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Carboxypeptidase Inhibition by Alkali-Treated Food Proteins

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Synthetic lysinoalanine is a more effective inhibitor of the zinc-containing enzyme carboxypeptidase A than is ethylenediaminetetraacetic acid (EDTA). The enzyme is also inactivated by alkali-treated lysinoalanine containing food proteins such as casein, high-lysine corn protein, lactalbumin, soy protein isolate, and wheat gluten and by alkali-treated zein, which contains no lysinoalanine. Zinc sulfate regenerates only part of the enzymatic activity after exposure of CPA to the treated proteins. The extent of inhibition increases with protein concentration and time of treatment. Any inhibition due to phytate is distinct from that due to the treatment. Molecular mechanisms involving lysinoalanine formation, racemization, zinc chelation, and protein unfolding are proposed to account for the inhibition of carboxypeptidase A by the treated proteins. The possible relevance of these findings to food safety and nutrition is also discussed.

Food processing conditions that use heat and alkali may result in compositional changes that include formation of lysinoalanine (LAL) cross-links and racemization of L-amino acid residues to D isomers (Masters and Friedman, 1979; Friedman et al., 1981; Liardon and Hurrell, 1983; Friedman et al., 1984a,b).

Feeding proteins containing LAL to rats induces changes in kidney cells. These changes are characterized by enlargement of the nucleus and cytoplasm, increased nucleoprotein content, and disturbances of DNA synthesis and mitosis [for a review, see Friedman et al. (1984a)]. The molecular mechanism of the observed cellular action is still not well understood. One possibility, however, is that since LAL contains three amino and two carboxyl groups and structurally resembles ethylenediaminetetraacetic acid (EDTA), a well-known metal chelator, LAL could chelate essential trace elements in vivo (Friedman, 1977). This prediction was partly confirmed by Hayashi (1982), who found that synthetic LAL has a strong affinity for metal ions and inactivates metalloenzymes such as carboxypeptidase A and B and alcohol dehydrogenase in vitro. These observations raise the question whether LAL-containing food proteins, in contrast to free LAL, also inhibit metalloenzymes.

The main objectives of the present study were to find out (a) whether alkali-treated food proteins inhibit the activity of the zinc-containing metalloenzyme, carboxypeptidase A (CPA), (b) whether the inhibition is related to the LAL content of the proteins, and (c) whether the inhibition can be reversed by zinc ions.

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EXPERIMENTAL SECTION

Materials. Carboxypeptidase A (bovine), lysinoalanine dihydrochloride (lot no. 53F0722), EDTA, hippuryl-L-phenylalanine, benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), and Tris base were from Sigma, St. Louis, MO. Casein, lactalbumin, soy protein isolate, and wheat gluten were from U.S. Biochemical Corp., Cleveland, OH. High lysine corn was from Crow's Hybrid Corn Co., Milford, IL. Zinc sulfate was from Fisher, Fair Lawn, NJ.

High-lysine corn protein was extracted by presolubilizing the protein in ethanol before extracting with sodium hydroxide, using the following procedure, adapted from Concon (1973). High-lysine corn was ground in an Alpine-Augsberg high-speed mill. The flour was sieved through a 150-mesh screen. The unsieved residue was alternately ground in a U.D. cyclone mill and resieved until all of the sample passed through a 150-mesh screen. Nine grams of the ground corn was combined with 270 mL of 70% ethanol in a 4-L beaker. The mixture was vigorously stirred for 4 min with an automatic stirrer. Next, 990 mL of 0.125 N NaOH was added to the mixture, which was then stirred for another 4 min. The suspension was then centrifuged at 8000–10 000 rpm for 5 min in containers with 250-mL capacity. The supernatant was dialyzed against distilled water in standard dialysis tubing in a cold room for 1 week. The solution was then lyophilized and ground for homogeneity. The yield was approximately 10%. Proximate analysis (%): N, 9.80; H_2O, 7.43; fat, 22.3; carbohydrate, 4.0.

