Straten and de Vrijer, 1973; Shaw and Wilson, 1982). These compounds possess strong, floral rose-like aromas and are probably responsible for some of the fruity floral aroma of carambolas (Arctander, 1969). The aroma of borneol has been described as dry, woody, and camphoraceous (Arctander, 1969). Quinoline, benzothiazole, and ethyl nicotinate, which are present in low concentrations, probably contribute to the aromatic aroma. Benzothiazole has been reported in mangoes, cranberries, vegetables, and animal products, and quinoline has been reported in coconuts, cocoa, nuts, and beer (Van Straten and de Vrijer, 1973; MacLeod and Pieris, 1984). Ethyl nicotinate has not been reported in foods, but the related compound, methyl nicotinate, has been found in nuts and coffee (Van Straten and de Vrijer, 1973). Although carambolas have been reported as apricot-like or apple-like in flavor and several of the constituents of this fruit are common to both apples and apricots (Chairote et al., 1981; Flath et al., 1967), the extract we prepared did not possess an aroma characteristic of either of those two fruit.

The identity of forty-one volatile components of one major carambola selection (Robert Newcomb, cv.) has been established for the first time. Several of these components are not common constituents of other fruit. This study provides a basis for comparison of other carambola cultivars to aid plant breeders and horticulturists in selecting varieties for further marketing and processing.

Registry No. Acetaldehyde, 75-07-0; ethyl acetate, 141-78-6; 2-methyl-1-propanol, 78-83-1; 1-pentanol, 71-41-0; 1-penten-3-ol, 616-25-1; 3-methyl-2-butanone, 563-80-4; 3-methyl-1-butanol, 123-51-3; ethyl butyrate, 105-54-4; hexanal, 66-25-1; *cis*-3-hexen-1-ol, 928-96-1; *trans*-3-hexen-1-ol, 928-97-2; hexanol, 111-27-3; α-pinene, 80-56-8; benzaldehyde, 100-52-7; 6-methyl-5-hepten-2-one, 110-93-0; 6-methyl-5-hepten-2-ol, 1569-60-4; β-pinene, 127-91-3; ethyl hexanoate, 123-66-0; 1,8-cineole, 470-82-6; limonene, 138-86-3; benzyl alcohol, 100-51-6; octanol, 111-87-5; acetophenone, 98-86-2; methyl benzoate, 93-58-3; ethyl sorbate, 2396-84-1; phenylethyl alcohol, 60-12-8; veratrole, 91-16-7; borneol, 507-70-0; diethyl succinate, 123-25-1; *o*-methylacetophenone, 577-16-2; 4-terpineol, 562-74-3; ethyl benzoate, 93-89-0; methyl salicylate, 119-36-8; ethyl nicotinate, 614-18-6; benzothiazole, 95-16-9; methyl anthranilate, 134-20-3; carvone, 99-49-0; phenylethyl acetate, 103-45-7; diethyl glutarate, 818-38-2; cinnamyl aldehyde, 104-55-2; quinoline, 91-22-5; cinnamyl acetate, 103-54-8; β-ionone, 79-77-6.

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Measurement of Amino Acid Racemization in Alkali-Treated Proteins Using an Immobilized D-Amino Acid Oxidase–Catalase Reactor

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An immobilized enzyme reactor system consisting of D-amino acid oxidase and catalase coimmobilized on porous succinamidopropyl-glass beads was examined with regard to its function in a method for determination of amino acid racemization in proteins. The extent of racemization was measured from the reduction in the amount of an amino acid, as determined by chromatographic analyses, following treatment of an acid hydrolysate with the immobilized enzyme reactor. Racemization rates resulting from alkaline heat treatments (0.2 N NaOH, 40 °C) of five proteins (soy isolate, wheat isolate, α-lactalbumin, bovine serum albumin, and β-lactoglobulin) indicated that, although the rates varied between proteins, the relative rate of phenylalanine racemization was constant and roughly double that for alanine. Racemization of tyrosine occurred at a rate similar to that of alanine; however, that for isoleucine, leucine, and valine was barely detectabl following a 20-h incubation.

