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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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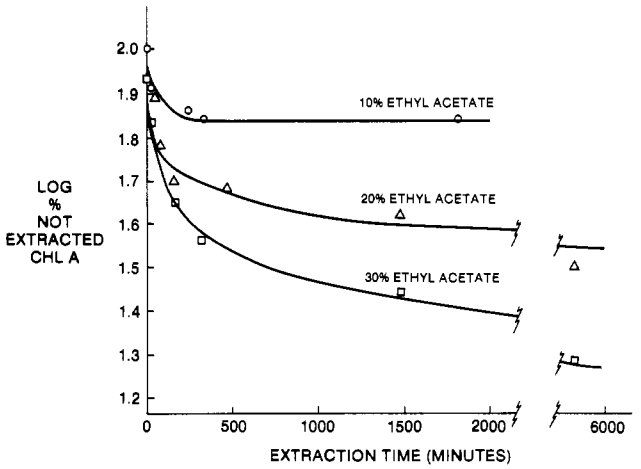

Figure 1. Extraction of chlorophyll *a* by solvent mixtures of ethyl acetate in hexane (v/v) at 2 °C. Lines fitted by regression calculations.

Thylakoid membranes were used for this study in order to have a well-defined, reproducible material with a high proportion of chlorophyll–protein. They were used in the lyophilized form in order to permit pigment extraction by relatively nonpolar solvent mixtures without the complications of having two liquid phases.

MATERIALS AND METHODS

Chloroplasts were prepared from field-grown spinach (cv. Early Smooth No. 424) by the method of Nakatani and Barber (1977). They were osmotically shocked in 0.0005 M $MgCl_2$, and the thylakoid membranes were collected by centrifugation at 20000 *g* for 30 min. The thylakoids were lyophilized and stored at −20 °C. For use in extractions, lyophilized thylakoids were suspended in hexane and portions taken for dilutions in ethyl acetate–hexane mixtures to give the mixtures noted in the text. Extractions were carried out at the indicated temperatures, 2, −20, and −40 °C, with near continuous stirring. Under these conditions there was no evidence of pheophytin formation as evaluated by the ratio of absorbances at 536 and 558 nm (Vernon, 1960). At intervals the extracting mixtures were centrifuged at the same temperature as the extraction, and single samples of the supernatant were taken for pigment composition analysis. One milliliter of extract was diluted to 25 mL with 80% acetone (v/v). Tests showed that this proportion of the extraction solvents did not significantly alter the absorbance values compared to those obtained in simple 80% acetone. Concentrations of chlorophyll *a* and chlorophyll *b* were calculated from spectrophotometric data by using the equations of Arnon (1949).

Statistical analysis were carried out by using the MINITAB computer package (Ryan et al., 1980).

RESULTS AND DISCUSSION

Figure 1 gives the time course of chlorophyll *a* extraction from lyophilized spinach chloroplast thylakoid membranes when solvent mixtures of different ethyl acetate concentrations were employed. These plots, using the logarithm of the percent chlorphyll not in solution as the ordinate, show that the extractions were always non first order. The weakly polar 10% ethyl acetate solvent mixture extracted only about 30% of the chlorophyll *a*. As the ethyl acetate concentration of the solvent mixture was increased, higher limiting percentages of chlorophyll *a* were extracted at the extended extraction times. Still more polar solvents, such as 80% acetone–20% water (v/v) or dimethylformamide (Moran and Porath, 1980), have been shown to completely remove chlorophylls from thylakoid membranes. These

