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AMMONIUM ION SALVAGE BY GLUTAMATE DEHYDROGENASE DURING DEFENCE RESPONSE IN MAIZE

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Key Word Index—*Zea mays*; Gramineae; maize; elicitor chitosan; glutamate dehydrogenase; amination electrode potential; energetic defence.

Abstract—When phytochemical defence response was elicited by treatment of maize with chitosan, the binomial isoenzymes of L-glutamate dehydrogenase (GDH, EC 1.4.1.2) isomerized. The amination electrode potential of the GDH charge isomers decreased by 10 mV in the chitosan-treated maize. GDH isomerization and amination are therefore spontaneous reactions. The free L-Glu and L-Gln were 3.84 and 3.68 μmol, respectively, per 10 g of the chitosan-treated maize. The amination activities of GDH showed that the 10 mV decrease in electrode potential accounted fully for the 3.84 μmol free L-Glu content of the chitosan-treated maize. Therefore the binomial GDH synthesized all the L-Glu, thereby salvaging at least 51% of the NH_4^+ liberated during phytochemical defence response, while glutamine synthetase salvaged the remaining NH_4^+. The chitosan-elicited isomerization of maize GDH and the accompanying 10 mV decrease in electrode potential widened the plant–pathogen amination energy barrier, thereby minimizing the amount of NH_4^+ the pathogen can absorb from the plant.

INTRODUCTION

The phenylpropanoid pathway embodies the major chemical defence mechanism consequent upon the infection of a plant by a pathogen. The committed step in the pathway [1] is the release of NH_3 from L-Phe by phenylalanine ammonia-lyase. The metabolic flux through the pathway is considerable because up to 40% of the plant's organic matter is derived from L-Phe during phytochemical defence response [2]. The salvage of the NH_4^+ released during phytochemical defence response has been ascribed to the activities of glutamine synthetase-glutamate synthase (GS-GOGAT) cycle [3]. Studies in which ^{15}N-labelled L-Phe was used showed that the NH_4^+ was recycled to the phenylpropanoid pathway [2]. The recent demonstration that NADH-glutamate dehydrogenase (GDH, EC 1.4.1.2) is more efficient than GOGAT in the synthesis of L-Glu in maize [4] suggests that the enzyme may be involved in the salvage of the NH_4^+ during phytochemical defence response. The charge-dependent isomerization of the subunits of GDH also suggests that the mechanisms by which the subunits salvage NH_4^+ may be part of the inducible defence response mechanism of plants against pathogens. Ammonium ion salvage is part of the general intermediary metabolism of the cell. Apart from the supply of some intermediates to the secondary metabolic pathways, the direct role of primary intermediary metabolism in phytochemical defence response has not been described before. Previous studies have also demonstrated that the GOGAT of chitosan-treated maize is inhibited by 2-ketoglutarate [5]. Chitosan treatment of maize therefore provides a unique opportunity for probing not only the general mechanism of NH_4^+ salvage, but in particular the role of GDH during phytochemical defense response.

Pathogenic fungal species are primarily found as contaminants of maize seeds. It has been demonstrated that the fungal infection induces chitosanase activities as a defense response by maize [6, 7]. Treatment of seeds with elicitor chitosan also triggered the phenylpropanoid pathway by inducing the synthesis of phenolic compounds and antifungal phytoalexins [8–10]. Chitosan is a de-*N*-acetylated derivative of chitin. Polysaccharides chitosan and chitin are structural components of the cell walls of many fungi [11]. Chitin and chitosan are thought to be released during plant–pathogen interaction by the actions of chitinase and chitosanase that cleave fungal wall polysaccharides [12]. Treatment of plants with chitosan therefore mimics fungal infection and thereby provides a method for avoiding the use of microbes in phytochemical defence response experiments.

Hereunder, we report that the elicitation of phytochemical defence response by treatment of maize with chitosan induced the isomerization of GDH and a lowering of the reductive amination potential of the enzyme by 10 mV, thereby enabling the enzyme to salvage at least 51% of the NH_4^+ released during the defence response.

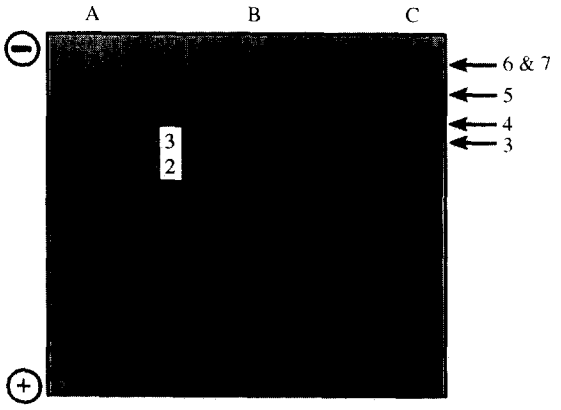

Fig. 1. One-dimensional native PAGE of GDH: native PAGE of the GDHs extracted from equal weights (130 mg) of the endosperms of (A) control maize seedlings, and (B) elicitor chitosan-treated seedlings. The electrophoresed gel was stained for NADH-GDH activity with tetrazolium blue reagent. Lane (C) is peanut GDH, which served as a marker.

