Changes in the Activities of Glutamine Synthetase and Glutamate Dehydrogenase of Asparagus Spears after Harvest

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Respiration rate, glutamine/glutamic acid levels and the activities of glutamine synthetase (GS, EC 6.3.1.2) and glutamate dehydrogenase (GDH, EC 1.4.1.2) in the top and bottom portions of harvested asparagus spears (Asparagus officinalis L. cv. Welcome) were examined. Spears were stored for up to 5 days at 25°C. Respiration rate (CO₂ production) of the spears declined during the first 2 days of storage and increased subsequently until the end of the storage period. The respiration rate of the top portion was over 2 or 3 times greater than that of the bottom portion or intact spears. GS activity increased in the top and bottom portions of the spears after 1 day and declined to about 38% and 65% of the initial levels by day 5, respectively. GDH activities in the top portion as well as the bottom portion showed an increasing trend until the end of the storage. Glutamine level increased, whereas glutamic acid decreased in concentration substantially during the first 24 h after harvest, in the top and bottom portions of the spears. There was a highly significant negative correlation observed between GS activity and the glutamine and glutamic acid content in the top and bottom portions of the spears. On the other hand, a highly significant positive correlation between GDH activity and the glutamine and glutamic acid content in the top and bottom portions of the spears was observed.

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Freshly harvested asparagus spears are highly perishable, having a postharvest life of 2-5 days at ambient temperatures¹. Tips of spears, comprised mainly of immature tissue, are particularly perishable and are usually the first part of the spear to show symptoms of postharvest deterioration, e.g. feathering and browning of bracts, tissue flaccidity and cellular breakdown ² ³ ⁴. The spears deteriorates rapidly with physiological changes including reduced respiration rate, toughening, flavor changes and losses of chlorophyll, ascorbic acid, soluble carbohydrate, protein, and amino acids ⁴ ⁵ ⁶ ⁷ ⁸ ⁹. Within 48 h after harvest, respiration rate of tips declines, protein was markedly lost, free amino acids increased, and ammonia started to accumulate ⁵ ⁷. Subsequent tissue deterioration rendered the tips unappealing to consumers. A better knowledge underlying changes occurring in tips of harvested spears may contribute to an understanding of the nature of the deterioration process that accompanies postharvest storage of asparagus spears.

Glutamate synthesis (GS) has been considered to be a focal point of nitrogen metabolism in living organisms. GS catalyses the first step in amino acid biosynthesis from ammonia¹⁰. Its product, glutamine, is the main source of nitrogen for the synthesis of all other nitrogen-containing compounds, including other amino acids and nucleotides (e.g. ATP). Glutamate dehydrogenase (GDH) has long been accepted as a major enzyme not only in amino acid biosynthesis but also in metabolic connection between the tricarboxylic
The discovery of glutamate synthase, however, sheds light on the pathway of ammonia assimilation in higher plants. It is now accepted that the incorporation of ammonia into amino acids occurs mainly via the GS/glutamate synthase (GOGAT) -catalyzed reactions\(^\text{12--13}\). On the other hand, the view persists that GDH acts as an alternative to the glutamate synthase cycle under conditions of high ammonia concentration\(^\text{14--16}\) or stress\(^\text{17}\), or it could act in the deamination of glutamate connecting the GDH with the carbon metabolism rather than nitrogen metabolism\(^\text{18}\).

To understand further the postharvest physiology of asparagus, this study has been undertaken to examine the activities of GS and GDH enzymes in the top and bottom portions of the spears and their amino acid content during their shelf-life at 25°C. Changes in the respiration rate and amino acid levels were reported and the results in relation to other metabolic changes in spear tips were discussed.

**Materials and Methods**

**Plant materials**

Green asparagus spears (Asparagus officinalis L. cv. Welcome) harvested from a commercial crop in Miki-cho, Ikenobe, Kagawa, Japan were obtained directly from a packing house. ‘Welcome’ is adapted to moderate cooler conditions and mainly grown in Japan. Spears were hand harvested and trimmed to approximately 25 cm length. The spears, which were of good quality, straight with closed bracts, were put in plastic bags and held at 25°C for up to 5 days. At harvest (day 0) and subsequent 24 h intervals, the spears were weighed and frozen at -30°C for amino acid and enzyme assays.

**Respiration rate measurement**

The spears were weighed and carefully placed in a 6-liter glass jar held at 25°C. Three replicates of 7 spears were used in the experiment. Carbon dioxide production was measured on intact spears, top and bottom portions at 24 h intervals. Production of CO\(_2\) was measured by taking 10 ml gas samples from the glass jar sealed for 1 h and injected to a TCD gas chromatograph equipped with a 1 m activated charcoal column at 65°C (GC-8 AIT, Shimadzu Co. Ltd.). The results were expressed as ml CO\(_2\) kg\(^{-1}\) h\(^{-1}\).

