Effect of Alkali Treatment on the Formation of Lysinoalanine in Corn

Quantitative analysis of lysinoalanine (LAL), a compound reported to produce nephrotoxic effects when fed to rats, was carried out in samples of corn after treatment with alkali under varied conditions. Sodium and potassium hydroxides produced much greater quantities of LAL than lime or calcium hydroxide when compared on an equimolar basis. The amounts of LAL formed increase with longer periods of alkali cooking. It is postulated that calcium ions may interfere in some manner with the mechanism of LAL formation.

A new amino acid, N^{ϵ}-(2-amino-2-carboxyethyl)lysine, was reported in alkali-treated proteins and given the trivial name "lysinoalanine" (LAL) (Bohak, 1964; Zeigler, 1964). It was suggested that this compound is formed through the addition of an amino group of a lysyl residue to the double bond of a dehydroalanyl residue formed by a β-elimination reaction and that it is readily formed whenever a -Cys-Lys sequence is present in a protein. The production of lysinoalanine upon severe alkaline treatment was found to be associated with loss of amino acids, impaired digestibility, reduced net protein utilization (De Groot and Slump, 1969), and nephrotoxic reactions, "renal cytomegalia" in rats (Woodard and Short, 1973). More recently, the presence of LAL has been reported in a variety of foods which had undergone heat treatment but had not been subjected to alkaline treatment (Steinberg et al., 1975).

Alkali treatment is frequently used in the preparation of proteins with desirable functional properties and for the destruction of aflatoxin, and "liming" of corn has been practiced for centuries by many societies in the preparation of "tortillas".

The present study was undertaken to relate certain parameters of the alkalization process with the amounts of lysinoalanine produced in corn.

EXPERIMENTAL SECTION

Alkalization. Samples of white corn (*Zea mays*), 100 g each, were treated with 150 ml of alkaline solution prepared by dissolving the required amount of alkali in distilled water. The corn–alkali mixtures were heated at 170 °F for 15 or 30 min, cooled to room temperature, and allowed to stand overnight. The mixtures were then drained through a sieve and washed by stirring each in 445 ml of distilled water. The washings were repeated six times before grinding for 90 s with the aid of a blender (Tekmar Co., Cincinnati, Ohio).

Analysis of Lysinoalanine and Lysine. The methods described by Woodard and Short (1973) were used with some modifications. Fifty milligrams of each washed ground sample was placed in an 18 × 150 mm tube and 3 ml of 6 N HCl was added. Each tube was drawn into a capillary 30 mm from the top. The capillary length was about 2 cm. The tubes were then frozen, evacuated, flushed twice with N_2 gas, reevacuated, sealed, and placed into an oven at 100 °C for a digestion period of 22 h.

After filtering through a plug of glass wool and washing with two 1-ml quantities of deionized water the samples were frozen and dried by applying high vacuum to a laboratory vacuum dessicator with the base packed with solid sodium hydroxide. The dried samples were washed twice with small quantities of deionized water and again taken to dryness in vacuo. Each dry sample was dissolved in 5 ml of a 0.2 M pH 2.2 citrate buffer and was filtered through a small fluted Whatman No. 1 filter paper. This solution was then analyzed for lysinoalanine and lysine using a Beckman Model 120 C amino acid analyzer (Spinco Division of Beckman Instrument, Inc., Palo Alto, Calif.).

A 2-ml sample was applied to a 20-cm column packed with Beckman PA35 spherical resin. The wall of the column was washed with several drops of the pH 2.2 buffer, and pressure applied to force the sample and buffer into the resin. Sodium citrate buffer (0.2 ml) (0.35 M, pH 5.28) was added and again forced into the top of the resin column. This process was repeated with the pH 5.28 buffer and connected with a pH 5.28 buffer pumping system which was allowed to operate for 10 min. The ninhydrin pump was then operated for 10 min after which the recorder was turned on. The switches were turned off automatically after 55 min when analysis was completed.

Under these conditions the LAL peak appeared 10 min earlier than that of lysine. Identification of the lysinoalanine peak as well as its quantitative determination was carried out with the aid of authentic samples of lysinoalanine (courtesy of Dr. J. C. Woodard, University of Florida). Calculation of peak areas was based on the height × width method.

Table I. Lysinoalanine (LAL) and Lysine Contents[a] in Alkali-Treated Corn

Treatment				
Alkali	Concn, mol/kg	Min heating at 170 °F	μg of LAL/g of protein	mg of Lys/g of protein
None	0	15	0	18.1
Lime	2.1	15	0	18.1
Lime	3.1	15	0	21.8
Lime	4.1	15	139.8	20.7
Lime	4.1	30	133.2	17.9
$Ca(OH)_2$	4.1	30	103.2	17.3
NaOH	1.5	30	0	22.6
NaOH	3.1	30	0	21.5
NaOH	4.1	30	1033.5	20.8
NaOH	4.1	30	1338.7	20.4
KOH	4.1	15	724.0	21.0

[a] Averages from duplicate determinations.