RESULTS AND DISCUSSION

Isomerization of GDH during phytochemical defence response

The GDH extracted from control maize endosperms and from the endosperms of chitosan-treated seeds were electrophoresed through native 7.5% polyacrylamide gel (PAG). In the result (Fig. 1), maize GDH displayed a 2-isoenzyme system in this traditional one-dimensional fractionation of the enzyme, compared with peanut GDH, which displayed the classical 6–7 system of isoenzymes. Furthermore, maize GDH isoenzyme 2 was more abundant in the chitosan-treated maize than in the control maize. Therefore, chitosan treatment of maize altered the relative abundance of the 2-isoenzyme system.

The GDH isolated from control maize endosperms and from the endosperms of chitosan-treated seeds were then subjected to isoelectric focusing (IEF) using Bio-Rad's Rotofor IEF cell [13]. Thereafter, the resulting 20 fractions were subjected to native 7.5% PAGE, followed by activity staining of the electrophoresed gel [4]. The resolution achieved is shown in Fig. 2. The GDHs from control maize and from chitosan-treated maize, focused in Rotofor tubes 5, 6, 7, 8, 9, 10, 11 and 12, corresponded to pH values of 5.8, 6.1, 6.3, 6.5, 6.8, 6.9, 7.1 and 7.3, respectively. This is therefore the typical 7 charge isomer system of GDH [14]. Native PAGE resolved each charge isomer to the constituent isoenzymes [4]. Therefore, Rotofor IEF elegantly fractionated the 2-isoenzyme system of maize GDH to the constituent isoenzymes of different charges. The resolution of maize GDH isoenzymes to their different charge isomers by Rotofor IEF was so efficient that isoenzyme 1, which was not detected in the traditional one-dimensional native PAGE became detectable in some charge isomers (Fig. 2). A comparison of Figs 1 and 2 therefore shows that the one-dimensional native PAGE of GDH did not resolve the enzyme into its population of binomial hexameric isoenzymes. The inability of the traditional one-dimensional native PAGE to resolve GDH isoenzymes into their respective charge isomers hampered the understanding of the isomerization property of the enzyme.

In the control maize, the GDH isoenzymes in row 3 were more abundant than those in row 2. However, in the chitosan-treated maize, the GDH isoenzymes in row 2 were more abundant than those in row 3. Therefore, the population distribution pattern of the GDH isoenzymes in the chitosan-treated maize differed from that of the control maize. Phytochemical defence response in maize therefore induced GDH subunit isomerization similar to those induced by NH_4^+ [4].

Many pathogenesis-related proteins, including chitosanase, chitinase and 1,3-β-glucanase are induced in plants during plant–pathogen interaction [15]. However, GDH differs from pathogenesis-related proteins because it is present in the plant prior to the infection of the plant by pathogen, but the NH_4^+ released by the

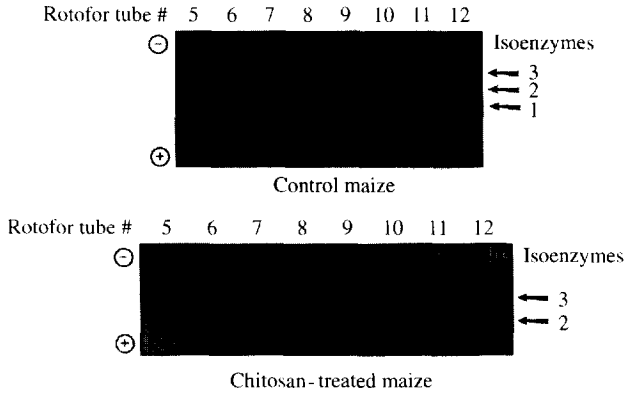

Fig. 2. Binomial isoenzymes of the GDH of control and chitosan-treated maize: the GDH isolated from maize seeds germinated in the presence of distilled water (control) and 0.1% elicitor chitosan solution were fractionated by Rotofor IEF and then by native 7.5% PAGE. GDH activity was detected by staining the electrophoresed PAG with tetrazolium blue reagent.