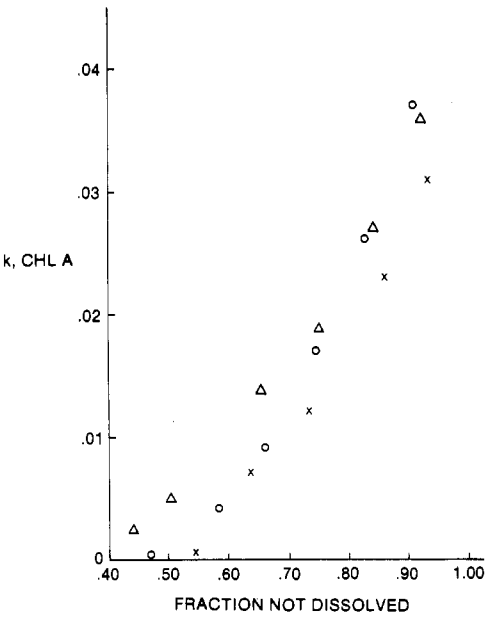

Figure 2. Relation of chlorophyll *a* specific extraction rate, *k*, to fractions not extracted. (△), (○), and (×) represent three different experiments in 25% ethyl acetate at 2 °C.

different limit extractabilities indicate chlorophyll binding sites with different solvent accessibilities.

While no attempt was made to establish the solubility of the chlorphylls in ethy acetate–hexane mixtures, a test showed that chlorophyll *a* at 161 μg/mL and chlorophyll *b* at 28 μg/mL remained completely in solution in a solvent mixture having 10% ethyl acetate in hexane (v/v) at −40 °C. Thus, it can be concluded that the limit extractabilities observed were not the result of a saturation of the solvents.

The lines plotted in Figure 1 and Figure 5 were calculated by using equations regressing the amount extracted on the time of extraction and were of the form

$$\text{amount extracted} = A + B[\log \log (\text{time} + 10)] + C/(\text{time} + 10) \quad (1)$$

where time was in minutes and *A*, *B*, and *C* were constants obtained from the statistical treatment.

The regression equations were used to obtain *k*, the specific extraction rate for micrograms of chlorophyll going into solution per minute per microgram of unextracted chlorophyll. It has the dimension 1/minutes, and it was calculated from the amount extracted in the 1-min interval starting 0.5 min before, and ending 0.5 min after, a selected extraction time.

It was evident that the specific extraction rate of chlorphyll *a* decreased as extraction proceeded and was related to the fraction of chlorophyll not extracted (Figure 2). This suggested that treatment effects on *k* should be compared at specific fractions of unextracted chlorophyll. At like fractions of unextracted chlorophyll, the extractions could be expected to involve similar types and numbers of chlorophyll-containing sites, making treatment comparisons more meaningful. Such comparisons were used to show the effect of increasing percentages of ethyl acetate giving increasing *k* values (Figure 3).

The data of Table I showed that an equilibrium between soluble and bound chlorophyll apparently had little influence in affecting the amounts of soluble pigment found at long extraction times since a single 3-day extraction brought nearly as much chlorophyll *a* into solution as did three separate 1-day extractions.

The effect of temperature on specific extraction rate is shown in the Arrhenius plot of log *k* vs. the reciprocal of

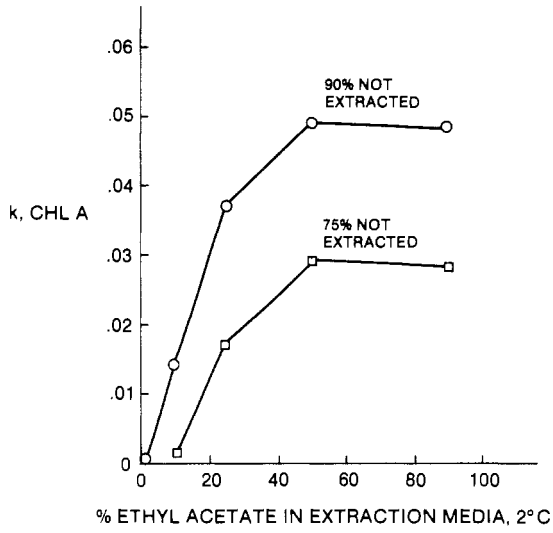

Figure 3. Influence of ethyl acetate concentration in the ethyl acetate–hexane extraction media on the specific extraction rate, k, for chlorophyll a at particular fractions of chlorophyll a not extracted at 2 °C.

