

Regulation of Glutamate Dehydrogenase and Glutamine Synthetase in Avocado Fruit during Development and Ripening

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The activity, protein, and isoenzymic profiles of glutamate dehydrogenase (GDH) and glutamine synthetase (GS) were studied during development and ripening of avocado (*Persea americana* Mill. cv Hass) fruit. During fruit development, the activity and protein content of both GDH and GS remained relatively constant. In contrast, considerable changes in these enzymes were observed during ripening of avocado fruit. The specific activity of GDH increased about 4-fold, coincident with a similar increase in GDH protein content and mRNA levels. On the other hand, GS specific activity showed a decline at the end of the ripening process. On the isoenzymic profile of GDH, changes in the prevalence of the seven isoenzymes were found, with a predominance of the more cathodal isoenzymes in the unripe and of the most anodal isoenzymes in the ripe fruit. Two-dimensional electrophoresis revealed that avocado fruit GDH consists of two subunits whose association gives rise to seven isoenzymes. The results support the view that the predominance of the more anodal isoenzymes in the overripe fruit was due to the accumulation of the α -polypeptide.

Ripening of climacteric fruits involves a series of coordinated metabolic events that alter their anatomy, biochemistry, physiology, and gene expression (Seymour et al., 1993). These alterations affect many characteristics, such as color, flavor, and texture of the fruit. It is well established that in order for ripening to proceed, expression of specific genes and subsequent synthesis of enzymes associated with normal ripening are required (Seymour et al., 1993).

Similarly, senescence of leaves is a physiological process in which not only the down-regulation of important genes but also the activation of a new set of genes occur (Watanabe et al., 1989). One of the main events taking place in senescing leaves is the remobilization of nitrogen and other important nutrients to storage organs and/or to a younger part of the plant. The main source of nitrogen comes from the hydrolysis of proteins, whose nitrogen moiety is translocated as the amides Gln and Asn (Zimmerman, 1960).

GDH (EC 1.4.1.2) catalyzes the amination of α -ketoglutarate (aminating or synthetic reaction) and the deamination of glutamate (deaminating or catabolic reaction). Today, the role of GDH remains obscure despite the fact that it is apparently

ubiquitous in plants. The enzyme could operate primarily in the assimilation or reassimilation of ammonium and play a role complementary to that of the glutamate synthase cycle in the synthesis of glutamate (Yamaya et al., 1984; Srivastava and Singh, 1987). Alternatively, it could catalyze the oxidation of glutamate, ensuring sufficient carbon skeletons for effective functioning of the tricarboxylic acid cycle (Thomas, 1978; Robinson et al., 1991).

GDH is a mitochondrial enzyme with a high K_m for NH_4^+ (Srivastava and Singh, 1987; Loulakis and Roubelakis-Angelakis, 1990a), which argues against an assimilatory role, even though Yamaya et al. (1984) have shown that the concentration of NH_4^+ in mitochondria is high enough to sustain appreciable levels of NADH-GDH activity. Yamaya and Oaks (1987) proposed that a possible function of mitochondrial GDH is the synthesis of glutamate from some of the NH_4^+ released within the mitochondria by photorespiration. Also, it has been suggested that GDH plays a role in ammonium reassimilation under conditions of stress (Srivastava and Singh, 1987). The observations that during darkness and natural senescence there is an increase in GDH activity (Kang and Titus, 1980; Kar and Feierabend, 1984; Srivastava and Singh, 1987), together with an alteration in its isoenzymes (Lauriere and Daussant, 1983), tend to support the above hypothesis.

On the other hand, GS (EC 6.3.1.2) activity is known to decrease during either natural or dark-induced senescence of leaves and cotyledons (Thomas, 1978; Kawakami and Watanabe, 1988; Kamachi et al., 1991). Decline of GS activity during senescence is a result of a decrease in the chloroplastic isoform GS₂, whereas the cytosolic isoform GS₁ remains relatively constant (Kamachi et al., 1991) or increases (Kawakami and Watanabe, 1988) during senescence.

