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MULTIPLE FORMS OF β-HEXOSAMINIDASE IN RIPENING TOMATO (*LYCOPERSICUM ESCULANTUM* L.)

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SUMMARY

The specific activity of β-hexosaminidase in tomato fruits increased during ripening with a peak at climacteric stage. The enzyme was purified from climacteric fruit for studying its properties. Two major isoforms (I and II) of β-hexosaminidase were separated by ion-exchange chromatography on DEAE-Sephadex A-50, with a percent abundance of 57 and 37, respectively, a third one being very minor. The isoforms I and II of β-hexosaminidase were individually subjected to gel permeation chromatography on Sephadex G-200 followed by PAGE. The pH optimum was 5.0 and 4.6 for isoforms -I and -II, respectively. The temperature optimum was 47^{0}C for isoform I, which had a thermostability of 72% at 57^{0}C for 15 min. Isoform II displayed a temperature optimum of 47 - 57^{0}C, with a thermostability of 100% under similar conditions. Both the isoforms of β-hexosaminidase were inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+}. The Km values for *p*-nitrophenyl-β-N-acetyl glucosaminopyranoside were 1.6 and 1.1mM, and molecular weights were > 94 & 64 kDa, respectively, for the isoforms -I and -II.

Key words: Fruit Ripening, β-Hexosaminidase, Multiple forms, Tomato

INTRODUCTION

β-Hexosaminidase (EC 3.2.1.52) is an important carbohydrate hydrolase classified as glycosidase which catalyzes the release of free N-glycans from polymeric/ oligomeric substrates containing hexosamine residues. The enzyme has been studied in several animal and microbial systems, where it is implicated in important physiological events like signal transduction, cell division and cell integrity (Karamanos *et al*. 1995, Wells *et al*. 2002, Wells *et al*. 2003). In higher plant systems, the enzyme has been purified from jack bean meal (Li and Li 1970), cotton seeds (Yi 1981), castor bean endosperm (Harley and Beevers 1985), water melon fruit (Nakagawa *et al*. 1988), wheat leaves (Barber and Ride 1989), lettuce (Posci *et al*. 1990), apple (Choi and Gross 1994) and cabbage (Chang *et al*. 1998). β-Hexosaminidase has not been widely studied in fruits in relation to ripening except in water melon (Nakagawa *et al*. 1988), apple (Choi and Gross, 1994) and our recent report in bell capsicum (Jagadeesh and Prabha 2002). Among glycosidases in fruits, β-hexosaminidase is the most active enzyme, especially in *Solanaceae* fruits, such as bell capsicum and tomato (Jagadeesh and Prabha 2002, Jagadeesh *et al*. 2004). In this communication, we report the presence of multiple forms of β-hexosaminidase in ripening tomato fruit and their characteristics. Despite the fact that tomato is a model fruit for many important biochemical and molecular studies in the area of fruit ripening, the information on β-hexosaminidase of tomato is novel. The occurrence of multiple forms of this enzyme in tomato during ripening is viewed in the context of fruit ripening.

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MATERIALS AND METHODS

Materials:

Freshly harvested tomato fruits were collected from a local farm. The four different developmental stages were selected based on size / weight and days (d) after fruit set, viz. stage: (1) 7d (2.5 - 3.5 g); (2) 14 d (8.5 - 9.5 g); (3) 21d (60 - 62 g) and (4) 28d (65 - 67 g). The four stages of ripening chosen were based on days after harvest, viz. stage: (5) mature dark green (0d); (6) light green (5d); (7) orange (10d) and (8) red ripe stage (15d). Acetone-dried powders were prepared from these tomato fruits at various stages of development and ripening using 3-volumes of chilled (-20^{0}C) acetone. The precipitate was filtered through a cheese cloth and washed with 500 ml chilled acetone. Finally, the precipitate was freed of acetone under shade and refrigerated.