**Enzyme extraction**

Each spear was cut into two equal halves (designated as the Top and Bottom portion) just before extraction. Approximately 5 g sample from each portion were homogenized under ice-cold conditions (ca. 0 ~ 4°C) with 1% polyvinylpolypyrrolidone (PVPP), proportional to the sample weight and 1 g of sea sand in Buffer A by using a mortar and pestle. One ml extraction buffer per g fresh weight of plant materials was used. Extraction was performed according to a method of HURST and CLARK\(^\text{19}\), in which Buffer A contained 50 mM Tris--HCl (pH 7.6), 10 mM MgSO\(_4\), 1 mM EDTA, 1 mM dithiothreitol (DTT), 12 mM 2-mercaptoethanol, 5 mM glutamate and 100 ml liter\(^{-1}\) glycerol. The homogenate was squeezed through a four-layer cotton cloth and the filtrate was centrifuged at 11 000 x g for 10 min. The residual tissues were re-extracted in 5 ml buffer A and dialyzed with 40 times dilution of the same buffer for 1 h and then centrifuged. The resulting supernatant were mixed together and used for the enzyme assay.

**Enzyme assays**

The enzymatic activities were assayed in a total volume of 1.0 ml. For GS, 80 mM L-glutamate--Na, 500 mM Tricine--KOH Buffer (pH 7.0), 600 mM NH\(_2\)OH, 200 mM MgSO\(_4\)7H\(_2\)O, 10 mM Diethylenetriamine pentaacetic acid (DTPA), 80 mM ATP and 800 mM mercaptoethanol were used. After incubating at 35°C for 8 min, the reaction was stopped by adding 1 ml ferric reagent (25 ml FeCl\(_3\). 6H\(_2\)O, 50 ml HCl and 20 ml TCA). The activities were monitored using a double--beam spectrophotometer (Shimadzu model UV-150-02) at 540 nm. The GDH activity was determined in both aminating and deaminating directions in a total volume of 1.0 ml. For GDH amination, 20 mM \(\alpha\)-ketoglutarate, 100 mM Tris--HCl (pH 8.0), 200 mM NH\(_4\)Cl, 1.0 mM CaCl\(_2\), 0.2 mM NAD(P)H and
100 µl enzyme solution were used. The GDH deamination activities were assayed with a mixture containing 100 mM L-Glutamate, 100 mM Tris–HCl (pH 9.3), 1.0 mM NAD(P)+, 0.5 mM CaCl2 and 100 µl enzyme solution. The GDH amination and deamination activities were monitored using a double-beam spectrophotometer (Shimadzu model UV-150-02) at 350 nm to NADH oxidation or NAD+ reduction. One unit of GDH activity is defined as the reduction or oxidation of 1 micromole of coenzyme (NADH / NADPH, respectively) per min at 30°C.

**Extraction and analysis of amino acids**

Triplicate samples of 10 g fresh weight were homogenized under ice-cold conditions with 15 ml of 75% ethanol then incubated in a water bath shaker at 45°C for 30 min. The solutions were filtered into a flask. The residues were added with 15 ml 75% ethanol and placed again in a water bath at 45°C for 30 min. The filtrate was evaporated under reduced pressure, adjusted to 10 ml with deionized water and centrifuged at 11 000 x g for 10 min. Following extraction, the aqueous solution (containing the amino acids) was loaded onto an Ion Exchange Resin (cation-exchange, H+- Amberlite IR 120) column (1.5 i.d. x 2 cm diameter), eluted and washed with water until pH 6~7. Finally, 25 ml 1N NH3 was poured through the column to elute the amino acids. Eluates were evaporated as described above and then made up to 10 ml with 0.02 N HCl and filtered through a nitrate cellulose membrane filter (0.45µm pore size). The ammoniacal eluates were stored in a vial at -20°C until analysis. The amino acid content was determined using an automatic amino acid analyzer (Hitachi L-8500) equipped with a Chromato-Integrator (Hitachi D-2850).

**Statistics**

A randomized complete block design was used with three replications. The level of significance was calculated from the F value of ANOVA. Linear correlation was used to evaluate the relationship between amino acid accumulation and enzyme activities.

**Results and Discussion**

**Respiration rate**

The respiration rate of asparagus (intact spears, top and bottom portions) stored at 25°C is shown in Fig 1. Respiration rates of all samples declined during the first 2 days of storage and increased subsequently until the end of the storage period. The respiration rates in asparagus spears were significantly different among intact and different portions of the spears. The rate the of top portion was over 2 or 3 times greater than that of the bottom portion or intact spears, respectively. This reflects a major gradient in metabolic activity along the spear, with apical tissue involved in active cell division and the initial stages of cell elongation showing the highest respiration rate.

