Regulation of Ammonium Ion Salvage and Enhancement of the Storage Protein Contents of Corn, Sweet Potato, and Yam Tuber by *N*-(Carboxymethyl)chitosan Application

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The biochemical approach to storage protein enhancement via NH_4^+ ion metabolism was investigated by treatment of growing yam tuber, sweet potato, and corn with *N*-(carboxymethyl)chitosan (NCMC). Application of NCMC to yam tuber gave rise to α-ketoglutarate (αKG)-dependent inhibition of the glutamate synthase (GOGAT) with an inhibition constant (K_i) of 3 mM but relieved the αKG-dependent inhibition of the glutamate dehydrogenase (GDH), with a concomitant 270% increase of the storage protein content. In sweet potato, NCMC application gave rise to glutamate-dependent inhibition of the glutamine synthetase (GS) with K_i of 15 mM but relieved the inhibition of the GDH by high αKG concentrations, with a concomitant doubling of the storage protein contents. In corn, NCMC application also gave rise to αKG-dependent inhibition of the GOGAT with K_i of 0.5 mM but relieved the αKG-dependent inhibition of the GDH, with a concomitant doubling of the storage protein content. NCMC treatment also reduced the levels of some of the high molecular weight polypeptides (deaminating) while it increased the levels of some of the low molecular weight polypeptides (aminating) of GDH. Therefore, NCMC enhanced the storage protein contents of the crops by enhancing NH_4^+ ion salvage.

INTRODUCTION

Previous studies in this laboratory had suggested that the protein deficiencies of the yam tuber and sweet potato were due to the α-ketoglutarate-dependent inhibition of the glutamate dehydrogenase and glutamate synthase of the storage organs of the crops (Osuji et al., 1991). The inhibition renders the protein-deficient crops inefficient in ammonium ion salvage, the evidence for which is the loss of ammonia by the crops during postharvest storage (Osuji and Ory, 1986, 1987). If food crops depend on their mechanisms of ammonium ion salvage for their storage protein contents, then the storage protein contents of the protein-deficient crops should be enhanced simply by physiologically relieving the relevant inhibitory influences from their ammonium ion salvage pathways.

N-(Carboxymethyl)chitosan (NCMC) is a derivative of the naturally occurring polysaccharide chitosan. It is readily absorbed by plants, and it enhances chlorophyll contents (Cuero et al., 1991). It has also been used to double the storage protein contents of corn (Osuji and Cuero, 1991a). Treatment of protein-deficient crops with NCMC therefore provides the alterations of physiological conditions suitable for investigating further the metabolic relationship between storage protein contents and the mechanisms of the major enzymes that salvage ammonium ion, viz. glutamate dehydrogenase (GDH), glutamate synthase (GOGAT), and glutamine synthetase (GS).

An understanding of the biochemical basis of the protein deficiencies of the tubers and cereals is a prerequisite for the development of new technologies for increasing the protein yields of the crops. Although the molecular basis of storage protein deficiency has been studied extensively in cereal crops (Okita et al., 1989; Inversen, 1983), only a few studies have been done on the biochemical approach to storage protein enhancement in food crops (Osuji and Ory, 1987; Osuji et al., 1991). Much work has also been done on the use of genetic engineering techniques to supplement the essential amino acid composition of the storage proteins of crops (Yang et al., 1989; Dodds, 1989), but very little has been done to enhance the protein yields of crops (Li, 1982). There is a greater preponderance of protein-deficient crops (70%) as compared to protein-rich crops (12%) (Meyer and Rask, 1984). There is therefore the need to understand the biochemical basis of storage protein deficiency in crops. In this paper we report that the treatment of protein-deficient crops with NCMC enhanced their storage protein contents by at least 100% and at the same time altered the ratios of the GDH polypeptides.

MATERIALS AND METHODS

Preparation of *N*-(Carboxymethyl)chitosan (NCMC). NCMC was prepared from native chitosan (Protan Lab, Redmond, WA) by modifications of the Muzzarelli (1988) method according to Vercellotti and St. Angelo (1989). The chitosan was reduced with sodium borohydride at pH 5 after it had been glyoxylated (Osuji and Cuero, 1991b). The NCMC was then purified by filtration through a 0.22-μm filter (Corning Laboratory Sciences, New York).

Treatment of Sweet Potato with NCMC in the Field. Field plot trials were carried out on the Prairie View A&M University Farm, Waller County, TX. A complete randomized block design (two treatments in three replicates per treatment) was used on a total area of 1300 m^2. Sweet potato (*Ipomoea batatas* L., cv. Puerto Rican) slips were planted 2 ft apart on ridges, in the last week of July, and were harvested in the first week of December. The experiment consisted of two treatments: (1) water treatment as control and (2) 0.1% NCMC treatment. The treatments were applied at 30 and 40 days after planting of the sweet potato. Each treatment consisted of the application of 250 mL of distilled water or of 0.1% NCMC solution to the soil immediately around the base of the plant. No fertilizers were applied to the plants. Four months after planting, five tubers were harvested randomly from each plot, peeled, cut into small cubes roughly 1 cm^3, mixed thoroughly, and frozen with liquid nitrogen before storage at −80 °C.

Treatment of Corn with NCMC in Greenhouse. Corn seeds (*Zea mays* L., cv. Pioneer 3369A) were planted in 5 L capacity

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pots filled with topsoil which was dug out from the Prairie View A&M University Farm, Waller County, TX. The crops were watered with tap water once daily throughout the experiment. The greenhouse temperature was 28 ± 3 °C, and the seeds were planted in the first week of March. At flowering stage (mid June), the crops were separated into two groups of 10 pots each, and the treatments were then applied. For the first group (control), 250 mL of distilled water was applied to the soil, while for the second group 250 mL of 0.1% NCMC solution was applied. The treatments were applied slowly and allowed to soak completely into the soil. The application of NCMC solution was repeated 1 week later. At the end of July, the matured corn ears were harvested and hand-shucked, and their endosperms were hand-dissected and then were mixed and stored at −20 °C.

Treatment of Yam Tuber with NCMC in Greenhouse. Healthy yam tubers (*Dioscorea alata* L.) were purchased from the green grocery, washed thoroughly with tap water, and cut into minisett sizes (30–40 g each piece) as recommended by Okoli et al. (1982). The minisetts were air-dried at ambient temperature (35 ± 2 °C) for 2 days and then planted in nursery pots filled with topsoil which was dug out from the Prairie View A&M University Farm, Waller County, TX. The nurseries were watered with tap water twice weekly, and after 3 weeks, the seedlings were transplanted into other pots (two seedlings per pot), of the same soil as that used in the nursery. Transplantation was done in the first week of April. The crops were watered two times weekly throughout the experiment. Twelve weeks after transplantation, the pots were separated into two groups. The first group (control) of five pots was treated with 250 mL of distilled water, but the second group (experimental) of five pots was watered with 250 mL of 0.1% NCMC solution per pot, as described for the corn experiment. The NCMC application was repeated at 13 weeks and finally at 20 weeks after tuber transplantation. In the first week of December, the tubers were harvested, peeled, and cut into small cubes, as was done for the sweet potato, and then frozen with liquid nitrogen and stored at −80 °C.

Extraction and Purification of Storage Proteins of Yam Tuber and Sweet Potato. The frozen cubes of tuber (200 g) were homogenized with 500 mL of ice-cold 0.05 M sodium borate buffer (pH 8.4) containing 1% (v/v) 2-mercaptoethanol at maximum speed for 3 min. The homogenate was centrifuged at 5000*g* for 15 min; the pellet was discarded while the supernatant liquid was recentrifuged at 12000*g* for 15 min. The supernatant liquid (crude protein extract) was chromatographed through a column (10 × 2 cm) of DEAE-cellulose which had been equilibrated with 0.05 M borate buffer (pH 8.4). The effluent was discarded. Nonstorage proteins were washed out of the column with 200 mL of 0.05 M Tris-HCl buffer (pH 8.4). The storage proteins were finally eluted from the column with 200 mL of 0.05 M Tris-HCl buffer (pH 8.4) containing 0.15 M NaCl (Harvey and Boulter, 1983).

