 J. Agric. Food Chem. 1983, 31, 46–50

Characterization of Some Oxidation Products of β-Sitosterol

Gail G. Daly, Eugene T. Finocchiaro, and Tom Richardson*

Commercial β-sitosterol was oxidzed at 100 °C for 48 h. Oxidation products were separated and isolated by using thin-layer chromatography (TLC) and preparative layer chromatography (PLC). Identification of individual oxidation products was based on mobility values from TLC and high-performance liquid chromatography (HPLC) separations, on specific color reactions, and on fragmentation patterns from mass spectra of the purified compounds. 7α,7β-Hydroxysitosterols, 7-ketositosterol, and the isomeric 5,6-epoxides were identified as oxidation products with lower R_f values than β-sitosterol. Δ^4-Sitosterol-3,6-dione, Δ^4-sitosterol-3-one, and Δ^5-sitosterol-3-one were identified as products with greater R_f values than β-sitosterol. Oxidation products of cholesterol structurally similar to those of β-sitosterol have been implicated in atherosclerosis and cancer. The biomedical significance of oxidized β-sitosterol and other oxidized phytosterols remains to be determined.

Several phytosterols including stigmasterol, campesterol, and the isomeric sitosterols, all structurally related to cholesterol, have been isolated from plant lipids and characterized (Verett et al., 1926; Bergman, 1953; Hunter et al., 1978).

Anderson et al. (1926a,b) demonstrated that crude sitosterol consists of at least three isomeric forms which were designated α, β, and γ. In its purified form, crystalline β-sitosterol (stigmast-5-en-3β-ol) is a white solid with a mp of 104 °C, $[\alpha]_D$ of −36°, and molecular weight of 414.69.

β-Sitosterol (Figure 1) is the predominant and most widely distributed phytosterol. It has been isolated from wheat germ oil (Anderson et al., 1926a), corn oil (Inglett, 1970), and cottonseed oil (Wallis and Chakrovorty, 1937). Ueno and Yamasaki (1935) found that β-sitosterol can be readily purified from Koryan (Kaoliang) corn oil, since no other phytosterols are found in this oil. β-Sitosterol and its fatty acid esters are known to occur in the lipids of many plant-derived food products such as wheat flour (Morrison, 1978).

Cholesterol, the principal sterol of higher animals, is closely related in structure to β-sitosterol (Figure 1). Cholesterol oxidizes spontaneously, photochemically, and during thermal processing of foods to yield a complex mixture of oxidation products, some of which have been found in various foods (Smith, 1981). Several of the oxidation products of cholesterol possess a number of biological effects and have been implicated in the etiology of atherosclerosis and cancer (Smith, 1981).

Mutagenic effects of oxidation products of cholesterol have been studied by Smith et al. (1979) using the Ames test. They observed mutagenic activity towards *Salmonella typhimurium* strains TA 1537, TA 1538, and TA 98. All active oxidation products of cholesterol were frame shift mutagens; however, high concentrations of oxidized cholesterol were necessary to observe mutagenesis. Their data indicated that the autoxidation products of cholesterol were direct mutagens rather than requiring metabolic activation by microsomal enzymes.

Because of the structural similarity between β-sitosterol and cholesterol, analogous oxidation products should be formed in plant-based foods. Thus, oxidation products of β-sitosterol may have similar health implications. However, the limited intestinal absorption of β-sitosterol by humans (5%) (Salen et al., 1970; Subbiah, 1973) may also be reflected in limited absorption of oxidized β-sitosterol products. Nevertheless, potential alkylating agents such as epoxides of β-sitosterol may be involved in toxic effects on intestinal tissue. Furthermore, microbial modifications of oxidized phytosterols in the colon may yield toxic products.

Yanishlieva et al. (1980) detected 10 major products in the autoxidation of sitosterol. Included among these were the stigmast-5,24-dien-3β-ol, 7α- and 7β-hydroperoxysitosterol, and 6α- and/or 6β-hydroperoxystigmast-4-en-3-one.

This research was designed to characterize as many oxidation products as possible in heated β-sitosterol. This study is preliminary to additional research on the occurrence and toxicity of oxidized phytosterols in various plant-derived foods.