Table 1. Aminating activities* of the GDH charge isomers of control and chitosan-treated germinating maize seeds

Rotofor tubes	pH	Control maize	Chitosan-treated maize
5	5.8	0.100±0.009	0.134±0.012
6	6.1	0.087±0.007	0.170±0.013
7	6.3	0.122±0.011	0.297±0.020
8	6.5	0.168±0.014	0.180±0.013
9	6.8	0.166±0.014	0.170±0.016
10	6.9	0.073±0.006	0.172±0.014
11	7.1	0.122±0.011	0.126±0.013
12	7.3	0.062±0.007	0.098±0.010

*Activity is expressed in NADH converted into NAD^- min^{-1} ml^{-1} of each Rotofor fraction. Activity is also numerically equal to mmol Glu produced.

plant during defence response induces the enzyme to isomerize. Therefore, GDH is a defence-related enzyme. Its inducible isomerization ensures the rapid salvage of the NH_4^+ released during the interaction of the plant with the pathogen.

Energetics of GDH isomerization

The amination activity of the GDH charge isomers permitted the calculation of the equilibrium constant (K_{eq}) of the amination reaction. The pH values of the charge isomers (Table 1) and the K_{eq} were then applied to the Nernst equation [16] in order to derive the amination electrode potentials of the charge isomers. A plot of the electrode potentials against the pH values is shown in Fig. 3. As expected, an increase of 1 pH unit was accompanied by a 59 mV decrease in amination electrode potentials of the GDH of both the control maize and of chitosan-treated maize. This is further evidence that amination is the thermodynamically favoured reaction of GDH [17]. Oxidative deamination activity of the GDH of chitosan-treated maize was found to be negligible, being less than 3 μmol min^{-1} ml^{-1} of the Rotofor fractions.

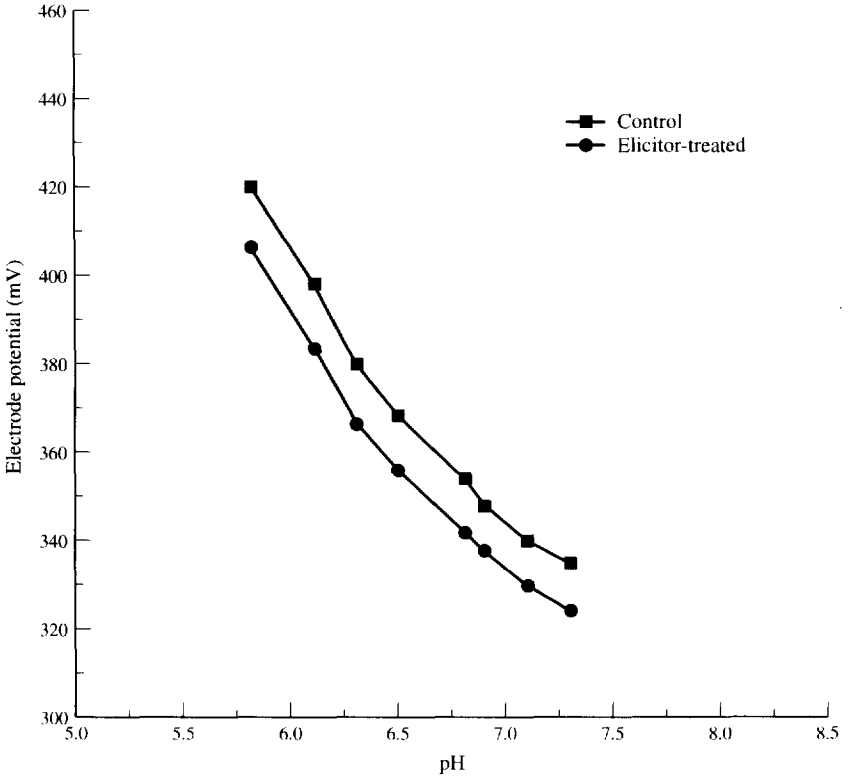

Fig. 3. Active defence response by the charge isomers of maize GDH: maize GDH was isolated from the endosperms of seeds germinated in the presence of distilled water (control) or 0.1% elicitor chitosan solution, followed by fractionation to the charge isomers by Rotofor IEF. The aminating activities of the charge isomers were converted into electrode potentials and then plotted against the pH values of the isomers.

The electrode potentials of the GDH charge isomers of chitosan-treated maize were 10 mV more negative than those of the control maize (Fig. 3). This means that the isomerization of, and amination by, GDH are spontaneous reactions. The lower electrode potential of the GDH of chitosan-treated maize widened the energy barrier between the plant and the pathogen, thereby minimizing the amount of NH_4^+ the pathogen can absorb from the plant. If the NH_4^+ released by the plant were readily available to the pathogen, the phenylpropanoid pathway would rapidly run out of L-Phe with consequent collapse of the phytochemical defence response. This means that the spontaneous isomerization of GDH is part of the active biochemical defence response by the plant. This energetic defence by GDH has hitherto not been recognized [15].