Enzyme extraction and purification:

Acetone-dried powder (10 g) of tomato at climacteric stage was extracted overnight at 4^{0}C with 200 ml of 0.05M sodium acetate buffer (pH 6.6) containing 0.5% polyvinylpyrrolidone. The enzyme extract was filtered, clarified and dialyzed against double distilled water. The enzyme extract was subjected to ion-exchange column chromatography (4 x 49 cm) on DEAE-Sephadex A-50. The enzyme proteins were eluted with a stepwise gradient of 0.05M - 0.2M NaCl in a total volume of 1200 ml (300 ml each of the four strengths employed). The enzyme active fractions were collected, appropriately pooled and dialyzed. Distinctly separable enzyme fractions were designated as isoforms-I, -II and –III (in order of their protein elution). The enzyme rich fractions corresponding to isoforms-I and -II were pooled, suitably concentrated using dry polyethylene glycol powder (Sigma Chemical Co.) after pouring the elutent into sterilized nitrocellulose tubing at 4^{0}C and used for further studies. The gel permeation chromatography using Sephadex G-200 (1.6 x 140 cm) was carried out for Isoforms-I and -II. The post-gel filtration chromatographic fractions were used for further studies.

Electrophoretic studies:

Native PAGE was carried out in 10% polyacrylamide gels according to the method of Laemmli (1970), and the proteins were visualized after silver staining according to the method of Porro *et al*. (1982).

Enzyme activity determinations:

Acetone-dried powders were extracted overnight at 4°C with 0.05 M sodium acetate buffer (pH 6.6) containing 0.5% polyvinylpyrrolidone. The enzyme extracts were filtered through Whatman No. 1 filter paper; clarified by centrifugation at 10,000 x g for 15 min at 4°C; dialyzed against double distilled water. The reaction mixture consisted of 1.25 mM *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside, 100 mM sodium acetate buffer (pH 5.0) and a suitable aliquot of the enzyme. Incubation was carried out for 15 min at 37^{0}C and the reaction was stopped with the addition of 20 µl 500 mM sodium bicarbonate to the reaction mixture. The colour intensity of the liberated *p*-nitrophenol was measured at 405 nm. One unit of the enzyme is defined as the amount of enzyme required to liberate 1 µmol *p*-nitrophenol per min. Protein content of the enzyme samples was determined by the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard.

Enzyme properties:

Optimum pH for the enzyme activity was determined by measuring the activity of β-hexosaminidase isoforms with *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate at 37°C over a wide range of pH in 100 mM KCl-HCl buffer (pH: 2.0 - 3.0) / sodium acetate buffer (pH: 3.0 - 6.0) / phosphate buffer (pH: 6.0 - 7.0). The reaction was stopped by the addition of 20 µl of 500 mM sodium bicarbonate to the reaction mixture. The colour intensity of the liberated *p*-nitrophenol was measured at 405 nm.

Optimum temperature for enzyme activity was determined by measuring the activity of β-hexosaminidase isoforms with *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside as substrate at different temperatures (27-77°C). The purified enzyme was incubated at varying temperatures in a reaction mixture containing 100 mM sodium acetate buffer, of respective optimum pH for 15 min. The reaction was stopped by the addition of 20 µl of 500 mM sodium bicarbonate to

the reaction mixture. The colour intensity of the liberated *p*-nitrophenol was measured at 405 nm. Temperature stability of the enzyme was determined by pre-incubating the same at varying temperatures (27 - 77°C) for 15 min before determining the enzyme activity at the respective optimum temperature in 100 mM sodium acetate buffer, of respective optimum pH.

Enzyme kinetic parameters - Km and Vmax were determined by carrying out the enzyme reaction with suitably diluted purified enzyme in 100 mM sodium acetate buffer at the optimum pH and optimum temperature of the respective enzyme isoform with varying substrate concentrations of *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside from 1 mM to 20 mM. Effect of divalent metal ions (Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) and EDTA on the enzyme activity was determined by including the respective salts at 1.0 mM concentration in the reaction mixture consisting of 100 mM sodium acetate buffer at optimal pH (of the respective enzyme isoform), 5 mM substrate *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside and incubating at the respective optimum temperature for 15 min.