Extraction of Storage Proteins from Corn Seeds. Corn storage proteins were extracted from 5 g of dry seeds with 100 mL of 70% ethanol containing 0.5% sodium acetate, as described by Wall et al. (1988). The extract (crude zein) was used for Kjeldahl nitrogen determination. In other extractions of the storage proteins of corn, globulins were first removed by homogenizing the corn seeds (5 g) with 100 mL of 0.5 M NaCl solution, and after centrifugation (10000*g* for 15 min), the pellet was homogenized with 100 mL of 70% ethanol containing 0.5% sodium acetate to obtain globulin-free zein extract. This extract was used for the SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Electrophoresis of Storage Proteins. SDS–PAGE was done according to the method of Laemmli (1970) using Tris–glycine buffer (pH 8.9) containing 0.05% SDS, at room temperature and at 100 V, using Bio-Rad Protean II cell. The sweet potato and yam tuber crude protein extracts (50 μL) and the globulin-free zein extracts of corn (50 μL) were added to equal volumes of sample buffer containing SDS (Davis et al., 1986) and heated in a boiling water bath for 2 min. After the samples cooled to room temperature, the volumes of the preparations were measured. Then aliquots of the sweet potato and yam tuber samples equivalent to proteins extracted from 3 mg of the tubers, and of the corn samples equivalent to proteins extracted from 0.3 mg of corn seed, were loaded into the wells of the polyacrylamide gels. For the sweet potato and yam tuber, 10% polyacrylamide gel was used; for corn, 12% polyacrylamide gel was used. The purified storage proteins of sweet potato and yam tuber were also prepared for electrophoresis and loaded into adjacent wells as reference standard markers. Protein molecular weight marker mixture (Sigma) [bovine serum albumin (66 000), chicken egg albumin (45 000), bovine erythrocyte carbonic anhydrase (29 000), and bovine milk α-lactalbumin (14 200)] was prepared, and samples were loaded adjacent to the storage protein wells of the polyacrylamide gel. At the end of the electrophoresis, the gel was silver stained according to the method given in Sigma Technical Bulletin (1989).

To determine the relative abundance of the storage protein bands after PAGE, the protein bands were subjected to densitometry at 450 nm using a Shimadzu dual-wavelength flying-spot scanning densitometer, Model CS 9000U.

Extraction and Assay of Enzymes of Ammonium Ion Salvage. Glutamate dehydrogenase was extracted from NCMC-treated sweet potato (25 g), yam tuber (2.6 g), and corn (5 g) and from control (untreated) corn (10 g) and was partially purified by fractional precipitation with solid ammonium sulfate as described previously (Osuji et al., 1991). Protein precipitates at the end of dialysis were dissolved by addition of 0.1 M Tris-HCl buffer (pH 8.2). The final volumes of the dialyzed enzyme after resolubilization of precipitates were 50, 10, and 7 mL for the NCMC-treated sweet potato, yam tuber, and corn, respectively, and 5.5 mL for the untreated corn. Glutamate dehydrogenase (GDH, EC 1.4.1.2) activity was determined according to the method of Loyola-Vargas and Jimenez (1984) using concentrations of 0.3–35.0 mM α-ketoglutarate (αKG), 3.0–530.0 mM NH_4Cl, 0.16 mM NADH, 1.3 mM $CaCl_2$, and the dialyzed enzyme extracted from 278, 500, and 360 mg of yam tuber, sweet potato, and corn, respectively, per assay. Total volume of assay was 3 mL (Osuji et al., 1991). In assays where NH_4Cl was the fixed varied substrate, NADH was used at the fixed concentration of 0.2 mM (King and Wu, 1971).

SDS–PAGE of the partially purified GDH extracts from the NCMC-treated and untreated crops was performed on 7.5% slab gel in Laemmli (1970) buffer. The GDH extracts were added to equal volumes of sample buffer containing SDS (Davis et al., 1986) and mixed thoroughly on a vortex mixer. Then aliquots of the samples equivalent to GDH extracted from 7.5 mg of crop were loaded into the wells of the PAG. Electrophoresis was carried out at 40 V for 12 h at 4 °C. At the end of the run, the gel was washed (gentle rocking action) with 200 mL of 15 mM Tris-HCl buffer (pH 8) containing 0.1% Triton X-100 for 15 min at room temperature to remove the SDS. The gel was then activity stained with tetrazolium bromide reagent (Garland and Dennis, 1977) as described previously (Osuji et al., 1991).

Glutamine synthetase was extracted from NCMC-treated sweet potato (25 g), yam tuber (2.8 g), and corn (5 g) and from the untreated crops (20 g) by homogenization with 3 times (w/v) their volume of ice-cold extraction buffer (0.1 M potassium phosphate, 25 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM glutamine, 10% glycerol) (Lea et al., 1990) and was partially purified by 40–60% solid ammonium sulfate precipitation (Lea et al., 1990). The enzyme pellet was suspended in a minimum volume of the extraction buffer and then dialyzed against four changes (2 L each change) of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 10% glycerol, and 0.1% 2-mercaptoethanol (Bennett and Cullimore, 1989). Protein precipitates at the end of dialysis were redissolved by doubling the volumes of the enzymes with 50 mM Tris-HCl buffer (pH 7.5). The final volumes of the dialyzed enzyme after resolubilization of precipitates were 50, 3.5, and 9.5 mL for the NCMC-treated sweet potato, yam tuber, and corn, respectively, and 10, 12.5, and 9 mL for the untreated sweet potato, yam tuber, and corn, respectively. The final buffer used for dialysis was tested for residual NH_4^+ ions according to the Berthelot method (Weatherburn, 1967); up to 0.5 mL of the buffer had no detectable NH_4^+ ion content. Glutamine synthetase (GS, EC 6.3.1.2) activity was determined according to the method of Lea et al. (1990) using 0.1–35.0 mM hydroxylamine, 1.0–75.0 mM L-glutamate, 25.0 mM ATP, and 50.0 mM $MgSO_4$, and for each assay the dialyzed enzyme extracted from 400 mg of crop was used. The reaction

was incubated at 35 °C for 30 min and stopped with 1 mL of $FeCl_3$ reagent (Loyola-Vargas and Jimenez, 1986). The final volume of the reaction mixture was 3 mL. Blank assays without ATP were set up; their absorbances at 540 nm were subtracted from those of the experiments. A calibration curve set up with authentic γ-glutamylhydroxamate was used to calculate the activities of the enzyme.

Glutamate synthase was extracted from NCMC-treated sweet potato (25 g), yam tuber (3.5 g), and corn (5 g) and from untreated corn (20 g) according to the method of Lea et al. (1990) and was partially purified by precipitation with solid ammonium sulfate as described previously (Osuji et al., 1991). Protein precipitates at the end of dialysis were redissolved by addition of 0.1 M potassium phosphate buffer (pH 7.5) as described for GS. The final volumes of the dialyzed enzyme were 50, 10.4, and 13.5 mL for the NCMC-treated sweet potato, yam tuber, and corn, respectively, and 8.5 for the untreated corn. Glutamate synthase (GOGAT, EC 1.4.1.13) activity was determined also as described by Lea et al. (1990) in a final volume of 3 mL, using for each assay the dialyzed enzyme extracted from 160, 370, and 470 mg of sweet potato, yam tuber, and corn, respectively, and the substrate concentrations described previously (Osuji et al., 1991).

Determination of Protein Contents. Storage proteins extracted from the crops were dialyzed against 0.05 M Tris-HCl buffer (pH 8.5) containing 0.1% 2-mercaptoethanol and 0.05% SDS to remove low molecular weight peptides, after which their protein contents were determined according to the Biuret method (Gornal et al., 1949) using bovine serum albumin as standard. Ethanol-soluble zein extracts of corn were further quantified according to the Kjeldahl method (Horwitz, 1975). All biochemicals were from Sigma Chemical Co. (St. Louis, MO).

