Role of Proteinaceous α-Amylase Enzyme Inhibitors in Preharvest Sprouting of Wheat Grain

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Proteins in wheat (*Triticum aestivum* L.) grain inhibit α-amylase enzyme from many sources. We compared proteinaceous α-amylase inhibitors in wheats that differ in preharvest sprouting susceptibility; determined involvement of the inhibitors in excised embryo germination; and assessed the relationship among α-amylase inhibition, embryo germination, and calcium. Water-soluble proteins were fractionated with ethanol, purified by ion-exchange chromatography, and tested for inhibition of α-amylase from unmalted grains. Only proteins from sprouting-resistant genotypes inhibited α-amylase in standard assays, but adding EDTA to chelate calcium induced inhibition by all genotypes. Chromatography yielded six inhibitor peaks that had molecular weights of 14000–68000 by electrophoresis. None of the inhibitors influenced germination of excised embryos. We concluded that proteinaceous α-amylase inhibitors interact with calcium but do not play primary roles in preharvest sprouting. They may have important secondary effects, however, by influencing α-amylase activity per unit of sprouting.

Preharvest sprouting of wheat and other cereals is a major problem in many regions of the world. Both agronomic attributes, particularly test weight and yield, and functional quality for milling and baking are adversely altered (Belderok, 1961, 1976; Bhatt et al., 1981). α-Amylase enzyme (E.C. 3.2.1:1) activity is closely associated with preharvest sprouting and is often used as a measure of sprouting damage (Mathewson and Pomeranz, 1977).

Proteinaceous compounds that inhibit α-amylase enzyme from animal sources have been widely studied because of their possible nutritional significance, retardation of insect infestation of grain, and protein–protein interaction properties (Richardson, 1981). Endogenous proteinaceous inhibitors of cereal α-amylase enzyme have been reported only recently (Weselake et al., 1985a). These or similar compounds may regulate α-amylase activity during sprouting and other processes (Warchalewski, 1977). The relationship between the level of inhibitor and α-amylase activity, however, is not clear. The inhibitor is reported to increase during grain maturation, when α-amylase activity is decreasing, and to decrease to a low level during germination, when α-amylase activity is increasing (Pace et al., 1978). Other reports indicate that the inhibitor remains active during germination (Weselake et al., 1985a).

Wheat α-amylase inhibitors occur in multiple molecular forms (O'Donnell and McGeeney, 1976). They belong to three main families having molecular weights of 12000, 24000, and 60000 (DePonte et al., 1976), and an α-amylase inhibitor of 30000 is described (Granum and Whitaker, 1977). Purothionins, basic low molecular weight proteins of 6000–12000, have also been reported (Balls et al., 1942); α-amylase enzyme might be inhibited by interactions of its Ca^{2+} cofactor with proteinaceous inhibitors, including purothionins (Lang et al., 1973; Jones and Meredith, 1982).

Susceptibility to preharvest sprouting and production of α-amylase enzyme differ markedly between red and white wheat classes and among genotypes within each class (McCrate et al., 1981). Reasons for these differences are not well understood, however. Objectives of the present investigations were to compare inhibitor levels in wheat classes and genotypes that differ in preharvest sprouting; determine effects of the inhibitors on germination of excised wheat embryos; and assess the relationship among α-amylase inhibition, calcium, and embryo germination.

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METHODS

Plant Material. Wheat genotypes used for the investigations were identified previously as having a wide range of dormancy and afterripening requirements (McCrate et al., 1981). Newton and Clark's Cream are hard red and white winter wheats, respectively, that resist preharvest sprouting, and Parker 76 and KS75216 are hard red and white winter wheats, respectively, that sprout readily under appropriate conditions (Nielsen et al., 1984).

The four genotypes and Lancota, a sprouting-resistant hard red winter wheat that provided excised embryos, were grown with recommended agronomic practices in replicated plots. Grain was harvested immediately when it ripened. After harvest, grain of the four former genotypes was held at room temperature, whereas grain of Lancota was frozen at −20 °C to arrest afterripening (Belderok, 1961).

