Amino Acids (2010) 38:1523–1532
DOI 10.1007/s00726-009-0373-1

ORIGINAL ARTICLE

Free amino acid production during tomato fruit ripening: a focus on L-glutamate

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Received: 27 July 2009 / Accepted: 12 October 2009 / Published online: 30 October 2009

Abstract In tomato, free amino acids increase dramatically during fruit ripening and their abundance changed differentially. More evident is L-glutamate which gives the characteristic "umami" flavor. Glutamate is the principal free amino acid of ripe fruits of cultivated varieties. In this paper, we examined the capacity of tomato fruits to process endogenous as well as exogenous polypeptides during the ripening transition, in order to analyze their contribution to the free amino acid pool. In addition, the activity of some enzymes involved in glutamate metabolism such as γ-glutamyl transpeptidase (γ-GTase), glutamate dehydrogenase (GDH), α-ketoglutarate-dependent γ-aminobutyrate transaminase (GABA-T), alanine and aspartate aminotransferases was evaluated. Results showed that peptidases were very active in ripening fruits, and they were able to release free amino acids from endogenous proteins and glutamate from exogenously added glutamate-containing peptides. In addition, red fruit contained enough γ-GTase activity to sustain glutamate liberation from endogenous substrates such as glutathione. From all the glutamate metabolizing enzymes, GDH and GABA-T showed the higher increase in activities when the ripening process starts. In summary, tomato fruits increase free amino acid content during ripening, most probably due to the raise of different peptidase activities. However, glutamate level of ripe fruit seems to be mostly related to GDH and GABA-T activities that could contribute to increase L-glutamate level during the ripening transition.

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Keywords Micro-Tom · Polyglutamate · Protease · *Solanum lycopersicum* · Glutamate synthesis

Introduction

Tomato (*Solanum lycopersicum*) is an important worldwide crop and represents one of the most highly consumed vegetables in Western countries. Tomato plays an important role in human diet and provides health benefits as source of vitamins (A and K), minerals (phosphorus and potassium; USDA National Nutrient Database 2008) and antioxidants (phenolics, folate, vitamins C and E, lycopene and β-carotene; Toor and Savage 2006). In the last years, interest in finding the way to improve the flavor of tomatoes is increasing, given that consumers are complaining about poor tomato organoleptic properties (Zanor et al. 2009). The ripening process involves drastic changes in fruit characteristics that improve palatability of fruit. The taste of tomato is enhanced during this process, which results from a combination of sugars, organic acids and free amino acids.

Free amino acid content of tomato fruit pericarp increases markedly during ripening transition of tomato fruit (Boggio et al. 2000), suggesting a high protein turnover. In particular, free glutamate content of ripe tomato fruit is much higher in all the cultivated varieties (Boggio et al. 2000; Pratta et al. 2004; Carrari et al. 2006; Mounet et al. 2007) than in tomato wild species (Schauer et al. 2005). The concentration of this amino acid is higher in tomato compared with many vegetables such as carrots, onions or pepper. In addition, glutamate provides the characteristic "umami taste" to foods with high free glutamate content such as cheese, tomato and mushrooms, which are major ingredients in cooking (Bellisle 1999).

Glutamate occupies a central role in the metabolism of amino acids in plants (Forde and Lea 2007). Biochemical and genetic approaches had been pursued to investigate the sources of glutamate accumulation in tomato fruit. Glutamate metabolizing enzymes were previously detected in vitro in the pericarp of ripening tomato fruit such as glutamate decarboxylase (GDC; EC 4.1.1.15), which catalyzes the decarboxylation of glutamate to γ-aminobutyrate (GABA; Boggio et al. 2000), glutamine synthetase (EC 6.3.1.2), involves in the synthesis of glutamine from glutamate (Scarpeci et al. 2007), and glutamate dehydrogenase (GDH; EC 1.4.1.3) that catalyzes the amination of 2-oxoglutarate (synthetic reaction) and the deamination of glutamate (catabolic reaction; Bortolotti et al. 2003; Pratta et al. 2004). Transgenic tomato plants with altered levels of glutamate were produced by introducing a gene for GDC in the anti-sense orientation (Kisaka et al. 2007a), by overexpressing *legdh1*, which encodes the beta subunit of tomato NADH-GDH (Kisaka et al. 2007b), and by expression of a *gdhA* gene for NADPH-dependent GDH of *Aspergillus nidulans* (Kisaka and Kida 2003). L-Glutamate is the main N-form of tomato phloem sap (Valle et al. 1998); therefore, it would also be supplied to the fruit, although to a lesser extent due to the low sink activity of ripe fruits (Ho 1988). Another potential source of glutamate in ripe fruit may be the degradation of endogenous peptides.

Tomato fruit ripening involves significant biological changes mainly due to the chloroplast to chromoplast transition, which leads to a decline in the amounts of many soluble and membrane-associated plastid proteins from chloroplast, while new chromoplast-specific proteins accumulate (Bathgate et al. 1985; Kahlau and Bock 2008). The observed changes in transcripts and metabolites during fruit ripening (Carrari et al. 2006) indicate that the ripening process involves substantial turnover of existing and newly synthesized proteins. Different carboxypeptidases had been already identified in tomato fruit (Mehta et al. 1996). In addition, two γ-glutamyl transpeptidases (γ-GTase; [5-L-glutamyl]-peptide:amino acid 5-glutamyl transferase; EC 2.3.2.2), which catalyze the glutamate transfer to either water (hydrolysis) or to an acceptor amino acid or peptide, were shown to increase during the tomato ripening transition (Martin et al. 1995). This fact implicates that glutamate could also be liberated from γ-glutamyl substrates such as glutathione or other molecules with a γ-linked terminal glutamate residue.

