

Behavior of Polymer-Supported Tomatine toward Cholesterol in the Presence or Absence of Butter Oil

5630

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Insoluble carboxy-functionalized polystyrene-divinylbenzene copolymers were bonded to tomatine at 0.03–0.11 mmol of tomatine/g of polymer. Tomatine was added either to the poly(styrenecarboxyl chloride) or directly to the (carboxymethyl)polystyrene through the mediation of dicyclohexylcarbodiimide. Tomatinized polymers removed cholesterol from hexane solutions of butter oil or cholesterol; however, the complex formed in the presence of butter oil is of low stability. Cholesterol was completely removed and the polymers were regenerated by benzene extraction at room temperature. Cholesterol bound to tomatinized polymers is not stable to 2-methyl-1-propanol extraction. Cholesterol uptake by tomatinized polymers was most efficient when hexane was the solvent.

INTRODUCTION

Heart disease, a singular manifestation of cardiovascular disorders, is associated with high blood cholesterol levels, high-fat diets, and the presence of cholesterol plaque deposits on arterial walls. Dairy products and all animal-derived foods contain low concentrations (<0.5%) of highly fat soluble cholesterol, which is a precursor in bile acid production and a normal cell component. Studies (Shorey et al., 1981) have shown that low-cholesterol diets (low fat) reduce blood cholesterol levels in humans. Since animal-derived foods represent a significant portion of the average diet, it would appear that cholesterol removal would constitute a first step in changing the public perception of these products as being somewhat less than wholesome because of their inherent cholesterol content. Butter oil being one of the simplest systems among dairy products was an ideal candidate for this program.

The selective removal of cholesterol from butter oil (Schwartz et al., 1967) was based on the formation of a digitonin-cholesterol complex during passage of a hexane solution of butter oil through a column of digitonin adsorbed on Celite. At a 7/1 weight ratio of digitonin to cholesterol, complete removal of cholesterol occurred with residual butter oil being washed from the column with solvent. Courregelonge and Maffrand (1987) reported the removal of cholesterol from butter oil by forming an inclusion complex with β -cyclodextrin. Sample recycling afforded 80% cholesterol removal and incomplete recovery of treated butter oil. Supercritical carbon dioxide extraction (Shishikura et al., 1986) of butter oil and passage of the extracted oil through silica gave comparable results. Subsequently, Kim (1989) disclosed that Shishikura et al. and a group at Cornell University refined the supercritical carbon dioxide fluid extraction process to the point where cholesterol removal and butter oil recovery exceeded 85%. The latest effort (Micich, 1990) showed that polymer-supported digitonin formed a reusable substrate to sequester cholesterol from hexane solutions of butter oil. In that study, digitonized polymers were regenerated by benzene extraction and were found to be somewhat less efficient cholesterol scavengers than digitonin on Celite. Approximately 10% of the polymer-bound digitonin actively complexed cholesterol from hexane solutions with even lower binding efficiencies for cholesterol in butter oil. The reduced efficiency of polymer-bound digitonin was ascribed to multiple bonding of digitonin to the

polymer or a high density of digitonin on the polymer. Subsequent steric distortions coupled with interference from butter oil constituents reduced complexation.

Tomatine is another steroidal saponin known to form a stable equimolar complex with cholesterol (Schulz and Sander, 1957). It is derived from tomato plants and is less toxic than digitonin. It is composed of a polycyclic steroidal secondary amine, tomatidine, and a tetrasaccharide containing glucose, galactose, and xylose. Thus, it can react with a carboxy-functionalized polymer to form an ester and/or an amide bond. In this study carboxypolystyrene, \ominus -CO₂H, was converted to the acid chloride and treated with tomatine in pyridine. (Carboxymethyl)polystyrene, \ominus -CH₂CO₂H, was reacted directly with tomatine in pyridine by using dicyclohexylcarbodiimide (DCC). Bonding tomatine to an insoluble polymer matrix permanently separates it from the homogeneous system and permits removal of complexed cholesterol by filtration or centrifugation. The known toxicity of digitonin stimulated this program to develop a less toxic polymer-bound complexing agent to remove cholesterol from butter oil and to demonstrate its reusability.

Csiky and Hansson (1986) performed the only similar study in which tomatine was chemically bonded to silica gel by using an epoxy-substituted silica as a stationary phase for the HPLC of sterols. The silica-supported tomatine was effective in separating several sterols including cholesterol, which could be removed by washing with hexane solutions of cyclohexanol.