Alkali Treatment. Five grams of soy protein was suspended in 500 mL of 0.1 N NaOH in a 1-L Erlenmeyer flask. Initial pH was checked. The flask was then stop-

pered with a Nalgene cap, covered with aluminum foil, and kept in a water bath at the appropriate temperature for the various time periods listed in the tables. At the end of the treatment, the flask was cooled under running water and the final pH was measured. The pH values of the protein suspensions ranged from 12.5 to 12.7 at 25 °C, which corresponds to an effective pH near 11.5 at 75 °C (Masters and Friedman, 1980). The material in Spectra/Por 4 dialysis tubing, molecular weight cutoff 12 000 (Spectrum Medical Industries, Los Angeles, CA), was then dialyzed against dilute acetic acid (0.25%) for the first day and thereafter against distilled water for another 2 days. The samples were then lyophilized.

Autoclaved Soy Protein. Soy protein (100 mg) in a 50-mL Erlenmeyer flask was heated in an autoclave at 121 °C for 10 min. Assay of inhibitory activity against CPA was carried out in the flask.

Denatured Soy Protein. One gram of soy protein was suspended in 10 mL of water and boiled for 2 min. Inhibitory activity against CPA was assayed with the cooled suspension.

Amino Acid Analysis. Amino acid analyses were carried out on a single-column Durrum 500 amino acid analyzer, as previously described (Friedman et al., 1981). In this system, LAL elutes as a single peak just before histidine.

Phytate Analysis. Low-phytate soy protein and soy flour were prepared as described by de Rham and Jost (1979). Phytic acid content of native and alkali-treated soy proteins and soy flours was measured by an HPLC procedure described by Knuckles et al. (1982).

Carboxypeptidase Assay. CPA activity was assayed by the method of Folk and Schirmer (1963) using hippuryl-L-phenylalanine as the substrate. The substrate (3.26 mg) was first dissolved in 100 μL of 95% ethanol and diluted to 10 mL in pH 7.5 Tris-HCl (0.025 M containing 0.5 M NaCl). Next, 2.9 mL of the substrate solution was pipetted into a cuvette and incubated at 25 °C for 3 min in a Cary 14 spectrophotometer with temperature control. To the substrate solution was then added 0.1 mL of the enzyme solution, prepared by dissolving 10 μM CPA in 2 mL of 10% LiCl. The resulting increase in absorbance at 254 nm was followed for 1 min. Enzyme activity was calculated as follows:

$$\text{units of activity/mg} = (A_{254}/\text{min})/(0.36 \times \text{mg of enzyme/mL of reaction mixture})$$

The undiluted stock solution contained 0.88 unit of enzyme/μL, or 47.37 units/mg of protein.

Trypsin Assay. Trypsin activity in the presence of alkali-treated (0.1 N NaOH; 75 °C; 3 h) soy protein and casein was assayed as follows: 300 mg of the protein was dissolved in 15 mL of 0.5 M Tris buffer, pH 8.5. The solution was then diluted with 0.05 M Tris, pH 8.2, containing 0.02 M $CaCl_2$ to a concentration of 1 mg of protein/0.5 mL. Duplicate test tubes containing 0.5 mL of the protein solution, 25 μg of trypsin, and 3 mL of the substrate (BAPNA) were then incubated in a 37 °C water bath for 10 min. Trypsin activity was measured as described previously (Friedman et al., 1982). Under these conditions, the treated soy protein did not affect trypsin activity and the treated casein lowered it by about 5%.

Effect of Synthetic LAL and EDTA. LAL solutions (5 mM) were prepared by dissolving 3.1-mg samples in 2 mL each of 0.025 M Tris-HCl buffers, pH 6.5, 7.0, 7.5, 8.0, and 8.5, containing 0.5 M NaCl. To each of these solutions was then added 100 μL of CPA stock solution, and the mixtures were left standing at room temperature for 3 min. Then, 100-μL aliquots were added to 2.9 mL of 0.5 or 1 mM substrate in pH 7.5 Tris buffer for assay of enzymatic activity. Identical experiments were carried out with EDTA (3.7 mg/2 mL) except that the CPA plus EDTA solutions were left standing for 60 min.