Synthesis of glutamate during phytochemical defence response

The free amino acid compositions of control maize endosperm and of maize treated with chitosan are shown in Table 2. The free L-Glu concentration of chitosan-treated maize was 384 nmol g^{-1} of maize. Since GOGAT is inhibited in chitosan-treated maize [5], GDH amination activity accounted for the free L-Glu concentration. On the other hand, GDH is inhibited in control maize [18]; therefore, GOGAT accounted for the 262 nmol of the free L-Glu in control maize [4].

Maize endosperm proteins are degraded to amino acids during seed germination [19–22]. To investigate the contribution which chitosan treatment of maize seedlings made to proteolysis, the proteolytic activities of endosperm extracts were determined. Proteolytic activities of the control, chitosan-treated and 25 mM NH_4Cl-treated maize were 15.3±1.3, 14.6±1.5 and 16.2±2.0 μg casein hr^{-1} g^{-1} endosperm, respectively. Therefore, the maize endosperms of the three experimental treatments had similar proteolytic activities, which were further demonstrated by the solid-phase digestion of PAG-immobilized casein (Fig. 4). The proteolytic enzymes from the three experimental treatments of germinating maize digested the casein in equal areas of the PAG, as determined by the densitometric measurements of the areas. Therefore, neither the chitosan nor the NH_4Cl treatment affected the proteolytic enzymes. Accordingly, the higher levels of L-Glu and metabolically related amino acids in the chitosan-treated maize were not due to the catabolism of the proteins. These results are in agreement with those on chitosan-mediated enhancement of the protein contents of maize [6, 18, 23] because if chitosan treatment of maize activated the proteolytic enzymes, the protein content of maize would have decreased instead.

If GDH is solely responsible for the synthesis of L-Glu in chitosan-treated maize, then the 10 mV decrease in the reductive amination potential of the GDH should account for the free L-Glu concentration. The electrode potentials (Fig. 3) and the amination activities of the GDH charge isomers (Table 1) showed that, on average, 360 mV produced 140 μmol of L-Glu. Protein extract from 10 g of maize endosperm was used for the Rotofor preparation of the GDH charge isomers pre-

Table 2. Free amino acid composition of control and chitosan-treated germinating maize seeds

Amino acids	Control seeds [nmol (g fr. wt)$^{-1}$]	Chitosan-treated seeds [nmol (g fr. wt)$^{-1}$]
Glu	262	384
Ser	366	420
Gly	526	390
Asn	552	512
Gln	242	368
His	192	238
Thr	300	312
Ala	1006	1340
Arg	294	280
Pro	544	778
Tyr	256	392
Val	450	464
Met	120	158
Ile	194	220
Leu	454	738
Phe	114	182
Trp	50	28
Lys	294	252
Asp	272	380

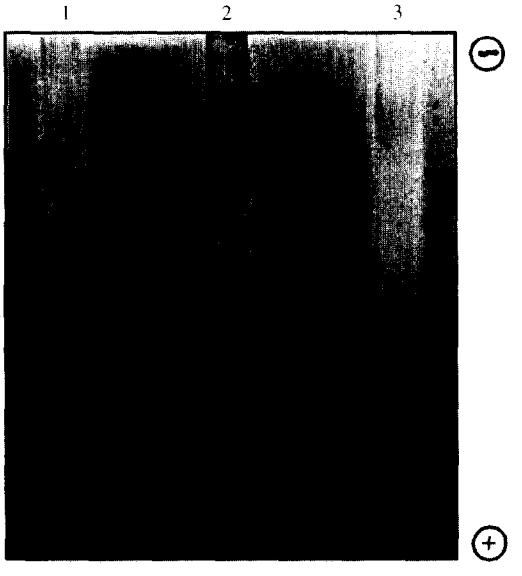

Fig. 4. Proteolytic activities of extracts from germinating maize endosperm: extracts from equal weights (0.5 g) of the endosperms of (1) control, (2) elicitor chitosan-treated and (3) 25 mM NH_4Cl-treated maize seedlings were electrophoresed through native PAG copolymerized with casein. The electrophoresed gel was incubated at 37° to permit proteolysis of the casein to take place. The gel was then stained with Coomassie Blue. Areas of proteolysis were quantified by densitometry.