RESULTS AND DISCUSSION

The LAL and lysine contents in corn flour after treatment with different alkaline reagents, at varied concentrations and heating periods, are given in Table I. It can be seen that concentrations at or below 3.1 mol of NaOH or lime per kilogram of corn did not produce detectable levels of lysinoalanine. However, when the concentration of sodium hydroxide was increased to 4.1 mol/kg significant amounts of lysinoalanine could be detected. In contrast to the effect of NaOH, only low amounts of LAL were formed when lime or $Ca(OH)_2$ were used. Although potassium hydroxide produced somewhat less LAL than did NaOH when applied on an equimolar basis, both of these reagents produced far more LAL than lime or $Ca(OH)_2$. Prolonging the NaOH cooking time from

15 to 30 min increased the amount of LAL.

The production of LAL in proteins by alkali treatment was reported to parallel a decrease in the content of lysine (Bohak, 1964; De Groot and Slump, 1969). In the present study, however, the lysine contents of alkali-treated samples were slightly higher than that of the control. A recent report by Katz and coworkers (1974) notes that in the lime-prepared tortillas a reduction occurs in the essential amino acids, with the exception of lysine. In view of the much smaller amounts of LAL produced (i.e., micrograms/gram) as compared with the quantities of lysine present (milligrams/gram), it is evident that a corresponding fall in lysine would not necessarily be obvious. In addition, changes in the lysine content, unrelated to the LAL reaction, may result from preferential effects of alkalization on digestibility by acid hydrolysis or on losses during the washing process.

The formation of very low amounts of LAL in the case of the treatments with lime or $Ca(OH)_2$ as compared with NaOH or KOH is intriguing. Perhaps the amount of $Ca(OH)_2$, a weak alkali, which can penetrate into the corn kernel is relatively low. However, preliminary semiquantitative experiments with NH_4OH resulted in the production of amounts of LAL approximately equal to those produced by KOH. It may be possible that calcium ions interfere with the mechanism of LAL formation by binding to certain portions of the amino acid sequence or blocking of certain function groups in the side chains of proteins.

It is interesting to note that lime has been traditionally used in Mesoamerica and the southwestern United States as the source of alkali in the preparation of "tortilla". Ashes and lye have been used elsewhere.

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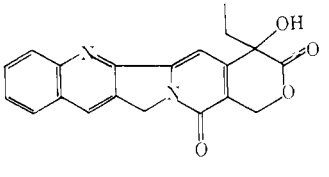
Camptothecin, a Selective Plant Growth Regulator

Camptothecin has been identified as a phytochemically active component of *Camptotheca acuminata*. Selective growth inhibition was found among the plant species tested. Tobacco and corn were retarded while no effect was noted on beans and sorghum when a 1×10^{-4} M emulsion was applied as a spray. Growth inhibition appeared to be confined to the meristematic portions of the test plants.

In a search for new naturally occurring plant growth regulants, an ethanolic extract of *Camptotheca acuminata* (Nyssaceae) was found to effectively control axillary bud growth of topped Xanthi tobacco plants. However, no effect was noted using a Greenpod bean assay. This activity was discovered in a screening program using extracts of plants collected as part of a search for tumor inhibiting compounds from plant sources.

Camptothecin, a novel alkaloid isolated from *Camptotheca*, has been intensively studied as an antitumor



CAMPTOTHECIN

reagent (Wall et al., 1966), but apparently has not been tested for plant growth regulating activity. Several extracts of *Camptotheca* have been fractionated to isolate the component affecting plant growth and to ascertain whether this component might be camptothecin. The effect of the plant growth regulating component was investigated using several species of mono- and dicotyledonous plants.

MATERIALS AND METHODS

Air-dried *Camptotheca* stem wood was ground in a Wiley mill and then exhaustively extracted with 95% EtOH. The initial sample for bioassay was fractionated via an 8-tube Craig countercurrent distribution (Tin-Wa et al., 1971) using $CHCl_3$–MeOH–H_2O (2:2:1). The phytochemically active fraction was located via bioassay on tobacco using a 10% concentration in lanolin (Marth and Mitchell, 1964). The active fraction was separated on TLC using silica gel H with acetone–$CHCl_3$ (1:1). The TLC plates were divided into sections and eluted with $CHCl_3$ and the isolated materials bioassayed using the tobacco seed germination assay (Mitchell and Livingston, 1968).

Camptothecin was isolated by silica column chromatography followed by recrystallization (Wall et al., 1966).

Camptothecin: mp 260–264 °C dec; λ_{max} (EtOH) 219, 253, 290, and 370 nm (ϵ 38000, 29000, 5000, 19000, respectively); ν_{max} (mull) 3440, 1750, 1660, 1610, 1570 cm^{-1}; δ 0.91 (3 H, triplet), 1.90 (2 H, multiplet), 5.26 (2 H, multiplet), 5.50 (2 H, multiplet); aromatic proton signals were not well resolved.

The NMR spectrum was obtained on a Model XL-100 spectrometer (Varian Associates) using Me_2SO-d_6 as a solvent. The purity of the isolated alkaloid was established by high-pressure liquid chromatography (HPLC). The HPLC analysis was performed on a Spectra Physics 3500 B liquid chromatograph [5 μm Spherisorb silica column (0.25 m × 4 mm), $CHCl_3$–EtOAc (70:30)].

Stable emulsions for spray application to test plants were prepared by dissolving camptothecin in tetrahydrofuran, addition of surfactant, Tween 80, and dilution to desired volume.