MATERIALS AND METHODS

The experimental design for the preparation, isolation, and characterization of major oxidation products of β-sitosterol is illustrated in Figure 2.

Sample Preparation. Commercial β-sitosterol (91% β-sitosterol, 9% campesterol) was obtained from Nutritional Biochemicals and was oxidized without further purification. Standard β-sitosterol was obtained by recrystallization of this mixture from hot absolute alcohol. Oxidation of the sterols was accomplished by heating 25 g in a 500-mL round-bottom flask in a dark oven for 48 h at 100 °C. The heat-treated sterols were dissolved in boiling absolute ethanol (reagent grade). This solution was concentrated under vacuum at room temperature until white crystals appeared and then cooled to 4 °C overnight. Crystals of unoxidized sterols were removed by filtration, and the filtrate was further concentrated until a thick yellow paste remained. The paste yielded 4.5 g of oxidized sterols when dried overnight in a vacuum desiccator. Cholesterol standards were purchased from Steraloids, Inc., and purified by PLC. Sitosterol 5,6α-epoxide was synthesized from recrystallized β-sitosterol (Fieser and Fieser, 1967) and purified by PLC and HPLC.

Thin-Layer Chromatography (TLC). Fifty milligrams of oxidized sterol was dissolved in 1 mL of redistilled ether (reagent grade, Fisher Scientific Co.). Fifty microliters of this solution was applied as an individual spot to 20 × 20 cm plastic sheets precoated with silica gel (Polygram SilG/UV_{254}, Macherey-Nagel Co.). Ordinarily, chromatograms were developed in ether, dried, and sprayed with 50% H_2SO_4, followed by heating for a few minutes at 100 °C to char the separated oxidation products. Mobility values of the various products relative to the solvent front (R_f) and to β-sitosterol (R_s) were determined for tentative identification. Reproducibility was established by repeating the foregoing procedures several times. Additional chromatograms of oxidation products

Department of Food Science, University of Wisconsin—Madison, Madison, Wisconsin 53706.

0021-8561/83/1431-0046$01.50/0 © 1983 American Chemical Society

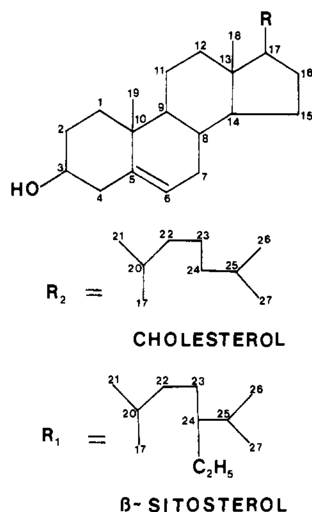

Figure 1. Similarity in structures of β-sitosterol and cholesterol.

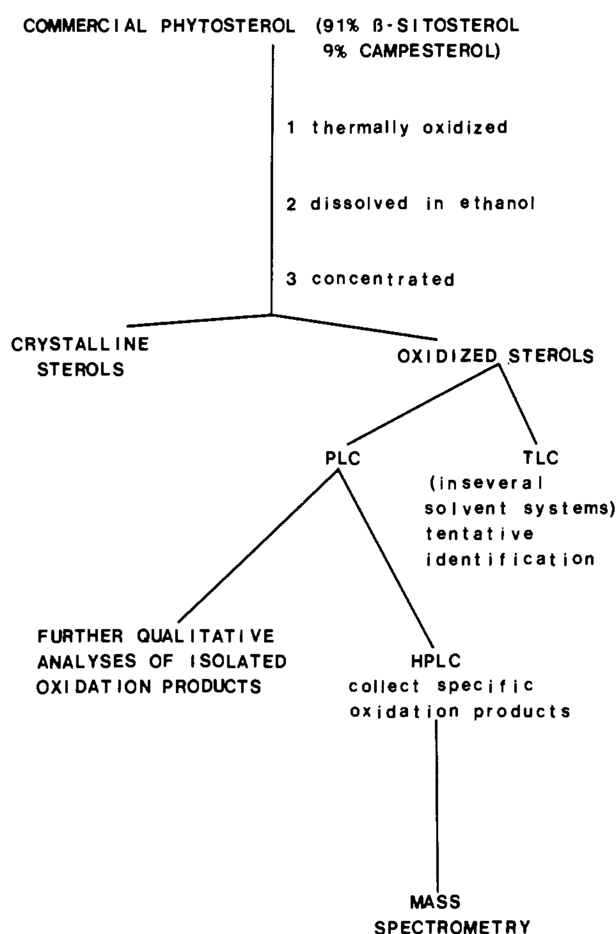

Figure 2. Scheme for the preparation, isolation, and characterization of major oxidation products of β-sitosterol.