In view of the lacunas in our knowledge concerning nitrogen-metabolizing enzymes during fruit development and ripening, the present study was initiated as an attempt to understand better the synthesis and regulation of GDH and GS during development and ripening of avocado (*Persea americana* Mill.) fruit. The data indicate that GDH activity increased significantly during ripening and especially during

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase.

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senescence, coinciding with elevated amounts of its immunoreactive protein and mRNA. This increase was due mainly to the accumulation of α -polypeptide of the anodal isoenzymes. In addition, a parallel decrease in GS both at activity and protein levels was observed.

MATERIALS AND METHODS

Plant Material

Avocado fruits (*Persea americana* Mill. cv Hass) were harvested at specified intervals during development (December, January, February, March, April, and May) and respiration, ethylene production, and firmness were determined according to Pesis et al. (1978). Lyophilized tissues were prepared and used for subsequent studies. Fruits from the January harvest were used for the ripening studies.

Protein Extraction

The extraction of proteins from avocado fruit was performed as described previously (Kanellis et al., 1989b). The extraction buffer contained 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 20 mM MgSO₄, 1 mM EDTA, 20 mM NaHCO₃, 5 mM β -mercaptoethanol, 0.5 mM PMSF, 10 μ M leupeptin, and 10% (v/v) glycerol. For native gel electrophoresis the crude protein extract was precipitated with ammonium sulfate and the 35 to 70% fraction was collected. The resultant precipitate was dissolved in a small volume of 200 mM Tris-HCl, pH 7.4, containing 14 mM β -mercaptoethanol, 10 μ M leupeptin, 0.5 mM PMSF, and 10% (v/v) glycerol and dialyzed (Loulakakis and Roubelakis-Angelakis, 1990a). For two-dimensional electrophoresis, protein was further purified by a Sephadex G-200 gel filtration column as previously described (Loulakakis and Roubelakis-Angelakis, 1990a).

Enzyme Assays

GDH activity was determined in the aminating direction by following the change in the A_{340} (Loulakakis and Roubelakis-Angelakis, 1990a). One unit of GDH activity was defined as the amount needed to oxidize 1.0 μ mol of NADH per min at 30°C.

GS activity was determined by the Mn²⁺-dependent transferase assay as described by Shapiro and Stadtman (1970). One unit of enzyme was defined as the amount that catalyzed the formation of 1 μ mol of γ -glutamylhydroxamate per min at 30°C.

Protein Determination

Protein concentration was measured by the method of Bradford (1976) using BSA as a standard.

PAGE and Protein Gel Blotting

SDS-PAGE was carried out according to Laemmli (1970). Native PAGE and activity staining of GDH and two-dimensional electrophoresis (native/SDS-PAGE and urea-IEF/SDS-PAGE) were performed as previously described (Loulakakis and Roubelakis-Angelakis, 1991). Electrophoretic transfer of proteins onto nitrocellulose membranes and immunodetection

methods were carried out according to Kanellis et al. (1989a). Antibodies used were prepared against grapevine GDH (Loulakakis and Roubelakis-Angelakis, 1990b) and *Phaseolus* root nodule GS (Cullimore and Mifflin, 1984).

Isolation of mRNA

Total RNA was isolated as described by Brown and Kafatos (1988). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose affinity chromatography. The RNA concentration was determined spectrophotometrically by measuring the A_{260} , and the purity and integrity were checked by nondenaturing agarose gel electrophoresis.

In Vitro Translation and Immunoprecipitation of Translation Products

In vitro translation of poly(A)⁺ RNA was performed by a nuclease-free rabbit reticulocyte lysate translation system (Stratagene) in the presence of [³⁵S]Met (New England Nuclear) according to the supplier's instructions. In vitro-synthesized GDH was immunoprecipitated and the immunocomplexes were collected as described previously (Sambrook et al., 1989).

RESULTS

Expression of GDH and GS during Avocado Fruit Development

Activities and immunoreactive protein content of GDH and GS were followed during avocado fruit development (Fig. 1). The aminating specific activity of GDH remained relatively constant from December to March and then showed a small increase (Fig. 1A). On the other hand, GS specific activity increased slightly during the first months and showed a gradual decrease thereafter.