sented in Fig. 2. From Table 2, the L-Glu content of 10 g of the endosperm of chitosan-treated maize was 3.84 μmol. Therefore, the 10 mV decrease in the reductive amination potential of GDH consequent upon the elicitation of phytochemical defence response in maize was sufficient to sustain the synthesis of the free L-Glu content of chitosan-treated maize. This is further evidence in support of the *in vivo* amination function of GDH. The above results show that the decrease in the electrode potential of the GDH of elicitor-treated maize is part of the energetic defence response for ensuring the efficient salvage of NH_4^+ and synthesis of L-Glu by the enzyme. Synthesis of L-Glu during phytochemical defence response ensures continued provision of L-Phe, which is the first substrate in the phenylpropanoid pathway [1, 2]. Synthesis of L-Glu is part of primary intermediary metabolism. The decrease in the reductive amination electrode potential of binomial GDH and the accompanying increase in the synthesis of L-Glu represent the contribution which primary intermediary metabolism makes in support of phytochemical defence response.

Ammonium ion salvage during phytochemical defence response

A comparison of the chitosan-dependent isomerization of GDH with those that were induced by NH_4Cl [4] shows that the effect of chitosan treatment was similar to that of 25 mM NH_4Cl treatment (Fig. 2). This suggests that the NH_4^+ released during defence response is considerable. The internal NH_4^+ concentrations of maize were 0.3 ± 0.02, 0.9 ± 0.1 and 0.8 ± 0.1 μmol g^{-1} of endosperm for the control, 25 mM NH_4Cl-treated maize and chitosan-treated maize, respectively. The fact that the internal NH_4^+ concentration of the 25 mM NH_4Cl-treated maize was equivalent to that of the chitosan-treated maize is further evidence that the NH_4^+ released during phytochemical defence response is considerable. The NH_4^+ content of actively photo-respirating plant cells [3, 24] is of the same order of magnitude as that of chitosan-treated maize. Therefore, NH_4^+ released during phytochemical defence response is efficiently salvaged. Since GOGAT is inhibited in chitosan-treated maize [5, 18], it follows that GDH activities accounted for the synthesis of all the L-Glu. However, GS was not inhibited in chitosan-treated maize. Therefore, NH_4^+ was salvaged by GDH and GS in chitosan-treated maize. Based on the concentrations of free L-Glu and L-Gln, which were 384 and 368 nmol g^{-1} of maize endosperm, respectively (Table 2), and on the fact that GDH synthesized all the L-Glu, it follows that GDH salvaged at least 51%, while GS salvaged at most 49% of the NH_4^+ released during phytochemical defence response. The combination of the GDH and GS activities is the basis of the efficient salvage of the NH_4^+ released during phytochemical defence response in maize.

Enhanced storage protein contents have been achieved by treatment of yam tuber, sweet potato and maize with chitosan [23, 25]. The induction of GDH isomerization by the NH_4^+ released in the chitosan-treated maize and the accompanying increase in the level of L-Glu now provide a further explanation for the biochemical basis of the protein enhancement. Chitosan treatment of suspension cell cultures of yam tuber and sweet potato also induced isomerization of their GDHs, and there were dramatic increases in the free L-Glu levels of the cells [26]. Yam tuber and sweet potato naturally carry heavy burdens of pathogenic fungi, which induce phytoalexin synthesis in the crops [27, 28]. Therefore, the active defence response by GDH ensures that the NH_4^+ released by the plant is efficiently and rapidly salvaged. This enables the plant to continue to operate the phenylpropanoid pathway in response to the presence of pathogen. A practical application of these results is that we have extended the post-harvest storage life of yam tubers [29] significantly, by washing the tubers with dilute solutions of chitosan (unpublished result).

The accumulating data on the isomerization of GDH are throwing more light on the possible physiological role of the enzyme. The isomerization of the enzyme has been demonstrated in plant senescence [30] and in fruit ripening [31]. Since the committed step in the GDH amination reaction is subunit isomerization [4], it follows that the observed isomerization of GDH during plant senescence and fruit ripening are indicative of the NH_4^+ salvage reaction of the enzyme. In senescence, just as in phytochemical defence response, NH_4^+ is released [1, 2, 30, 31] by the plant. Therefore, senescence and plant–pathogen interaction are the ideal physiological states of the plant most suitable for probing the role of GDH in NH_4^+ salvage. The use of chitosan instead of pathogen in this study assured the absence of pathogen's GDH in the assays [30].

The role of NADH-GDH in the salvage of the NH_4^+ released during phytochemical defence response could have been investigated further by treatment of the maize seeds with mixtures of methionine sulphoximine (MSX) and chitosan. Since MSX inhibits the subunit isomerization of maize NADH-GDH [4], it is unsatisfactory to apply it to the crop when specific inhibition of GS [32, 33] is desired. A plant growth retardant like MSX [33], which requires many days for its effects to be manifested physiologically, does have the potential additional problem of exercising some undesirable side effects [34]. One of the side effects of MSX in maize is, unfortunately, that it inhibits the isomerization of the NADH-GDH. Since the committed step in the catalytic mechanism of GDH is its subunit isomerization [4, 14, 18, 30, 31, 35], it is necessary not only to demonstrate the GDH isoenzyme population distribution, but also the activities of the GDH charge isomers in experiments pertaining to NH_4^+ salvage. Many previous excellent studies on the redox activities of the enzyme did not carry out this analysis because there was no preparative scale IEF equipment.