were developed in diethyl ether–cyclohexane, 9:1 (v/v) (Aringer and Eneroth, 1974), and ethyl acetate–heptane, 1:3 (v/v) (Ansari and Smith, 1978). Because of potential structural similarities, oxidation products of β-sitosterol were tentatively identified by comparison with TLC data on oxidized cholesterol (Smith et al., 1967; Aringer, 1979).

Preparative Layer Chromatography (PLC). A suspension of 200 g of silica gel 60 PF-254 (E-M reagents) in 470 mL of water was allowed to stand for 1 h. The suspension was spread onto 20 × 20 cm glass paltes with a Desaga-Brinkmann spreader set at a thickness of 2 mm. The plates were dried for 2 days at room temperature, heat activated at 120 °C for 2 h, and stored in a desiccator. Fifty milligrams of oxidized sterol, dissolved in 1 mL of redistilled ether, was applied as a band across the bottom of the plate which was developed in ether. When the solvent front had reached the top of the plate, it was removed from the ether and dried, the edges were sprayed with 50% H_2SO_4, and it was heated at 100 °C for 5 min to locate the oxidation products. Bands thus located were scraped off the plates and adsorbent was extracted with redistilled ether. The ether was evaporated at room temperature with a stream of prepurified nitrogen to obtain the oxidation products. The products in the individual bands were subjected to further purification using high-performance liquid chromatography as employed by Ansari and Smith (1979) to characterize oxidation products of cholesterol.

High-Performance Liquid Chromatography (HPLC). Oxidation products of β-sitosterol were further purified by using a μBondapak C_{18} column (3.9 mm × 30 cm) with acetonitrile–water, 9:1 (v/v), as the mobile phase. The column, pump, sample injector, and solvents were all products of Waters Associates. Column effluent was monitored with a Perkin-Elmer LC-55 spectrophotometer set at 240 nm for general use and with a Waters differential refractometer (Model 401) for the detection of the 5,6-epoxysitosterols. An OmniScribe recorder (Houston Instruments) recorded the elution patterns. Forty microliters of ether as a control or oxidation products dissolved in ether was injected by using a Hamilton syringe. Major peaks were collected and elution times compared to those of Ansari and Smith (1979) for oxidized cholesterol and were used for tentative identification. The elution solvents were evaporated by using a stream of prepurified nitrogen at room temperature, and the resulting products subjected to mass spectrometry.

Mass Spectrometry (MS). Mass spectra were obtained with an AEIMS9 mass spectrometer equipped for electron impact (70 ev) and a source temperature of 100–125 °C above ambient. Samples were introduced as solids via the direct inlet probe and spectra recorded by using an AEI DS-50 data system.

Additional Analytical Procedures. The suspected 5,6-epoxide (spot 4, Figure 3) was dissolved in ether and HCl gas was passed through the solution to yield the putative chlorohydrin adduct. Ether was evaporated, and the resulting product was chromatographed, together with the untreated, suspected epoxide, as a reference.

The suspected epoxide was also subjected to alkaline picration (Fioriti et al., 1966) and perchloric acid hydrolysis (Knapp et al., 1976). Reaction products of both the sitosterol epoxide and a reference 5,6-epoxycholesterol were examined by using TLC.

Alkaline ethanol was added to the suspected Δ^4-3,6-dione (spot 6, Figure 3), and its absorption pattern was recorded by scanning wavelengths 200–450 nm with a Beckmann Model 25 spectrophotometer (Meyer, 1955).

A chromatogram of the suspected Δ^4-3-ketone (spot 7, Figure 3) was sprayed with nicotinic acid hydrazide, dried at room temperature, and observed for fluorescence under long wave ultraviolet radiation (Neher, 1969).