The changes in enzyme protein were monitored by immunoblotting. Proteins were separated in SDS-PAGE, transferred onto nitrocellulose membranes, and probed with polyclonal antibodies raised against GDH (Fig. 1B) and GS (Fig. 1C). The immunoblots of GDH showed that the level of GDH protein remained relatively constant during fruit development (Fig. 1B). On the other hand, the levels of the 44-kD immunoreactive antigen of GS decreased slightly, whereas those of the 41-kD antigen did not change during this 6-month period (Fig. 1C).

Regulation of GDH and GS during Avocado Fruit Ripening

The activity profiles for GDH and GS during the ripening process of the fruits are shown in Figure 2A. NADH-GDH specific activity increased significantly (about 4-fold) between the 6th and 8th d of ripening. This increase in NADH-GDH followed the climacteric rise in ethylene production and respiration and reached its highest value when the firmness of the fruits approached almost zero (not shown). By contrast, the specific activity of GS decreased. The rate of this decrease, however, was slower than the increase in NADH-GDH specific activity (Fig. 2A). The above variation in the specific activity of GDH was reflected in the corresponding changes

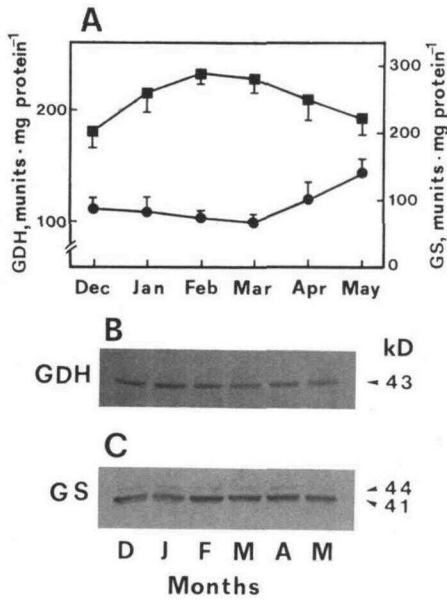


Figure 1. Developmental changes in avocado fruit GDH and GS. A, Specific activities of NADH-GDH (●) and GS (■). Each value is the average of four determinations. Bars indicate s.d. B and C, Protein blot analysis of GDH and GS, respectively. Twenty micrograms of total protein were resolved in 10% acrylamide SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-GDH (B) or anti-GS (C) serum.

in the enzyme protein content. GDH immunoreactive protein was fairly constant during the first 6 d after harvest but increased considerably on the 7th and 8th d of the ripening process (Fig. 2B). This increase in protein content of GDH was correlated with an accumulation of the steady-state amount of GDH mRNA, as evidenced by immunoprecipitating the *in vitro* translation products of avocado poly(A)⁺ mRNA (Fig. 2C). On the other hand, a slight decline in the 44-kD immunoreactive GS antigen was observed (Fig. 2D). It seems that these changes are too small to be correlated with the trends of change in enzyme activity.

Changes in the Profile of GDH Isoenzymes

The changes in the isoenzyme forms of GDH during development on the tree and ripening off the tree were analyzed by activity staining of native PAGE gels. The enzyme was separated into seven isoenzymes (Fig. 3) showing a pattern similar to that of grapevine GDH (Loulakakis and Roubelakis-Angelakis, 1990a). It was evident that during development on the tree and ripening off the tree, major qualitative alterations in the isoenzymes of GDH occurred (Fig. 3). More specifically, during early development the more cathodal isoenzymes predominated and the more anodal isoenzymes were not detectable. Later, this pattern gradually changed, and in the fruits harvested in April and May nearly all the isoenzymes of GDH were present (Fig. 3A). During ripening this transition from the cathodal to anodal isoenzymes was more pronounced (Fig. 3B); that is, the more cathodal isoenzymes decreased while the staining intensities of the middle