RESULTS

The initial velocities of each enzyme were used to construct Lineweaver–Burk double-reciprocal plots, the results of which were then used for the construction of replots as described by Michal (1973) and Segel (1975) to derive the true kinetic constants of the enzyme. The substrate concentrations used in the enzyme assays were those deduced previously for the normal kinetic behavior of the enzymes (Osuji et al., 1991). Enzymes extracted quantitatively from equal weights of each treated and untreated crop were used for assays, and in that way the enzyme kinetic results from a treated and the corresponding untreated crop were made directly comparable. Treated and untreated crops were also harvested on the same day and were immediately processed and stored in a freezer. The results of the analyses from the NCMC-treated and untreated crops are arranged in pairs as necessary in the figures and tables to illustrate vividly the effects of NCMC on the NH_4^+ ion metabolism of the crops.

NCMC-Mediated Alteration of GDH Activities. The double-reciprocal plot (Figure 1a) of the GDH from untreated corn intersected on the $1/V$ axis, and α-ketoglutarate (αKG) inhibited the enzyme. The replot of slopes against the αKG concentrations gave an inhibition constant (K_i) of 7.0 mM. The double-reciprocal plot (Figure 1b) of the GDH from NCMC-treated corn intersected on the $1/S$ axis, and there was no inhibition of the enzyme by αKG. The replot of the $1/V$ axis intercepts against the reciprocals of αKG concentrations gave a Michaelis constant (K_m) of 2.7 mM. The double-reciprocal plot (Figure 2) of the GDH from NCMC-treated yam tuber intersected on the $1/S$ axis, and there was no inhibition of the enzyme by αKG as was the case in the untreated yam tuber (Osuji et al., 1991). The replot of the $1/V$ axis intercepts against the reciprocals of αKG concentrations gave a K_m of 1.9 mM αKG, which is typical of the GDH from high-protein storage organs (Osuji et al., 1991). Figure 3 shows that the GDH from NCMC-treated sweet potato was not inhibited by high concentrations of αKG as was the case with the GDH from the untreated crop (Osuji et al., 1991). Replot of the $1/V$ axis intercepts

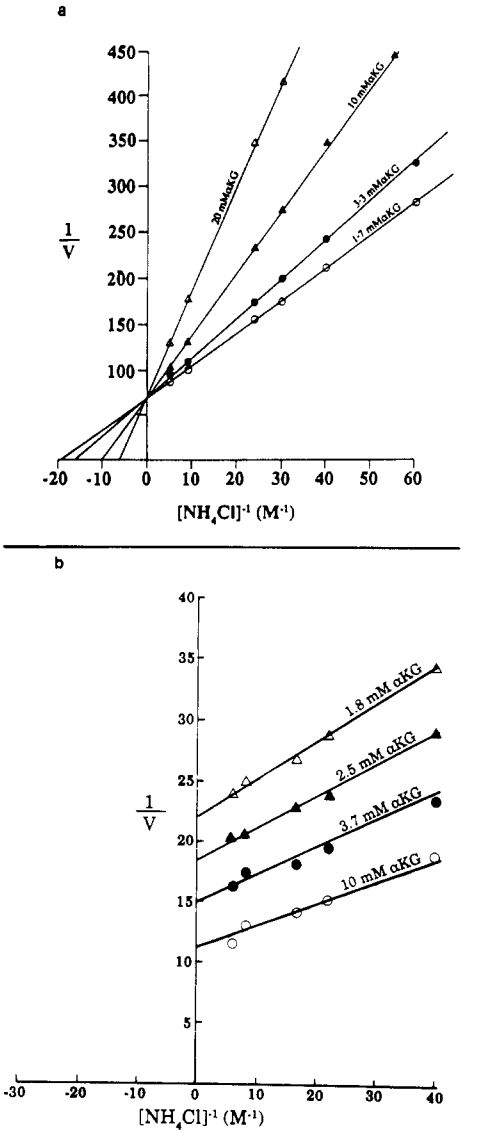

Figure 1. Double-reciprocal plots of the velocity of GDH against varied NH_4Cl concentrations in the presence of a constant concentration of NADH (0.1 mM) with αKG held at varied fixed levels. Assays were carried out with $(NH_4)_2SO_4$-precipitated GDH from (a) untreated corn and (b) NCMC-treated corn. Velocities are mmol min^{-1} (g of fw)$^{-1}$. GDH extracted from 360 mg of corn was used in each assay in a final volume of 3.0 mL.

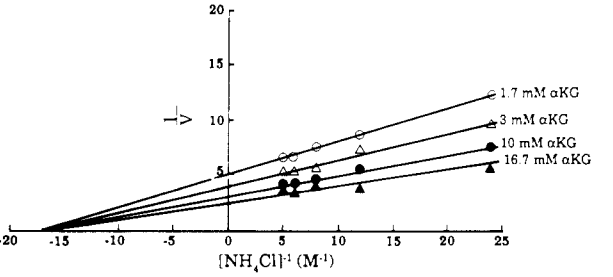

Figure 2. Double-reciprocal plots of the velocity of GDH against varied NH_4Cl concentrations in the presence of a constant concentration of NADH (0.1 mM) with αKG held at varied fixed levels. Each assay was carried out with $(NH_4)_2SO_4$-precipitated GDH from 270 mg of NCMC-treated yam tuber. Velocities are mmol min^{-1} (g of fw)$^{-1}$. The total volume of each assay was 3.0 mL.

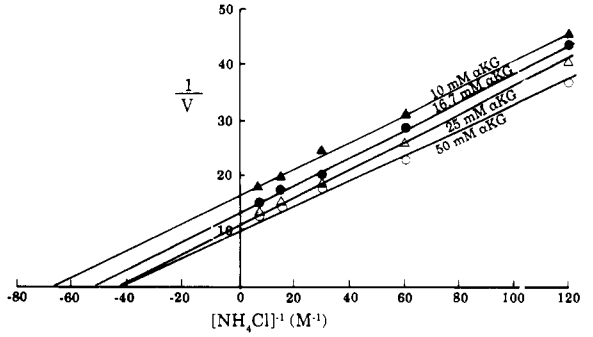

Figure 3. Double-reciprocal plots of the velocity of GDH against varied NH_4Cl concentrations in the presence of a constant concentration of NADH (0.1 mM) with αKG held at varied fixed levels. Each assay was carried out with $(NH_4)_2SO_4$-precipitated GDH from 500 mg of NCMC-treated sweet potato. Velocities are mmol min^{-1} (g of fw)$^{-1}$. The total volume of each enzyme assay was 3.0 mL.

against the reciprocals of αKG concentrations gave a K_m of 10 mM αKG (Table I).

The effects of the NCMC treatment of the crops on the K_m values of GDH for NH_4^+ ion were deduced by construction of Lineweaver–Burk double-reciprocal plots at fixed varied NH_4Cl and fixed αKG concentrations. For the GDHs of the NCMC-treated crops, replots of the $1/V$ axis intercepts against the reciprocals of NH_4Cl concentrations gave straight lines (Figure 4a). Similarly, for the GDHs of the untreated crops, replots of the slopes against the reciprocals of NH_4Cl concentrations also gave straight lines (Figure 4b). Figure 4 shows that NH_4^+ ion, unlike αKG, did not inhibit the GDHs of the crops, but the NCMC treatment of the crops dramatically lowered the K_m values of the enzyme for NH_4^+ ion: from 83.0 mM in the untreated yam to 44.0 mM in the treated yam; from 67.0 mM in the untreated sweet potato to 27.0 mM in the treated sweet potato; and from 50.0 mM in the untreated corn to 12.0 mM in the treated corn (Table I). The K_m of 12.0 mM NH_4^+ ion for the GDH of NCMC-treated corn is very close to the 10 mM NH_4^+ ion concentration of mitochondria (Yamaya et al., 1984). The application of NCMC to the crops therefore enhanced the amination activity of GDH.