Table I. Effect of Concentration of Alkali-Treated and Untreated Soybean Protein on the Inhibition of Carboxypeptidase A[a]

protein concn, mg	carboxypeptidase A inhibited, % without $ZnSO_4$[b]	with $ZnSO_4$[c]
25	48.3 ± 2.07[d]	21.5 ± 0.82
50	75.6 ± 1.65	23.2 ± 1.64
100	88.9 ± 0.93	49.0 ± 1.85
200	92.4 ± 0.83	52.9 ± 2.47
untreated control, 100 mg	21.5 ± 0.20	13.7 ± 3.66
dialyzed control, 100 mg	24.2 ± 0.80	17.6 ± 1.71

[a] Conditions of alkali treatment: 1% protein; 75 °C; 3 h. [b] The protein and enzyme were incubated for 35 min. [c] The protein and enzyme were incubated for 5 min, zinc sulfate was added, and incubation was continued for another 30 min. [d] Average from two separate determinations ± standard deviation.

Effect of Proteins. One hundred milligrams of ground protein was dissolved or suspended in 2 mL of Tris buffer at pH 7.5. The solution or suspension was then added to 10 μL of undiluted enzyme solution and the mixture shaken for various periods, usually 35 min, in a 25 °C water bath. The reaction was terminated by immersing the flasks in crushed ice for 5 min and then centrifuging them at 0 °C for 5 min in a Beckman micro B centrifuge. Enzymatic activity was determined with the supernatant solution.

The influence of $ZnSO_4$ was studied by replacing 1 mL of the buffer with 1 mL of 1 mM $ZnSO_4$. Additional details are given in the footnotes to the tables.

RESULTS AND DISCUSSION

Effect of Protein Concentration. To assess the influence of protein concentration on the extent of inhibition of CPA by alkali-treated soy protein, four experiments were carried out in which the concentration varied from 25 to 200 mg. The results in Table I show that the extent of inhibition was 48.3% with 25 mg of protein, 75.6% with 50 mg, 88.9% with 100 mg, and 92.4% with 200 mg. All of the values were higher than those observed with the untreated (21.5%) and dialyzed (24.2%) controls. Since 100 mg of alkali-treated soy protein produced nearly maximal inhibition, this amount was used in all further studies.

Effect of Time. To assess the influence of time of exposure on the extent of inhibition of CPA by alkali-treated soy protein, a series of experiments were carried out with reaction times of 10–120 min. The results in Table II suggest that inactivation takes place relatively rapidly since time of exposure had a relatively minor influence on inhibition (which ranged from 85–94%). A 35-min time period was adopted as standard for most of the experiments.

Effect of Phytate. Since soy products contain phytic acid, which is known to chelate zinc and other transition metal salts (Clydesdale and Camire, 1983; Graf and Eaton, 1984), we tested the influence of phytate removal on the inhibitory activities of both soy protein isolate and soy flour. Table III shows that (a) subjecting commercial soy protein isolate to the phytate removal procedure of de Rham and Jost (1979) lowered the phytate content from 0.81 to 0.68%, (b) the corresponding values for soy flour were 2.62 and 0.48%, (c) phytate removal lessened the inhibition of CPA from 23.8 to 1.5% for the protein and from 28.7 to 14.7% for the soy flour, (d) alkali treatment of either product increased inhibition to 88.9% for the soy protein and 100% for the soy flour, (e) the presence of zinc

Table II. Effect of Time on the Inhibition of Carboxypeptidase A Activity by Alkali-Treated Soybean Protein[a]

time, min	carboxypeptidase inhibited, % without $ZnSO_4$	with $ZnSO_4$[b]
10	85.0 ± 0.92[c]	41.2 ± 1.85
20	86.9 ± 1.85	42.5 ± 1.85
30	88.9 ± 0.93	49.0 ± 1.85
60	92.2 ± 0.00	47.0 ± 1.02
120	94.2 ± 0.93	58.8 ± 2.77

[a] Conditions of alkali treatment: 1% protein; 75 °C; 3 h. [b] Incubation with $ZnSO_4$ was for an additional 30 min after the indicated exposures without $ZnSO_4$. [c] Average from two separate determinations ± standard deviation.