RESULTS AND DISCUSSION

Activity of β-hexosaminidase in tomato fruits at different stages of fruit development and ripening is displayed in Fig. 1. The specific activity of the enzyme increased towards ripening with a peak at climacteric stage (Fruit stage-6). β-Hexosaminidase of tomato at climacteric stage was purified by ion-exchange chromatography on DEAE- Sephadex A-50, followed by gel permeation chromatography on Sephadex G-200. The purification profile of the enzyme on DEAE-Sephadex-A50 is depicted in Fig. 2. The β-hexosaminidase enzyme activity resolved into three distinct isoforms (I, II and III) by this ion-exchange chromatography upon stepwise elution with 0.05 - 0.2 M sodium chloride, with relative abundance of 57, 35 and 8%, respectively. The elution profile of the isoforms-I and -II when they were further subjected to gel permeation chromatography on Sephadex G-200 are presented in Fig. 3A and 3B. The specific activity of the enzyme increased by about 3-fold for isoform-I and 5.5-fold for isoform II during these

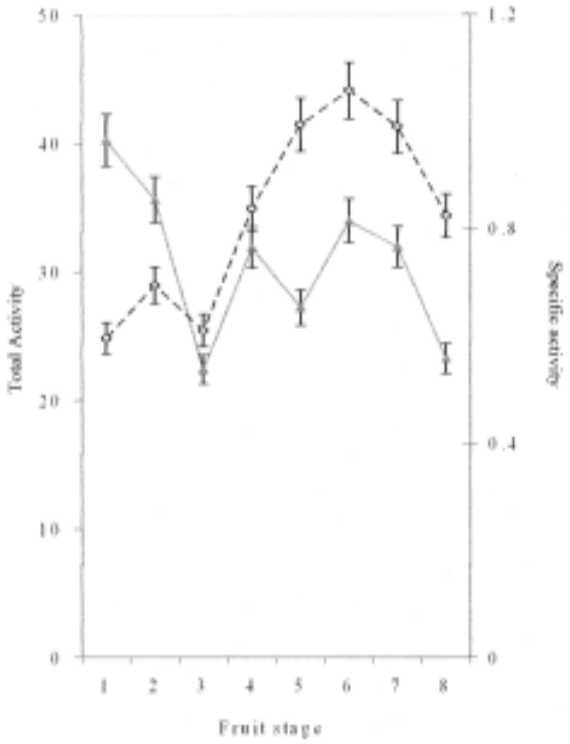

Fig. 1. Activity of β-hexosaminidase at different stages of fruit development and ripening in tomato
(-▲-) Total activity; (-o-) Specific activity
Stages 1-4: Fruit development; Stages 5-8: Fruit ripening

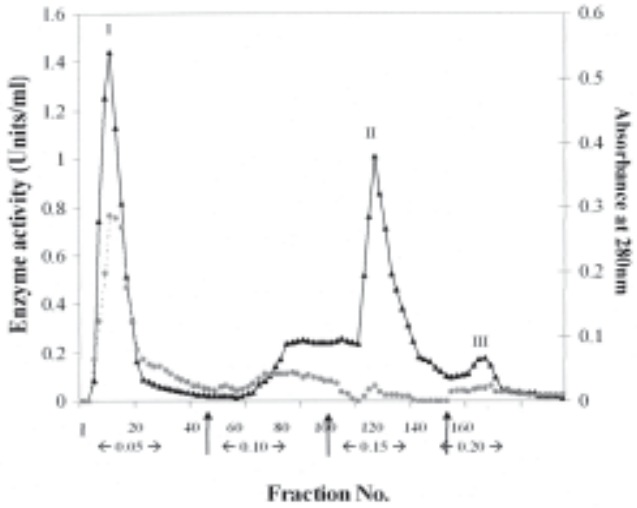

Fig. 2. DEAE-Sephadex A-50 column chromatographic elution profile of β-hexosaminidase isoforms from tomato
Column equilibrated with double distilled water and eluted with increasing concentrations of 0.05, 0.10, 0.15, 0.20 and 0.25M NaCl; ph 6.8 (Flow rate: 0.66 ml/min.) Isoform -I, -II and -III eluted with 0.10, 0.15 and 0.20M NaCl respectively, Arrows indicate the change of NaCl gradient.
(-●-) Absorbance at 280 nm; (-△-) Enzyme activity