Since the amination reaction of GDH is considered the thermodynamically more favourable reaction than

the deamination reaction, it is somewhat contradictory that the staining reaction of the enzyme in PAG is in the deamination direction. This formazan precipitating reaction is indeed very slow, normally requiring the PAGE of milligram quantities of the GDH extract, followed by overnight incubation of the gel in tetrazolium blue solution. The staining reaction is very slow because NAD^+ and L-Glu bind to the enzyme to form dead-end complexes [36]. The staining reaction is faster in the amination direction, but more bands appear in the gel due to the presence in plant extracts of other NADH-dependent dehydrogenases. Although the direction in which an enzyme reaction is assayed does not necessarily indicate the physiological function of the enzyme, we have developed a Western blot method which employs chemiluminescent substrates for the detection of nanogram quantities of Rotofor-fractionated GDH isoenzyme population in 1 min (unpublished results). The over-riding chemical evidence in support of a physiological function for the redox activity of NADH-GDH is the spontaneity of the reaction in the amination direction [34], as again demonstrated in Fig. 3.

EXPERIMENTAL

Germination of maize seeds. Maize seeds (*Zea mays* L. Pioneer 3369A var.) were soaked overnight in, 0.1% native chitosan soln (Protein Lab., Redmond, WA, U.S.A.) or 25 mM NH_4Cl. They were then germinated between filter papers in 9-cm diam. Petri dishes, in a growth chamber (30°, 12 hr day/night cycle). The filter papers were moistened daily with H_2O, chitosan or 25 mM NH_4Cl. Germination was stopped at 102 hr by freezing the seedlings on dry ice. There was 100% germination in each treatment. The endosperms were dissected out by hand and immediately stored at −70°.

Extraction of GDH. GDH was extracted from the endosperms (20 g) and partially purified by pptn with solid $(NH_4)_2SO_4$ [5]. The protein pellet was dissolved in a minimum vol. of GDH extraction buffer [5] and dialysed against 3 changes of 3.5 l 10 mM Tris–HCl buffer (pH 8.2) at 4°. Protein ppts at the end of dialysis were removed by centrifugation (10 000 *g*, 15 min, 4°). The vol. of dialysed extract was made up to 60 ml with 10 mM Tris–HCl buffer (pH 8.2).

Free soln IEF of GDH. Partially purified GDH (*ca* 0.5 g protein) extracted from 10 g endosperm was made 4 M with de-ionized urea, and 2% with Bio-Lyte ampholyte (pH 3–10, 40% w/v). This soln (40 ml) was applied to the Rotofor cell [13] and focused [4]. After IEF, the 20 Rotofor frs were collected and their pH values measured. Removal of ampholytes was accomplished by making each fr. 1 M with NaCl and then dialysing at 4° against 3 changes of 3.5 l 10 mM Tris–HCl (pH 8.2).

Assay of GDH activity. GDH activity of the Rotofor frs was determined by photometry at 340 nm [4, 5]. For the aminating reaction, all substrates were prepd in 0.1 M Tris–HCl (pH 8.2). Concs of 16 mM 2-ketoglutarate, 50 mM NH_4Cl, 1 mM $CaCl_2$ and 5 mM NADH were used. The reaction was started by addition of 0.3 ml dialysed Rotofor fr. The total reaction vol. was 3 ml.

In the deaminating direction [14], all substrates were prepd in 0.1 M Tris–HCl (pH 9.4). Concns of 50 mM L-Glu, 0.5 mM $CaCl_2$, 1 mM NAD^+ and 0.3 ml dialysed Rotofor fr. were used. The total reaction vol. was 3 ml.

Protein concns were determined by the method of ref. [37], using BSA as standard. GDH activities presented are the average of 3 independent experiments, each with 2 replicate assays of GDH activity.

Native PAGE. Equal vols (400 μl) of partially purified GDH extracts from control and chitosan-treated maize were prepd [4] and electrophoresed through native 7.5% slab PAG at 4°. GDH extracted from peanut (*Arachis hypogaea, L.* var. Runner) cotyledons was also loaded in an adjacent well and electrophoresed, to serve as a marker. After Rotofor fractionation of the GDH extract, equal vols (250 μl) of the Rotofor frs containing the GDH activities were prepd [4] and similarly electrophoresed at 4° through native 7.5% slab PAG.