Table III. Effect of Phytate and Alkali Treatment of Soy Products on Carboxypeptidase A Activity[a]

soy product	carboxypeptidase A activity inhibited, % without zinc sulfate[b]	with zinc sulfate[c]	phytate content, %
untreated soy protein	23.8	0.90	0.81
low-phytate soybean protein	1.5	0.90	0.68
alkali-treated soy protein	88.9	49.0	0.83
untreated soy flour	28.7	2.9	2.62
low-phytate soy flour	14.7	0.0	0.48
alkali-treated soy flour	100.0	100.0	3.94
sodium phytate, 4 mg[d]	0.0		
sodium phytate, 40 mg[e]	19.1		

[a] Conditions of alkali treatment: 1% protein or flour; 0.1 N NaOH; 75 °C; 3 h. [b] The soybean protein or flour was incubated with the enzyme for 35 min. [c] The soybean protein or flour was incubated with the enyzme for 5 min. zinc sulfate was added, and incubation was continued for another 30 min. [d] 4 mg of sodium phytate and 10 μL of CPA stock enzyme solution were incubated at 25 °C for 1 h. [e] As above, except used 40 mg of sodium phytate.

sulfate prevented the inhibitory action of native soy products, and (f) the presence of zinc sulfate only partly prevented the inhibition by alkali-treated soy protein but did not prevent that by treated soy flour.

The reduction of the phytate content of the soy protein by the trichloroacetic acid (TCA) extraction procedure of de Rham and Jost (1979) seems rather small. Whether this slight change in phytate content accounts for the substantial change in inhibition awaits further study. In addition, alkali treatment seems to increase the phytate content of soy flour (Table III). The treatment possibly renders additional, tightly bound phytate extractable by TCA. In spite of these uncertainties, the data in Table III nevertheless show that any action of phytate on the metalloenzyme is distinct from that of alkali treatment.

Additional evidence for this conclusion comes from the observed noninhibition of CPA by sodium phytate at a level (4 mg) equivalent to the maximum amount present in the protein (Table III). However, 19% of CPA activity was lost when the amount of free phytate was increased 10-fold, to 40 mg (Table III).

Effect of Free Lysinoalanine. Preliminary results in Table IV show that (a) 5 mM LAL completely inhibited CPA activity in the pH range 6.5–7.0, (b) inhibition at pH 8.0 requires about twice the amount of LAL and depends on substrate concentration, (c) LAL does not inhibit CPA at pH 8.5, and (d) LAL is a more potent inhibitor than EDTA in the pH range 6.5–7.5 since additional rate studies revealed that it takes about 60 min for EDTA to completely inactivate the enzyme compared to only about 3 min for LAL.

These results and additional studies on the concentration dependence of the inactivation suggest that inhibitory

Table IV. Effect of Lysinoalanine and EDTA on Carboxypeptidase Activity[a]

substrate concn, mM	% carboxypeptidase inhibited at pH 6.5	7.0	7.5	8.0	8.5
			LAL		
0.5	100	100	100	0.0 (11.5)	0.0 (0.0)
1.0	100	100	96.2	0.0 (98.6)	0.0 (0.0)
			EDTA		
0.5	100	100	100	68.6 (100)	16.1 (61.5)
1.0	100	100	100	71.3 (100)	36.3 (68.7)

[a] Listed values were obtained with 5 mM LAL or EDTA except those in parentheses, which were obtained with 10 mM inhibitor concentration.

effectiveness of LAL against CPA decreases with increasing pH and that inhibition requires a threshold concentration of LAL of about 5 mM at 2.6 μM CPA. They also support the suggestion that LAL-induced cytomegaly in the rat kidney could be due to binding of LAL or LAL-containing peptides to structural and functional metalloproteins in the kidney cells (Friedman, 1977).

Effect of Protein-Bound Lysinoalanine. Two sets of experiments were carried out to assess the influence of protein-bound lysinoalanine on inhibition of CPA by alkali-treated soy protein. In the first, alkali treatment was carried out at 10 °C intervals in the temperature range 25–95 °C. The LAL content of these samples ranged from about 2 to 10 mmol/100 g (Table V). In the second, the time of treatment varied from 10 to 480 min. The LAL content of these samples ranged from about 3 to 12 mmol/100 g.

Table V shows that the inhibition of CPA by both sets of samples increased with LAL content, reaching a maximum at about 10 mmol of LAL/100 g of protein. Significant inhibition occurred even with relatively low levels (2–3 mmol/100 g) of LAL.