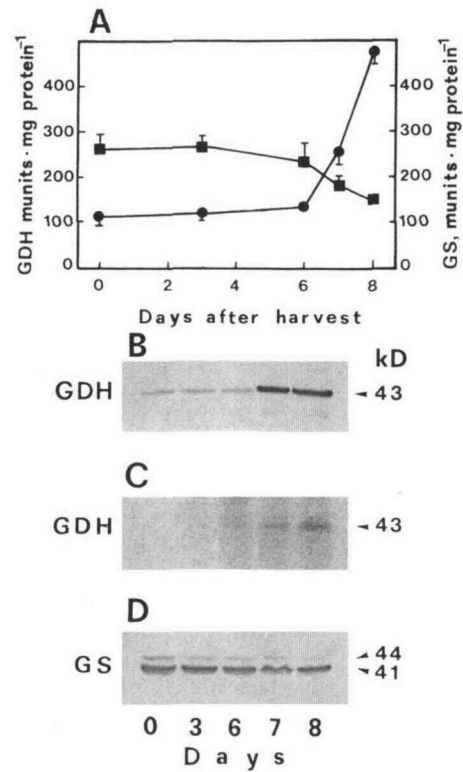


Figure 2. Ripening-related changes in avocado fruit GDH and GS. A, Specific activities of NADH-GDH (●) and GS (■). Each value is the average of four determinations. Bars indicate s.d. B, Protein blot analysis and immunodetection using anti-GDH serum. C, Autoradiogram of the *in vitro*-synthesized GDH. Poly(A)⁺ RNA was *in vitro* translated and GDH was immunoprecipitated. Immunocomplexes were separated by SDS-PAGE on 10% acrylamide gels that were subjected to autoradiography. D, Protein blot analysis and immunodetection using anti-GS serum.

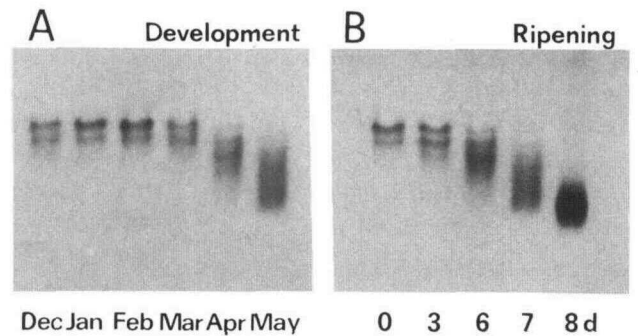


Figure 3. Isoenzymic profile of GDH during development (A) and ripening (B) of avocado fruit. Samples were resolved in 5% acrylamide native gel electrophoresis and the gels were stained for GDH activity.

and more anodal isoenzymes increased. On the 6th and 7th d of ripening, all seven isoenzymes were seen, and, finally, on the 8th d, the isoenzyme 7 predominated, coinciding with advanced senescence of fruits.

Polypeptide Composition of the Avocado Fruit GDH

Previous work from this laboratory has shown that grapevine GDH consists of two subunit polypeptides, α and β , whose association in hexamers give rise to seven isoenzymes. To elucidate the polypeptide composition of the avocado enzyme, protein extracts were analyzed in long acrylamide gels (30×16 cm, 7.0% concentration of acrylamide) followed by immunodetection of the GDH protein. These conditions, which were successful for the analysis of grapevine GDH, failed to separate avocado fruit enzyme into two discrete polypeptide bands (data not shown). This problem was resolved by using urea-IEF/SDS-PAGE in combination with protein blotting. Partially purified protein samples from fruits that expressed all isoforms of GDH (Fig. 3B) contained two almost equally distributed polypeptides of GDH (Fig. 4A) exhibiting slightly different molecular masses (Fig. 4B). These two polypeptides were termed α and β subunits in accordance with the nomenclature for grapevine GDH (Loulakakis and Roubelakis-Angelakis, 1991).