Inhibitor Extraction. A modification of the O'Donnell and McGeeney (1976) procedure was used for extracting and separating α-amylase inhibitors (Figure 1). Grain of the four genotypes was ground through a 1-mm sieve to produce whole wheat flours, which were suspended in three volumes of water and centrifuged at 4000*g* at 0 °C for 15 min. The residue was washed with one volume of water and centrifuged. The supernatants from the first and second centrifugation were filtered and lyophilized. The resulting powder was dissolved in water heated at 70 °C for 30 min to inactivate α- and β-amylase enzymes (Warchalewski, 1977), cooled, and centrifuged at 8000*g* at 0 °C for 10 min.

Absolute ethanol was added to 60% (v/v) volume of the aqueous extract at 20 °C, and the precipitate was discarded after centrifuging at 8000*g* at 0 °C for 30 min. The ethanol concentration was raised to 90% (v/v), and the resulting precipitate, which contained the α-amylase inhibitory activity (O'Donnell and McGeeney, 1976), was separated by centrifugation as above. The precipitate was washed with absolute ethanol three times and dried at 30 °C overnight. The precipitate was dissolved in 0.01 M Tris-HCl buffer (pH 9.2) containing 1 mM NaCl and dialyzed against the same buffer at 4 °C overnight. Any cloudiness that developed was removed by centrifugation.

The extract was applied to a 1.5 × 40 cm DEAE-Sephacel column that was equilibrated previously with the buffer. Four column volumes of buffer were eluted, and then the extract was eluted with a continuous linear gradient of 0–0.5 M NaCl. The flow rate was 0.33 mL min^{-1},

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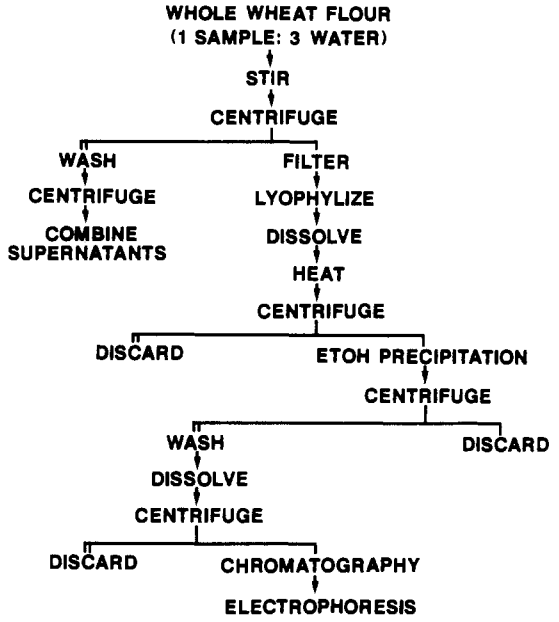

Figure 1. Separation and purification procedure for endogenous wheat α-amylase enzyme inhibitors.

and 6-mL fractions were collected. Protein in the effluent was monitored continuously at 280 nm.

All extracts and fractions were tested for inhibition of α-amylase (see below). Chromatography effluents that were inhibitory were combined within genotypes, dialyzed against 0.03 M NH_4HCO_3, lyophilized to dryness, and held for electrophoresis. In a separate study, fractions obtained from chromatography of Newton inhibitor were assayed individually for α-amylase inhibition, calcium content, and effect on embryo germination.

α-Amylase Inhibition. α-Amylase enzyme inhibitor activity in crude and purified extracts was measured by a modification of the methods of Barnes and Blakeney (1974) and Pace et al. (1978). All assays used enzyme obtained by NaCl extraction of KS75216 wheat seed germinated in 10^{-3} mM GA_3 (gibberellic acid) for 5 h.