A number of methods for determination of the extent of amino acid racemization in a protein has been reported. These include use of enzymes (L-lysine decarboxylase) and microorganisms (Provansal et al., 1975), gas chromatographic/mass spectrometric analysis following deuterium labeling (Liardon and Jost, 1981; Liardon and Hurrell, 1983), and tritium–hydrogen exchange techniques (Hayashi and Kameda, 1980). Most of these methods, however, require the use of sophisticated instrumentation not readily available to many laboratories. Racemization resulting from alkaline food processing conditions could seriously impair the digestibility of a protein. Therefore, we sought to develop a simplified technique for measuring

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this parameter by using instrumentation more commonly available. Thus, a method involving comparison of amino acid analyses of acid hydrolysates with an without treatment with coimmobilized D-amino acid oxidase (DAAO)–catalase was developed.

DAAO [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] is a flavoenzyme requiring FAD for activity. It catalyzes the oxidation of certain D-amino acids to the corresponding α-keto acid (Parkin and Hultin, 1979):

$$\text{D-amino acid} + H_2O + O_2 \rightarrow \alpha\text{-keto acid} + H_2O_2 + NH_3 \quad (1)$$

The H_2O_2 formed can be dismuted with catalase, thus preventing any potential reversible or irreversible inhibition by this oxidant:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

Thus, treatment of an acid hydrolysate of a protein with coimmobilized DAAO–catalase reduces the concentrations of those acids that are substrates for the enzyme by an amount equivalent to the level of the D-amino acid originally present.

EXPERIMENTAL SECTION

Chemicals. Succinic anhydride, lactate dehydrogenase (LDH, 300 units/mg), NADH, FAD, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), D forms of amino acids, and DAAO were purchased from Sigma Chemical Co. (St. Louis, MO). Controlled-pore glass (CPG) beads (53-nm pore diameter, 120–200 mesh) were obtained from Electro-nucleonics (Fairfield, NJ), amino acid calibration standards were from Pierce Chemical Co. (Rockford, IL), (3-aminopropyl)triethoxysilane was from Aldrich Chemical Co. (Milwaukee, WI), and catalase (77 826 units/mg) was from Millipore Corp. (Bedford, MA).

Glass Derivatization. The glass surface was converted to aminopropyl-glass according to procedures given by Taylor and Swaisgood (1980) using (3-aminopropyl)triethoxysilane. The amino groups were then succinylated by reaction with succinic anhydride in chloroform to yield a succinamidopropyl-glass surface (Merrifield, 1963).

Enzyme Immobilization. Prior to immobilization, 2 mL of DAAO solution (5 mg/mL) was dialyzed against 4 L of 0.05 M sodium phosphate, pH 6.6, for 24 h at 6 °C. The dialyzed DAAO solution was mixed with a catalase solution (0.2 mg/mL) in a ratio of activities of DAAO:catalase (1:4) and made to a final volume of 5 mL. Coimmobilization was carried out by using the sequential method of Janolino and Swaisgood (1982) by recirculation of the enzyme mixture through a column of EDC-activated succinamidopropyl-CPG beads for 24 h at 6 °C. The immobilized enzymes were washed with 500 mL of 1 M NaCl in 0.05 M sodium phosphate, pH 6.6, containing 0.3 μM FAD and 0.02% sodium azide.

Activity Assay. Activity of immobilized DAAO was assayed by coupling the reaction with LDH-catalyzed reduction of pyruvate (Boehringer Mannheim, 1973):

$$\text{D-Ala} + {}^1/_2O_2 \xrightarrow[\text{catalase}]{\text{DAAO}} \text{pyruvate} + NH_3 \quad (3)$$

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+ \quad (4)$$

The immobilized enzyme was assayed at pH 7.5 and 25 °C by using the microrecirculation reactor system described by Taylor and Swaisgood (1980), and the absorbance at 340 nm was monitored with a flow-through cuvette in a Gilford spectrophotometer. One unit of activity is defined as 1 absorbance unit/min. The immobilized catalyst contained 4.3 units of activity/mL of packed bead volume.

Determination of the Time Required for Treatment of the Acid Hydrolysate with Immobilized DAAO–Catalase. The time required to approach completion of the catalyzed oxidation of D-amino acids with an immobilized DAAO–catalase reactor was determined by using standard DL-amino acid (1:1) solutions. Completion of the reaction was taken as the point at which the amount of the amino acid decreased by half. Thus, with this system, oxidation of the D-amino acid was complete within experimental error of amino acid analysis.