Finally, to clarify the participation of the two polypeptides in the composition of GDH isoenzymes of avocado fruit, two-dimensional native/SDS-PAGE and immunoblotting were used (Fig. 5). Each of the isoenzymes consisted of a different ratio of the two polypeptides. From the first to the seventh isoenzyme, the relative amount of the α subunit increased while the β subunit decreased. The ripening process produced a transition in the synthesis of the two subunit polypeptides of GDH and consequently in the expression of different isoenzymes (Fig. 5, A–D). The GDH β subunit resulted in the accumulation of the more cathodal isoenzymes during early ripening (Fig. 5A), whereas the α subunit resulted in the

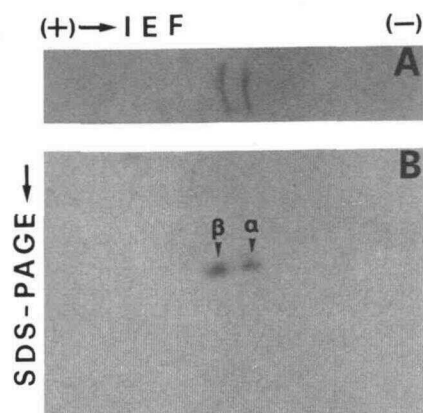


Figure 4. One- (urea-IEF) and two-dimensional analysis (urea-IEF/SDS-PAGE) of GDH from avocado fruit. A, IEF electrophoresis in the presence of 9.5 M urea. B, Second dimension on 7.0% SDS-polyacrylamide slab gel. The proteins were transferred onto nitrocellulose membranes and probed with anti-GDH serum. The protein sample from the 7th d of the ripening process was analyzed.

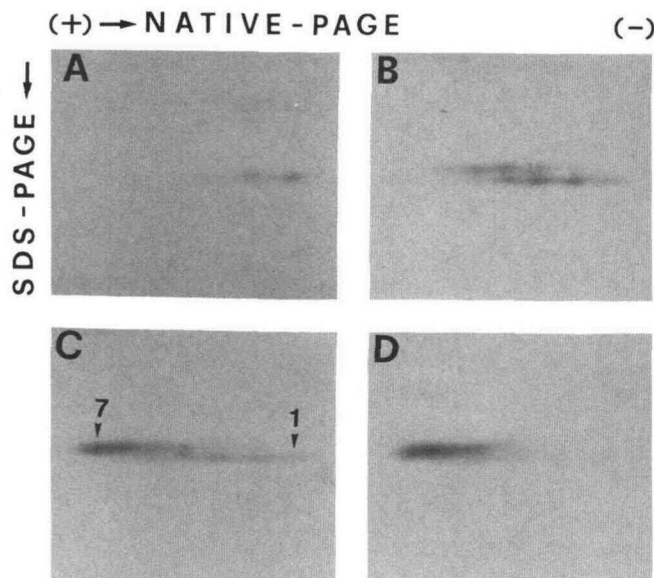


Figure 5. Two-dimensional analysis (native/SDS-PAGE) of GDH from avocado fruit. Separation in the first dimension was performed by 5% acrylamide native electrophoresis and in the second dimension on 7.0% SDS-polyacrylamide slab gels. The proteins were transferred onto nitrocellulose membranes and probed with anti-GDH serum. Protein samples from the 0 (A), 6th (B), 7th (C), and 8th (D) d of the ripening process were analyzed.

assembly of the more anodal isoenzymes late in ripening (Fig. 5D).

DISCUSSION

The changes in activity and protein content of GDH and GS in avocado fruit during the prolonged period of maturation on the tree and during the short postharvest period off the tree were studied. During development of the fruit on the tree, a small variation in the activity and the protein content of GDH and GS was noticed during the later stages of development (Fig. 1). This change in GDH reflected a shift in its isoenzymic composition from a more cathodal pattern to a pattern in which both cathodal and anodal isoenzymes were expressed (Fig. 3A). In fruits that were harvested in April and May all seven isoenzymes of GDH were present, with the anodal isoenzymes predominating.