GDH activity was detected by staining the electrophoresed gel in glutamate–NAD^+–phenazine methosulphate–tetrazolium blue soln [4, 38]. GDH bands appeared after incubation of the reaction mixt. at room temp. overnight. L-Glu was omitted in the blank test. Gels stained without L-Glu showed no bands.

Free amino acid analysis by HPLC. Free amino acids were extracted from maize endosperms by homogenization with twice their vol. (w/v) of 1% picric acid [4]. The supernatant was passed through a 2-cm column of Dowex-1 to remove the picric acid. Effluents (10 μl) were used for free amino acid analysis by HPLC [4].

Ammonium ion contents of maize endosperm. Frozen endosperms (5 g) were homogenized with 40 ml extraction reagent (0.3 mM H_2SO_4 containing 20 g PVP) for 3 min and centrifuged [6]. As controls, different aliquots (50–200 μl) of 0.3 mM L-Gln were added to the endosperms, and homogenized with extraction reagent [6]. NH_4^+ contents of endosperm extracts were determined by the phenol–hypochlorite method [39]. The controls with added L-Gln had *ca* 6% increase *A*; therefore, interference by amino acids on the NH_4^+ assay was negligible [6].

Extraction and assay of proteolytic enzymes. Endosperms (5 g) were defatted by homogenization with 50 ml cold Me_2CO [19]. Proteolytic enzymes were then extracted from the Me_2CO powder by homogenization with 40 ml 0.05 M Na-Pi buffer (pH 7) containing 0.01% NaN_3. After centrifuging (15 000 *g*, 15 min, 4°) the homogenate, the supernatant was treated with solid $(NH_4)_2SO_4$. Protein which pptd between 30 and 65% $(NH_4)_2SO_4$ satn was collected by centrifugation (15 000 *g*, 15 min, 4°), dissolved in the minimum vol. of extraction buffer and dialysed at 4° overnight against 3 changes of the same buffer, each change being 3.5 l.

The dialysed extract was brought to a vol. of 5 ml with proteolysis assay buffer (50 mM NaOAc, pH 4, containing 5 mM 2-mercapto-ethanol).

Proteolytic activity was determined [19] by incubation of 0.5 ml dialysed extract with 1 ml proteolysis assay buffer, containing 5 mg casein, for 4 hr at 37°. The reaction was stopped by addition of 1.5 ml 10% TCA. In the control experiments, TCA was added without incubation at 37°. The TCA pptn of proteins was allowed to take place at 4° for several hr. TCA-insoluble protein was removed by centrifugation (15 000 *g*, 15 min). Protein content of the supernatant was determined by the method of ref. [37]. Proteolytic assays were done in triplicate.

Proteolytic activity was further demonstrated by PAGE [19] in which 0.05% casein was copolymerized in native 7.5% PAG. Equal vols (40 μl) of dialysed protease extracts were prepd and loaded into gel wells, and electrophoresed as described above at 4°, 100 V and 3.5 hr. The gel was then incubated overnight at 37° in proteolysis assay buffer. After incubation, the gel was stained with Coomassie Blue R. Areas of PAG in which proteolytic enzymes digested casein were quantified by densitometry.