Inhibition was measured by diluting 0.5 mL of the test solution (adjusted to pH 5.5) with 3.5 mL of NaCl extraction solution and adding 5 mL of α-amylase extract. The mixture was held at room temperature for 20 min and equilibrated at 50 °C for 10 min, and a Cibacron Blue labeled amylose tablet was added to each tube. The mixture was agitated vigorously every 2.5 min during incubation at 50 °C for 30 min. The reaction was stopped by adding 1 mL of 0.5 N NaOH, and the tube contents were filtered through Whatman No. 4 paper. Absorbance of the filtrate was measured at 620 nm against a blank from which inhibitor was excluded. Percentage α-amylase inhibition was calculated by the formula $(A_B - A_S)/A_B \times 100$, where A_B = absorbance of the blank and A_S = absorbance of the sample. Inhibition was expressed either as percentage as described or as percentage per unit protein as measured by the Bradford (1976) procedure.

PAGE-SDS Electrophoresis. Molecular weights of the α-amylase inhibitors were determined by the electrophoretic method of Weber and Osborn (1969). Chromatographic fractions that were inhibitory and standard proteins (bovine albumin, trypsinogen, β-lactoglobulin, egg albumin, lysozyme) were run on a continuous system. The 10% (w/v) gel and buffer contained 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.01% (v/v) bromophenol blue, and 0.2 M phosphate (pH 7.0). Samples (10–80 μL) were layered on the gel and run at 2 mA for 30 min and at 8 mA for 5–6 h. Gels were fixed in methanol–acetic acid–water (3:1:6), stained in methanol–acetic acid–Coomassie Brilliant Blue R (8:2:0.05), and destained in methanol–acetic acid–water (5:7.5:87.5). Molecular weights of α-amylase inhibitors were calculated from their electrophoretic mobilities relative to those of the standard proteins.

Embryo Bioassay of α-Amylase Inhibitors. Frozen Lancota seeds were warmed to room temperature, surface-sterilized with 70% (v/v) ethanol for 10 min and 0.1 mM sodium hypochlorite for 10 min, and rinsed thoroughly with sterile distilled water. Embryos were excised by sectioning seeds with a sharp blade; one side of the embryos contained a small amount of pericarp and seed coat, and the other, a small amount of endosperm.

For bioassays, 25 embryos were placed endosperm side down on double layers of sterile filter disks in 15 × 100 mm Petri dishes. The filter disks were moistened with 4 mL of sterilized water or 1 mL of Newton chromatography effluent plus 3 mL of sterilized water. Germination response of embryos was recorded daily, and a promptness index (PI) (George, 1967) was calculated from the formula $4D_1 + 3D_2 + 2D_3 + D_4$, where D_n = the number of embryos germinating that day. All tests were replicated four times.

Calcium Content and Depletion. Water extracts of the four genotypes were tested for α-amylase inhibition and calcium content before and after adding 0.2 mM EDTA. Treated extracts were stirred at room temperature for 30 min and dialyzed against distilled water at 4 °C overnight. α-Amylase inhibition was measured as described above.

Calcium content was determined by digesting extracts in nitric acid–perchloric acid solutions, diluting them with lanthanum chloride, and reading them by atomic absorption spectrophotometry at 422.7 nm.

Other studies determined the effect of dialysis of KS75216 genotype extracts on α-amylase inhibition. Chromatographic fractions of the sprouting-susceptible genotype were collected as described above, dialyzed against 0.01 mM Tris-HCl at 4 °C overnight, and assayed for α-amylase inhibition and protein content as described previously.

RESULTS

Levels of α-amylase inhibition in extracts obtained from the four genotypes by procedures outlined in Figure 1 are shown in Table I. Inhibition was observed only in extracts from the two genotypes that resist preharvest sprouting, Newton and Clark's Cream, and not in the sprouting-susceptible genotypes, Parker 76 and KS75216. The low inhibitory activity in crude water extracts was not increased by heating. Ethanol precipitation, however, greatly increased the concentration of inhibitor in the two genotypes in which it occurred. Ion-exchange chromatography further concentrated the inhibitor into six peaks (Figure 2) that varied in activity (Table I). Chromatography of the same extracts from the two sprouting-susceptible genotypes, on the other hand, yielded only one major protein peak, which was not inhibitory.