NCMC-Mediated Alteration of GOGAT Activities. The double-reciprocal plot of the GOGAT of the endosperms of NCMC-treated corn shows that αKG inhibited the enzyme (Figure 5a); the replot of slopes against the concentrations of αKG gave a K_i of 0.5 mM. The GOGAT of the endosperms of untreated corn was not inhibited by αKG (Figure 5b); the replot of slopes against the reciprocals of the αKG concentrations gave a K_m of 0.25 mM. The double-reciprocal plot of the GOGAT of NCMC-treated yam tuber shows that αKG inhibited the enzyme (Figure 6); the replot of slopes against the concentrations of αKG gave a K_i of 3.0 mM. The GOGAT of untreated yam tuber was not inhibited by αKG (Osuji et al., 1991). Therefore, as in the NCMC-treated corn, the GOGAT of the NCMC-treated yam tuber was inhibited by αKG. The double-reciprocal plot of the GOGAT from NCMC-treated sweet potato shows that αKG did not inhibit the enzyme (Figure 7); the replot of slopes against the reciprocals of αKG concentrations gave a K_m of 8.0 mM. The GOGAT of untreated sweet potato was inhibited by αKG with a K_i of 1.0 mM (Osuji et al., 1991). The treatment of sweet potato with NCMC therefore relieved the inhibition of GOGAT by αKG. Generally, the above results show that if GOGAT was inhibited in the untreated crop, the NCMC treatment of the crop relieved the inhibition, but if the GOGAT was not inhibited in the untreated crop, the NCMC treatment of the crop gave rise to an inhibition by αKG.

NCMC-Mediated Alteration of GS Activities. The Lineweaver–Burk double-reciprocal plots of the GSs of the endosperms of NCMC-treated and untreated corn are shown in parts a and b, respectively, of Figure 8. Figure 8a shows that L-glutamate did not inhibit the enzyme, and a replot of the $1/V$ axis intercepts vs the reciprocals of the L-glutamate concentrations gave a K_m of 2.3 mM. Figure 8b shows the L-glutamate inhibited the enzyme, and the replot of the $1/V$ axis intercepts vs the L-glutamate concentrations gave a K_i of 6.0 mM. This is typical of the GS of corn (Muhitch, 1989). The double-reciprocal plots of the GSs of NCMC-treated and untreated yam tuber are shown in parts a and b, respectively, of Figure 9. Figure 9a shows that L-glutamate inhibited the enzyme, and the replot of the $1/V$ axis intercepts against the concentrations of L-glutamate gave a K_i of 13.5 mM. Figure 9b shows that L-glutamate did not inhibit the GS of the untreated yam tuber, and the replot of slopes against the reciprocals of L-glutamate concentrations gave a K_m of 25.0 mM. The double-reciprocal plots of the GSs of NCMC-treated and untreated sweet potato are shown in parts a and b, respectively, of Figure 10. Figure 10a shows that L-glutamate inhibited the enzyme, and the replot of slopes against the L-glutamate concentrations gave a K_i of 15.0 mM. Figure 10b shows that L-glutamate did not inhibit the GS of untreated sweet potato, and the replot of slopes against the reciprocals of L-glutamate concentrations gave a K_m of 33.0 mM (Table I). The effects of the NCMC treatment of crops on GS activities were therefore similar to those on GOGAT activities, because if the GS activity was not inhibited by L-glutamate in the untreated crop, the NCMC treatment of the crop gave rise to an inhibition by L-glutamate; conversely, if the GS was inhibited by L-glutamate in the untreated crop, the NCMC treatment of the crop relieved the inhibition.

Polypeptides of GDH. The overall effects (Table I) of NCMC on the kinetic properties of the three enzymes indicated that NCMC was consistent only in the enhancement of the amination activity of the GDHs (Figures 1–4). Therefore, the effect of NCMC on the relative abundance of the amination-catalyzing and the deamination-catalyzing polypeptides of GDH was investigated. SDS–PAGE of the GDHs followed by activity stain showed that the enzyme was dissociated into polypeptides that retained enzymatic activity (Figure 11). The gel was fully loaded to demonstrate the number of GDH polypeptides clearly. The anodal polypeptides of corn GDH [molecular weight (MW) from 14 400 to 31 000] were not well resolved when GDH extracted from 7.5 mg of corn was electrophoresed, but the resolution improved to three polypeptides (MW of 14 400, 25 500, and 31 000) when GDH extracted from 4 mg of corn was electrophoresed, although the cathodal polypeptides were then not distinct. Figure 11 shows that the corn GDH polypeptide of 45 000 MW was less abundant in the NCMC-treated than in the untreated corn. Densitometric traces of the corn GDH after PAGE showed that the anodal polypeptides were present at about the same levels in the treated and untreated corn. Figure 11 shows that the yam tuber GDH polypeptide of 66 000 MW was less abundant in the NCMC-treated than in the untreated crop, but the anodal polypeptide of 31 000 MW was more abundant in the treated crop. NCMC did not affect the relative abundance of the highest molecular weight polypeptide of GDH (polypeptide 1 in Figure 11) in both corn and yam tuber. Similar results to those of

Table I. Some Kinetic Constants of the GDH, GS, and GOGAT of NCMC-Treated and Untreated Yam Tuber, Sweet Potato, and Corn

enzyme	varied fixed substrate[a]	kinetic constants	yam tuber		sweet potato		corn	
			treated	untreated	treated	untreated	treated	untreated
GDH	NH_4^+, αKG	V_{max}[b]	417.0 ± 23.0	200.0 ± 20.0[c]	167.0 ± 12.0	100.0 ± 10.0[c]	111.0 ± 8.0	14.0 ± 1.1
GDH	αKG	K_i, mM		1.3 ± 0.1[c]				7.0 ± 0.7
GDH	αKG	K_m, mM	1.9 ± 0.1		10.0 ± 0.9	8.0 ± 0.9[c]	2.7 ± 0.2	
GDH	NH_4^+	K_m, mM	44.0 ± 5.5	83.0 ± 10.0	27.0 ± 3.0	67.0 ± 8.5	12.0 ± 1.0	50.0 ± 2.5
GOGAT	Gln, αKG	V_{max}[b]	71.0 ± 5.0	50.0 ± 4.0[c]	200.0 ± 14.0	100.0 ± 10.0[c]	15.4 ± 1.2	25.0 ± 2.0
GOGAT	αKG	K_i, mM	3.0 ± 0.2			1.0 ± 0.05[c]	0.5 ± 0.04	
GOGAT	αKG	K_m, mM		11.0 ± 1.5[c]	8.0 ± 0.6			0.25 ± 0.02
GS	NH_2OH, Glu	V_{max}[b]	60.0 ± 4.0	25.0 ± 2.5	110.0 ± 9.0	130.0 ± 7.0	15.0 ± 1.2	5.5 ± 0.5
GS	Glu	K_i, mM	13.5 ± 1.2		15.0 ± 1.3			6.0 ± 0.6
GS	Glu	K_m, mM		25.0 ± 2.0		33.0 ± 4.5	2.3 ± 0.2	

[a] In the GDH assay, where αKG was the fixed varied substrate, NH_4^+ was the varied substrate and vice versa; in GOGAT assay, where αKG was the fixed varied substrate, L-Gln was the varied substrate; in GS assay, where L-Glu was the fixed varied substrate, hydroxylamine was the varied substrate. [b] V_{max} is expressed as μmol min^{-1} g^{-1}. [c] Osuji et al. (1991).

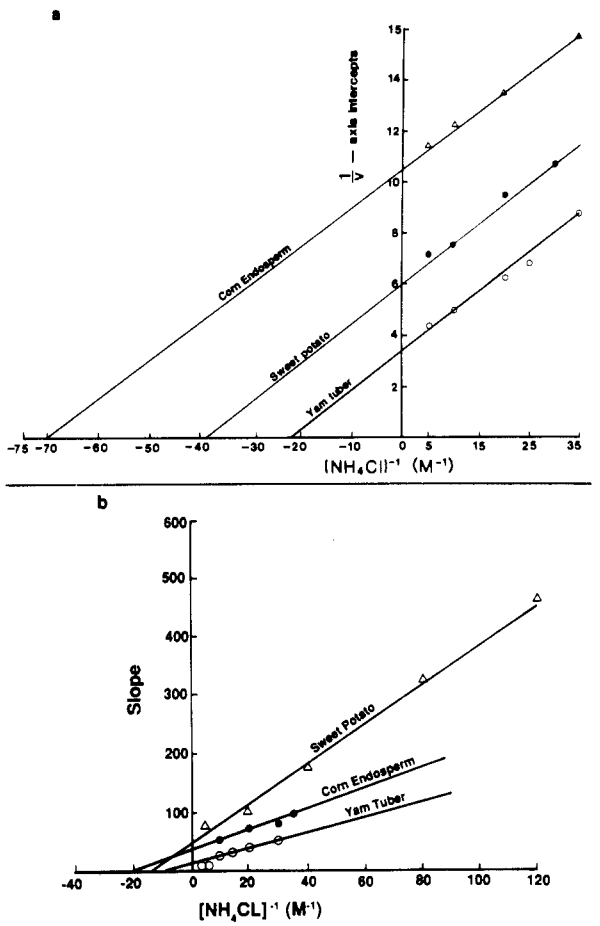

Figure 4. Replots of the data from double-reciprocal plots for the determination of the K_m values of GDH for NH_4^+ ion. For the NCMC-treated crops (a), the $1/V$ axis intercepts were plotted vs the reciprocals of NH_4Cl concentrations. For the untreated crops (b), the slopes were plotted against the reciprocals of NH_4Cl concentrations. The GDHs precipitated with $(NH_4)_2SO_4$ from 270, 500, and 360 mg of yam, sweet potato, and corn, respectively, were assayed at varied αKG concentrations in the presence of a constant (0.2 mM) NADH concentration with NH_4Cl held at varied fixed levels, in a total volume of 3.0 mL per assay.