Among the pathways that could produce glutamate depicted in Fig. 1, the activity of 2-oxoglutarate-dependent L-α-amino acid transaminases (EC 2.6.1) and GABA transaminase (GABA-T; EC 2.6.1.19) was previously detected in ripening tomato fruits (Boggio et al. 2000; Akihiro et al. 2008).

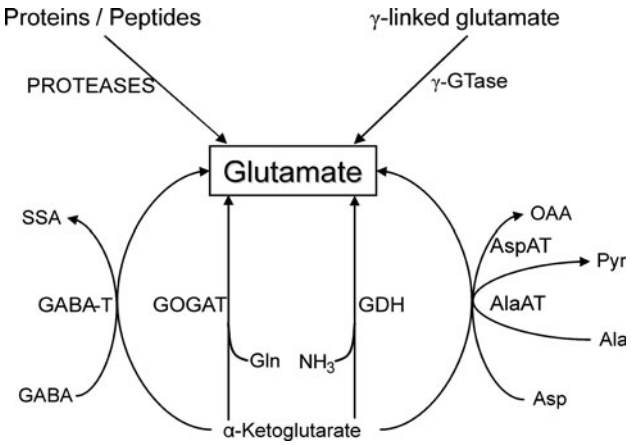

Fig. 1 Proposed pathways of glutamate production in tomato fruits

In this work, we investigated the contribution of different enzymatic activities to increase the level of free amino acids during the ripening transition of the Micro-Tom fruit pericarps. For that purpose, we tested peptidase activities with endogenous as well as exogenously added peptides, and L-glutamate metabolizing enzymes. In addition, we used poly-L-glutamate (PGA) as substrate in order to determine the existence of peptidase activities of fruit pericarp, which could be able to release glutamate from endogenous substrates having terminal glutamate residues.

Materials and methods

Plant material

Tomato (*S. lycopersicum*) plants cv. Micro-Tom were grown in a controlled environment cabinet under 400 μmol s^{-1} m^{-2} light intensity at the top of a fruit-containing plant. The temperature ranged from 23°C during the light period (14 h) to 18°C in the dark, and the relative humidity was 70 ± 10%. Plants were grown in soil, continuously maintained under optimal irrigation and supplied weekly with half strength Hoagland solution (Malacrida et al. 2006). Fruits were harvested starting 4 h after the light was on at the mature green stage (G), yellow (Y), orange (O) or red (R) stages (Malacrida et al. 2006). Pericarp tissue of the harvested fruits was obtained by removing the locule tissues and seeds and immediately processed or frozen in liquid nitrogen and stored at −80°C until analysis.

Determination of free amino acid content

Frozen pericarp tissues were extracted with chloroform/methanol (Valle et al. 1998). The amino acid composition in the methanolic phase was determined by derivatization

with *o*-phthaldialdehyde and analyzed in a HPLC system (Boggio et al. 2000). All determinations were done in triplicate.

Enzyme extraction

For the extraction of azocaseinolytic activity, 1 g of frozen tissue was ground in 0.3 mL of 0.5 M HEPES–KOH (pH 7.5), 100 mM $MgCl_2$, 10 mM EDTA, 0.3 mL of glycerol and 2% (w/v) polyvinylpolypyrrolidone, using mortar and pestle in ice. The extracts were centrifuged at 15,300×*g* for 10 min at 4°C.

For all other enzymes activities, the extraction buffer contained 0.75 M Tricine–KOH (pH 8), 100 mM $MgCl_2$, 10 mM EDTA, 50 mM β-mercaptoethanol, 15 mM phenylmethylsulfonylfluoride, and 0.3 mL of glycerol and 2% (w/v) polyvinylpolypyrrolidone, and for the transaminases 1 mM pyridoxal phosphate (PLP) was also added. The supernatants were desalted using Sephadex G-25 columns equilibrated with the same extraction buffer (10 times diluted) plus 10% (v/v) glycerol.

Enzyme activities

Endopeptidase activity was measured using azocasein, a chromogenic substrate, whose hydrolysis releases the azo dye into the media where it is detected by absorbance at 440 nm, as previously described (Ramakrishna and Rao 2005) with the following modifications. The reaction was carried out in a mixture containing 50 mM sodium acetate (pH 5.5), 0.65% (w/v) azocasein, 1.25 mM β-mercaptoethanol and 7.5 mM cysteine. The reaction mixture was incubated at 37°C for 3 h. One unit (U) of azocaseinolytic activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 at 440 nm in a 1 cm cuvette under the assay conditions.

The γ-GTase activity was determined at 30°C in an assay mixture containing 100 mM Tris–HCl (pH 8), 5 mM γ-glutamyl 3-carboxy-4-nitroanilide and 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC), and followed by the increase in absorbance at 405 nm (molar extinction coefficient of 3-carboxy-4-nitroanilide: 9,500 M^{-1} cm^{-1}).

GABA-T activity was determined as the GABA-dependent production of glutamate with α-ketoglutarate as amino acceptor (Breitkreuz and Shelp 1995). The assay mixture contained 100 mM Tris–HCl (pH 8.5), 2 mM GABA, 20 μM PLP, 10 mM α-ketoglutarate and desalted extract. The reaction mixture was incubated for 3 h at 30°C, then sulfosalicylic acid was added to a final concentration of 60 mM and the precipitate was removed by centrifugation. The supernatant was neutralized with NaOH, and glutamate content was determined with a spectrophotometric enzymatic cycling method (see below). The complete reaction mixture minus substrates served as controls for glutamate-independent GABA-T production.