Stock solutions (1 μmol/mL) were prepared for each of the amino acids D-Ala, D-Phe, D-Asp, and D-Glu. Ten milliliters of each stock solution was added to 4 mL of an amino acid calibration standard solution containing 2.5 μmol of each/mL of 0.01 N HCl. To this mixture were added 2 mL of 0.5 M sodium phosphate, pH 6.6, 60 μL of 12 mM FAD, and 70 μL of 6% sodium azide. The resulting solution was diluted with distilled water and adjusted to pH 7.5 and a final volume of 20 mL. Ten milliliters of this solution was treated with immobilized DAAO–catalase while the remaining 10 mL served as a control.

Alkali Treatment and Protein Sample Preparation. Stock solutions (20 mg/mL) of the following proteins were made in 0.2 N NaOH: β-lactoglobulin (isolated and purified in this laboratory), bovine serum albumin (BSA, Sigma Chemical Co.), α-lactalbumin (Sigma Chemical Co.), soy isolate (Supro 620, Ralston Purina Co., St. Louis, MO), and wheat isolate (No. 1200, Manildra Protein Corp., Myrtle Beach, SC). Two milliliters of these solutions was transferred to 10-mL ampules, sealed, and heated in a water bath at 40 °C for 2, 8, or 20 h. Following racemization, 20 μL 0.2 mM L-norleucine was added as an internal standard, the mixture was transferred to an empty ampule, and 2 mL of concentrated (12 N) HCl was added. The ampule was evacuated and sealed and hydrolysis was performed in a heating block at a temperature of 110 °C for 24 h. The acid-hydrolyzed sample was neutralized with 6 N NaOH, and the following components were added: 5 mL of 0.2 M sodium phosphate, pH 6.6, 70 μL of 6% sodium azide, and 40 μL of 15 mM FAD. The mixture was then adjusted to pH 7.5 and diluted to a final volume of 20 mL. Ten milliliters of this solution was used for treatment with immobilized DAAO–catalase and 10 mL served as the control.

Treatment of Samples with an Immobilized DAAO–Catalase Reactor. The immobilized enzyme beads (0.8 mL) were placed in a jacketed column and operated in a fixed-bed configuration. The reactor was first washed with 6 mL of the sample, and then the remaining 4 mL was recirculated at 25 °C. At the indicated time (4 h for protein hydrolysates), a 0.2-mL portion was withdrawn and diluted to 1 mL with citrate buffer, pH 2.2, and 500 μL was used for amino acid analysis. For the case of the amino acid standard solution, 500 μL containing 50 nmol of each was applied to the Beckman 116 amino acid analyzer. After each use the reactor was washed with 1 M NaCl in phosphate buffer, 0.05 M sodium phosphate buffer, pH 6.6, containing FAD and then with the same buffer without NaCl and subsequently stored in 0.05 M sodium phosphate, pH 6.6, containing 0.3 μM FAD and 0.02% sodium azide.

Determination of Racemization. The percent racemization was calculated by using the relationship

$$\% \text{ racemization} = 2(P_i' - P_i'')100/P_i'$$

where P_i' is the peak area for a given amino acid in the control chromatogram and P_i'' is that for the amino acid in the treated sample. All peak areas were calculated

Table I. Percent Oxidation of D-Amino Acids by the Immobilized DAAO-Catalase Reactor following a 2-h Treatment

amino acid	peak area of control sample, arbitrary units	peak area of treated sample, arbitrary units	% oxidation[a] of the D-amino acid
Ala	4.2	2.0	100
Phe	32.6	16.1	100
Asp	3.8	3.6	10
Glu	4.5	4.4	4

[a] The sample was a 1:1 mixture of the D and L forms. Control experiments indicated that no oxidation of L-amino acids occurred. The calculation assumes an equal contribution to the area of the control sample by both forms.

Table II. Percent Racemization[a] of Alanine and Phenylalanine in Alkali-Treated Proteins

protein	Ala			Phe			k_{Phe}/k_{Ala}[c]
	2 h[b]	8 h[b]	20 h[b]	2 h[b]	8 h[b]	20 h[b]	
soy isolate	10	18	27	21	40	53	2.5
wheat isolate	7	16	26	14	32	48	2.3
α-lactalbumin	8	16	26	10	20	45	2.3
bovine serum albumin	8	14	24	10	16	41	2.3
β-lactoglobulin	6	13	22	8	15	40	2.5

[a] Calculated according to the expression 2D/(D + L) × 100. [b] Treatment time; the protein was incubated in 0.2 N NaOH at 40 °C for the time indicated. [c] The first-order rate constants were evaluated from plots of ln [(1 + D/L)/(1 − D/L)] vs. time.

relative to that for the internal standard, L-norleucine.

