Fluorometric Biosensing of the Total Amino Acid Content and the Glutamate Content of Green Tea Infusions Using an Automated Multi-channel Flow System

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An automated multi-channel continuous-flow analyzing system was constructed for the rapid measurement of both the total amino acid content and the L-glutamate content of green tea infusions. L-Glutamate in samples was oxidized and deaminated enzymatically in a channel with a mini-reactor that was packed with l-glutamate oxidase immobilized on glass beads, and the evolved ammonium ions were measured by a fluorogenic reaction with o-phthalaldehyde and β-mercaptoethanol. The total amino acid content was measured in a channel with another mini-reactor which was filled with immobilized l-amino acid oxidase. The fluorometric method is not subject to interference by reducing substances such as tea catechins and ascorbate, and the analytes in tea infusions were measured without pretreatment of samples. The use of this system for evaluation of tea quality was investigated.

Key words: fluorometric biosensing; multi-channel flow system; L-glutamate; total amino acid; green tea

Information about the total amino acid content of green tea infusions is significant for the evaluation of the quality of the tea. 1) and several analytical methods have been developed for this purpose. 2) An approach using a flow-through sensor based on l-amino acid oxidase 3) was reported recently to provide an alternative method for measuring the amino acid content of tea infusions amperometrically (with an oxygen electrode) without pretreatment of samples.

The L-glutamate content is another important variable for the evaluation of tea quality. Compared with infusions of stems, infusions of tea leaves are rich in L-glutamate. 4) Adulteration of commercial tea beverages by addition of L-glutamate has been reported to be a trick for improving their taste. 5) Methods to measure both the total amino acid content and the glutamate content of green tea infusions are not yet available and are urgently required.

Approaches using an enzyme electrode have been restricted to single-component studies. However, approaches that incorporate a bioreactor in the manifold of a multi-channel flow-injection system have the potential for multi-component measurements. 5) An automated multi-channel flow system developed previously was characterized by improved performance. 5) This report describes a modification of this system, whereby both the total amino acid content and the L-glutamate content of green tea infusions can be measured without pretreatment of samples.

Materials and Methods

Chemicals. L-Glutamate oxidase (Gl ox: EC 1.4.3.11; from Streptomyces sp. X119-6; 6.8 U/mg) was obtained from Yamaha Shoya (Chiba, Japan). Catalase (EC 1.11.1.6; from bovine liver; 6500 U/mg) and L-amino acid oxidase (L-AOD; EC 1.4.3.2; from Crotalus durissus; 7 U/mg) were purchased from Boehringer Mannheim Yamanouchi Co. (Tokyo, Japan). Aminopropyl glass beads of controlled pore size (aminopropyl-CPG; 80-120 mesh, average pore diameter, 70 nm: amine content, 100 μmol/g) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glutaraldehyde (25%, w/w), o-phthalaldehyde (OPA), and 2-mercaptoethanol (99%) were from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of analytical-reagent grade and were used without further purification.

Preparation of green tea infusions. Tea leaves of various grades were kindly donated by the Fukuoka Institute for Tea Production. Tea infusions were prepared by the method typically used for sensory tests. In brief, 1.5 g of tea leaves were infused for 5 min in 90 ml of hot water (75 °C) and the infusions were filtered through standard filter paper (No. 2 filter paper: Advantec Co., Tokyo, Japan).

Preparation of the OPA reagent. The OPA labeling reagent was prepared by dissolving 0.84 g of OPA (in 10 ml of ethanol) and 1.32 ml of 2-mercaptoethanol in 1 liter of 0.1 M phosphate buffer (pH 6.8). The reagent was generally prepared about 24 h before use, covered, and left at room temperature under normal laboratory lighting conditions.

Preparation of the immobilized enzyme reactors. L-Glutamate oxidase (20 U) and catalase (260 × 10^3 U) were successively immobilized on 0.06 g (dry weight) of aminopropyl-CPG as described previously, 6) and the immobilized enzyme gel was packed into a glass tube (5 cm × 2 mm i.d.) to serve as the L-GluOD reactor. L-amino acid oxidase (15 U) and catalase (260 × 10^3 U) were successively immobilized on 0.18 g (dry weight) of aminopropyl-CPG by the same procedures, and the gel was packed into a spindle-shaped glass tube (0.5 ml) to serve as the L-AOD reactor. The unreacted functional groups of glutaraldehyde that attached on the immobilized L-AOD gel were not masked by glycine 7) since L-AOD reacts slightly with glycine. Both reactors were stable for more than 4 months when immersed in 0.1 M phosphate buffer (pH 6.8) at 4 °C.

Instrument. The automated multi-channel flow system that was described previously 6) was used and the flow (0.7 ml min; controlled by a peristaltic pump) from the outlet of the flow system (Fig. 1) was merged with the stream of the OPA reagent (1.2 ml min; controlled by another peristaltic pump). The merged flow was forced through the reaction coil (tapered stainless tube, 100 cm × 1 mm i.d.) which was immersed in a water bath (63 °C). The fluorescence of the resultant OPA-derivatives was detected with a flow-through fluorometer (FS-8000; Tosoh, Tokyo, Japan). The excitation and the emission wavelengths were 410 nm and 470 nm, respectively.

