apply, because food cholesterol is tightly associated with lipoprotein, biomembranes, or fatty granules, therefore limiting their accessibility (Saito and Ohuchi 1998).

The method proposed here is selective and suitable for complexed cholesterol. It has the particular advantage that, in addition to reducing cholesterol, it enhances the supply of pro-vitamin D sterols.

Protein degradation may be adequate for individuals with egg protein hypersensitivity. If not desired, proteolysis could be prevented by mutagenesis and by selection of strains with low protease activity, as it has been done for other hydrolases (Hunseler and others 1987).

In conclusion, this work shows a simple biological way to process eggs in which cholesterol content becomes a source of provitamin D. The method might have application for food enrichment utilizing the full potential of eggs.

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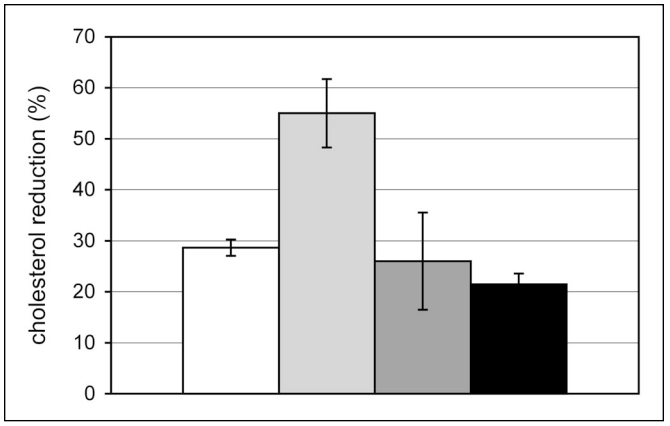

Figure 5—The influence of culture parameters on egg-cholesterol reduction by *Tetrahymena*. Control □; aeration ■; agitation ■; baffles ■. Aeration was the only parameter significantly ($p < 0.05$) increasing cholesterol bioconversion with respect to control cultures.

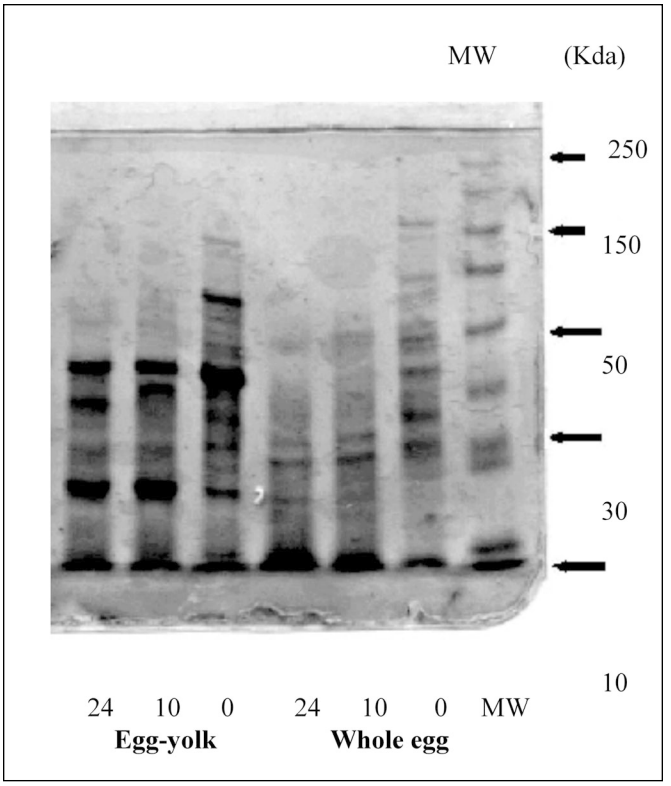

Figure 6—Protein digestion in egg and egg yolk suspensions treated with Tetrahymena cells. Phast gels were used to separate proteins upon incubation for 0, 10, and 24 hours.

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