RESULTS AND DISCUSSION

Treatment of mixtures of standard L- and D-amino acids with a reactor containing immobilized DAAO–catalase indicated that essentially complete oxidation of D-alanine and D-phenylalanine occurred within 2 h since the original chromatographic peak area for the 1:1 mixture was reduced by half in that time period (Table I). Further changes in the peak area for these amino acids were not observed following a 10- or a 20-h treatment. However, extensive oxidation of D-aspartate or D-glutamate was not observed during such treatments, indicating that these amino acids are not good substrates for the oxidase. Similar observations concerning the specificity of DAAO have been made previously (Naoi et al., 1978; Tosa et al., 1974). D-Tyrosine, D-isoleucine, D-leucine, and D-valine are also substrates (Naoi et al., 1978; Tosa et al., 1974); consequently, some data for these amino acids are included in this study.

Analysis of alkali-treated proteins using the immobilized enzyme reactor indicated that racemizaion of alanine and phenylalanine occurred in all cases (Table II). Following a 20-h incubation at 40 °C in 0.2 N NaOH, D-Phe accounted for more than 20% (>40% racemization) of the total for that residue, while D-Ala represented more than 11% (>22% racemization) of that amino acid. These values may be slightly higher than the actual racemization due to alkali treatment since some racemization occurs during acid hydrolysis. However, calculations based on the rate constants reported by Liardon and Jost (1981) indicate that the amount of acid-catalyzed racemization occurring for alanine and phenylalanine would be <2% and <6%, respectively.

Values observed for the racemization of tyrosine in these proteins were essentially the same as those listed in Table II for alanine. However, racemization of leucine, isoleucine, and valine was quite low, 5% or less at 20 h, which is at the lower limit of detection for the method. Thus, the relative rates of racemization of these redisues are consistent with previous observations, e.g., Masters and Friedman (1979) and Liardon and Hurrell (1983).

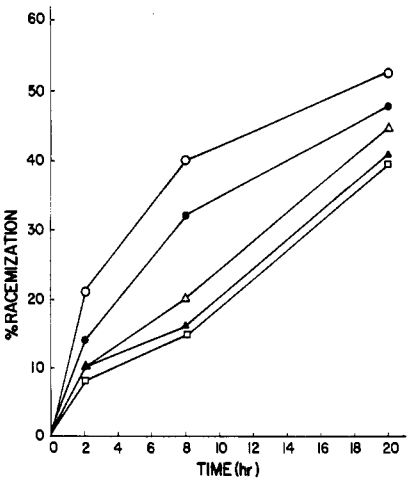

Figure 1. Percent racemization of phenylalanine in alkali-treated proteins. The proteins were incubated in 0.2 N NaOH at 40 °C for the time indicated. Legend: (○) soy isolate; (●) wheat isolate; (△) α-lactalbumin; (▲) bovine serum albumin; (□) β-lactoglobulin.

These data show that phenylalanine racemized at a rate roughly double that for alanine. When the data in Table II were plotted in the form of ln [(1 + D/L)/(1 − D/L)] vs. time (plot not shown), linear first-order kinetics were obtained, as has also been reported by Masters and Friedman (1979). The ratio of the resulting rate constants, k_{Phe}/k_{Ala}, ranged from 2.3 to 2.5. Similarly, Masters and Friedman (1979) and Bunjapamai et al. (1982) observed that the rate of phenylalanine racemization was roughly twice that for alanine when proteins were incubated in 0.1 N NaOH at 65 °C. The former authors also observed that although the absolute racemization rates of various amino acids may vary among proteins, the relative rates for different amino acids within a single protein remained constant, which is consistent with our results. It appears that the rate of racemization for alkali-treated proteins is largely determined by the R substituents and is correlated with the electron-withdrawing character as reflected by the σ^* value (Masters and Friedman, 1979; Liardon and Hurrell, 1983). Thus, the relative order of racemization rates are aspartic acid > phenylalanine > alanine with valine, isoleucine, and leucine exhibiting the lowest rates.