MATERIALS AND METHODS

Caution. Benzene is a suspected carcinogen; exercise care in its use. Styrene and divinylbenzene (DVB) were distilled under dry nitrogen prior to use. Dicyclohexylcarbodiimide was vacuum distilled and stored in a drybox under nitrogen. *p*-Vinylbenzoic acid was recrystallized from 20% aqueous ethanol and vacuum dried prior to use. Pyridine was distilled from calcium hydride and stored over 4-Å molecular sieves under dry nitrogen. Tomatine, cholesterol, and Merrifield resin [chloromethylated polystyrene (2% DVB), 1.04 mequiv of chlorine/g of polymer] were from Sigma Chemical Co. (St. Louis, MO). Phthaldehyde and anhydrous dimethylformamide were from Aldrich Chemical Co. (Metuchen, NJ). Infrared spectra were taken from KBr disks by using a Perkin-Elmer 1310 microprocessor-controlled infrared spectrophotometer. Visible spectra were obtained with a Beckman DU Series 70 UV-vis spectrophotometer using 1-cm matched cells. Cholesterol was determined according to the *o*-phthalde-

hyde sulfuric acid colorimetric method for which the violet complex has a molar absorptivity of $24\,100\text{ L mol}^{-1}\text{ cm}^{-1}$ (Bachman et al., 1976). The method is thus sensitive to much better than $0.1\ \mu\text{mol}$ of cholesterol. On the basis of absorbance variations of calibration values from 0 to $80\text{-}\mu\text{g}$ of cholesterol, the relative error of cholesterol concentrations in Tables I–VII is $\pm 9\%$. Butter oil was prepared from locally purchased sweet unsalted butter. The butter was liquefied and centrifuged and the oily layer separated from the aqueous phase. The oily layer was vacuum filtered through double paper, yielding a clear filtrate of anhydrous butter oil which assayed at 0.28% cholesterol. The sample was stored at $-20\text{ }^\circ\text{C}$ under nitrogen.

Carboxy Polymers. Carboxypolystyrene ($<0.1\%$ DVB) popcorn polymer contained 0.38 mmol of $\text{CO}_2\text{H/g}$ of polymer with $\text{DF} = 0.04$. (Carboxymethyl)polystyrene (2% DVB) Merrifield resin contained 0.95 mmol of $\text{CO}_2\text{H/g}$ of polymer with $\text{DF} = 0.10$. The methods of preparation were previously described (Micich, 1990). The degree of functionalization (DF) is the mole fraction of phenyl groups carrying the indicated functionality.

Tomatinized Polymer Acid Chloride Method. Tomatinized polymer syntheses are variations of previously described methods (Micich, 1990). Carboxypolystyrene, popcorn polymer, 4.0 g (1.51 mmol of CO_2H) was added to 35 mL of benzene and dried azeotropically by distilling off 10 mL of benzene. To the cool dried polymer under nitrogen was added $5\ \mu\text{L}$ of anhydrous dimethylformamide and 4.2 g (33 mmol) of oxalyl chloride in 5 mL of anhydrous benzene. The mixture was stirred under gentle reflux overnight. Solvent and excess oxalyl chloride were removed at $1\text{ mm}/2.5\text{ h}/25\text{ }^\circ\text{C}$ followed by final removal at $0.1\text{ mm}/1\text{ h}/50\text{ }^\circ\text{C}$. Tomatine 0.80 g (0.79 mmol) was dried at $0.1\text{ mm}/1\text{ h}/105\text{ }^\circ\text{C}$ and then dissolved in 25 mL of anhydrous pyridine. The solution was added to the dry polymer acid chloride and stirred for 72 h at $105\text{ }^\circ\text{C}$. The reaction mixture was cooled, treated with 2 mL of methanol, and stirred for 4 h . The mixture was quantitatively transferred to a coarse sintered glass funnel and filtered. To the polymer was added 10 mL of pyridine, and the dispersion was allowed to stand for 5 min . The mixture was filtered and the process repeated twice again with a total of 30 mL of pyridine. This washing procedure was repeated with 30 mL of each of the following warm solvents: benzene, benzene/ethanol (1:2, 1:1, 2:1), ethanol, and finally methanol. The tan polymer was dried to a constant weight of 4.13 g , giving a product with 0.03 mmol of tomatine/g of polymer. An IR spectrum of the polymer showed a broad intense OH band at 3440 cm^{-1} and shoulder carbonyl bands at 1730 and 1670 cm^{-1} .