The results of the second set of experiments, summarized in Table VI, show that different amounts of LAL are produced when structurally different proteins are exposed to the same alkaline conditions [cf. also Friedman (1979)]. For casein, the alkaline treatment (0.1 N NaOH; 65 °C; 3 h) generated 17.4 mmol of LAL/100 g of protein. The corresponding value for lactalbumin was 18.0, for soybean protein 7.9, for wheat gluten 4.4, for high-lysine corn protein 4.25, and for zein 0. The data in this table also show no direct relationship between the LAL contents of these proteins and their extent of inhibition of CPA. For example, alkali-treated wheat gluten, with a low LAL content, completely inhibited CPA, whereas alkali-treated lactalbumin, with a high LAL content, inhibited only 57.5% of the enzyme activity.

Additional evidence for the hypothesis that protein-bound LAL, in contrast to the free amino acid, may not be directly involved in the mechanism of inhibition comes from the observation with the corn protein, zein. This protein has no lysine and, therefore, does not form LAL during exposure to alkali. Nevertheless, the data in Table VI show that exposure of zein to 0.1 N NaOH for 3 h at 65, 75, or 85 °C increased the inhibitory capacity from 16.0% for the untreated protein to 21.4, 50.3, and 71.0% for the treated zeins. These results strikingly demonstrate the complex nature of the inhibitory process.

Effect of Zinc Sulfate. The effect of addition of $ZnSO_4$ was investigated to ascertain whether the inhibition is the result of chelation or abstraction of the zinc atom that is part of the active site of CPA (Kaiser et al., 1974; Chlebowski and Coleman, 1976). Since concurrent or sequential addition of zinc sulfate prevented or reversed the

Table V. Relationship between Lysinoalanine Content and Carboxypeptidase Inhibitory Activities of Alkali-Treated Soybean Proteins

treatment	carboxypeptidase A inhibited, % without $ZnSO_4$[a]	carboxypeptidase A inhibited, % with $ZnSO_4$[b]	lysinoalanine content, mmol/100 g
untreated protein	21.5 ± 0.20[c]	13.7 ± 3.66	0
dialyzed protein	24.2 ± 0.80	17.6 ± 1.71	0
1% protein; 0.1 N NaOH; 3 h			
25 °C	60.3 ± 1.02	7.25 ± 2.05	2.05
35 °C	75.4 ± 2.04	8.71 ± 2.04	3.24
45 °C	75.4 ± 0.00	27.2 ± 2.60	3.95
55 °C	89.9 ± 0.00	34.8 ± 2.68	6.09
65 °C	89.9 ± 0.00	48.1 ± 0.00	7.90
75 °C	88.6 ± 1.79	58.2 ± 1.80	9.33
85 °C	100.00 ± 0.00	76.8 ± 0.98	10.73
95 °C	100.0 ± 0.00	89.0 ± 0.00	9.69
1% protein; 0.1 N NaOH; 75 °C			
10 min	34.4 ± 0.97	12.4 ± 0.97	3.22
30 min	61.4 ± 0.00	15.9 ± 1.95	6.78
60 min	79.6 ± 0.99	33.8 ± 2.00	6.65
180 min	88.9 ± 0.93	49.0 ± 1.85	9.63
300 min	93.1 ± 1.72	57.3 ± 0.00	10.52
480 min	100.0 ± 0.00	66.2 ± 2.00	12.09

[a] The protein and enzyme were incubated for 35 min. [b] The protein and enzyme were incubated for 5 min, zinc sulfate was added, and incubation was continued for another 30 min. [c] Average from two separate determinations ± standard deviation.