![Fig. 1 Respiration rate of intact spears, top and bottom portions of the spears held at 25°C.](image)

The pattern of respiration rate changes of the spear in this study is very similar to that reported in whole spears, some other vegetables, and plants put in conditions that lead to depletion of carbohydrate reserves. TRIPPI et al. associated a decline in respiratory activity with senescence in cut flowers and suggested it was related to nucleotide metabolism. Harvested asparagus immediately removes the source of respiratory substrates and may lead to substrate limitation of respiration rate. It was observed that CO2 production of asparagus spears increased after 3 days until the end of the storage period. This
finding was confirmed by PLATENIUS\textsuperscript{5,23} and KING \textit{et al.}\textsuperscript{2} where more CO$_2$ was released from asparagus spears over 3~5 days. The increased respiration rate was significantly higher in the top portion by about 24\% and 45\% than the bottom portion and intact spears, respectively, at the end of 5 days storage. The results may be characterized by the type of the tissue. BURTON\textsuperscript{24} reported that in asparagus the pattern of deterioration is influenced by the heterogeneity of the tissues: the tip comprises actively dividing meristematic cells grading into a zone of cellular elongation, whereas the bottom portion comprises more mature tissue where cell elongation has ceased and the vascular tissues is lignifying. The changes in quality, such as feathering, desiccation or toughening were noted during the storage experiment.

\textbf{Enzyme activities}

GS and GDH activities in the top and bottom portions of the spears during storage at 25\degree C are shown in Figs. 2 and 3, respectively. The GS activity increased in the top and bottom portions of the spears after 1 day and declined to about 38 \% and 65 \% of the initial levels by day 5, respectively. A decline of the GS activity was also found in asparagus spears held at 20\degree C\textsuperscript{19}. The decline in GS activity is a response to harvest and may be the consequence of autophagic processes\textsuperscript{25} induced by carbohydrate deprivation in harvested asparagus spears\textsuperscript{26}. GS showed significantly higher activities in the top portion than the bottom portion of the spears. GS activity would be expected to be highest in the tip of the spear since this is the region of the highest protein content and cell division\textsuperscript{1,4}. KING \textit{et al.}\textsuperscript{26} considered the possibility that ammonia accumulation may be a factor in the perishability of asparagus, and that it may occur due to changes in GS activity or other nitrogen metabolizing enzymes after harvest.

The GDH amination (NADH-dependant) activity of the top portion was significantly higher than the bottom portion, while a significant higher activity in the GDH deamination (NAD$^+$-dependant) was found after 1, 3 and 5 days storage in the top portion than the bottom portion of the spears. Both GDH amination (NADH) and GDH deamination (NAD$^+$) activities almost double over the 5 day postharvest period both in the top and bottom portions of the spears, but GDH amination was relatively higher than GDH deamination. GIVAN\textsuperscript{27} and LAURIERE and DAUSSAN\textsuperscript{28} suggested that ammonia produced by proteolysis could be responsible for the observed increase in the aminating (NADH) activity of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Glutamine synthetase activity in the top and bottom portions of the spears held at 25\degree C. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbol.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Changes in the activities of (A) amination and (B) deamination glutamate dehydrogenase enzyme in the top and bottom portions of the spears held at 25\degree C. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbol.}
\end{figure}
The considerable increase of deamination (NAD⁺) GDH activity during storage showed that the enzyme also operates in the direction of energy generation. It is believed that GDH amination (NADH) dealt with high levels of nitrogen produced e.g. stress, and GDH deamination (NAD⁺) can provide the cell with energy ensuring the continued operation of the citric acid cycle in the face of carbohydrate starvation [39]. The increasing GDH activities after harvest, followed a similar control mechanism which governs the course of senescence [30-32] and in some cases parallels ammonium accumulation. The results of the present study showed that a rise in GDH activity is linked with a corresponding decline in GS activity which confirmed a similar result in asparagus spears [30] and in Pisum sativum [31]. Since GS enzyme decreases during senescence [31], the activity of GDH may possibly serve as an adaptation to detoxify the ammonium generated during senescence.

Glutamine and glutamic acid content

Fig. 4 shows the glutamine and glutamic acid levels of asparagus spears in the top and bottom portions of the spears held at 25°C for 5 days. Both glutamine and glutamic acid contents were significantly higher in the top portion than in the bottom portion of the spears during storage up to the 3rd and 4th day after harvest. Glutamine content increased after 1 day of storage and then declined until the end of the storage period while glutamic acid declined continuously during storage both in the top and bottom portions of the spears. Postharvest losses of protein and amino acid have been described in asparagus [30,31]. Though glutamine is known to be the principal N-translocator, the rise in glutamine level measured here during the first 24 h storage at 25°C was of little importance quantitatively (Fig. 4), which corresponds with the decrease in GS activity (Fig. 2). In some cases glutamine did not accumulate substantially [30]. The glutamine and glutamic acid content ranging from 118.53 to 1373.7 nmol/g and from 138.21 to 1041.67 nmol/g fresh weight, respectively were observed after 24 ~ 72 h of storage. At the end of the storage period, both glutamine and glutamic acid declined to almost the same lower level both in the top and bottom portions of the spears.