Aspartate amino transferase (AspAT; EC 2.6.1.1), alanine aminotransferase (AlaAT; EC 2.6.1.2), GDH and GDC were measured as previously described (Boggio et al. 2000) with slight modifications using desalted extracts. AspAT and AlaAT were determined at 30°C by the decrease in absorbance at 340 nm of an assay mixture containing 100 mM Tricine–KOH (pH 8), 2.5 mM aspartate, 2 mM EDTA, 40 μM PLP, 0.2 mM NADH, 2 U mL^{-1} malate dehydrogenase (EC 1.1.1.37) and 2.5 mM α-ketoglutarate for AspAT activity and 100 mM HEPES–KOH (pH 7.5), 10 mM alanine, 2 mM EDTA, 40 μM PLP, 0.2 mM NADH, 5 U mL^{-1} lactate dehydrogenase (EC 1.1.1.27) and 2.5 mM α-ketoglutarate for AlaAT activity. In both cases, a 15-min preincubation of the desalted extract plus the reaction mixture without NADH, malate dehydrogenase/lactate dehydrogenase and α-ketoglutarate was performed for transaminase activation. The reaction was started by addition of α-ketoglutarate. For GDH activity, a reaction mixture of 150 mM Tris–HCl (pH 8), 200 mM NH_4Cl, 1 mM $CaCl_2$, 0.2 mM NADH and 20 mM α-ketoglutarate was used for the aminating activity and a mixture of 150 mM glycine (pH 9.3), 0.5 mM $CaCl_2$, 10 mM NAD^+ and 100 mM glutamate for the deaminating activity. The reaction was started by addition of α-ketoglutarate or glutamate in each case at 25°C and followed by the decrease/increase in absorbance at 340 nm (molar extinction coefficient of NADH: 6,220 M^{-1} cm^{-1}). GDC activity was followed by a discontinuous method as the glutamate-dependent production of GABA. The assay mixture contained 100 mM MES (pH 5.8), 20 mM glutamate, 500 μM PLP, 10 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and desalted extract. The reaction mixture was incubated for 0, 1, 3, 5 and 7 min at 30°C. The reaction was stopped with 5% (v/v) perchloric acid, neutralized with KOH and the precipitate was removed by centrifugation. GABA content was determined in the supernatants as previously described (Boggio et al. 2000). The complete reaction mixture minus glutamate served as control.

One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzes the transformation of 1 μmol of substrate per min.

Immunoblot analysis

Ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1) protein was determined in leaf and fruit extracts of Micro-Tom plants as described in Boggio et al. (2000). Protein extracts (46 g) from deribbed leaves, green and red fruits were fractioned by SDS-PAGE (8%) and blotted on nitrocellulose membrane, immunoassayed using

antiserum raised against Fd-GOGAT of *Zea mays* and detected with ECL system (Amersham).

Protein determinations

Protein concentrations of fruit pericarp extracts were determined according to Bradford (1976) using bovine serum albumin as standard.

Endogenous proteins and exogenous polyglutamate degradation

Fruit pericarp extracts were prepared as described for enzyme extraction of azocaseinolytic activity, but using 0.5 M sodium acetate (pH 5.5) as extraction buffer. 50 μg of protein (final volume 1 mL) was incubated for 18 h at 37°C to test endogenous protein degradation or with the addition of 50 μg poly-α-L-glutamate (PGA: 50–100 kDa) to test exogenous PGA degradation. These extracts were precipitated with 10% (v/v) trichloroacetic acid, resuspended in sample buffer and subjected to 10% SDS-PAGE (10%, w/v acrylamide gel). The supernatants were reserved to assay total amino acids and L-glutamate concentration. The gels were fixed with 3% acetic acid and stained with Coomasie Brilliant Blue. Gels were photographed using a digital camera and the percentage of degradation was quantified by densitometry (ImageJ program).

Total amino acids assay

Total free amino acids released from endogenous protein degradation were determined by a modified Cd-Ninhydrin method. An equimolar mixture of alanine, glutamine, glutamic acid and glycine was used as standard. A longer heating time (15 min) was used to reach the maximum color for free amino acids (Doi et al. 1981).

L-Glutamate assay

Glutamate was determined by a spectrophotometric enzymatic cycling method using 2 U GDH from bovine liver (Fluka) and 0.1 U recombinant D-phenylglycine aminotransferase (EC 2.6.1.72) from *Pseudomonas stutzeri* per mL and the progress of the reaction was monitored for 3 min (Khampha et al. 2004). Briefly, L-glutamate is first converted to α-ketoglutarate and ammonia by the action of GDH with a concomitant reduction of NAD^+ to NADH. The α-ketoglutarate is recycled back into L-glutamate in the transamination reaction catalyzed by D-phenylglycine aminotransferase using D-4-hydroxyphenylglycine as an amino donor, which itself is converted to 4-hydroxybenzoylformate. Both NADH and 4-hydroxybenzoylformate strongly absorb UV light at 340 nm. The working range is 0.2–20 μM of L-glutamate.

RNA extraction and reverse transcription

Fruits were collected at two development stages (mature green and red) from different plants. For each RNA sample, a piece of pericarp from five fruits was excised to form the bulk for total RNA extraction. Total RNA was extracted from the frozen pericarps using the Plant RNeasy extraction kit (QIAGEN) according to the manufacturer's instructions. Nucleic acid concentrations were measured at 260 nm. Purity of the total RNA extracted was determined as the 260/280 nm ratio and the integrity was checked by electrophoresis in 1.5% (w/v) agarose gel. To eliminate the residual genomic DNA present in the preparation, 0.75–1 μg of RNA was treated with RNAse-free DNAse RQ1 (Promega) according to the manufacturer's instructions. For cDNA synthesis, oligo(dT) primer and SuperScript III Reverse Transcriptase (Invitrogen) were used according to the manufacturer's instructions.