An electrophoretogram of the chromatography peaks of one of the sprouting-resistant genotypes, Newton, is illustrated in Figure 3. Electrophoresis separated two to five bands in each chromatography peak; estimated molecular weights of the proteins in the different bands ranged from 14 000 to 68 000 (Table II).

The relationship among α-amylase inhibitor activity in chromatography fractions of Newton wheat, calcium content of the fractions, and germination of excised embryos in the presence of the fractions is shown in Table III. Inhibition ranged from 0% by fractions that contained 0.93 μg g^{-1} calcium to 36.6% by fractions that contained 0.18

Table I. Percentage α-Amylase Inhibition per Milligram of Protein and Protein Content of Fractions Extracted from Four Wheat Genotypes

	genotype							
	Newton		Parker 76		Clark's Cream		KS75216	
fraction	inhibn, % mg^{-1} ± SE	protein, mg ± SE	inhibn, % mg^{-1}	protein, mg	inhibn, % mg^{-1} ± SE	protein, mg ± SE	inhibn, % mg^{-1}	protein, mg
water extract	0	5558 ± 331	0	2879	0	2300 ± 125	0	3417
heated extract	0	850 ● 97	0	535	0	546 ± 43	0	858
ethanol ppt								
before dialysis	2 ± 0	19 ± 2	0	41	0.8 ± 0.1	26 ± 2	0	49
after dialysis	4 ± 0	3 ± 0	0	39	0.8 ± 0.1	15 ± 1	0	39
chromatography								
peak 1	75 ± 7	0.1 ● 0	0		53 ± 4	0.1 ● 0	0	
peak 2	109 ± 9	0.2 ± 0	0		20 ± 1	0.3 ● 0	0	
peak 3	43 ± 4	0.1 ± 0	0		9 ± 1	0.5 ± 0	0	
peak 4	24 ± 2	0.2 ● 0	0		17 ± 1	0.2 ± 0	0	
peak 5	48 ± 4	0.1 ± 0	0		91 ± 7	0.1 ± 0	0	
peak 6	632 ± 83	0.1 ± 0	0		22 ± 1	0.1 ± 0	0	

Table II. Estimated Molecular Weights of Proteins Separated by SDS Gel Electrophoresis of Chromatography Peaks from Newton Wheat Relative to Standard Proteins

chromatogr peak	electrophoresis band				
	1	2	3	4	5
1	68 000	65 000	63 000		
2	65 000	63 000			
3	59 000	55 000	53 000		
4	60 000	58 000	55 000	17 000	14 000
5	60 000	58 000	55 000	14 000	
6	65 000	63 000	58 000	57 000	