Tomatinized Polymer Dicyclohexylcarbodiimide Method. (Carboxymethyl)polystyrene (2.00 g , 1.90 mmol of CO_2H) and tomatine (0.36 g , 0.36 mmol) were dried together as a mixture at $105\text{ }^\circ\text{C}/1.5\text{ h}/<0.1\text{ mm}$. Under dry nitrogen was added 16 mL of anhydrous pyridine and 1.02 g (5.0 mmol) of dicyclohexylcarbodiimide. The polymer suspension was stirred for 24 h at $5\text{ }^\circ\text{C}$ and then treated with 10 mL of water and stirred overnight at $25\text{ }^\circ\text{C}$. The supernatant was removed with the aid of a filter stick. The residual polymer was treated with 10 mL of warm solvent and stirred for 5 min , and the solvent was removed. This washing procedure was repeated twice again with a total of 30 mL of each of the following warm solvents: pyridine, benzene, benzene/ethanol (2:1, 1:1, 1:2), and ethanol. The product was transferred to a micro-Büchner funnel with methanol and dried to a constant weight of 2.18 g . The product contained 0.09 mmol of tomatine/g of polymer. An IR spectrum of a KBr disk showed a broad OH band at 3440 cm^{-1} and ester and amide carbonyls appearing as bands at 1730 and 1660 cm^{-1} , respectively.

Cholesterol Removal Procedures. Procedures A, treatment of polymer-supported tomatine with hexane solutions of butter oil or cholesterol, and B, cholesterol removal from polymer-supported tomatine by benzene extraction, were developed for polymer-supported digitonin, and in this work were applied without modification to tomatinized polymers (Micich, 1990).

RESULTS AND DISCUSSION

Tomatine reacted directly with (carboxymethyl)polystyrene, $\text{O-CH}_2\text{CO}_2\text{H}$, in the presence of DCC and pyridine at $5\text{ }^\circ\text{C}$. Coupling occurred in $\sim 24\text{ h}$ with IR bands showing both ester and amide linkages. After removal of

Table I. Cholesterol Uptake from Hexane Solution by Tomatinized Polymers after 48 h

sample ^a	mmol of $\text{CO}_2\text{H/g}$ of polymer	mmol of tomatine/g of polymer	mg of cholesterol uptake ^a /g of polymer	wt of tomatine/wt of cholesterol
1	0.38	0.03	3.2	10
2	0.95	0.06	2.7	22
3	0.95	0.09	2.0	47
4	0.95	0.11	2.9	36
5	0.95	0.12	2.6	46
control	0.95	0	0.9	0

^a Sample 1 is carboxypolystyrene from acid chloride; the remaining samples are (carboxymethyl)polystyrene obtained by DCC coupling.

monomeric materials through a series of solvent extractions, the samples contained $0.03\text{--}0.12\text{ mmol}$ of tomatine/g of polymer with most of the values at $\sim 0.10\text{ mmol}$ of tomatine. Variation in the molar ratios of tomatine to polymer of $0.15\text{--}1.0$ or DCC to polymer of $1\text{--}10$ had no obvious effect on the concentration of bound tomatine. Concentrations of bound tomatine increased with reaction time beyond 24 h . Carboxypolystyrene, $\text{O-CO}_2\text{H}$, did not add to tomatine in the presence of DCC. The inactivity is probably the result of steric factors because in this case the carboxyl group is attached directly to the benzene ring. In this study, tomatine was found to add to $\text{O-CO}_2\text{H}$ only at the 0.03-mmol level under the rather vigorous conditions of the acid chloride method. Previously digitonin was bound to this polymer at levels no better than 0.05 mmol/g of polymer (Micich, 1990).

(Carboxymethyl)polystyrene shows a broad carbonyl absorption at 1700 cm^{-1} , while carboxypolystyrene exhibits two carbonyl bands at 1730 and 1680 cm^{-1} associated respectively with free and hydrogen-bonded carboxyl groups. Tomatinized polymers were characterized by a weight gain, a broad intense OH band centered at $\sim 3440\text{ cm}^{-1}$, and carbonyl bands at 1730 , 1670 , and 1660 cm^{-1} associated with ester and amide bonds.

Cholesterol uptake from a hexane solution after 48 h is summarized in Table I for polymers containing $0.03\text{--}0.12\text{ mmol}$ of tomatine/g of polymer. Bound tomatine amounts to about 10% of the available carboxyl groups in the polymers. Sample 1 containing 0.38 mmol of $\text{CO}_2\text{H/g}$ of polymer was obtained via the acid chloride method, while the remaining samples were obtained by DCC coupling. Cholesterol uptake is a maximum at the lowest tomatine concentration and tends to plateau with further increases in bound tomatine. Configurational distortions of the saponin beyond a specific concentration are probably responsible for the ineffectiveness of higher tomatine concentrations. Schwartz et al. (1967) showed that free digitonin on Celite completely binds cholesterol at a $7/1$ weight ratio. The efficiency of cholesterol uptake was gauged by the weight ratio of tomatine to cholesterol. These values range from 10 to 50 and clearly show the effectiveness of sample 1 as a cholesterol scavenger. The values of cholesterol uptake are comparable to those found in digitonized polymers (Micich, 1990). These polymer samples were used to obtain the results in Tables II–VII.