As previously noted, the rate of racemization varies between proteins (Masters and Friedman, 1979; Hayashi and Kameda, 1980). Data in Figure 1 suggest that soy isolate and wheat isolate are particularly susceptible to the alkali treatment. However, the rates of racemization for milk whey proteins were lower. Masters and Friedman (1979) also observed slower rates of racemization in α-lactalbumin as compared to wheat gluten.

Use of an immobilized DAAO–catalase reactor system provides a relatively simple method for determination of racemization. The only instrumentation required is a means for amino acid analysis. The two reactors used in this study retained 72% of the initial DAAO activity after treatment of 17 samples over a 4-month period. Consequently, the stability should permit use of the same reactor over a relatively long period of time.

Registry No. DAAO, 9000-88-8; catalase, 9001-05-2.

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Extraction of Chlorophylls *a* and *b* from Different Binding Sites on Thylakoid Chlorophyll-Proteins

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Chlorophyll pigments interfere with the utilization of leaf proteins in fabricated food systems intended for human use. The extraction of the pigments from lyophilized spinach leaf thylakoid membranes was studied in ethyl acetate–hexane mixtures at temperatures of 2, −20, and −40 °C. As the ethyl acetate concentration of the solvent mixture was increased, higher limiting percentages of chlorophyll were extracted at extended extraction times. Results were analyzed with regard to the specific extraction rate, k, which decreased as the fraction of nonextracted chlorophyll decreased. The apparent activation energies for the extractions indicated breakages of noncovalent bonds. The results suggest a variety of binding sites for the chlorophylls and a protein conformational change prior to the pigment dissociation.

One of the most abundant sources of proteins is leaves, and this fact has prompted many studies on the possibilities of using leaf proteins as food or animal feed (Telek and Graham, 1983). Much of the leaf protein consists of chlorophyll–protein located in the thylakoid membranes of the chloroplasts. A difficulty in its utilization in human food is its chlorophyll content (Edwards et al., 1975), rendering the initial products green and then changing in hue as the chlorophyll transforms into other products (Bacon and Holden, 1967). While output of processed whole leaf protein is small and used mainly for poultry feed, the potential production is very large.

Studies on the nature of pigment binding mechanisms should provide supporting information useful in the technology of pigment removal and adapting these chlorophyll–proteins and their functional properties to human food use. There is a further interest in chlorophyll binding in that the organization of chlorophyll molecules in photosynthetic membranes determines the nature of the energy transfer process from sites of light absorption to photochemical reaction centers (Lutz, 1977).

A variety of proteins can bind chlorophyll (Criddle, 1966), but not to the levels of 20% found with thylakoid chlorophyll–proteins. With casein the complexes do not contain much more than 1% chlorophyll (Giller, 1970). An interesting example of an artificial pigment–protein is the chlorophillide–apomyoglobin complex that contains a ratio of 1:1 of chlorophyllide and apomyoglobin. It was prepared by Boxer and Wright (1979), who showed that the chlorophyllide molecule was bound in the heme pocket of the apomyoglobin.

Binding of chlorophyll to thylakoid protein is through the porphyrin part of the molecule. Lutz (1977), making use of resonance enhanced Raman spectroscopy, showed that hydrogen bonds associated with the C9 keto C=O were prominently involved in binding of chlorophyll *a* and this same group as well as the C3 formyl group was involved in hydrogen bonding of chlorophyll *b*. Mg was also involved in binding as had been indicated by an analysis of the triplet state (Clarke et al., 1982). Suggestions that the phytyl groups are freely mobile in the liquid phase of the thylakoid membrane (Eigenberg et al., 1981) lessen the chances for these long nonpolar chains to form hydrophobic bonds to the protein.

Removal of chlorophyll from chlorphyll–proteins has been studied by using two kinds of extracting solvents, very nonpolar solvents such as hexane or heptane, and water-miscible solvents such as acetone and dimethylformamide. The nonpolar solvents do little damage to the biochemical functionality of the lipoproteins (Cox and Bendall, 1974) but extract chlorophyll poorly (Oquist and Samuelsson, 1980). The water-miscible solvents are extremely good extracting materials but damage or alter the lipoproteins (Davis et al., 1981).

The work presented here used mixtures of hexane and ethyl acetate to provide solvents of differing polarities. The object was to see if the bound chlorophyll behaved as if it were bound on one or a variety of sites. This was to be done by determining the effects of time, temperature, solvent composition, and fraction of unextracted chlorophyll on the rate of pigment dissociation from the protein complex.

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