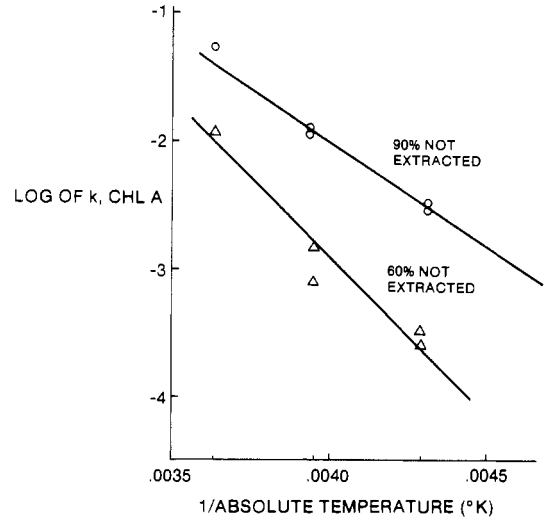

Figure 4. Arrhenius plot of chlorophyll a specific extraction rate, k, against the reciprocal of the absolute temperature. Extractions carried out in 50% ethyl acetate in hexane (v/v). Straight lines fitted by regression calculations.

Table I. Comparison of a Single and a Three Times Extraction of Chlorophyll *a* Using an Ethyl Acetate–Hexane Mixture[a]

type of extraction	% extracted
single 3-day extraction	69.6 a
successive extractions	
first 1-day extraction	57.6 b
second 1-day extraction	9.2 c
third 1-day extraction	4.1 d
sum of 1-day extractions	70.9 a

[a] Extractions were at −40 °C in 40% ethyl acetate in hexane (v/v). Values not followed by the same letter were significantly different at $P < 0.05$.

the absolute temperature (Figure 4). If the specific extraction rate, k, at a particular fraction not extracted was considered to have many of the properties of a specific extraction rate constant, then the Arrhenius plot should provide some insight into the apparent activation energies of the extractions. The slope of the best fitting line connecting the points was obtained by least-squares regression. This slope, when multiplied by $2.303R$ ($R = 1.987$ cal/deg), gave an apparent activation energy for the extraction of

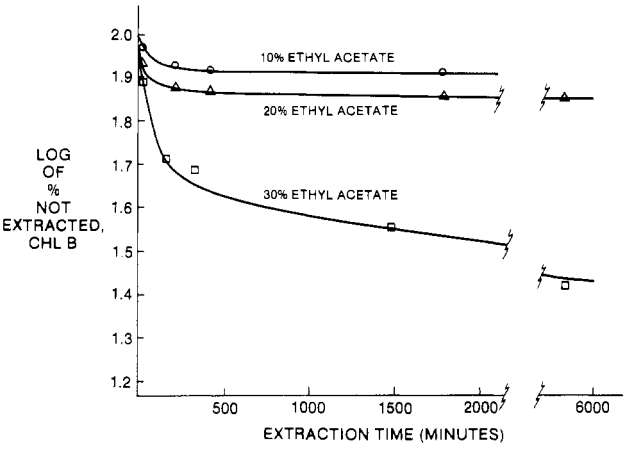

Figure 5. Extraction of chlorophyll b by solvent mixtures of ethyl acetate in hexane (v/v) at 2 °C. Lines fitted by regression calculations.