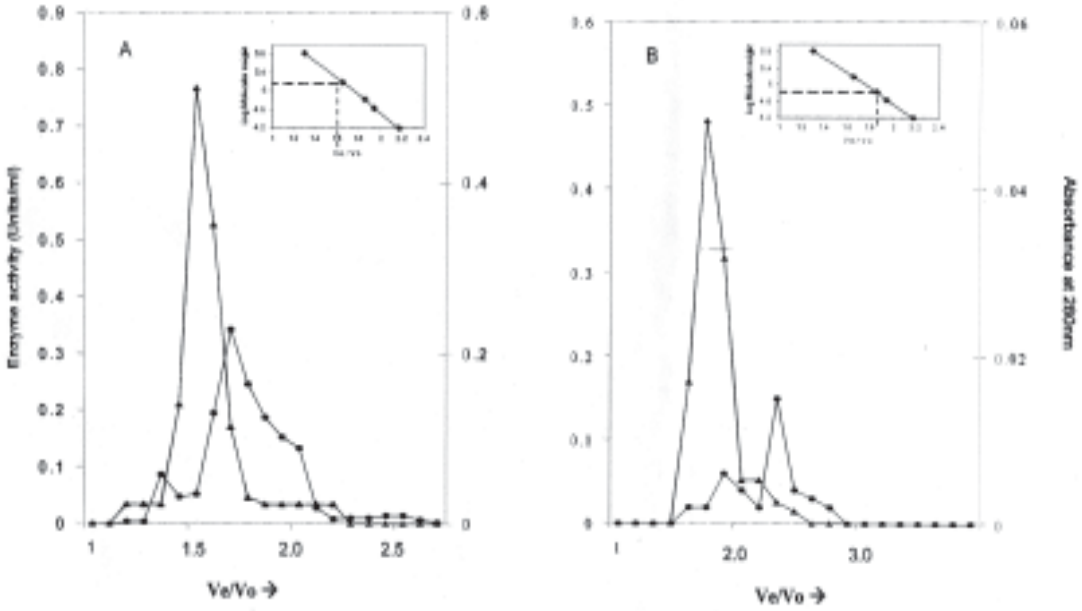

Fig. 3. Gel permeation chromatography profile of β-hexosaminidase isoform I activity from tomato
A: Isoform - I and B: Isoform - II
(-●-) Absorbance at 280 nm; (-△-) Enzyme activity

purification steps with a percent recovery of 24.7 and 16.6, respectively (Table 1).

Table 2 summarizes the properties of β-hexosaminidase isoforms-I and -II as determined in their

Table 1. Purification profile of β-hexosaminidase from tomato fruit

Purification steps	Total protein (mg)	Total activity* (EU)	Specific activity (EU/mg prot.)	Purification fold	Recovery (%)
1. Crude extraction	146.6	368.9	2.52	1.00	100
2. DEAE-Sephadex A-50 Chromatography					
Isoform-I	24.7	153.7	6.23	2.47	41.7
Isoform-II	13.2	95.3	7.22	2.86	25.8
Isoform-III	3.0	20.4	6.70	2.65	5.5
3. Sephadex G-200 Chromatography					
Isoform-I	12.0	91.8	7.63	3.03	24.7
Isoform-II	4.4	61.4	13.9	5.54	16.6

* 1 EU is equivalent to 1 mmol *p*-nitrophenol released per min.

* Activity expressed per 10 g acetone dried powder.

Table 2. Properties of β-hexosaminidase isoforms from tomato

Property	Isoform-I	Isoform-II
pH optimum	5.00	4.60
Temperature optimum	47^{0}C	47-57^{0}C
Thermal stability at 57°C for 15 min	72%	100%
Km for *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside	1.60 mM	1.10 mM
% activity with *p*-nitrophenyl-b-D-N-acetyl galactosaminopyranoside	34.7	35.7
Molecular weight	>94 kDa	~64 kDa