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REFERENCES

1. Dixon, R. A. and Dey, P. M. (1983) in *Advances in Enzymology* (Miester, A., ed.), Vol. 55, pp. 1–136. John Wiley, New York.
2. Razal, R., Ellis, S., Singh, S., Lewis, N. G. and Towers, G. H. N. (1995) *Annual Meeting of the Phytochemical Society of North America (Book of Abstracts)*. p. 37.
3. Lea, P. J., Blackwell, R. D., Murray, A. J. S. and Joy, K. W. (1989) in *Plant Nitrogen Metabolism* (Poulton, J. E., Romeo, J. and Conn, E. E., eds), Vol. 23, pp. 157–189. Plenum Press, New York.
4. Osuji, G. O. and Madu, W. C. (1995) *Phytochemistry* **39**, 495.
5. Osuji, G. O. and Cuero, R. G. (1992) *J. Agric. Food Chem.* **40**, 728.
6. Cuero, R. G. and Osuji, G. O. (1991) *Med. Fac. Landbouwe. Rijksuniv. Gent.* **56**, 1415.
7. Cuero, R. G., Duffus, E., Osuji, G. O. and Pettit, R. E. (1991) *J. Agric. Sci.* **117**, 165.
8. Fajardo, J. E., Waniska, R. D., Cuero, R. G. and Pettit, R. E. (1994) *Food Biotechnol.* **8**, 213.
9. Walker-Simmons, M., Hadwiger, L. and Ryan, C. A. (1983). *Biochem. Biophys. Res. Commun.* **110**, 194.
10. Kombrink, E., Beerhues, L., Garcia-Garcia, F., Hahlbrock, K., Muller, M., Schroder, M., Witte, B. and Schmelzer, E. (1993) in *Mechanisms of Plant Defense Responses* (Fritig, B. and Legrand, M., eds), pp. 236–249. Kluwer Academic Press, Boston, MA.
11. Hahn, M. G., Cheong, J., Alba, R., Enkerli, J. and Cote, F. (1993) in *Mechanisms of Plant Defense Responses* (Fritig, B. and Legrand, M., eds), pp. 99–116. Kluwer Academic Press, Boston, MA.
12. Kendra, D. F., Christian, D. and Hadwiger, L. A. (1989) *Physiol Mol. Plant Pathol.* **35**, 215.
13. Rotofor System Instruction Manual (1990) Bio-Rad Laboratories, Hercules, CA, U.S.A.
14. Loulakakis, A. K. and Roubelakis-Angelakis, K. A. (1991) *Plant Physiol.* **97**, 104.
15. Kombrink, E., Hahlbrock, K., Hinze, K. and Schroder, M. (1991) in *Biochemistry and Molecular Biology of Plant–Pathogen Interactions* (Smith, C. J., ed.), pp. 237–254. Clarendon Press, Oxford, U.K.
16. Segel, I. H. (1976) *Biochemical Calculations*, 2nd Edn, pp. 172–179. John Wiley, New York.
17. King, J. and Wu, Y. (1971) *Phytochemistry* **10**, 915.
18. Osuji, G. O., Cuero, R. G. and Washington, A. C. (1991) *J. Agric. Food Chem.* **39**, 1590.
19. Barros, E. G. and Larkins, B. A. (1990) *Plant Physiol.* **94**, 297.
20. Fujimaki, M., Abe, M. and Arai, S. (1977) *Agric. Biol. Chem.* **41**, 887.
21. Harvey, B. M. R. and Oaks, A. (1974) *Plant Physiol.* **53**, 453.
22. Moureaux, T. (1979) *Phytochemistry* **18**, 1113.
23. Osuji, G. O. and Cuero, R. G. (1992) *Food Biotechnol.* **6**, 105.
24. Joy, K. W. (1988) *Can. J. Botany* **66**, 2103.
25. Osuji, G. O. and Cuero, R. G. (1992) in *Sweetpotato Technology for the 21st Century* (Hill, W. A., Bonsi, C. K. and Loretan, P. A., eds), pp. 78–86. Tuskegee University Press, Tuskegee, AL, U.S.A.
26. Madu, W. C. (1995) PhD Thesis. Abia State University, Uturu, Nigeria.
27. Ogundana, S. K. (1993) in *Advances in Yam Research* (Osuji, G. O., ed.), Vol. 2, pp. 124–134. CARC, Prairie View A&M University, Prairie View, TX, U.S.A.
28. Peterson, J. K. and Harrison, H. F. (1992) in *Sweetpotato Technology for the 21st Century* (Hill, W. A., Bonsi, C. K. and Loretan, P. A., eds), pp. 263–272. Tuskegee University Press, Tuskegee, AL, U.S.A.
29. Osuji, G. O. and Umezurike, G. (1985) in *Advances in Yam Research* (Osuji, G. O., ed.), Vol. 1, pp. 259–276. Anambra State University of Technology Press, Enugu, Nigeria.
30. Srivastava, H. S. and Singh, R. P. (1987) *Phytochemistry* **26**, 597.
31. Loulakakis, K. A., Roubelakis-Angelakis, K. A. and Kanellis, A. K. (1994) *Plant Physiol.* **106**, 217.
32. Rhodes, D., Brunk, D. G. and Magalhaes, J. R. (1989) in *Plant Nitrogen Metabolism* (Poulton, J.

E., Romeo, J. T. and Conn, E. E., eds), pp. 191–226. Plenum Press, London.
33. Shaner, D. L. (1989) in *Plant Nitrogen Metabolism* (Poulton, J. E., Romeo, J. T. and Conn, E. E., eds), pp. 227–261. Plenum Press, London.
34. Oaks, A. (1995) *Can. J. Botany* **73**, 1116.
35. Oaks, A. (1994) *Can. J. Botany* **72**, 739.
36. Stone, S. R., Heyde, E. and Copeland, L. (1980) *Arch. Biochim. Biophys.* **199**, 560.
37. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
38. Cammaerts, D. and Jacobs, M. (1983) *Plant Sci. Letters* **31**, 65.
39. Weatherburn, M. W. (1967) *Analyt. Chem.* **39**, 971.