RESULTS AND DISCUSSION

The complex nature of the oxidation products of β-sitosterol required a combination of separation techniques to obtain the major oxidation products of sufficient purity for mass spectral analysis. The presence of minor unidentified oxidation products, even though undetected on the PLC plates, was evident when HPLC was performed on the individually isolated bands. At least three peaks were observed during HPLC analyses of each PLC band.

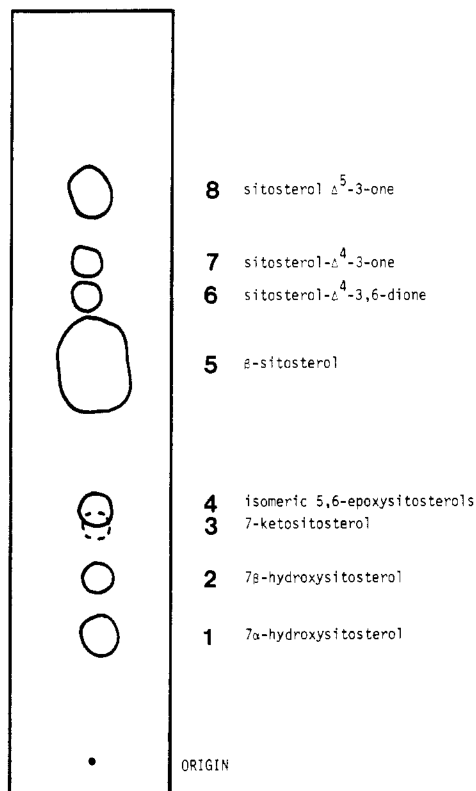

Figure 3. Oxidation products of β-sitosterol observed on silica gel UV_{254} TLC plates developed in ether, sprayed with 50% H_2SO_4, and then heated. Unsaturated ketosteroids were detected before heating by quenching of fluorescence upon irradiation of plates with ultraviolet light.

Since the PLC method used is dependent on adsorption effects whereas the HPLC used is partition chromatography, it is not unreasonable that a combination of the two techniques would yield different separation patterns that could be exploited to purify the major oxidized components. Purified, oxidized samples were stored in the dark, under nitrogen at −20 °C, to minimize changes prior to mass spectral analyses.

A typical TLC chromatogram of the oxidation products of β-sitosterol is shown in Figure 3. There were at least five oxidation products (spots 1–4) with lower R_f values than β-sitosterol (spot 5) and about three products with higher R_f values (spots 6–8). When the analytical TLC plates were sprayed with 50% H_2SO_4, spots 1 and 2 instantly became blue. This color development is typical for 7α- and 7β-hydroxycholesterols (Chicoye et al., 1968). The R_s values (Table I) and the blue colors indicated spots 1 and 2 were 7α- and 7β-hydroxysitosterol, respectively. The epimeric 7α- and 7β-hydroxysitosterols would be expected as major oxidation products since a free radical allylic to the double bond at the 5–6 position would be stabilized by resonance, yielding the 7α- and 7β-hydroperoxides since oxygen could attack on either side of the alicyclic ring. Subsequent reduction of these hydroperoxides would result in the 7α- and 7β-hydroxy epimers. Alternately, the thermal homolysis of the peroxide bonds could yield the 7-oxyl radicals which could then abstract hydrogen from other molecules to give the 7-hydroxy epimers. Furthermore, the 7-oxyl radicals could disproportionate to form the 7-ketone (Smith, 1981). These observations are consistent with those of Yanishlieva et al. (1980), who detected the 7α- and 7β-hydroperoxides in oxidized sitosterol.

Further TLC studies utilizing the techniques of Smith and Price (1967) confirmed the presence of a 7-ketone which cochromatographed with the epoxides (spots 3 and

Table I. Relative Migration of β-Sitosterol and Cholesterol Oxidation Products upon Thin-Layer Chromatography

sterol oxidation product	TLC R_c[a]	TLC R_s[a]
7α-hydroxycholesterol[d]	0.35[b]	
7α-hydroxysitosterol[f]		0.35[b]
7β-hydroxycholesterol[d]	0.50[b]	
7β-hydroxysitosterol[f]		0.48[b]
7-ketocholesterol[b]	0.60	
7-ketositosterol[f]		0.60[b]
5,6-epoxycholesterol[b]	0.65	
5,6-epoxysitosterol[f]		0.65[b]
cholest-4-ene-3,6-dione[e]	1.29[c]	
sitos-4-ene-3,6-dione[f]		1.28–1.31[c]
cholest-4-en-3-one[e]	1.46[c]	
sitos-4-en-3-one[f]		1.41–1.46[c]
cholest-5-en-3-one[e]	1.93[c]	
sitos-5-en-3-one[f]		1.92[c]