RHODES et al. [30] suggested that glutamine could regulate the levels of GDH and GS, which implies that a single metabolite of ammonia assimilation is responsible for directing the flow of ammonia into amino acids via the two alternative routes of ammonia assimilation and they are concurrently regulated by a common metabolite. These results seem to agree with the findings of RHODES et al. [30] that the inverse relationship demonstrated between GS and GDH suggests that glutamine acts as a repressor of GS and an inducer of GDH. The mechanism whereby, when the reduction in the potential to assimilate toxic concentrations of ammonia was reduced by repression of GS, this was compensated for by an increase in GDH level. Thus the combined operation of these two ammonia assimilating enzymes could not only provide the necessary glutamate for biosynthesis but also maintain a potential to prevent excessive

Fig. 4 Glutamine and glutamic acid contents in the top and bottom portions of the spears held at 25°C. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbol.
Correlation between the enzyme activities of GS and GDH and the glutamine and glutamic acid contents

Table 1 shows the correlation coefficients ($r$) between the activities of GS and GDH and the glutamine and glutamic acid contents in the top and bottom portions of the spears. There was a highly significant negative correlation observed between the GS and the glutamine and glutamic acid content in the top and bottom portions of the spears. On the other hand, a highly significant positive relationship was evident between GDH and the glutamine and glutamic acid content in the top and bottom portions of the spears with a correlation coefficient of $r=0.667 \sim 0.831$ and $0.641 \sim 0.868$, respectively. These observations showed that the increased GDH activity during storage with a declined GS activity suggests that both pathways for ammonia assimilation were independent of the nitrogen source and maybe regulated through an interaction of carbon supply and glutamate concentrations. It has been suggested that glutamine would exert a positive control on GDH but a negative one on GS and therefore it may determine the entry of ammonia via either pathway.\textsuperscript{20,36}

Table 1 Correlation coefficients ($r$) between the activities of glutamine synthetase and glutamate dehydrogenase and some amino acid content in the top and bottom portions of asparagus spears held at 25°C for 5 days.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Portions</th>
<th>Glutamine</th>
<th>Glutamate</th>
<th>Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Synthetase</td>
<td>Amination</td>
<td>Deamination</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Top</td>
<td>$-0.705^{**}$</td>
<td>0.667^{**}</td>
<td>0.698^{**}</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>$-0.719^{**}$</td>
<td>0.861^{**}</td>
<td>0.641^{**}</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Top</td>
<td>$-0.963^{**}$</td>
<td>0.831^{**}</td>
<td>0.791^{**}</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>$-0.752^{**}$</td>
<td>0.868^{**}</td>
<td>0.626^{**}</td>
</tr>
</tbody>
</table>

** denote significant at $p<0.01$, $n=18$

References

5) PLATENIUS, H.: Plant Physiol., 17, 179 (1942)
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23) PLATENIUS, H.: *Plant Physiol.*, 18, 671 (1943)
27) GIVAN, C.: *Phytochemistry*, 18, 375 (1979)

(35) (Article) Changes in the Activities of GS

ASASPARAGUS 若葉収穫後のグルタミン合成酵素とグルタミン酸脱水素酵素活性变化

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収穫されたアスパラガス若葉（Asparagus Officinalis L.）は、上部と下部の呼吸速度、グルタミンとグルタミン酸濃度とグルタミン合成酵素（GS, EC 6.3.1.2）、グルタミン酸脱水素酵素（GDH, EC 1.4.1.2）活性について検討した。若葉は25℃で5日まで貯蔵された。若葉の呼吸速度（炭酸ガス生成）は貯蔵2日目に減少し、次いで貯蔵最終日まで増加した。上部の呼吸速度は下部成虫の各々の各々2倍か3倍以上であった。GS活性は1日後上部と下部の若葉で増大したが、5日に初の38%と約65%に各々減少した。上部と同様に下部におけるGDH活性は、貯蔵最終日まで増加傾向を示した。若葉上・下部のグルタミン濃度は増加し、一方グルタミン酸は収穫後初めの24時間で本質的に濃度が減少した。若葉の上・下部のGS活性とグルタミン、グルタミン酸濃度の間に負の相関が認められた。他方、若葉の上・下部のGDH活性とグルタミン、グルタミン酸濃度の間に正の相関が認められた。

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