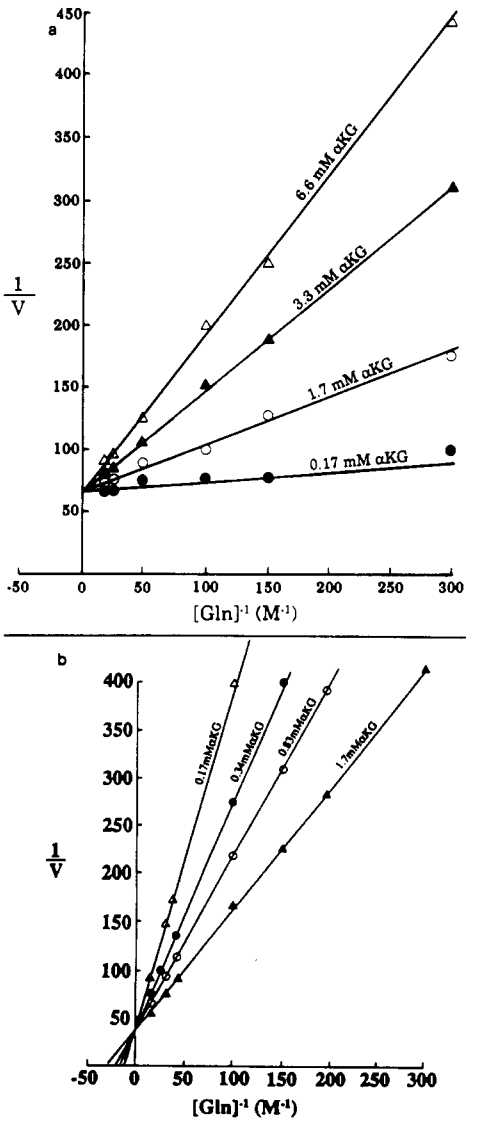

Figure 5. Double-reciprocal plots of velocity of GOGAT against varied L-Gln concentrations in the presence of a fixed NADH concentration (0.1 mM) with αKG held at varied fixed levels. GOGAT precipitated with $(NH_4)_2SO_4$ from (a) NCMC-treated corn and (b) untreated corn was used for the assays. Velocities are mmol min^{-1} (g of fw)$^{-1}$. GOGAT extracted from 470 mg of corn was used in a total volume of 3.0 mL per assay.

yam tuber were obtained for sweet potato GDH (not shown), whose anodal polypeptides were also more abundant in the NCMC-treated than in the untreated crop. Loulakakis and Roubelakis-Angelakis (1991) have also reported the disaggregation of grapevine GDH with SDS into two major and some minor polypeptide. The above results suggest that one of the high molecular weight polypeptides of GDH catalyzes the deamination reaction, because NCMC reduced the relative abundance of one of the high molecular weight polypeptides (the second highest molecular weight polypeptides in Figure 11) with concomitant relief of the αKG-dependent inhibition of the enzyme (Figures 1–3).

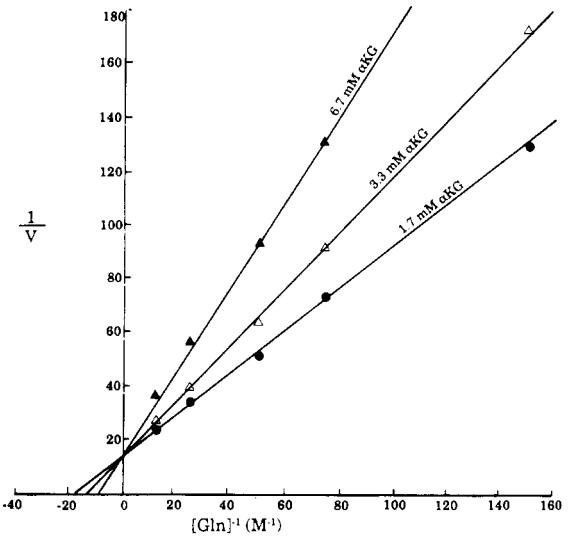

Figure 6. Double-reciprocal plots of velocity of GOGAT against varied L-Gln concentrations in the presence of a fixed NADH concentration (0.1 mM) with αKG held at varied fixed levels. GOGAT precipitated with $(NH_4)_2SO_4$ from 370 mg of NCMC-treated yam tuber was used for each assay, in a total volume of 3.0 mL. Velocities are mmol min^{-1} (g of fw)$^{-1}$.

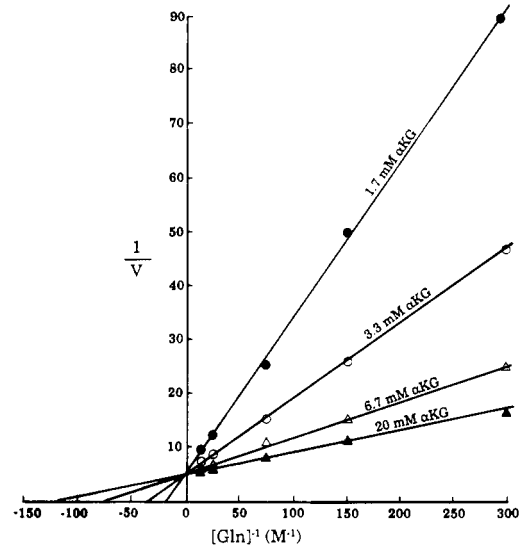

Figure 7. Double-reciprocal plots of velocity of GOGAT against varied L-Gln concentrations in the presence of a fixed NADH concentration (0.1 mM) with αKG held at varied fixed levels. GOGAT precipitated with $(NH_4)_2SO_4$ from 160 mg of NCMC-treated sweet potato was used for each assay, in a total volume of 3.0 mL. Velocities are mmol min^{-1} (g of fw)$^{-1}$.

NCMC-Mediated Enhancement of Storage Protein Contents. Figure 12 shows that the major storage protein of the yam tuber has a molecular weight of about 14 000 and that the NCMC-treated crop has more of that protein than the untreated crop. The relative abundance of the different protein bands (Figure 12) in the treated and untreated yam tuber was quantified by densitometry (not shown). The results showed that the major storage protein band constituted 22% of the crude storage protein extract of the untreated tuber, while it constituted 55% of the crude protein extract of the NCMC-treated tuber. NCMC treatment therefore altered the ratio between the major storage protein and the other soluble proteins of the yam. The Biuret analysis of the total soluble protein extracts showed that the untreated yam tuber contained 13 mg of saline-soluble proteins, while the NCMC-treated yam tuber contained 36 mg of saline-soluble proteins/g of tuber (Table II). Therefore, the NCMC treatment also increased