[a] R_c = mobility value of products relative to cholesterol. R_s = mobility value of products relative to β-sitosterol. [b] TLC system ethyl ether at room temperature. [c] TLC system ethyl acetate–heptane, 1:3 (v/v), at room temperature. [d] Taken from Chicoye et al. (1968). [e] Taken from Ansari and Smith (1978). [f] Oxidation products of β-sitosterol from PLC.

4, Figure 3). Although these oxidation products have slightly different R_s values (Table I), the PLC isolate of the epoxide spot also contained some of the 7-ketone. Additionally, the chromatogram of the 7-ketone indicated an R_s identical with that observed for the analogous R_c of 7-ketocholesterol (Table I).

Additional oxidation products became visible after the TLC plates were subjected to heating. Spot 4 (Figure 3), tentatively identified as an epoxide of β-sitosterol, had an R_f value of 0.46 [diethyl ether–cyclohexane, 9:1 (v/v)] which corresponded closely to R_f values of 0.48 reported in the literature for 24α-ethyl-5α,6α-epoxycholestan-3β-ol (5α,6α-epoxide of β-sitosterol) and 5α,6α-epoxycholestan-3β-ol (Aringer and Eneroth, 1974). Furthermore, both cholesterol and β-sitosterol epoxides had identical R_c and R_s values, respectively, when developed on the same TLC plate (Table I). The epoxide might have arisen from epoxidation of the 5,6 double bond by an adjacent 7α- or 7β-hydroperoxide or possibly by its peroxyl radical. According to Smith (1981) a mixture of α- and β-epoxides of sitosterol should arise with a predominance of the β-epimer. Although there have been numerous studies on the biological properties of the 5α,6α-epoxide of cholesterol, very little is known of the biological effects of the β epimer. To our knowledge, there has been no published research on the toxicity of either the α- or β-epoxides of β-sitosterol.

Hydrochlorination of the suspected β-sitosterol epoxide yielded a product with an R_f value lower than that of the original epoxide. The R_f of a reference epoxide obtained from the original PLC plate was compared to that of the suspected chlorohydrin adduct. The reference epoxide produced one spot on the TLC. The HCl-treated epoxide yielded two spots, the less polar one (possibly the 7-ketosterol) having the same R_f as the reference epoxide and the second more polar spot presumably being the chlorohydrin adduct.

Alkaline picration of both the reference epoxycholesterol and the suspected epoxide yielded a yellow spot on Sil G TLC plates developed in chloroform–methanol–acetic acid, 100:20:1 (v/v/v). The yellow color of the picrate adduct was indicative of an epoxide. Futhermore, the resultant hydrolysis products of the perchloric acid treated epoxides were examined by using Sil G TLC plates developed in ethyl ether. Both epoxide preparations yielded major spots

Table II. Relative Retention Times of Oxidation Products of β-Sitosterol Separated Using HPLC

sterol	relative retention time[a]
7α-hydroxysitosterol	0.25
7β-hydroxysitosterol	0.26
7-ketositosterol	0.35
5,6β-epoxysitosterol	0.45
5,6α-epoxysitosterol	0.49
Δ^4-sitosterol-3,6-dione	0.28
Δ^4-sitosterol-3-one	0.91
Δ^5-sitosterol-3-one	1.06

[a] System = μBondapak C_{18} column; acetonitrile-water, 9:1 (v/v); 1 mL/min; 0.01 S. Retention times relative to that of β-sitosterol.

with R_f values identical with those of a reference cholesterol triol. Thus, the TLC data strongly support the presence of a sterol epoxide. Although the isomeric 5,6-epoxides of cholesterol are not mutagenic in the Ames assay (Smith et al., 1979; Ansari et al., 1982) the cholesterol α-epoxide can apprently alkylate DNA in vitro (Blackburn et al., 1979). Therefore, the existence of these compounds in plant-derived foods requires further study.