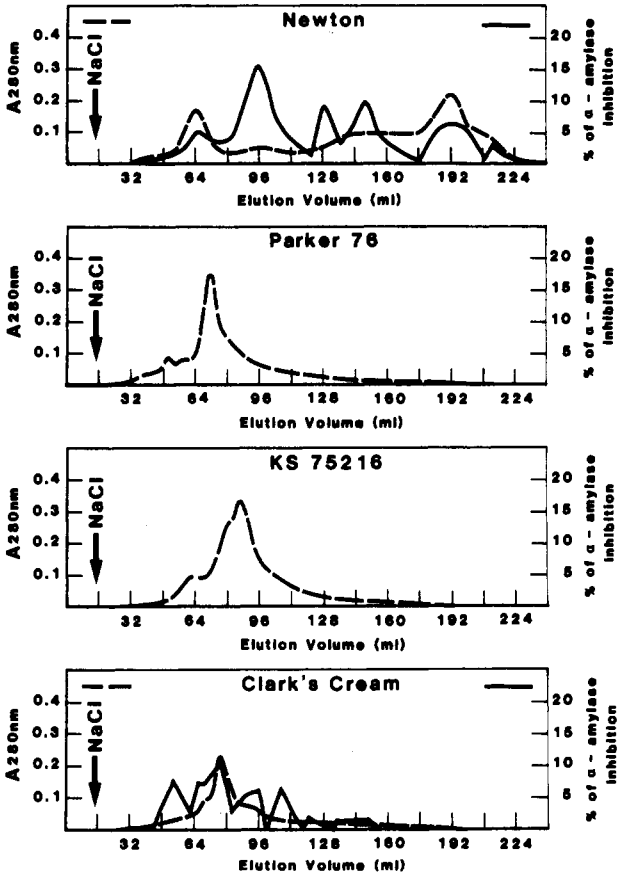

Figure 2. DEAE-Sephacel chromatograms of the alcohol fraction of four wheat genotypes after elution with an NaCl gradient.

$\mu g\ g^{-1}$ calcium. The r value between α-amylase inhibition and calcium content was negative and significant. Germination PI of embryos treated with the chromatography fractions ranged widely but correlated neither with α-amylase inhibition nor with calcium content.

Table III. Percentage α-Amylase Inhibition, Calcium Content, and Effect on Excised Embryo Germination Promptness Index (PI) of Chromatography Fractions from Newton Wheat

chromatogr fraction, no.	α-amylase inhibn, % ± SE	Ca content, $\mu g\ g^{-1}$	embryo germination, PI ± SE
22	0	0.9 ± 0.1	7 ± 1
23	0	0.9 ● 0.1	12 ± 1
24	11 ± 1	0.4 ± 0.1	4 ± 0
25	20 ± 2	0.6 ± 0.1	4 ± 0
26	16 ± 1	0.6 ± 0.1	4 ± 0
27	8 ± 1	0.7 ± 0.1	4 ± 0
28	23 ± 2	0.3 ± 0.0	10 ± 1
29	9 ± 1	0.4 ± 0.1	4 ± 0
30	37 ± 2	0.2 ● 0.0	6 ± 1
31	22 ± 2	0.5 ± 0.1	6 ± 1
32	13 ± 1	0.9 ± 0.2	5 ± 1
33	6 ± 1	0.3 ± 0.0	2 ± 0
r, α-amylase inhibn		−0.60[a]	−0.09
Ca content			0.29

[a] Significant at $P = 0.05$ level.

Table IV. Percentage α-Amylase Inhibition and Calcium Content of Water Extracts from Four Wheat Genotypes before and after Adding EDTA

	before adding EDTA		after adding EDTA	
genotype	inhibn, % ● SE	Ca content, $\mu g\ g^{-1}$ ● SE	inhibn, % ± SE	Ca content, $\mu g\ g^{-1}$ ± SE
Newton	23 ± 2	26 ● 2	37.3	6.1
Parker 76	0 ± 0	32 ± 2	3 ± 0	6 ± 1
Clark's Cream	6 ± 1	26 ± 2	13 ± 1	8 ± 1
KS75216	0 ± 0	33 ± 2	7 ± 1	6 ± 1

Table V. Percentage α-Amylase Inhibition per Milligram of Protein and Protein Content of KS75216 Wheat Chromatography Peaks after Dialysis against Tris-HCl Buffer

chromatogr peak no.	α-amylase inhibn, % mg $protein^{-1}$ ± SE	protein content, mg ± SE
1	37 ± 2	0.1 ± 0
2	61 ● 4	0.1 ● 0
3	90 ± 5	0.1 ± 0
4	80 ± 4	0.1 ● 0
5	193 ± 8	0.1 ± 0

Adding EDTA to crude water extracts increased the α-amylase inhibition in wheat genotypes in which it was previously noted (Table IV). It also prompted inhibition by extracts from Parker 76 and KS75216, genotypes that were not inhibitory before adding EDTA. Calcium content of crude extracts without EDTA was slightly higher in the two sprouting-susceptible genotypes than in the two resistant genotypes.