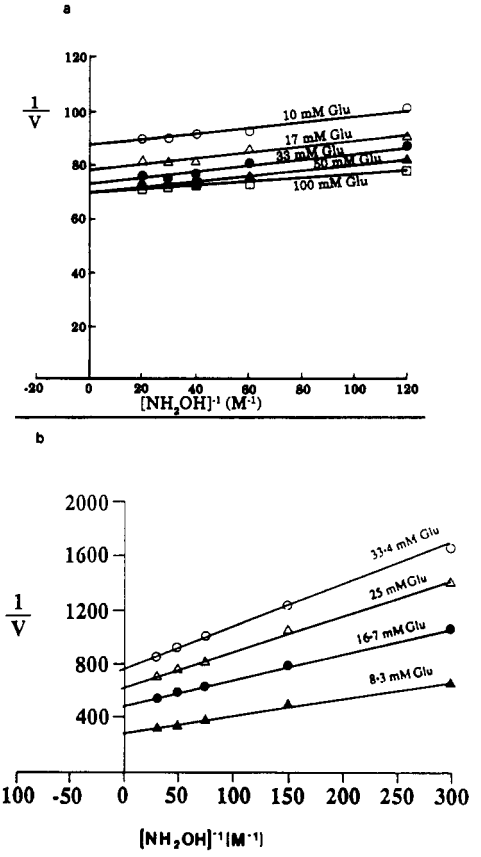

Figure 8. Double-reciprocal plots of velocity of GS against varied hydroxylamine concentrations in the presence of a fixed concentration of ATP (25 mM) with L-Glu held at various fixed levels. GS precipitated with $(NH_4)_2SO_4$ from (a) NCMC-treated corn and (b) untreated corn was used for the assays. Velocities are mmol min^{-1} (g of fw)$^{-1}$. GS extracted from 400 mg of corn was used in a total volume of 3.0 mL per assay.

the total soluble protein contents of yam tuber, by about 270% (fresh weight).

The SDS–PAGE of the storage proteins of sweet potato (Figure 13) shows that the major storage protein has a molecular weight of about 35 000 and that the NCMC-treated crop has more of that protein than the untreated crop. The relative abundance of the protein bands (Figure 13) of the treated and untreated sweet potato was determined by densitometry, and the results (not shown) showed that the densitometric area of the major storage protein in the NCMC-treated sweet potato was double that of the untreated crop. The Biuret analysis of the soluble protein extracts showed that the untreated sweet potato contained 18 mg of the saline-soluble proteins/g of fresh tuber, as compared to 39 mg of the saline-soluble proteins/g of the NCMC-treated tuber (Table II). Therefore, the NCMC treatment increased the storage protein contents of sweet potato by 100% (fresh weight).

Figure 14 shows the SDS–PAGE patterns of the ethanol-soluble proteins (zein) of the endosperms of NCMC-treated and untreated corn. Quantitation of the relative abundance of the zein bands by densitometry showed that the area of the zein in the treated corn was double that in the untreated corn (not shown). The Kjeldahl nitrogen assays of the ethanol-soluble protein extracts showed that the untreated corn contained 60 mg, while the NCMC-treated corn contained 130 mg of the proteins/g of fresh endosperm. The NCMC treatment of the corn therefore only doubled the zein content of the corn endosperm (Table II), as has been reported previously (Osuji and Cuero, 1991b).

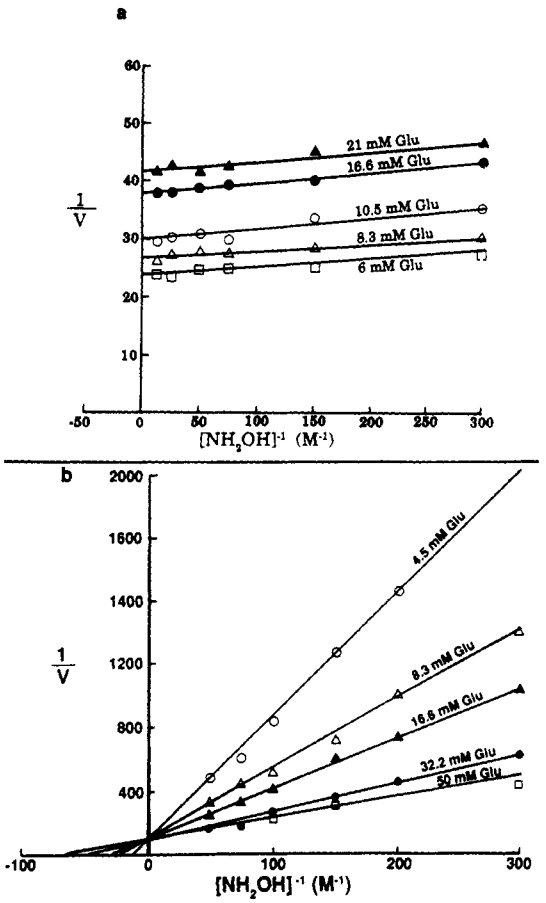

Figure 9. Double-reciprocal plots of velocity of GS against varied hydroxylamine concentrations in the presence of a fixed concentration of ATP (25 mM) with L-Glu held at various fixed levels. GS precipitated with $(NH_4)_2SO_4$ from (a) NCMC-treated yam tuber and (b) untreated yam tuber was used for the assays. Velocities are mmol min^{-1} (g of fw)$^{-1}$. GS extracted from 400 mg of yam tuber was used in a total volume of 3.0 mL per assay.

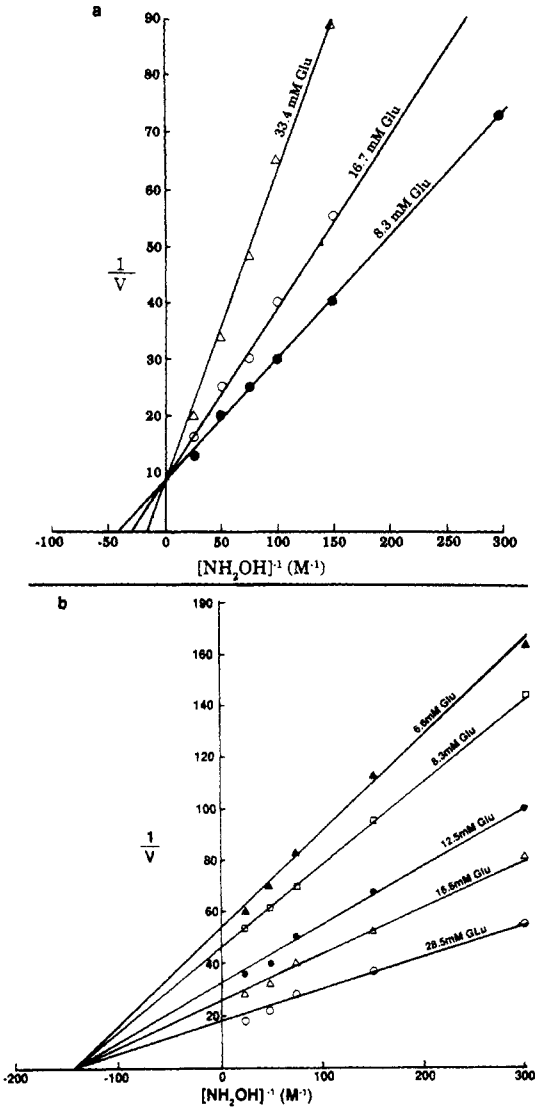

Figure 10. Double-reciprocal plots of velocity of GS against varied hydroxylamine concentrations in the presence of a fixed concentration of ATP (25 mM) with L-Glu held at varied fixed levels. GS precipitated with $(NH_4)_2SO_4$ from (a) NCMC-treated sweet potato and (b) untreated sweet potato was used for the assays. Velocities are mmol min^{-1} (g of fw)$^{-1}$. GS extracted from 400 mg of sweet potato was used in a total volume of 3.0 mL per assay.

DISCUSSION

Regulation of NH_4^+ Ion Salvage by NCMC Application to Crops. Figures 1–10 clearly show that the NCMC treatment of the crops dramatically altered the kinetics of the three enzymes of NH_4^+ ion salvage. The enzyme kinetic results (Table I) for NCMC-treated yam tuber show that the GOGAT and GS were inhibited while the GDH was not inhibited. Previous results (Osuji et al., 1991) had shown that in the untreated yam tuber the converse was the case; i.e., the GDH was inhibited while the GOGAT was not inhibited. Since the GS of the untreated yam tuber was not inhibited (Figure 9b), the application of NCMC to the tuber, therefore, altered the pathway of NH_4^+ ion salvage of the crop. NCMC achieved this regulation by relieving the αKG-dependent inhibition of the GDH (Osuji et al., 1991).