The parent compound β-sitosterol (spot 5, Figure 3), which turned reddish brown when the TLC plates were heated after being sprayed with sulfuric acid, was identified by comparison with the migration of a β-sitosterol standard on the same plate.

Oxidation products of β-sitosterol with higher R_s values were tentatively identified as Δ^4-sitosterol-3,6-dione (spot 6, Figure 3), Δ^4-sitosterol-3-one (spot 7, Figure 3), and Δ^5-sitsterol-3-one (spot 8, Figure 3) based on the similarities between R_s and R_c values listed in Table I. Possible mechanisms for the formation of these ketones can be found in the comprehensive volume by Smith (1981).

The suspected Δ^4-sitosterol-3,6-dione isolated from PLC plates turned yellow immediately after contact with alkaline ethanol; this solution exhibited a maximum absorption near 250 nm and a second absorption band at 380 nm with an intensity of approximately 75% that of the major band (Meyer, 1955) indicative of the diketone.

Under long wave UV radiation, a yellow fluorescent spot with the proper R_f was observed after spraying the TLC plate with nicotinic acid hydrazide, suggesting the presence of Δ^4-sitosterol-3-one (Neher, 1969).

Retention times from HPLC of the oxidation products of β-sitosterol relative to β-sitosterol (RT) are shown in Table II. Although most of the HPLC analyses were conducted by utilizing an ultraviolet detection system, detection of the 5,6-epoxides, even at wavelengths as low as 210 nm, is not readily achieved (Smith, 1981). Monitoring of ultraviolet absorption of column effluent during HPLC of the epoxide isolate indicated a major peak with an R_T value of 0.35. This value is close to that observed for the analogous 7-ketocholesterol (Ansari and Smith, 1979) and is consistent with our TLC data which indicated the presence of the 7-ketosterol in the epoxide isolate. Subsequent HPLC studies of this isolate utilizing a refractive index detector resulted in the isolation of a major effluent peak with an R_T value of 0.45. Mass spectral analysis of the resultant product identified it as the 5,6-epoxide of β-sitosterol. In addition, a minor HPLC peak (R_T = 0.49) of the PLC isolate chromatographed with a reference 5α,6α-epoxysitosterol. Thus, the combined HPLC, TLC, and MS data confirm these two peaks as the 5,6β- and 5,6α-epoxysitosterols, respectively. The remaining R_T values are also close to those presented by Arsari and Smith (1979) using the same separation system for analogous oxidation products of cholesterol.

Table III. Fragmentation Patterns from Mass Spectra of β-Sitosterol and Some of Its Oxidation Products

sterol	m/e	relative abundance
7α- or 7β-hydroxysitosterol	430 (M^+)	67 (91)
	431 (M + 1)	17 (27)
	415 ($-CH_3$)	67 (82)
	412 ($-H_2O$)	83 (91)
	289 (−R)	33 (55)
	271 [−(R + H_2O)]	33 (46)
	247 [−(R + X)]	50 (73)
	229 [−(R + H_2O + X)]	100 (100)
sitosterol 5,6-epoxide	430 (M^+)	100
	431 (M + 1)	38
	415 ($-CH_3$)	10
	412 ($-H_2O$)	24
	397 [−(H_2O + CH_3)]	11
	394 ($-2H_2O$)	8
	289 (−R)	10
	271 [−(R + H_2O)]	10
	247 [−(R + X)]	10
	229 [−(R + H_2O + X)]	9
β-sitosterol	414 (M^+)	100
	415 (M + 1)	30
	399 ($-CH_3$)	18
	396 ($-H_2O$)	28
	273 (−R)	30
	255 [−(R + H_2O)]	30
	231 [−(R + X)]	32
	201 [−(R + H_2O + X)]	30
Δ^4-sitosterol-3,6-dione	426 (M^+)	
	427 (M + 1)	
	411 ($-CH_3$)	50
	285 (−R)	100
	243 [−(R + X)]	100
Δ^4-sitosterol-3-one	412 (M^+)	42
	413 (M + 1)	
	397 ($-CH_3$)	100
	271 (−R)	25
	229 [−(R + X)]	50
Δ^5-sitosterol-3-one	412 (M^+)	100
	413 (M + 1)	27
	397 ($-CH_3$)	13
	271 (−R)	
	229 [−(R + X)]	13