Analysis of gene expression by real-time PCR

Real-time PCR (qPCR) reactions were performed in a Mastercycler® ep realplex thermal cycler (Eppendorf) using the intercalation dye SYBR Green I (Roche) as fluorescent reporter to monitor dsDNA synthesis. A master mix for each qPCR run was prepared. Final concentrations, in a total volume of 20 μL, were as follows: 1× qPCR buffer minus Mg, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4× SYBR Green I and 0.5 U Platinum Taq DNA Polymerase (Invitrogen). 5 μL of 1:10 diluted cDNA was added. 1 μM each for specific sense and anti-sense primers was used. Gene-specific primers used were as follows: *GDH* (GenBank accession U48695), forward 5′-AAGGAGT CACCATCCTACCG-3′ and reverse 5′-TGTGAGTCTTG CACATATCCTTG-3′; *GABA-T1* (GenBank accession AY240229), forward 5′-CCTTGCCACAGAGTTTGCG-3′ and reverse 5′-CAAGTTCTTCTGGAGTAACTAC-3′; *GABA-T2* (GenBank accession AY240230), forward 5′-GTACTTTCTACAGAGTTTGTAG-3′ and reverse 5′-A GTTCTTCAAGACTCAAGGTG-3′; *GABA-T3* (GenBank accession AY240231), forward 5′-TGAGAAGCATGGAG TGTTGG-3′ and reverse 5′-TCATGAGCTTTTTATC TTCTTCTGA-3′, and *RpL2* (GenBank accession X64562), forward 5′-CGTGGTGTTGCTATGAATCC-3′ and reverse 5′-GTCAGCTTTGGCAGCAGTAG-3′. All tubes were subjected to 1 min at 95°C, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 40 s. SYBR Green I absorbance was detected at 72°C. qPCR for each gene was done on three biological replicates. To ensure amplification of one specific gene product, melting curves were

performed as follows: 95°C for 15 s, 60°C for 15 s, a 20 min melting cycle of 60–95°C (T_m calculation) and 5°C for 15 s. *GDH* and *GABA-T* expression levels in each sample were normalized to the level of RpL2 (Balbi and Lomax 2003). A comparative Ct method using $2^{-\Delta\Delta Ct}$ formula was used to obtain relative quantitative levels of *GDH* and *GABA-T* transcripts in green and red fruit as described (Livak and Schmittgen 2001).

Statistical analysis

The experimental data were subjected to statistical Student's *t* test and analysis of variance (ANOVA). When *F* test data were significant in ANOVA, individual means were further tested by LSD. A *P* value less than 0.05 was considered statistically significant. Model assumptions were tested by analysis of residuals. Logarithmic transformation was used in free amino acid determinations and square root was used in glutamate determination after proteolysis and PGA digestion. Significant difference in qPCR data was determined at $P < 0.05$ using an unpaired, two-tailed Student's *t* test on normalized Ct values.

Table 1 Relative molar content of free amino acids of the pericarp of Micro-Tom ripening fruits

Amino acids	Relative molar content (%)		
	Green	Yellow	Red
Aspartate	1.4 ± 0.9	1.5 ± 0.2	0.8 ± 0.2
Threonine	5.9 ± 4.7	7.9 ± 5.9	1.4 ± 0.7
Serine	2.8 ± 2.0	6.0 ± 1.8	2.1 ± 0.3
Glutamate	12.7 ± 5.0	20.2 ± 7.6	55.0 ± 0.6
Glutamine	14.7 ± 3.7	20.0 ± 5.1	15.7 ± 0.8
Glycine	2.6 ± 0.9	1.7 ± 0.2	1.5 ± 0.4
Alanine	2.0 ± 1.8	1.1 ± 0.5	1.8 ± 0.6
Cysteine	3.9 ± 3.2	2.4 ± 1.7	2.0 ± 0.9
Valine	2.8 ± 1.4	2.4 ± 1.7	1.8 ± 0.3
Tyrosine	2.2 ± 0.5	0.6 ± 0.5	0.8 ± 0.7
Phenylalanine	2.1 ± 0.5	1.6 ± 1.1	1.4 ± 0.2
GABA	40.4 ± 5.2	30.1 ± 8.2	8.3 ± 1.8
1-Methyl histidine	0.6 ± 0.1	1.4 ± 0.5	2.2 ± 0.2
Histidine	2.0 ± 1.0	0.9 ± 0.5	1.3 ± 0.6
Arginine	1.0 ± 0.2	1.3 ± 0.6	2.4 ± 0.2
Total (μmol per g of FW)	3.6 ± 0.2	11.6 ± 4.4	19.2 ± 5.1

Results are presented as means of at least three independent experiments ± SE. The experimental data were subjected to ANOVA ($P < 0.05$)

Results

Variation of free amino acid composition of mature fruit during the ripening transition

At ripening, the total free amino acid content of mature red fruit increased almost fivefold relative to green fruit on a fresh weight basis (Table 1). From all free amino acids, GABA, glutamate, threonine and tyrosine contents showed statistically significant variations ($P < 0.05$) during the ripening transition (Table 1). GABA was found to be the most abundant free amino acid in the pericarp of green and yellow fruits (Table 1), decreasing sharply at red stage. The relative proportion of other amino acids, such as threonine and tyrosine, declined during ripening, while the others did not show significant variation. Glutamine content was one of the highest at all ripening stages, although it was almost invariable during the green–red transition (Table 1). The glutamate content of ripening fruits increased during ripening reaching 55% of the relative molar content of total free amino acids ($P < 0.05$) becoming the most abundant free amino acid of ripe Micro-Tom fruits (10.56 μmol L-glutamate per g fresh weight).