The removal of cholesterol from simple hexane solutions and from hexane solutions of butter oil and the regeneration of tomatinized polymers derived from both types of polymer carboxylic acids are summarized in Tables II–IV. Cholesterol uptake by identical polymer samples is shown in Table II. Cholesterol uptake was measured in three series of determinations after 24 , 48 , and 120 h . After each series, cholesterol was stripped from the samples and the control by benzene extraction. The samples were

Table II. Reusability of Tomatinized Polymer To Remove Pure Cholesterol from Hexane Solution

sample ^a	mmol of tomatine/ g of polymer	mg of cholesterol uptake/g of polymer for series A-C after ^b								
		A			B			C		
		24 h	48 h	120 h	24 h	48 h	120 h	24 h	48 h	120 h
1	0.03	3.0	3.2	3.6	3.2	2.7	2.7	2.5	3.1	2.6
2	0.03	2.6	3.1	4.0	3.5	3.2	3.1	2.5	3.3	3.3
control	0	0.5	0.2	1.0	0.5	1.0	1.3	0.8	1.0	1.6

^a Used 0.40-g polymer sample derived from \ominus -CO₂H. ^b Reaction time (hours) after which cholesterol uptake was determined. The same notation is used in Tables III and IV.

Table III. Reusability of Tomatinized Polymers To Remove Pure Cholesterol from Hexane Solution

sample ^a	mmol of tomatine/ g of polymer	mg of cholesterol uptake/g of polymer for series A-C after								
		A			B			C		
		24 h	48 h	144 h	24 h	48 h	72 h	24 h	72 h	144 h
1	0.06	2.2	2.7	3.3	3.3	3.3	3.5	1.1	1.6	3.9
2	0.09	1.4	2.0	2.4	1.6	2.1	2.7	0	1.8	2.2
3	0.11	2.5	2.9	2.8	2.5	3.1	3.3	0.7	2.3	2.0
4	0.12	3.2	2.6	3.3	2.3	2.3	3.5	0.1	2.2	3.9
control	0	0.9	0.9	2.5	0.5	1.0	1.2	2.3	1.9	2.6

^a Used 0.40-g polymer samples derived from \ominus -CH₂CO₂H.

Table IV. Reusability of Tomatinized Polymers To Remove Cholesterol from Hexane Solutions of Butter Oil

sample ^a	mmol of tomatine/ g of polymer	mg of cholesterol uptake/g of polymer for series A-C after								
		A			B			C		
		24 h	48 h	120 h	24 h	48 h	120 h	24 h	48 h	120 h
1	0.03	0	0.7	0.7	0.5	0.8	1.1	0.4	0.8	1.1
2	0.03	0	0.6	1.0	0.7	0.8	1.0	0.4	0.8	0.7
3	0.09	0	0.4	0.4	0.3	0.5	0.8	0.2	0	0.3
4	0.11	0	0.3	1.3	0.3	0.5	1.0	0	0.7	1.1
control	0	0.8	0.7	0.2	0.3	0.4	0.8	0	0	0.5

^a Used 0.40 g of polymer; samples 1 and 2 were derived from \ominus -CO₂H and samples 3 and 4 from \ominus -CH₂CO₂H.

Table V. Cholesterol Removal by Benzene Extraction of Tomatinized Polymers

sample ^a	mmol of tomatine/ g of polymer	mg of cholesterol/g of polymer for series A-C					
		A		B		C	
		present	extracted	present	extracted	present	extracted
1	0.06	3.3	3.2	3.5	3.3	3.9	3.7
2	0.09	2.4	2.5	2.6	2.2	2.2	2.5
3	0.11	2.8	2.3	3.2	2.9	2.0	3.3
4	0.12	3.3	2.4	3.4	2.4	3.9	3.2
control	0	2.5	2.5	1.3	2.0	2.6	3.8

^a Polymer samples were derived from \ominus -CH₂CO₂H.