Mass spectrometry fragmentation patterns of β-sitosterol and its oxidation products (excluding the 7-ketone) are given in Table III. Characteristic fragments resulted from the parent ion M^+ and the isotopic M + 1, loss of H_2O (18 mass units), loss of methyl CH_3 (15 mass units), loss of side chain R (141 mass units), loss of R X − next three carbons from the five-membered ring (183 mass units), loss of R and H_2O (159 mass units), and loss of R, H_2O, and X (201 mass units). Loss of H_2O was not observed for the ketones. The mass spectra are consistent with the preliminary structural assignments based on TLC and HPLC. Thus, the major oxidation products of β-sitosterol have been characterized by utilizing several complementary methods. The eight compounds which include the 7α,β-hydroxysterols, the isomeric 5,6-epoxides, and four ketones must be studied further to determine whether they present potentially adverse effects on humans as has been proposed for some of their cholesterol analogues.

ACKNOWLEDGMENT

We are grateful to M. Micke for mass spectra, to J. H. von Elbe and E. Attoe for assistance with HPLC, and to P. Gordon for helpful discussions.

Registry No. β-Sitosterol, 83-46-5; 7α-hydroxy-β-sitosterol, 34427-61-7; 7β-hydroxy-β-sitosterol, 15140-59-7; 7-keto-β-sitosterol, 2034-74-4; 5,6β-epoxy-β-sitosterol, 20835-90-9; 5,6α-epoxy-β-sitosterol, 53034-66-5; Δ^4-sitosterol-3,6-dione, 23670-94-2; Δ^4-sitosterol-3-one, 1058-61-3; Δ^5-sitosterol-3-one, 51529-11-4.

LITERATURE CITED

Anderson, R. J.; Nabenhauer, F. P.; Shriner, R. L. *J. Biol. Chem.* **1926a**, *71*, 389.
Anderson, R. J.; Shriner, R. L.; Burr, G. O. *J. Am. Chem. Soc.* **1926b**, *48*, 2987.
Ansari, G. A. S.; Smith, L. L. *Chem. Phys. Lipids* **1978**, *22*, 55.
Ansari, G. A. S.; Smith, L. L. *Chromatographia* **1979**, *11*, 307.
Ansari, G. A. S.; Walker, R. D.; Smart, V. B.; Smith, L. L. *Food Chem. Toxicol.* **1982**, *20*, 35.
Aringer, L. *Lipids* **1979**, *15*, 563.
Aringer, L.; Eneroth, P. *J. Lipid Res.* **1974**, *15*, 389.
Bergman, W. *Annu. Rev. Plant Physiol.* **1953**, *4*, 383.
Blackburn, G. M.; Rashid, A.; Thompson, M. H. *J. Chem. Soc., Chem. Commun.* **1979**, 420.
Chicoye, E.; Powrie, W. D.; Fennema, O. R. *J. Food Sci.* **1968**, *33*, 581.
Fieser, M.; Fieser, L. F. "Reagents for Organic Synthesis"; Wiley: New York, 1967; Vol. 1, p 136.
Fioriti, J. A.; Bentz, A. P.; Sims, R. J. *J. Am. Oil Chem. Soc.* **1966**, *43*, 487.
Hunter, I. R.; Walden, M. K.; Heftmann, E. *J. Chromatogr.* **1978**, *153*, 57.
Inglett, G. E. "Corn, Culture, Processing, Products"; Avi Publishing Co.: Westport, CT, 1970; pp 246–247.
Knapp, F. F.; Wilson, M. S.; Schroepfer, G. J. *Chem. Phys. Lipids* **1976**, *16*, 31.
Meyer, A. S. *J. Org. Chem.* **1955**, *20*, 1240.
Morrison, W. R. *Cereal Chem.* **1978**, *55*, 548.
Neher, R. "Thin Layer Chromatography", 2nd ed.; Stahl, E., Ed.; Springer-Verlag: New York, 1969; p 324.
Salen, G.; Ahrens, E. H., Jr.; Grundy, S. M. *J. Clin. Invest.* **1970**, *49*, 952.
Smith, L. L. "Cholesterol Autoxidation"; Plenum Press: New York, 1981.
Smith, L. L.; Mathews, W. S.; Price, J. C. *J. Chromatogr.* **1967**, *27*, 187.
Smith, L. L.; Price, J. C. *J. Chromatogr.* **1967**, *26*, 509.
Smith, L. L.; Smart, V. B.; Ansari, G. A. S. *Mutat. Res.* **1979**, *68*, 23.
Subbiah, R. M. T. *Am. J. Clin. Nutr.* **1973**, *26*, 219.
Ueno, S.; Yamasaki, R. *J. Soc. Chem. Ind., Jpn., Suppl.* **1935**, *38*, 113.
Verett, E.; Wallis, S.; Fernholz, E. *J. Am. Chem. Soc.* **1926**, *48*, 2446.
Wallis, E. S.; Chakrovorty, P. N. *J. Org. Chem.* **1937**, *2*, 335.
Yanishlieva, N.; Schiller, H.; Marinova, E. *Riv. Ital. Sostanze Grasse* **1980**, *57*, 572; *Chem. Abstr.* **1981**, *95*, 98142g.