Amino acids production by proteolytic activities of ripening fruits

Peptidase activities from whole pericarp extracts were assayed using two substrates, fruit pericarp endogenous proteins and azocasein. Proteolysis of endogenous fruit proteins was followed by incubating the pericarp fruit extracts 18 h at 37°C and subjected to SDS-PAGE, as described in "Materials and methods". Results indicate that endogenous protein degradation occurred at all maturation stages (Fig. 2), showing the green fruit the highest degree of degradation (55%), as revealed by densitometric analysis ($P < 0.05$; Fig. 2, bottom line). The contribution of peptidases to the free amino acid level of ripening fruit was also analyzed (Fig. 3a). The results indicate that the amino acids released from endogenous substrate degradation of ripening fruits were much higher in red fruits compared to other ripening stages ($P < 0.05$). When azocasein, a chemically modified protein commonly used as a non-specific substrate of peptidases, was used for the peptidase assay, tomato fruit showed similar tendency to increase during the fruit ripening transition ($P < 0.05$; Fig. 3b). On the other hand, in vitro degradation of endogenous red fruit protein reached 28% (Fig. 2), which corresponded to around 50 nmol total free amino acid per mg red fruit protein (Fig. 3a).

Glutamate production activities of ripening fruits

To test the capability of fruit extracts to release free glutamate residues, peptidases of fruit extracts were also assayed with exogenous PGA as substrate. Free glutamate

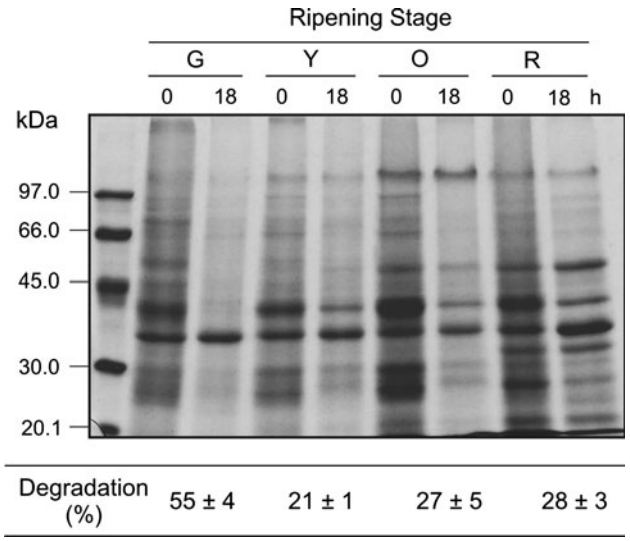

Fig. 2 Endogenous pericarp protein digestion of ripening Micro-Tom fruits. Autoproteolysis of protein extracts (50 μg) from fruits at different ripening stages (*G*, *Y*, *O* and *R*) by incubation at 37°C for 0 or 18 h, and analyzed by SDS-PAGE. The gel was stained with Coomasie Brilliant Blue R-250. The *first lane* corresponds to molecular mass markers (given in kilodaltons to the left of the gel). A picture of a representative stained SDS-polyacrylamide gel is presented. *Numbers* in the *bottom line* indicate the percentage of degradation of pericarp proteins after 18 h of autoproteolysis. The values were calculated after densitometric analysis of stained SDS-polyacrylamide gels of three biological replicates. The experimental data were subjected to ANOVA ($P < 0.05$)

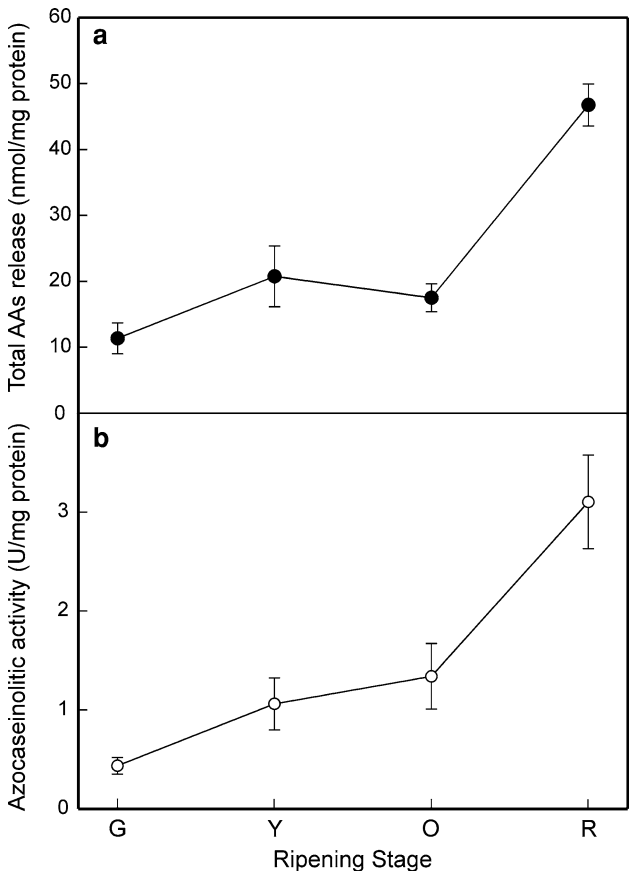

Fig. 3 Changes in proteolytic activity during Micro-Tom fruit ripening. **a** Total free amino acids liberated from the pericarp proteins after 18 h autodigestion were determined in the supernatant obtained from trichloroacetic acid precipitation of the incubation mixture. **b** Proteolytic activity was measured in pericarp extracts employing azocasein as substrate. One unit (U) of azocaseinolytic activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 at 440 nm in a 1 cm cuvette under the assay conditions. Results are presented as means of at least three independent experiments ± SE. The experimental data were subjected to ANOVA ($P < 0.05$)

released from endogenous proteins and from PGA was evaluated and the results are shown in Fig. 4. These data indicate that the glutamate released after PGA digestion was significantly higher in orange and red fruits than in green and yellow fruits ($P < 0.05$). However, L-glutamate liberated from endogenous protein degradation of ripening fruits was very low ($P < 0.05$). These results indicate that ripe fruit contains proteases that are able to release glutamate from endogenous proteins or peptides when they are available.