NCMC treatment of the sweet potato relieved the αKG-dependent inhibition of the GOGAT, but simultaneously, it imposed a glutamate-dependent inhibition on the GS activity. The GDH of the NCMC-treated sweet potato was not inhibited by high concentrations of αKG. Therefore, the NCMC application to sweet potato regulated the mechanisms of NH_4^+ ion salvage by relieving the αKG-dependent inhibition of GOGAT and GDH as was suggested previously (Osuji et al., 1991).

In the corn, Table I also shows that NCMC treatment regulated the mechanisms of NH_4^+ ion salvage by relieving the inhibition on the GDH and GS and by imposing an αKG-dependent inhibition on the GOGAT. Therefore, the NCMC treatment of corn generally enhanced the salvage of NH_4^+ ion as was observed in the NCMC-treated yam tuber and sweet potato.

The above results show that whereas the amination activity of GDH was enhanced in all of the treated crops, the activities of GS and GOGAT either were inhibited simultaneously as in the treated yam tuber or were inhibited in a reciprocating manner as in the treated sweet potato and corn. Since GS and GOGAT operate as a metabolic cycle (Lea et al., 1989), the simultaneous or reciprocating inhibition achieved the same goal, which was the reduction of the efficiency of the cycle in NH_4^+ ion salvage in the NCMC-treated crops. Apart from the induction of chitosanase activity following the treatment of seeds with chitosan (Hirano et al., 1988), no other metabolic pathway has been reported to be altered following the application of NCMC to crops.

The lowering of the K_m values of GDH for NH_4^+ ion in the NCMC-treated crops (Figure 4) is further support for the relief of the αKG-dependent inhibition of the enzyme

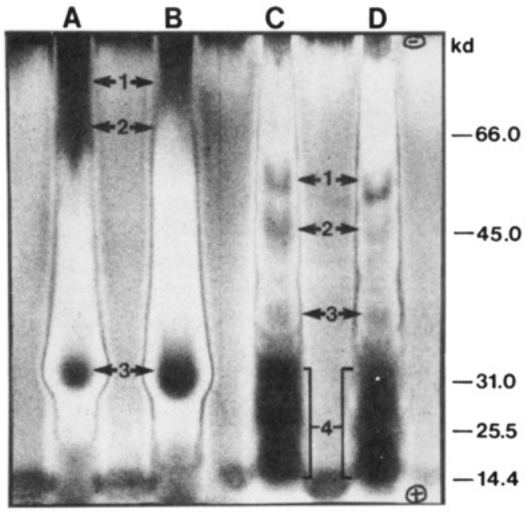

Figure 11. SDS–7.5% PAGE of the GDHs extracted from (a) untreated yam tuber, (b) NCMC-treated yam tuber, (c) untreated corn, and (d) NCMC-treated corn. GDH extracted from 7.5 mg of each crop was loaded into each well; after electrophoresis, the gel was activity stained with tetrazolium bromide reagent.

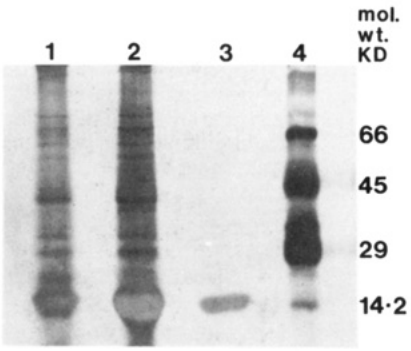

Figure 12. SDS–PAGE of the saline-soluble proteins of (1) untreated yam tuber, (2) NCMC-treated yam tuber, and (3) purified major storage protein of yam tuber. Proteins extracted from 3 mg of fresh yam tubers were applied into lanes 1 and 2. Protein molecular weight markers are in lane 4.

Table II. Storage Protein Contents of NCMC-Treated and Untreated Yam Tuber, Sweet Potato, and Corn

crop	storage proteins, mg (g of fw)$^{-1}$ ± SE
untreated yam tuber	13.0 ± 1.0
NCMC-treated yam tuber	36.0 ± 4.0
untreated sweet potato	18.0 ± 1.0
NCMC-treated sweet potato	39.0 ± 4.0
untreated corn	60.0 ± 5.0
NCMC-treated corn	130.0 ± 15.0

by NCMC. Loulakakis and Roubelakis-Angelakis (1991) have suggested that GDH isoenzymes are made up of two types of polypeptides: one catalyzes the amination, while the other catalyzes the deamination reaction. The reduction of the abundance of the corn GDH polypeptide of 45 000 MW (Figure 11) in the NCMC-treated crop, with concomitant relief of the αKG-dependent inhibition, suggests that the polypeptide catalyzes the deamination reaction. Similarly, the reduction of the abundance of the yam tuber GDH polypeptide of 66 000 MW in the NCMC-treated crop (Figure 11), with concomitant relief of the αKG-dependent inhibition, also suggests that the polypeptide catalyzes the deamination reaction, while the increase in the abundance of the 31 000 MW polypeptide of GDH in the NCMC-treated yam with concomitant increase in the amination activity suggests that the polypeptide catalyzes the amination reaction. Likewise, the

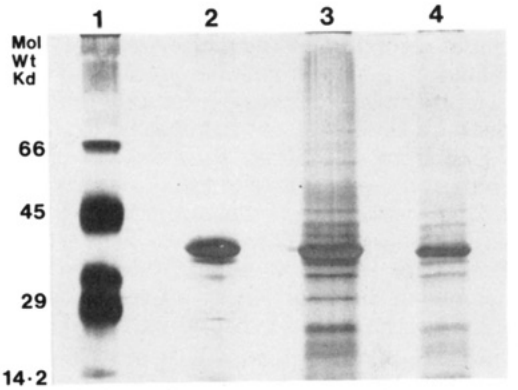

Figure 13. SDS–PAGE of the saline-soluble proteins of (3) NCMC-treated sweet potato and (4) untreated sweet potato. Lane 2 is the purified major storage protein of the sweet potato. Proteins extracted from 3 mg of freshly harvested sweet potato tubers were applied into lanes 3 and 4. Protein molecular weight markers are in lane 1.

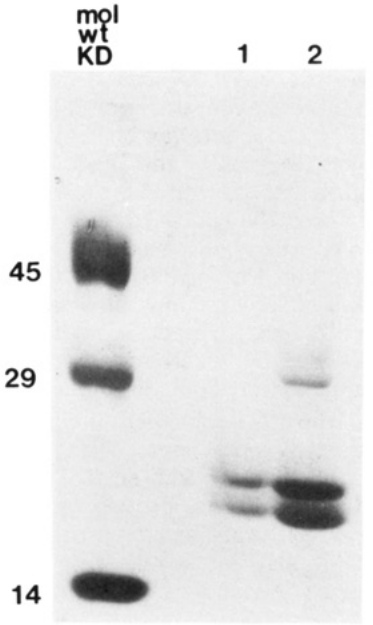

Figure 14. SDS–PAGE of the ethanol-soluble proteins (zeins) of (1) endosperms of untreated corn and (2) endosperms of NCMC-treated corn. Proteins extracted from 0.3 mg of the endosperms were applied into lanes 1 and 2.

increase in the abundance of the anodal polypeptides of the GDH of NCMC-treated sweet potato (not shown) suggests that they catalyze the amination reaction. These results indicate that the 66 000 GDH polypeptide of yam and the 45 000 GDH polypeptide of corn are responsible for the αKG-dependent inhibition of GDH and possibly also for the hydrolysis of the α-immunoglutarate–GDH intermediate Schiff base back to αKG in protein-deficient crops, as was proposed previously (Osuji et al., 1991). The selective alteration of the abundance of some GDH polypeptides in NCMC-treated crops suggests that the NCMC exercised its regulatory effect at the molecular level of gene expression.