Received for review November 16, 1981. Revised manuscript received August 30, 1982. Accepted September 16, 1982. This research was supported by the Food Research Institute, University of Wisconsin—Madison, by a grant from the Co-operative Research Service, U.S. Department of Agriculture, and by the College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, WI 53706.

Debittering Mechanism of Bitter Peptides from Milk Casein by Wheat Carboxypeptidase

Hironori Umetsu,* Hiroatsu Matsuoka, and Eiji Ichishima

The bitter peptide fraction obtained from the peptic hydrolysate of casein was treated with crystalline wheat carboxypeptidase. The bitterness of the bitter fraction lessened with an increase in free amino acids. The enzymatic hydrolysate obtained from the digest of the bitter peptide fraction by wheat carboxypeptidase was chromatographed on Sephadex G-15, and the eluate was subjected to amino acid analysis. When the release percentage of total free amino acids was approximately 30%, those of hydrophobic amino acids with a Δf value (cal/mol) >1600 were 32–76%, and amino acids with a Δf value <1600 were scarcely released except for alanine and threonine. The wheat carboxypeptidase seems to have an ability to eliminate bitter taste in enzymatic protein hydrolysate by releasing hydrophobic amino acids from bitter peptides.

Enzymatic hydrolysates of various proteins have a bitter taste which may be one of the main hindrances to their utilization in food. Though unutilized protein from soybean (Fujimaki et al., 1968), fish (Fujimaki et al., 1973; Hevia et al., 1976), etc. have been modified by the treatment with proteolytic enzymes for the purpose of improving their solubility, heat stability, and resistance to precipitation in acidic environments, the use of their enzymatic hydrolysates has been limited owing to the presence of bitter flavor components. Some kinds of cheese such as Cheddar cheese rarely have a bitter taste in matured or finished products (Nelson, 1975; Hamilton et al., 1974).

Many bitter peptides have been identified and their structures estimated in enzymatic hydrolysates of soybean protein (Fujimaki et al., 1970b) and casein (Matoba et al., 1970; Clegg et al., 1974). The bitterness of peptides appears to be closely related to the contents and sequence of hydrophobic amino acids (Clegg et al., 1974).

Arai et al. (1970) showed that the bitterness of peptides from soybean protein hydrolysates was decreased by treatment of *Aspergillius* acid carboxypeptidase. It was reported that an endopeptidase-catalyzed reverse reaction, usually called the plastein reaction, was effective for debittering (Fujimaki et al., 1970a). Two debittering methods based on the removal of hydrophobic peptides from enzymatic protein hydrolysates have been proposed. Studies by Roland et al. (1978) showed that a nonbitter peptide fraction was selectively prepared from soy and casein protein hydrolysates by hydrophobic chromatography.

Food Research Laboratory, Pentel Co., Ltd., Soka, Saitama 340, Japan (H.U.), Laboratory of Food Chemistry, Tachikawa College of Tokyo, Akishima, Tokyo 196, Japan (H.M.), and Laboratory of Enzymology and Microbial Chemistry, Tokyo Nōkō University, Fuchu Tokyo 183, Japan (E.I.).

0021-8561/83/1431-0050$01.50/0 © 1983 American Chemical Society