Another way to release free L-glutamate is by the action of γ-GTase on γ-linked terminal glutamate residue from endogenous peptides such as glutathione. γ-GTase activity was measured in tomato fruit extracts. The γ-GTase activity decreased during fruit ripening (Fig. 5a). Although γ-GTase activity was lower in red fruit than in green fruit, γ-GTase still could liberate around 40 nmol of L-glutamate per min per mg of protein from γ-linked residues in red fruit.

Other enzymes involved in L-glutamate synthesis were evaluated in ripening Micro-Tom fruits. Activities of GDH, AspAT, AlaAT and GABA-T were tested (Fig. 5). Results show steadily increase of the ratio of aminating/deaminating GDH activity (Fig. 5c) and GABA-T activity (Fig. 5f) during the ripening transition, reaching around 20 and 40 nmol of L-glutamate per min per mg of protein in red fruit, respectively. The ratio of aminating/deaminating GDH activity was significantly higher in red fruit ($P < 0.05$) than in other fruit ripening stages. On the other hand, GABA-T activity was significantly different only in green fruit ($P < 0.05$). In addition, activity of Fd-GOGAT, an enzyme present in chloroplast, was measured in fruits during ripening without success; therefore, immunoblot analysis was performed (Fig. 6). As previously described (Bortolotti et al. 2003), Fd-GOGAT was evident only in green fruits. Another enzyme involved in glutamate metabolism is GDC. Activity of this enzyme was measured in ripening Micro-Tom fruit and results showed a sharp decrease of GDC activity during this process (Fig. 7).

Expression analysis of GDH and GABA-T, enzymes that increased their activities during fruit ripening, was

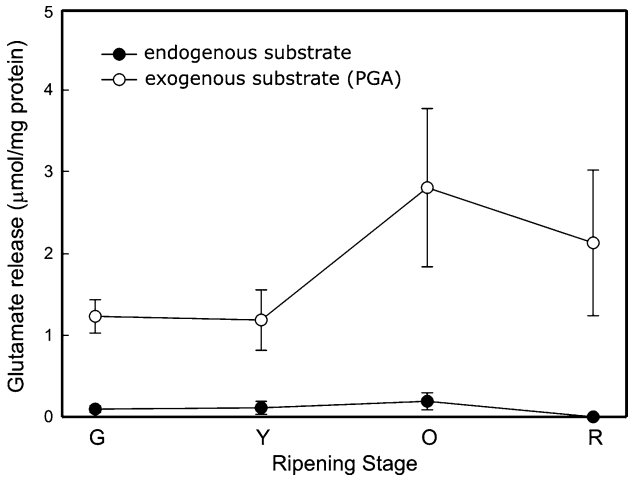

Fig. 4 Quantification of free glutamate liberated after peptidase activity of fruit pericarp using endogenous proteins or PGA as substrates. Glutamate content liberated from the pericarp proteins autodigestion and from PGA degradation was determined in the supernatant obtained from trichloroacetic acid precipitation of the incubation mixture (see Fig. 3a). Results are the means of at least three independent experiments ± SE. The experimental data were subjected to ANOVA ($P < 0.05$)

performed by real-time PCR. Transcript levels of *GDH* and the three isoforms of *GABA-T* in green and red fruits were significantly lower ($P < 0.05$) in red fruit, except for *GABA-T3* (Fig. 8). These results indicate no correlation between GDH and GABA-T activities and transcript levels (Figs. 5, 8).

Discussion

In tomato (*S. lycopersicum*), free L-glutamate increases dramatically in the fruit during ripening (Boggio et al. 2000) in all the cultivated varieties tested so far (Pratta et al. 2004; Table 1), becoming the most abundant amino acid of ripe fruits. By contrast, wild species of the *S. lycopersicum* complex have less glutamate content than those of the domesticated species (Schauer et al. 2005). These data suggest that during tomato domestication glutamate metabolic pathways that contribute to increase glutamate content in ripe fruits were selected together with valuable traits leading to enhance fruit color intensity, shape and/or size. A negative correlation between free glutamate content and fruit shelf-life had been observed (Pratta et al. 2004). Glutamate is a direct precursor for chlorophyll biosynthesis (Pontoppidan and Kannangara 1994). Considering that down-regulation of chlorophyll biosynthesis occurs at ripening stage, it was postulated that glutamate accumulation was, at least in part, due to this process at this time point (Carrari et al. 2006). However, in fruits of *green flesh*, a tomato mutant defective in