Table VI. Inhibition of Carboxypeptidase A by Structurally Different Untreated and Alkali-Treated Food Proteins[a]

protein	carboxypeptidase inhibited, % without zinc sulfate[b]	carboxypeptidase inhibited, % with zinc sulfate[c]	lysinoalanine content, mmol/100 g
casein, untreated	28.3 ± 0.0[d]	31.8 ± 0.0	0.0
casein, alkali treated	50.3 ± 1.63	12.5 ± 1.85	17.4
lactalbumin, untreated	16.7 ± 0.76	4.58 ± 0.78	0.0
lactalbumin, alkali treated	57.5 ± 0.76	36.0 ± 2.99	18.0
soy protein, untreated	21.5 ± 0.20	13.7 ± 3.66	0.0
soy protein, alkali treated	89.9 ± 0.00	48.1 ± 0.00	7.90
wheat gluten, untreated	23.7 ± 1.65	16.8 ± 1.63	0.0
wheat gluten, alkali treated	100.0 ± 0.0	85.0 ± 1.63	4.40
corn protein, high lysine, untreated	17.6 ± 2.61	14.5 ± 0.60	0.0
corn protein, high lysine, alkali treated	62.4 ± 7.45	56.7 ± 1.69	4.85
zein, untreated	16.0 ± 1.33	15.5 ± 1.44	0.0
zein, alkali treated, 65 °C	21.4 ± 0.97	24.9 ± 1.44	0.0
zein, alkali treated, 75 °C	50.3 ± 1.44	34.5 ± 0.72	0.0
zein, alkali treated, 85 °C	71.0 ± 0.81	57.4 ± 0.81	0.0

[a] Alkali treatment: 1% protein; 65 °C; 3 h except for zein, which was also treated at 75 and 85 °C. [b] The protein and enzyme were incubated for 35 min. [c] The protein and enzyme were incubated for 5 min, zinc sulfate was added, and incubation was continued for another 30 min. [d] Averages from two separate determinations ± standard deviation.

inhibition of metalloenzymes by LAL (Hayashi, 1982), it was of interest to establish whether this process also operates with alkali-treated proteins. Tables I–III, V, and VI show that zinc sulfate minimized but did not completely prevent inhibition of CPA by all of the alkali-treated food proteins tested.

Zinc sulfate alone seems to have an inhibitory effect on the enzyme since exposure of 5 μL of CPA of stock to 0.5 mL of 1 mM zinc sulfate lowered CPA activity by about 6%. The listed values were not corrected for this effect. Since the reversible inhibition by free LAL presumably involves abstraction or removal of the zinc moiety from the active site of the metalloenzymes, followed by restoration of activity after readdition of zinc to the active sites, our studies imply that free LAL and the alkali-treated proteins may operate by different mechanisms.

Effect of Heat Denaturation. Using autoclaved or heated soy protein had no or only a minor influence on inhibitory activity against CPA (results not shown). This result implies that the inhibition by the treated proteins are not caused by heat-induced conformational changes in the protein macrostructure.

Mechanistic Possibilities. Carboxypeptidase A hydrolyzes amino acids one at a time from the C-terminal end of the polypeptide chain. The enzyme differs from most other proteases by having zinc as part of its active site and by undergoing a large conformational change during the binding of the substrate in order to bring together the components of the active site for efficient activity (Zubay, 1983).

Inhibition of CPA activity could occur by any of several mechanisms: (a) competitive inhibition by a substrate-like molecule, including the carboxyl end of the polypeptide chain; (b) blockage (masking) of the active site of CPA by noncompetitive inhibition; (c) removal of the cofactor essential for catalytic activity, namely, Zn^{2+}. Free LAL appears to act, at least in part, by mechanism c through chelation of Zn^{2+} (Hayashi, 1982).

Our results suggest, however, that LAL bound in the polypeptide chain acts predominantly by the other mechanism(s). Nonterminal LAL differs from free LAL in that four of the five potential groups that can participate in metal binding (two COOH, two NH_2) participate in amide (peptide) linkages. The inability of Zn^{2+} to completely reverse inhibition of CPA activity by alkali-treated soy protein suggests that the above-named groups are, indeed, important for Zn^{2+} binding.

Denaturation of protein by alkali or heat or both exposes a number of side chain groups that might react with CPA. The COOH-terminal region may be rendered accessible

to the active site of CPA. If this sequence binds to the active site but is slowly hydrolyzed or binds with much greater affinity to the active site than to the substrate, it could hinder substrate access and inhibit activity competitively (mechanism a). This mechanism appears to resemble the action of the naturally occurring carboxypeptidase inhibitors from potatoes (Pearce et al., 1983) and kidney beans (Hojima et al., 1979).