purified forms obtained from gel permeation chromatography. The pH optima for isoform-I and -II were 5.0 and 4.6, respectively. The temperature optimum for isoform-I was 47^{0}C, while for isoform-II it was any temperature in the range 47 - 57^{0}C. The temperature stability at 57^{0}C for 15 min was 72 and 100% for the two isoforms, respectively. The Km of the enzyme for *p*-nitrophenyl-β-N-acetyl glucosamino-pyranoside was 1.6 and 1.1 mM, respectively, for isoform-I and -II. The substrate specificity for these β-hexosaminidase isoforms was comparatively higher for N-acetylglucosamine substrates than for N-acetylgalactosamine substrates (Table 3). Isoform-II of β-hexosaminidase was also observed to possess α-mannosidase activity to a considerable extent. The molecular weights of isoform-I and -II were >94 kD and 64 kD, respectively, as determined by native PAGE (Fig. 4).

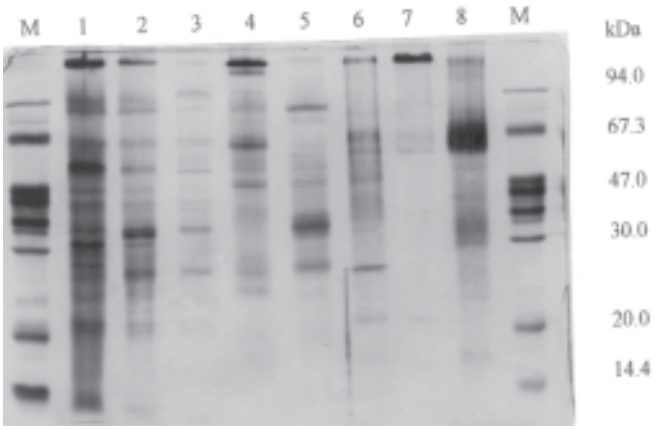

Fig. 4. Native PAGE profile of proteins from tomato fruit at different steps of purification
Lane 1, crude; lane 2, dialyzed; lane 3, neutral unheld proteins; lane 4, isoform-I; lane 5, 0.10M NaCl eluted; lane 6, isoform II (lane 4-6 are from IEC); lane 7, isoform I; lane 8, isoform II (lane 7 and 8 are from GPC) and lane M, m.w. markers

Table 3. Activity of β-hexosaminidase isoforms with various substrates

Substrate	Reflecting enzyme activity	β-hexosaminidase Isoform-I	β-hexosaminidase Isoform-II
1. *p*-nitrophenyl-β-D-N-acetyl glucosaminopyranoside	β-N-acetylhexosaminidase	100	100
2. *p*-nitrophenyl-β-D-N-acetyl galactosaminopyranoside	β-N-acetylhexosaminidase	34.7	35.7
3. *p*-nitrophenyl-α-D-mannopyranoside	α-Mannosidase	0.51	12.6
4. *p*-nitrophenyl-α-D-glucopyranoside	α-Glucosidase	4.0	8.0
5. *p*-nitrophenyl-β-D-glucopyranoside	α-Glucosidase	1.7	17.8
6. *p*-nitrophenyl-α-D-galactopyranoside	α-Galactosidase	8.0	41.0
7. *p*-nitrophenyl-β-D-galactopyranoside	β-Galactosidase	5.2	9.9

Values represent enzyme activity expressed as relative % activity obtained with *p*-nitrophenyl-b-D-N-acetyl glucosaminopyranoside

The effect of divalent metal ions on β-hexosaminidase isoforms is given in Table 4. Divalent metal ions were generally inhibitory to these isoforms of β-hexosaminidase. At 1mM level, Cu^{2+} and Hg^{2+} showed nearly 100% inhibition of both the isoforms of β-hexosaminidase. Zn^{2+} at 1 mM level in the reaction medium showed nearly 95% inhibition for isoform-I and 82% inhibition for isoform-II. Fe^{2+} at 1 mM level in the reaction medium showed 35% inhibition of isoform-I and about 9% inhibition of isoform-II. Mn^{2+} at the same concentration, inhibited isoform-I by 5% while it was 15% for isoform-II. Mg^{2+} and Ca^{2+} did not appear to inhibit b-hexosaminidase activity at 1 mM level in the reaction medium. EDTA (a metal chelator) showed nearly 1.5-fold activation of isoform-I when included at 1 mM level in the reaction medium.