checked for residual cholesterol by performing the color reaction (Micich, 1990) on the extracted polymers. Polymer sample loss incurred during benzene extraction (10–15 mg) and that used in the color reaction (25 mg) were replaced with original polymer to a final sample weight of 0.40 g (Micich, 1990). The results show excellent agreement between the samples in each series. Cholesterol uptake is uniform after two regenerations and appears complete within ~24 h. Similar data are shown in Table III for tomatinized polymers obtained by DCC coupling. These samples show comparable cholesterol uptake after one regeneration but are less efficient scavengers after the second regeneration, requiring longer reaction for maximum uptake. Cholesterol uptake by both types of polymers from hexane solutions of butter oil and regeneration of the samples are shown in Table IV. The results show (a) the presence of induction periods (zero uptake after 24 h in series A), (b) slow cholesterol uptake for samples in each series, (c) cholesterol uptake reversal with sample 3 in series C, and (d) cholesterol uptake is about 25% of that found with hexane solutions of pure cholesterol. This behavior reflects not only interference from butter oil components but also indicates that the choles-

terol-polymer complex is weaker than that formed with digitonized polymers (Micich, 1990).

The efficiency of benzene extraction in removing cholesterol from tomatinized polymers is summarized in Table V. When each set of cholesterol uptake experiments shown in Table III was concluded, the samples and control were extracted with a series of benzene aliquots, and the cholesterol content was determined. The agreement between recovered cholesterol and that bound to the polymer sample is excellent in each series. Extracted polymers were found to be free of cholesterol.

A previous study using polymer-supported digitonin (Micich, 1990) showed that 2-methyl-1-propanol extraction of cholesterol bound to these polymers removed only free cholesterol. This suggested the presence of a stable cholesterol-digitonin polymer complex. The stability of cholesterol bound to tomatinized polymer, derived from DCC coupling, to 2-methyl-1-propanol extraction is shown in Table VI. According to the procedure developed to remove cholesterol from digitonized polymers, several cholesterol-containing polymer samples were repeatedly extracted first with 2-methyl-1-propanol and then with benzene. 2-Methyl-1-propanol removed substantial quan-

Table VI. 2-Methyl-1-propanol Extraction of Cholesterol from Tomatinized Polymers

sample ^a	mg of cholesterol/g of polymer		
	present ^b	extracted	
		2-methyl-1-propanol	benzene
1	4.3	1.5	3.5
2	1.2	1.3	0.7
3	2.2	1.3	2.1
4	1.1	1.4	0.7
5	3.2	0.7	2.8

^a Samples contained 0.11 mmol of tomatine/g of polymer. ^b These values include the control value.

Table VII. Effect of Solvent on Cholesterol Uptake by Tomatinized Polymer

sample	solvent ^a	mg of cholesterol/g of polymer ^b	
		found	control
1	hexane	2.9	0.9
2	methanol	1.0	1.6
3	ethanol	0.8	0.3
4	90% aqueous ethanol	0.9	1.3

^a Cholesterol uptake with cyclohexane, 2,3-dimethylbutane, benzene, ethyl acetate, 2-methyl-1-propanol, acetonitrile, and acetone was 0–0.3 mg/g of polymer. ^b Polymer samples contained 0.11 mmol of tomatine/g of polymer.

ties of cholesterol from every sample with complete removal occurring after subsequent benzene extractions. Since the final 2-methyl-1-propanol extract still contained a significant concentration of cholesterol, it is conceivable that continued extractions would eventually lead to complete removal of cholesterol. The somewhat high values of total extracted vs present may be due to an inherent procedural error.

The effect of solvents on cholesterol uptake by tomatized polymers is shown in Table VII. A single polymer sample containing 0.11 mmol of tomatine/g of polymer was used with exposure times of at least 72 h. The poorest interacting solvent, hexane, led to the highest cholesterol uptake. Methanol and ethanol are equivalent and result in only one-third the cholesterol uptake. All other solvents gave essentially zero cholesterol uptake. Curiously, the hexane isomers, cyclohexane and 2,3-dimethylbutane, are in this class. Essentially the same behavior was noted with polymer-supported digitonin (Micich, 1990).

On the basis of the information developed in this study the most efficient cholesterol-complexing polymers contained the lowest levels (0.03 mmol) of tomatine. Polymer-

supported tomatine removed cholesterol from hexane solutions and from hexane solutions of butter oil. The polymers were reactivated by benzene extraction and reused at essentially the same level of activity. Cholesterol uptake efficiency in butter oil is ~25% of that with hexane solutions of pure cholesterol. Hexane would be the solvent of choice for cholesterol uptake. Cholesterol-tomatinized polymer complexes are not stable to 2-methyl-1-propanol extraction. Tomatinized polymers form cholesterol complexes that are less stable than digitonized polymers (Micich, 1990) but exhibit comparable cholesterol uptake.

The apparent efficiency of low tomatine concentrations and its lower toxicity than digitonin are incentives to develop more effective polymer-supported systems.

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