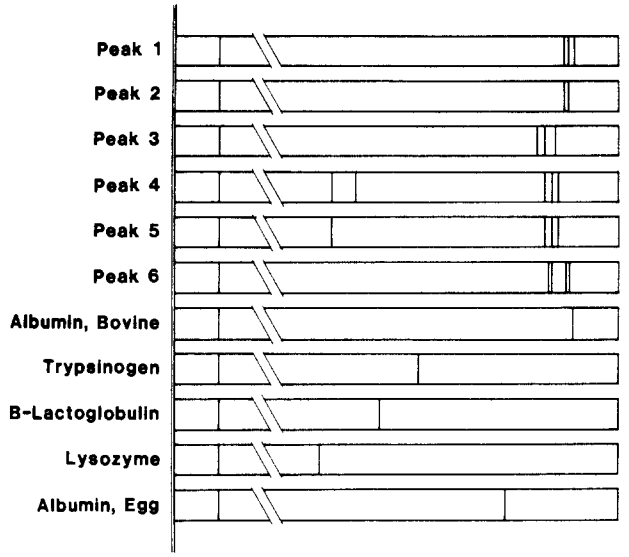

Figure 3. SDS-PAGE electrophoretograms of α-amylase inhibitor chromatography fractions of Newton wheat. The lower line represents the marker, and other lines represent movement of the separated bands relative to the marker.

Dialysis of the chromatography effluent of KS75216 extract against Tris-HCl also caused substantial inhibition of α-amylase (Table V) compared with undialyzed effluent (Table I). Activity separated into five distinct peaks that were similar in magnitude to activity of extracts from sprouting-resistant genotypes shown in Table I.

DISCUSSION

Preharvest sprouting resistance of wheat has been attributed to endogenous catechin–tannin compounds, caryopsis structure, and embryo responsiveness to inhibitors (Belderok, 1976; McCrate et al., 1982; Stoy and Olsen, 1980). Present results suggest that proteinaceous α-amylase inhibitors may influence preharvest sprouting but probably are not primary determinants of genotypic variation in sprouting susceptibility. Proteinaceous inhibitors may exert an important secondary effect, however, by regulating α-amylase enzyme activity per unit of sprouting. Genotypes such as Clark's Cream that produce little α-amylase activity per unit of sprouting and also resist sprouting are doubly useful for alleviating adverse effects of inclement conditions on wheat functional properties (Nielsen et al., 1984; Upadhyay et al., 1984).

Endogenous inhibitors of preharvest sprouting slow germination and production of α-amylase enzyme concurrently (McCrate et al., 1982). The lack of any discernable effect of proteinaceous α-amylase inhibitors on excised embryos indicates that these compounds are not a major factor in preharvest sprouting. Sprouting tests that are based on α-amylase activity in whole flour extracts may lead to contrary conclusions, however (Mathewson and Pomeranz, 1977). They do not distinguish between germination per se and activity of enzymes that are induced by germination.

An association of proteinaceous α-amylase inhibitors with calcium is indicated by the negative correlation between the two parameters and effects of treatments to chelate or remove calcium. Calcium is required for stabilizing α-amylase enzyme, and chelation of the metal by EDTA has been shown to inactivate malt α-amylase (Fisher and Haselback, 1951). The effect of proteinaceous inhibitors appears to be similar to that of MHP (myoinositol hexaphosphate), which suppresses α-amylase activity in sprouted wheat meal by chelating the calcium that is needed for enzyme activity or by interacting directly with the enzyme protein moiety (Jacobsen and Slotfeldt-Ellingsen, 1983; Sharma et al., 1978). Calcium may also mediate the interaction between α-amylase enzyme and proteinaceous inhibitors by electrostatic effects as reported for NaCl in barley by Weselake et al. (1985b). Removing calcium by EDTA or dialysis may enhance inhibition of α-amylase in the four wheat genotypes that we studied by facilitating interactions between the proteinaceous inhibitors and the enzyme.