Table 4. Effect of divalent metal ions and metal chelator on β-hexosaminidase activity

Metal ions (1mM)	Activity retained (%) Isoform I	Isoform II
Control	100.0 ± 0.19	100.0 ± 0.30
Cu^{2+}	1.50 ± 0.12	1.7 ± 0.10
Fe^{2+}	65.3 ± 0.21	91.3 ± 0.15
Hg^{2+}	2.0 ± 0.30	2.0 ± 0.32
Mg^{2+}	103.3 ± 0.23	97.3 ± 0.25
Ca^{2+}	106.1 ± 0.30	87.7 ± 0.17
Mn^{2+}	94.7 ± 0.13	84.3 ± 0.13
Zn^{2+}	4.5 ± 0.18	18.0 ± 0.22
EDTA	143.0 ± 0.32	100.0 ± 0.12

Though β-hexosaminidase is shown to be important in animal and microbial systems, its importance in higher plant systems and its significance in fruit ripening has not received any attention. β-hexosaminidase showed a prominent activity with a climacteric peak during ripening in several ripening fruits, it was thought relevant to study this enzyme and its properties in the light of 'fruit ripening'. The study of the enzyme has revealed presence of two major isoforms of β-hexosaminidase during ripening in tomato similar to what was observed by us in bell capsicum (Jagadeesh and Prabha 2002).

The present study has revealed that the enzyme properties of β-hexosaminidase from tomato, viz., optimum pH, optimum temperature, thermal stability and inhibition by metal ions have considerable similarity with those of bell pepper reported earlier (Jagadeesh and Prabha 2002). The only deviation is that, while β-hexosaminidase isoform-I of tomato is resolved with 0.05 M NaCl with an abundance of 57%, the same was resolved with 0.10 M in bell capsicum with an abundance of 38%. The inhibition by the metal ion Cu^{2+} is 100% for isoform-II of tomato. While the inhibition was only 22% for isoform-II of bell capsicum (Jagadeesh and Prabha 2002). The Km value for *p*-nitrophenyl-β-N-acetyl glucosamino-pyranoside was generally higher for the β-hexosaminidase isoforms in bell capsicum than for the corresponding enzyme isoforms of tomato, indicating higher affinity for this substrate in the latter fruit. β-Hexosaminidase enzyme from cabbage has recently been reported to contain three subunits of 51, 57 and 64 kD on SDS-PAGE. Its Km for *p*-nitrophenyl-b-D-N-acetyl-glucosamine was reported to be 0.94 mM, with an optimum pH of 4.0 and optimum temperature of 60^0C (Chang *et al*. 1998). β-Hexosaminidase from lettuce has been partially purified and its relative molecular mass was shown to be 69 kD on SDS-PAGE (Posci *et al*. 1990).

β-Hexosaminidase generates free N-glycans from glycoproteins or any hetero-oligomers / polymers containing hexosamine residues. Presence of free N-glycans is reported in ripe tomato with a presumable role as signal molecules (Priem *et al*. 1993). Both isoform-I and -II of β-hexosaminidase of tomato here were also found to hydrolyze chitin (a large polymer) at pH < 4.0, which suggests that these enzyme isoforms may act as both glycosidase and glycanase (Figure not shown). Whether such types of oligo- or polymeric fractions (glycoproteins or otherwise) are present in the *in vivo* situation of the fruit remains to be established. α-Mannosidase is a glycoprotein enzyme containing N-acetyl-glucosamine and mannose residues, and is a glycosidase implicated in fruit ripening. Mannosidase enzyme from jack bean is reported to be a glycoprotein containing N-glycan residues (Bowles *et al*. 1983). Interestingly, isoforms of α-mannosidase from both capsicum and tomato were also found to be glycoproteins (Priyasethu and Prabha 1997, Jagadeesh *et al*. 2004,

2004a). α-Mannosidase as a glycoprotein could itself be a susceptible substrate for β-hexosaminidase. This is suggested from the somewhat close interrelationship between b-hexosaminidase and α-mannosidase enzymes in fruits like bell capsicum and tomato during ripening.

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