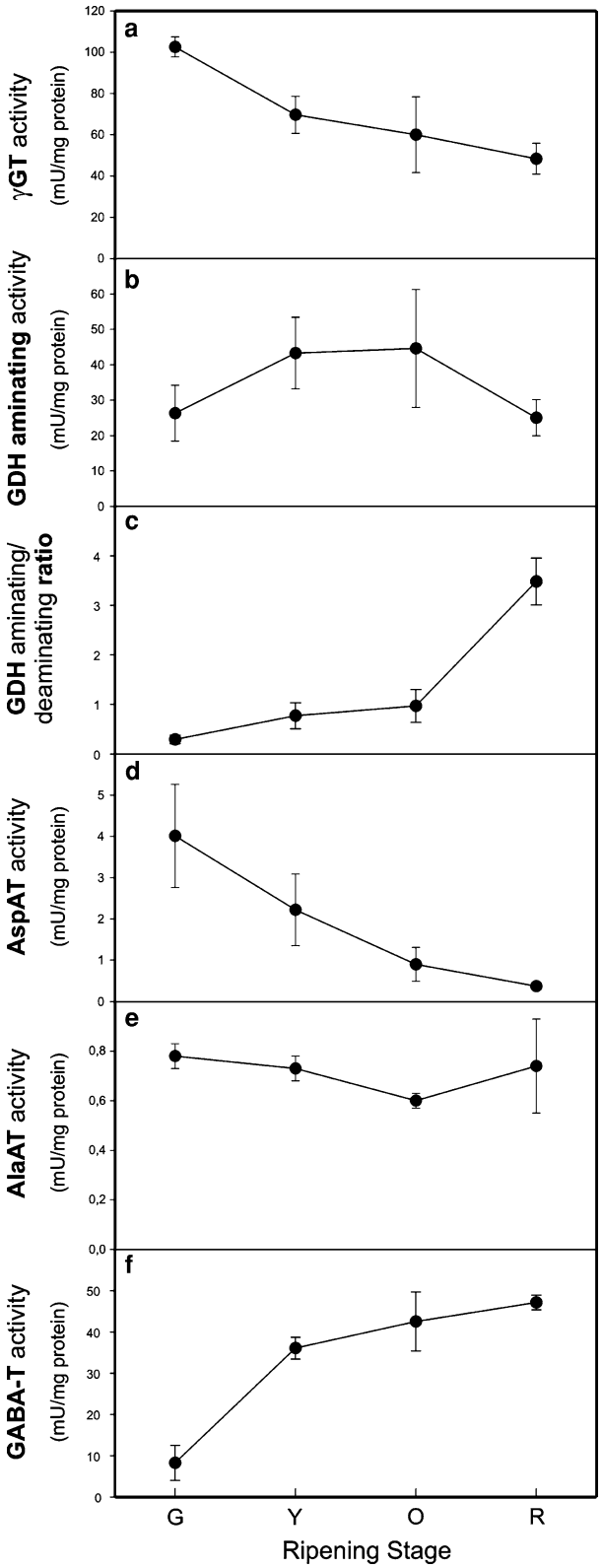

Fig. 5 Enzyme activities that produce glutamate during fruit ripening. The activities were measured in pericarp extracts as described in "Materials and methods". Results are the means of at least three independent experiments ± SE. The experimental data were subjected to ANOVA ($P < 0.05$)

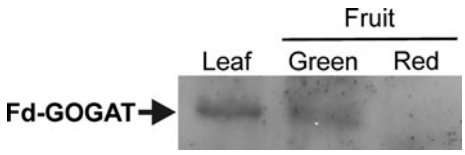

Fig. 6 Immunoblot analysis of ferredoxin-dependent glutamate synthase in leaf and fruit extracts of Micro-Tom plants. Protein extracts (46 g) from deribbed leaves, mature green and red fruits were immunoassayed as described in "Materials and methods"

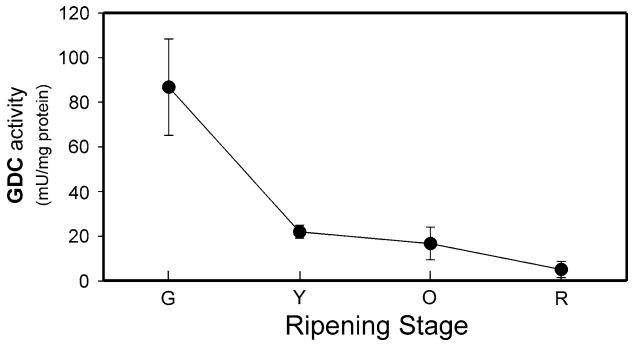

Fig. 7 Glutamate decarboxylase activity during fruit ripening. The activity was determined as described in "Materials and methods". Results are the means of at least three independent experiments ± SE. The experimental data were subjected to Student's *t* test analysis ($P < 0.05$)

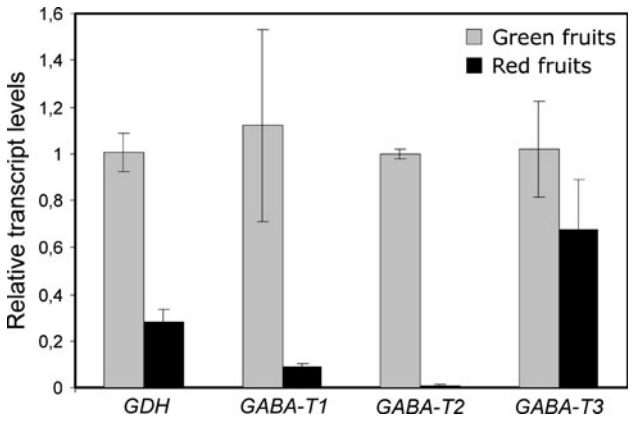

Fig. 8 Relative transcript levels of *GDH* and *GABA-T* in green and red fruits. Total RNA of fruits were retrotranscribed using a poly-dT primer, then subjected to real-time PCR analysis using specific oligonucleotides. Red fruit transcripts were related to green fruit transcripts, which were arbitrarily fixed to 1. Results are the means of at least three independent experiments ± SE. The experimental data were subjected to Student's *t* test analysis ($P < 0.05$)

chlorophyll degradation, glutamate content also increased in the ripening process (Bortolotti et al. 2003), suggesting that the accumulation of free glutamate in the pericarp of ripe tomato fruits is not associated with the chloroplast–chromoplast transition.