It is apparent that several molecular species of proteinaceous compounds inhibit α-amylase in wheat. Most of them are in the range of molecular weight 60 000 ± 5000, the largest reported to date (DePonte et al., 1976). None of them resemble the intermediate species of molecular weight 30 000 (Granum and Whitaker, 1977), and only a few approach the low molecular weight of purothionins (Balls et al., 1942; Jones and Meredith, 1982). The several species of inhibitors that occur may contribute to the heterogeneity of different wheat genotypes and even different chromatography fractions. It seems likely, however, that the most quantitatively important proteinaceous inhibitors in wheat lie in the upper range of molecular weights.

Genotypic differences in the amounts and distribution of proteinaceous α-amylase inhibitors suggest that they can be manipulated genetically. Altering inhibitor levels could reduce the level of α-amylase per percentage of sprouting, if not the susceptibility to preharvest sprouting. This could improve the functional quality of wheat flour and perhaps even the nutritional properties of leavened products.

Registry No. Ca, 7440-70-2; α-amylase, 9000-90-2.

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DNA Breakage by Flavan-3-ols and Procyanidins in the Presence of Cupric Ion

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DNA breakage by flavan-3-ols and procyanidins in the presence of cupric ion was investigated by gel electrophoretic and computer analysis. Flavan-3-ols and procyanidins cleaved λ-DNA in a concentration-dependent manner. The DNA-breaking activity was remarkably enhanced approximately in connection with the increase of the number of dihydroxy or trihydroxy groups in a molecule. Although the presence of cupric ions was indispensable for DNA cleavage by (−)-epicatechin (EPI), excess amounts of cupric ions inhibited the activity. A number of single-strand breaks occurred prior to double-strand breaks. A maximum rate of DNA cleavage by EPI occurred at pH 7.0. A correlation was not observed between the oxidation rate of EPI and the cleavage activity. DNA breakage by EPI was strongly inhibited by the addition of catalase or various kinds of radical scavengers. From the results obtained, a breaking mechanism was proposed that DNA chains were broken by oxygen radicals generated by a local oxidation of EPI in the vicinity of DNA via DNA–Cu^{2+}–EPI complexes.

Polyphenols including hydrolyzable, condensed, and other tannins are widely distributed in the plant kingdom and consumed daily by humans in milligram to gram quantities (Brown, 1980). It has been reported that tannic acid has antimutagenic effects (Gichner et al., 1987), and hydrolyzable tannins have antitumor activities (Miyamoto et al., 1987). Contrary to these reports, tannic acid is known to be a naturally occurring hepatoxin (Handler and Baker, 1944) and to induce nucleolar changes in hepacytes (Rao et al., 1987) and liver cancer (Korpâssy, 1959).

Such in vitro genotoxic assays as the DNA breakage test will be helpful to assess the potential genetic hazard by food components to humans. In the previous paper (Shirahata et al., 1985), we reported that hydrolyzable tannins can cleave DNA chains in the presence of Cu^{2+}. In order to clarify the relationship between the structures of hydrolyzable and condensed tannins and DNA breaking activity, we developed a rapid and exact method for quantitative determination of nucleic acid breaking activity with a combination of gel electrophoresis and microcomputer analysis.

Flavan-3-ols are constituent units of condensed tannins, and procyanidins belong to condensed tannins. This paper describes the DNA-breaking activity of flavan-3-ols and procyanidins in the presence of Cu^{2+} analyzed by this new method.

MATERIALS AND METHODS

DNA and Reagents. Double-stranded DNA from λ-phage was purchased from Biotech Co. Plasmid pBR322 DNA was prepared as described before (Shirahata et al., 1985). Catalase was obtained from Sigma Chemical Co. Superoxide dismutase was purchased from Toyobo Co. Various radical scavengers were all commercial grade. All flavan-3-ols and procyanidins were isolated from various kinds of plants, and their chemical structures were determined as reported previously. Flavan-3-ols: (+)-catechin (CAT) (Nonaka and Nishioka, 1982), (−)-epicatechin (EPI) (Nonaka et al., 1983), (+)-gallocatechin

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