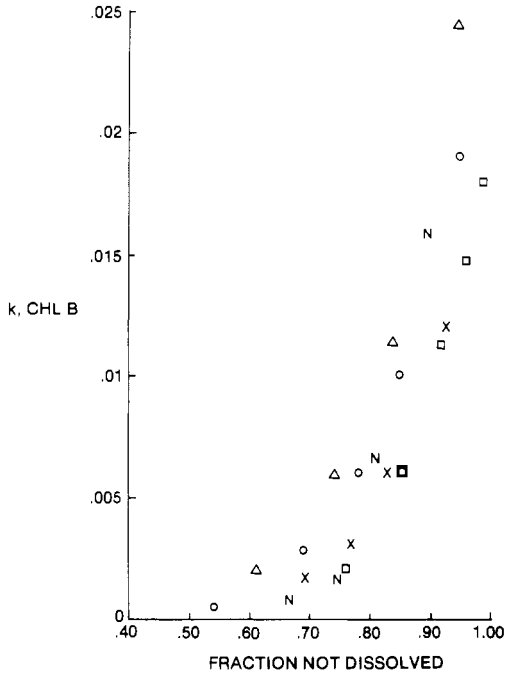

Figure 6. Relation of chlorophyll b specific extraction rate, k, to fractions not extracted. (△), (○), (×), and N represent four different experiments in 25% ethyl acetate at 2 °C.

Table II. Apparent Activation Energies for the Extraction of Chlorophyll *a* and *b* from Lyophilized Thylakoid Membranes[a]

	chlorophyll a		chlorophyll b	
% not extracted	activation energy, cal/mol	R^2	activation energy, cal/mol	R^2
90	8200	99	4100	92
60	10600	94	4200	99

[a] Extractions carried out in 50% ethyl acetate in hexane (v/v) at 2, −20, and −40 °C.

chlorophyll a at particular fractions of unextracted chlorophyll a. These apparent activation energies, along with the R^2 values of the regression lines, are given in Table II.

The extraction behavior of chlorophyll b followed a similar pattern to that of chlorophyll a. Long-time extractions of chlorophyll b (Figure 5) solubilized less pigment than was seen for chlorophyll a. The effect on k of increasing ethyl acetate concentrations continued beyond 50% ethyl acetate (Figure 6). Lower k values were found

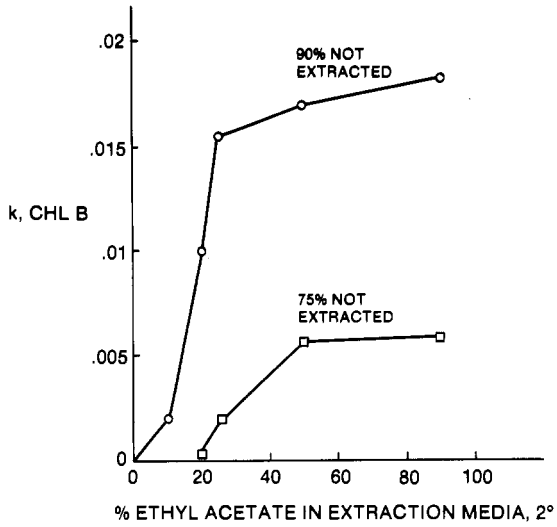

Figure 7. Influence of ethyl acetate concentration in the ethyl acetate–hexane extraction media on the specific extraction rate, *k*, for chlorophyll *b* at particular fractions of chlorophyll *b* not extracted at 2 °C.

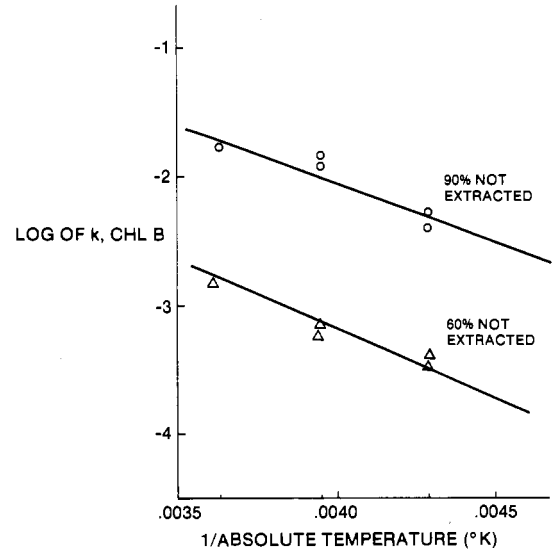

Figure 8. Arrhenius plot of chlorophyll *b* specific extraction rate, *k*, against the reciprocal of the absolute temperature. Extractions carried out in 50% ethyl acetate in hexane (v/v). Straight lines fitted by regression calculations.