The GS and GOGAT of plants are known to be multiisoenzymic in structure (Bennett and Cullimore, 1989; Chen and Cullimore, 1989). The detailed kinetic properties of the individual polypeptides of GS, as well as the regulation of their genes, have been reported (Muhitch, 1989; Cock et al., 1991). The subunit structures of GS and GOGAT might have rendered them susceptible to the regulatory actions of NCMC (Figures 5–10) as was the case with GDH (Figures 1–4). Since the subunits and

polypeptides of GDH, GS, and GOGAT are susceptible to differential regulation at the molecular level by various nitrogenous metabolites, it follows that each enzyme is really a multienzyme complex consisting of different polypeptides with closely related but specific catalytic functions. Therefore, to evaluate the effects of nitrogenous metabolites on the activities of the three enzymes, their complete polypeptides and subunits must be quantitatively utilized in the assays. There is now the need to focus attention at the individual polypeptides of these three enzymes, and in this direction, we are sequencing the cDNAs of some of the GDH polypeptides affected by NCMC.

Enhancement of Storage Protein Contents by NCMC. Whereas the Biuret and Kjeldahl analyses demonstrated that total soluble proteins were increased in the NCMC-treated crops (Table II), the densitometric analyses following the PAGE of the proteins permitted the quantitation of the percentages of the major storage proteins in the crops. Except in the yam tuber (Figure 12), where the NCMC treatment not only altered the ratio between the major storage protein and the other soluble proteins but also increased the concentration of the major storage protein, the NCMC treatment of sweet potato and corn only enhanced the concentration of the major storage protein bands without altering the ratios between the various proteins (Figures 13 and 14). NCMC treatment of corn seedlings germinating at different temperatures, however, enhanced the concentrations of the zeins and also altered the ratios between the different zein polypeptides (Osuji and Cuero, 1991a). But no new major storage proteins different in molecular weight from those in the untreated crops were produced in the NCMC-treated crops (Figures 12–14). Therefore, the NCMC did not activate any proteolytic degradation of the storage proteins.

The relief of the αKG-dependent inhibition of GDH in the NCMC-treated crops, coupled with the depression of the activities of GOGAT and/or GS, resulted in the enhancement of the storage protein contents (Tables I and II). These results are in agreement with the ability of αKG to inhibit NH_4^+ ion salvage with resultant storage protein deficiency in yam and sweet potato (Osuji et al., 1991). The above results also suggest that GDH is the key enzyme which salvages the NH_4^+ ion required for storage protein enhancement in the three crops studied. Figure 15 shows that the protein-deficient crops are generally inefficient in NH_4^+ ion salvage because of the inhibition of GDH. The lowering of the K_m of GDH for NH_4^+ ion (Figure 4) implies that the amination capabilities of the NCMC-treated crops were enhanced. The enhanced amination activity of GDH would in turn lead to efficient production of L-glutamate for enhanced amino acid transamination.

To probe the molecular basis of the effects of NCMC on storage proteins, the mRNA contents of NCMC-treated corn were found to be increased (Osuji and Cuero, 1991a). This suggests that NCMC enhanced transcription, thereby providing more storage protein mRNA. In the NCMC-treated crops, therefore, the enhanced amino acid metabolism coupling with the enhanced mRNA production gave rise to the enhanced protein levels (Figures 12–15). Hadwiger and Loschke (1981) suggested possible DNA–chitosan interaction in microorganisms. NCMC is a more soluble derivative of chitosan (Cuero et al., 1991) and so is likely to retain the DNA binding ability. The GS of NCMC-treated corn was not inhibited and so could couple with GDH to increase the efficiency of L-glutamine production. L-Glutamine is an amino donor in nucleotide biosynthesis (Miflin et al., 1981). Therefore, the enhanced

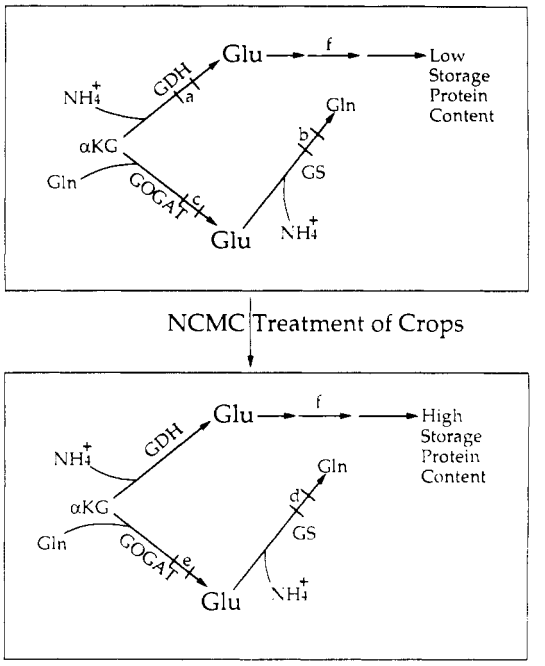

Figure 15. Switching of the NH_4^+ ion salvage mechanisms from the GS–GOGAT pathway in the untreated crops to the GDH pathway in the NCMC-treated crops, with concomitant enhancement of storage protein contents in the NCMC-treated crops. (a) Inhibition in all three crops; (b) inhibition in corn; (c) inhibition in sweet potato; (d) inhibition in yam tuber and sweet potato; (e) inhibition in yam tuber and corn; (f) steps in storage protein synthesis.

GS and GDH activities in NCMC-treated corn also account, in part, for the increased RNA contents. These considerations lend further support to the suggested relationship between NH_4^+ ion salvage and storage protein contents of crops (Osuji and Ory, 1986).

Since the ratios of GDH polypeptides were altered in the NCMC-treated crops, the catalytically active conformation of the enzyme would also change accordingly (Figures 1–4). One of the mechanisms of enzyme regulation is the alteration of enzyme catalytic conformation (Fersht, 1985), but the results (Figure 11) did not indicate whether the alteration of GDH was at the posttranslational or posttranscriptional level. However, the fact that the storage proteins were not degraded in the NCMC-treated crops indicates that NCMC did not active the proteolytic enzymes. The degradation of the NCMC in the crops might also have produced a continuous but low level of some nitrogenous intermediates. The NCMC treatments increased the chitosanase levels in the crops (Cuero and Osuji, 1991). The resultant nitrogenous intermediates might have also affected not only the ratios of the enzyme polypeptides as reported by Loulakakis and Roubelakis-Angelakis (1991) but also their activities as reported by Ratajczak et al. (1981). We have, therefore, compared the effects of NCMC, NH_4Cl, and combined NCMC/NH_4Cl treatments on the storage protein contents and the activities of the enzyme of NH_4^+ ion salvage in corn (Osuji and Cuero, 1991d). The results showed that NH_4Cl increased the GDH activities similar to the effects of NCMC, while the combined NH_4Cl/NCMC treatments increased the activities of the GOGAT–GS cycle but inhibited the GDH activities and decreased the zein contents. These results lend support to the concept of a metabolic linkage between NH_4^+ ion, GDH activities, and the storage protein contents of crops, as summarized in Figure 15.

The conventional approaches to the enhancement of

the storage protein yields of corn and potato are the classical plant breeding (Inversen, 1983; Li, 1982) and the recombinant DNA techniques (Yang, 1989). The advantages of the biochemical approach via NCMC treatment of the crops include the following: the high-protein crops produced are not phenotypically altered; the production of the high-protein crop is achieved in one crop season only and so the biochemical approach is rapid; and most importantly, the technology has been applied with success to a wide range of protein-deficient food crops (Osuji and Cuero, 1991c). Yam tubers are recalcitrant crops in genetic studies (Ene and Okoli, 1985), and so significant progress has not been made in the improvement of their storage protein yields even by classical breeding techniques (Osuji, 1988). The biochemical approach to storage protein enhancement through the application of NCMC to the growing yam crop is therefore a shortcut to the high-protein yam tubers. Yam tubers are the staples of millions of people in parts of the world who suffer from dietary protein deficiency (IITA, 1988; Hahn et al., 1987). The high-protein yam tuber would, therefore, make some nutritional impact in the yam zones of the world.

NCMC has been reported to increase the chlorophyll contents of tomato plants (Cuero et al., 1991). The ability of NCMC to regulate the activities of GDH, GS, and GOGAT and thereby to increase the storage protein contents of the protein-deficient crops suggests that it is a bioregulator of plant metabolism. However, chitosan and its derivatives have not been found in higher plants; they are the major constituents of the exoskeletons of crabs, crayfish, and shrimp and other seafood wastes (Arai et al., 1968).

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