If the alkali treatment partially hydrolyzes the test protein, the new number of COOH termini will be increased in proportion. This will increase the probability that one or more can bind to CPA but be hydrolyzed slowly. Groups exposed by unfolding may interact with side chains on CPA external to the active site. If such interactions between denatured protein and CPA result in masking of the active site of CPA, noncompetitive inhibition may be observed (mechanism b).

Zinc binding involving amino acid side chains exposed or generated by alkali treatment is theoretically possible due to disruption of hydrogen bonds or salt bridges or to deamidation of asparagine or glutamine to produce new COOH groups. Extensive studies of the mechanism of CPA action and structure of the active site [see Kaiser et al. (1974), Lipscomb (1982), and Vallee and Galdes (1984) for reviews] indicate that zinc is coordinated by a Glu, two His, and H_2O and that Glu, Arg, and Tyr residues are involved in substrate binding or catalysis. If these or other side chains in the treated protein can achieve proper orientation to furnish multiple ligands for Zn^{2+}, the protein could compete with CPA for the metal ion.

Plots of reciprocal velocities ($1/v$) against inhibitor (treated protein) concentration depicted in Figure 1 permit some conclusions about the general aspects of the CPA inhibition (Dixon, 1953). Figure 1 shows a greater inhibitory effect of treated soy protein on CPA in the absence of zinc ions. Limits on K_i (the inhibition constant) can be determined from the plots in Figure 1. These are $K_i < 10$ mg in the absence of Zn^{2+} ions and $K_i > 100$ mg in the presence of Zn^{2+} ions. These results suggest that zinc ions eliminate one mode of inhibition or greatly weaken the binding of the protein to the enzyme.

Another possibility is that the D-amino acid residues formed by the alkali treatment bind nonspecifically to the active site of CPA, thus inhibiting the enzyme. This could occur since (a) CPA has its primary specificity for aromatic amino acid side chains such as Phe and Tyr and (b) these residues rapidly racemize to D isomers under the influence of alkali and heat (Masters and Friedman, 1979, 1980; Liardon and Hurrell, 1983). The extent of this type of inhibition should depend on the number and location of such residues formed during the alkaline treatment.

This suggestion is reinforced by our observation that the extent of inhibition may not depend on protein-bound LAL, since inhibition by alkali-treated zein, which has no lysine and thus cannot produce LAL, progressively increased with increasing temperature of the alkali treatment from 65 to 85 °C (Table VI). The dependence of the inactivation on the temperature of the alkali treatment may be due to enhanced racemization of amino acid residues at the higher temperature (Masters and Friedman, 1979).

Nutritional Implications. Inhibition of CPA by treated proteins may have nutritional implications. Inhibition would hinder further digestion of a food peptide by CPA or other carboxypeptidases so that action of aminopeptidase or endopeptidase would be required. Specifically, during in vivo digestion of food proteins, the protein is cleaved by endopeptidases (pepsin, trypsin, chymotrypsin, elastase), yielding large peptide fragments that are then further degraded by a variety of exopeptidases such as CPA and carboxypeptidase B (CPB), aminopeptidases, and dipeptidases (Floch, 1981; Schwimmer, 1981). CPA removes the C-terminal amino acid residue. Its primary specificity is for aromatic amino acids such as Phe, Tyr, and Trp. These considerations imply that if alkali-treated proteins inhibit CPA, the negative feedback mechanism that accounts for the in vivo effects of naturally occurring carboxypeptidase (Pearce et al., 1983) and soybean trypsin inhibitors (Gallaher and Schneeman, 1984) could also operate with the CPA inhibitors.