In parallel to this, GABA and threonine levels decreased significantly in ripening fruits (Table 1). The lower GABA contents could be the consequence of the decrease in GDC activity (Fig. 7) and the increase in GABA-T activity of ripe fruit (Fig. 5f). Threonine, by contrast, could be metabolized to pyruvate, which together with glyceraldehyde 3-phosphate is needed for the synthesis of isopentenyl pyrophosphate, a precursor of the carotenoids (Cunningham and Gantt 1998). As in other tomato fruit varieties, the carotenoid content of Micro-Tom mature fruits increased significantly during ripening (Malacrida et al. 2006), suggesting that both substrates were present in the pericarp of these fruits.

While protein content decreased from 0.67 to 0.19 mg per g fruit fresh weight during ripening, the total free amino acids increased more than five times per gram of fresh fruits (Table 1). These data suggested high proteolytic activity of these fruits. In vitro degradation of endogenous proteins decreased during ripening (Fig. 2), although the free amino acid pool released from tomato pericarp proteins increased in ripening fruits (Fig. 3a). This could be related with an increment in the exopeptidase activity during ripening, suggested by the non-specific protease activity pattern (Fig. 3b). It is worthwhile to mention that peptidases present in these extracts could have different intracellular localizations.

In Micro-Tom fruits, we detected glutamate peptidase activity using poly-α-L-glutamate as exogenous substrate at all ripening stages of mature fruit (Fig. 4). Carboxypeptidases and endopeptidases are enzymes able to cleave specifically C-terminal glutamate residues in peptides (Bown and Gatehouse 2004; Qi et al. 1994). The capacity of fruit to degrade exogenously added glutamic peptide indicates that carboxypeptidases and endopeptidases present in fruit would be able to cleave specifically C-terminal glutamate residues of endogenous fruit polypeptides. These enzymes also cleave the glutamate residue linked via its α-amino group to pteroic acid in folic acid and folate analogs. During ripening, folate level in tomato fruits decreased markedly (Basset et al. 2002), maybe due to the action of this glutamate peptidase. Nevertheless, the drop in folate content of ripening fruit was 0.4 nmol g^{-1} (fresh weight; Basset et al. 2002), suggesting that its contribution to free glutamate pool, around 10 µmol g^{-1} (fresh weight; Table 1), is low.

Another enzyme that catalyzes glutamate liberation is γ-GTase. In a previous report, γ-GTase activity was shown to increase during ripening of tomato fruits (cv. Ailsa Craig) on a fresh weight basis using ACC and reduced glutathione as substrates (Martin et al. 1995). The total glutathione content of ripe red fruit (cv. Rutgers) also increased. In Micro-Tom fruits, however, γ-GTase activity decreased more than twice (Fig. 5), maybe due to the use of different tomato cultivars and substrates. Nevertheless, red fruit still

contained enough γ-GTase activity to sustain glutamate liberation (40 nmol per min per mg of protein) from peptides with a γ-linked terminal glutamate residue. It is worth to note that the presence of these peptides had already been reported in plants (Kasai et al. 1986; Lancaster and Shaw 1994). These reports concluded that the physiological function of γ-GTase was as a hydrolase and not as a transferase.

These results indicate that various peptidase activities are clearly present in ripening Micro-Tom and they are able to release free amino acids from tomato fruit pericarp proteins. However, the contribution to increase the free glutamate content from endogenous peptides during protein recycling occurring in the ripening transition seemed to be low (Fig. 4).

Additional sources of glutamate for ripening fruits would be the import from the source leaves and the reactions catalyzed by glutamate synthase, GDH or aminotransferases. In mature green fruit, Fd-GOGAT was observed, but it was undetectable in red fruits (Fig. 6). Although free L-glutamate is the main N-form of the tomato phloem sap (Valle et al. 1998), the fruit sink strength decreased during ripening (Ho 1988) indicating that import of glutamate in ripe fruit from phloem system is unlikely. Previous reports indicated induction of GDH and GABA-T during the fruit ripening process (Boggio et al. 2000; Akihiro et al. 2008) and transgenic tomato plants overexpressing *legdh1* showed that the level of glutamate was about twice that in the control fruits (Kisaka et al. 2007b). Therefore, enzymes involved in glutamate metabolism were tested in this work. Results showed that aminating to deaminating GDH activities are favoured and increased concomitantly with GABA-T activity during fruit ripening (Fig. 5c, f). The apparent disconnect between transcript level (Fig. 8) and enzyme activity was previously observed in tomato fruit for GDH (Scarpeci et al. 2007) and GABA-T (Akihiro et al. 2008). These data indicate that both enzymes would be good candidates to contribute to increase the free L-glutamate content of Micro-Tom ripening fruits, emphasizing the importance of post-translational regulation of L-glutamate metabolism.

In summary, the rise in free glutamate content of tomato fruit could be due to the markedly increase of GDH and GABA-T activities during the ripening transition, and to the avoidance of glutamate consumption through the reaction catalyzed by GDC, which was almost undetectable in ripe fruits. To a minor extent, the hydrolase activity of γ-GTase could liberate free glutamate from peptides containing γ-linked terminal glutamate residue. The proteolytic activity observed during fruit ripening seemed to contribute more to increase the total free amino acid content than the free glutamate level of ripe tomato fruit.

Acknowledgments This research was supported by grants from the National Agency for the Promotion of Science and Technology (ANPCyT, PICTs nos. 08-13549 and 2006-01358) and the National Institute of Agricultural Technology (INTA, grant no. AEBIO3451). We are very grateful to Dr. Suthep Wiyakrutta, Faculty of Science Mahidol University, Bangkok, Thailand, for kindly provided the recombinant D-phenylglycine aminotransferase enzyme, and Mercedes Leiva and Hebe Bottai for statistical analyses.

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