(Figure 7), and these were less influenced by temperature (Figure 8) than was seen with chlorophyll *a*. Consequently, the apparent activation energy for chlorophyll *b* extraction (Table II) was less than for chlorophyll *a* extraction.

The rate of release of chlorophyll into solutions seems to be controlled by a process other than the immediate dissociation of chlorophyll from binding sites. This is suggested by the relatively small changes in apparent activation energies with large differences in extraction rate and the low reversibility of the chlorophyll binding (data not given). An alternative mechanism for a rate controlling reaction would be a conformational alteration of the lipoprotein, proceeding with time and enhanced by higher ethyl acetate concentration, exposing bound chlorophyll to the disassociating effects of the solvent mixtures. The magnitudes of the apparent activation energies were consistent with breakage of noncovalent bonds accompanying protein rearrangement and chlorophyll dissociation.

The possible involvement of conformation alterations as a prelude to chlorophyll solubilization was indicated when the effects of heating were observed. Very little

Table III. Effect of Heating Membranes on the Extraction of Chlorophyll *a* into Soybean Oil and an Ethyl Acetate–Hexane Mixture[a]

	% of chlorophyll *a* extracted		
		in hexane (9)–ethyl acetate (1)–soybean oil (1)	
	in soybean oil, 20 °C	0 °C	20 °C
not heated	18.4 d	32.5 c	39.8 c
heated 10 min at 100 °C	62.1 b	85.3 a	92.4 a

[a] Soybean oil suspensions of thylakoid membranes (0.5 mL, 0.25 mg of chlorophyll/mL) were heated or not heated as indicated. After being cooled, the suspensions were diluted to 5.0 mL to give the indicated liquids and held for 2 days at the indicated temperatures with occasional stirring before measurement of the soluble chlorophyll. Values not followed by the same letter were significantly different at $P < 0.05$.

chlorophyll could be extracted from lyophilized thylakoid membranes by soybean oil (Wesson Oil). When suspensions of thylakoids in oil were heated at 100 °C, some chlorophyll went into solution. When such preparations, along with unheated suspensions, were placed in hexane–ethyl acetate (9:1), there was less solubilization of chlorophyll from the unheated samples (Table III). One might suggest that a protein alteration took place during heating that exposed chlorophyll to the mild solubilizing action of soybean oil or the ethyl acetate–hexane mixture. However, more tests directly measuring protein structure would be desirable to establish conformational changes as necessary for pigment extraction.

The chlorophyll can be visualized as being bound to the lipoprotein by hydrogen bonds involving its carbonyl functions (Lutz, 1977) in a region toward the interior of the folded lipoprotein. The folding of peptide chains in lipid media (Rao et al., 1983), creating interior polar regions, may well be stabilized by hydrogen bonds in analogy to the stabilization of peptide folding in aqueous milieu by hydrophobic influences (Kauzman, 1959; Tanford, 1970). The hydrocarbon phytyl group would then extend outward, between peptide chains, and be associated with the lipophilic exterior of the protein and with the membrane lipids. Solvents that readily extracted chlorophyll, such as acetone and dimethylformamide, have oxygen functions that compete for hydrogen-bond association. The present work suggests that their effectiveness resides in their ability both to alter the protein conformation and to disrupt the chlorophyll–protein bonding.

The range of *k* values was evidence for the heterogeneity of binding of chlorophyll to the protein. For instance, they indicate that the population of sites adding to the soluble chlorophyll when there was 90% unextracted pigment was different from the population contributing when there was 50% unextracted pigment. At a higher percent unextracted chlorophyll, the dissociating sites had a weaker chlorophyll–protein binding, or were more accessible to the solvent, than was the case at lower percent unextracted chlorophyll.

This examination of extraction rates with different ethyl acetate concentrations in the extracting media and different extraction temperatures provides several lines of evidence that the chlorophyll *a* and chlorophyll *b* were bound to the lipoproteins at a variety of types of sites.

Registry No. Chlorophyll *a*, 479-61-8; chlorophyll *b*, 519-62-0.

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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-