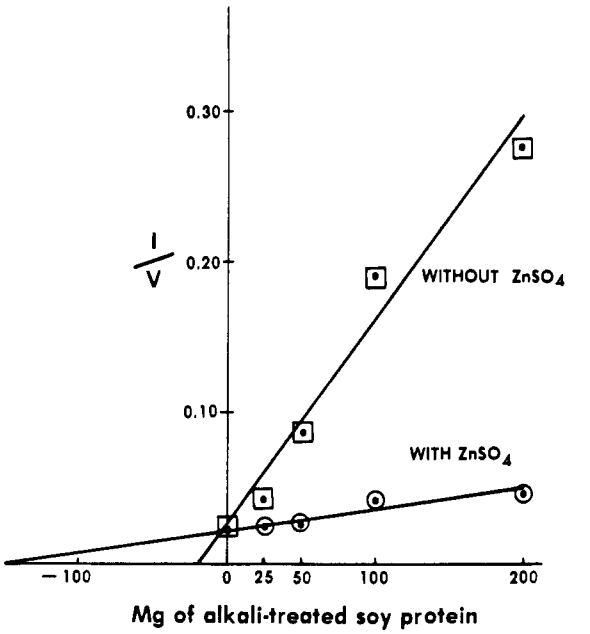

Figure 1. Plot of the reciprocal velocity ($1/v$) against protein concentration for the inhibition of carboxypeptidase A by alkali-treated soy protein in the presence and absence of zinc sulfate. Data are from Table I without correction of inhibition by untreated control. The velocity, v, is in units/mg, where 100% activity = 47.37 units/mg.

Although alkali treatment is widely used in food processing (Sternberg et al., 1975; Sternberg and Kim, 1977; Haagsma and Slump, 1978; Raymond, 1980; Friedman and Masters, 1982), such treatments may not always be as severe as those shown in Tables V and VI. The possible inhibition of metalloenzymes by commercially processed food proteins merits further study, especially since some D-amino acids may have antinutritional properties (Friedman and Gumbmann, 1984a,b).

Finally, our studies show no inhibition of trypsin by alkali-treated soy protein and slight (less than 5%) inhibition by alkali-treated casein. In related studies, Vimont-Rpisoli et al. (1980) reported that nondigestible peptides isolated from alkali-treated casein can bind and inactivate trypsin in vitro, and Percival and Schneeman (1979) demonstrated that poorly digested casein–glucose browning products do not stimulate increased trypsin production by the rat pancreas. Additional studies are needed to better define the possible antinutritional significance of these findings.

In summary, the results of this study demonstrate for the first time that alkali-treated lysinoalanine-containing and lysinoalanine-free food proteins inactivate metalloenzymes such as carboxypeptidase A. Additional studies are needed to ascertain whether such inactivation is related to the observed biological effects of alkali-treated food proteins and whether alkali-treated food proteins affect

mineral nutrition.

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Registry No. LAL, 18810-04-3; CPA, 11075-17-5; EDTA, 60-00-4; Zn, 7440-66-6.

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Phenolic Constituents in the Leaves of Northern Willows: Methods for the Analysis of Certain Phenolics

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Several phenolic components were extracted from willow samples by using the same extraction procedure, purified, and analyzed by spectrophotometry and gas chromatography. The components analyzed were total phenolics, condensed tannins, and phenolic glycosides. Methods for drying the material, extraction, and analysis are tested and discussed.

Higher plants may produce a great variety of secondary phenolic compounds, the role of which in the metabolism of the plant itself has not been adequately explained. Many of these compounds are bound with sugars in living plants. Sugar conjugates of phenolic aglycons may decrease the toxicity or reactivity and increase the solubility of the compounds to make it easier for them to be transported or stored without harm to the plant producing them (Vickery and Vickery, 1981).

In Finland, the genus *Salix* contains about 20 native species and numerous hybridized forms. The secondary chemistry of Salicaceae has been studied for taxonomic purposes because morphological identification is often very difficult. Because certain phenolics are bitter tasting and/or have the ability to precipitate plant and animal proteins, they have been considered as defense compounds against animal predators and microbes [e.g., Markham (1971) and Feeny (1976)].

The present work was carried out as part of an investigation on the relationship between willow feeding herbivores and the secondary chemistry of Salicaceae. The object of this study was to determine the distribution and seasonal variation of certain phenolics and their dependence on fertilization in laboratory and field conditions. This paper describes the methods developed and tested for isolation and characterization of certain phenolic components in *Salix* extracts.

EXPERIMENTAL SECTION

Equipment. For colorimetric determinations a Hewlett-Packard dual-wave spectrophotometer was used and for column chromatography an LKB fraction collector equipped with a UV-detector. The gas chromatograph used to analyze the trimethylsilyl derivatives of willow samples was a Packard 433 equipped with a flame ionization detector. A fused silica SE-52 capillary column 25

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