Ripening of avocado fruit is accompanied by distinct changes in protein synthesis, mRNA accumulation, and gene expression of specific enzymes associated with normal ripening (Christoffersen et al., 1982, 1984; Tucker and Laties, 1984; Kanellis et al., 1989a). These changes take place mainly during the initial stages of ripening. The present data demonstrated that an enzyme of nitrogen metabolism, namely GDH, exhibited elevated levels in its specific activity and immunoreactive protein during avocado fruit ripening (Fig. 2). In contrast, GS activity and protein content of the 44-kD immunoreactive antigen decreased slightly during avocado ripening (Fig. 2), following a pattern of regulation opposite to that of GDH. The above increase in GDH specific activity and protein content was also reflected in the abundance of

its mRNA transcripts, as evidenced by immunoprecipitation with anti-GDH serum of *in vitro* translation products of avocado poly(A)⁺ mRNA (Fig. 2). Furthermore, the above changes in GDH activity and protein and mRNA abundance were accompanied by a noticeable shift in the pattern of GDH isoenzymes (Fig. 3), with the anodal isoforms predominating during ripening.

These isoforms were composed of two subunit polypeptides, α and β , with small but distinct differences in their molecular mass and charge (Fig. 4). Native/SDS-PAGE analysis confirmed the existence of the two subunits of GDH and revealed their differential participation in the seven isoenzymes (Fig. 5). These results, in combination with the data reported for grapevine, indicate that GDHs from both species share similarities in their subunit content (Loulakakis and Roubelakis-Angelakis, 1991). The two subunits are associated in an ordered ratio so that isoenzymes 1 and 7 are homopolymers of the β and α subunits, respectively, and isoenzymes 2 through 6 are hybrids consisting of different ratios of α and β subunits (Fig. 5). The differential appearance of GDH isoenzymes during ripening (Fig. 3) was due to the differential accumulation of α and β subunits (Fig. 5). More specifically, in unripe fruit, the β subunit was more abundant, leading to a cathodal isoenzymic pattern, whereas in overripe and senescent fruit the α subunit predominated, giving rise to the more anodal isoenzyme(s) (Figs. 3 and 5). Whether this differential accumulation of the α and β subunits of GDH during avocado ripening is due to differential gene expression of GDH or to posttranslational modifications is not known.

GDH seems to be a developmentally regulated protein whose increased levels of synthesis coincided with the late events of ripening. However, its physiological role in avocado ripening is not known. A similar pattern of changes in the GDH enzyme system was also noticed in oat leaves and grapevine callus when they were supplied with exogenous ammonium (Barash et al., 1975; Loulakakis and Roubelakis-Angelakis, 1991, 1992), as well as during natural or dark-induced senescence of leaves (Lauriere and Doussant, 1983). Barash et al. (1975) showed that the synthesis of a new isoenzyme of GDH was dependent on ammonium. Similarly, *de novo* synthesis of the GDH α subunit and subsequent formation of the more anodal isoenzymes were also induced by ammonium ions in grapevine callus (Loulakakis and Roubelakis-Angelakis, 1991, 1992). In addition, Lauriere and Daussant (1983) identified the ammonium-inducible isoenzyme as being identical with the form that was induced by darkness or senescence in wheat leaves.

These findings suggested that the inducing factor of GDH during leaf senescence was the ammonium ion produced by proteolysis (Givan, 1979). Thus, GDH induction was possibly due to ammonium and not to senescence *per se*. This situation may be true in senescing avocado fruits, since the induction of GDH occurred late during ripening, and proteolysis and subsequent production of ammonium ions is expected to occur. In fact, recent evidence indicates that carboxypeptidase is induced in tomato fruit during ripening, accompanied by an enhancement of protein degradation (Mehta and Mattoo, 1994).

The pattern of changes in GDH during ripening of avocado fruits, although similar in many respects to that of senescing

leaves, may not, however, reflect identical metabolic needs, because reallocation of nitrogen cannot be involved in ripening avocado fruits. It may be suggested that the oxidation of glutamate by GDH may supply intermediates to the tricarboxylic acid cycle, thereby ensuring its function (Robinson et al., 1991). It is obvious that the role of GDH during ripening of avocado fruit is at present completely unknown.

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