Analysis of the L-glutamate content by HPLC. A tea infusion (diluted 10-fold with 10 mM phosphate buffer, pH 6.8) was filtered through a membrane filter (0.45 μm; Advantec Co.), and the filtrate was used directly without any pretreatment. A pre-column labeling HPLC method was used to measure the glutamate content, as described previously. 7)

Results and Discussion

The pH of the system was maintained within the optimum
Fig. 1. Schematic Representation of the System.

Fig. 2. Response Signals for Measurements of the L-Glutamate.

Table 1. Course of the Flow-line Switching Protocol Used in the Measurements of L-Glutamate and Total Amino Acids

<table>
<thead>
<tr>
<th>Operating conditions</th>
<th>Valve 1 position</th>
<th>Valve 2 Position (channel)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 2</td>
<td>Phosphate buffer</td>
<td>Waste</td>
<td>System purging and feeding of sample</td>
</tr>
<tr>
<td>2 6</td>
<td>Glutamate standard, samples 1, 2, 3, sequentially</td>
<td>Blank</td>
<td>Channel purging and baseline reading</td>
</tr>
<tr>
<td>6 26</td>
<td>Glutamate standard, samples 1, 2, and 3, sequentially</td>
<td>Blank</td>
<td>Blank reading</td>
</tr>
<tr>
<td>26 28</td>
<td>Phosphate buffer</td>
<td>Blank</td>
<td>Channel purging</td>
</tr>
<tr>
<td>28 30</td>
<td>Glutamate standard, samples 1, 2, and 3, sequentially</td>
<td>GluOD</td>
<td>Baseline reading</td>
</tr>
<tr>
<td>30 46</td>
<td>Glutamate standard, samples 1, 2, and 3, sequentially</td>
<td>GluOD</td>
<td>Determination of glutamate</td>
</tr>
<tr>
<td>46 48</td>
<td>Phosphate buffer</td>
<td>GluOD</td>
<td>Channel purging</td>
</tr>
<tr>
<td>48 50</td>
<td>Phosphate buffer</td>
<td>AOD</td>
<td>Baseline reading</td>
</tr>
<tr>
<td>50 66</td>
<td>Theanine standard samples 1, 2, and 3, sequentially</td>
<td>AOD</td>
<td>Determination of total amino acids</td>
</tr>
<tr>
<td>66 70</td>
<td>Phosphate buffer</td>
<td>AOD</td>
<td>Channel purging</td>
</tr>
</tbody>
</table>

pH range for both the immobilized amino acid oxidase and the immobilized glutamate oxidase by 0.1 M phosphate buffer (pH 6.8). The pH of all the test solutions was adjusted with the same buffer. The flow of the test solutions was directed by the two rotary flow-line switching valves (Fig. 1) and programmed as described previously. In brief, different samples were sequentially aspirated into the Blank channel and the GluOD channel for measurements of L-glutamate.

Typical response curves obtained from the “Blank” channel and the “L-GluOD” channel for the measurement of L-glutamate are shown in Fig. 2.
Quantitation of glutamate

Glutamate oxidase catalyzes the following reaction and its substrate specificity is high. \(^{10}\)

\[
\begin{align*}
1-\text{Glutamate} + O_2 & \rightarrow 1-\text{GluOD} \\
& \rightarrow 2-\text{Ketoglutarate} + H_2O_2 + NH_4^+
\end{align*}
\]

Measurement of the evolved hydrogen peroxide is the general method for calculating the starting concentration of glutamate. However, reducing substances, such as catechins and ascorbate, in green tea infusions disturb such measurements. The proposed method makes use of the fluorogenic reaction of o-phthalaldehyde (OPA) with ammonium ions (and also primary amines), which is immune to these reducing agents. At neutral pH, ammonium ions react well with OPA in the presence of β-mercaptoethanol to yield an isosindole derivative with a high quantum yield of fluorescence. \(^{13}\) By contrast, amines and amino acids produce much less fluorescence and the selectivity for ammonium ions can be further improved when on-line derivatization in the reported flow system is included. \(^{11}\) The gain in fluorescence intensity after the treatment with the enzyme is a function of the amount of glutamate consumed (and also the amount of ammonium ions generated), as described by the following formula:

\[
dF = (Y_{\text{NH}_4} - Y_{\text{glutamate}}) \times N
\]

Where \(dF\) is the difference in the fluorescence intensity before after the enzymatic reaction and \(Y_{\text{NH}_4}\) and \(Y_{\text{glutamate}}\) represent the numerical products of the reaction rates of the fluorogenic reaction and the quantum yields of the isosindole derivatives for ammonium ion and glutamate, respectively. \(N\) is the number of glutamate molecules consumed in the enzymatic reaction.

Since the data obtained by the signal-subtraction procedure are directly proportional to the extent of the enzymatic reaction, the selectivity of this method relies basically on that of the enzyme. Although other chemical methods with higher selectivity for ammonium ions are available, \(^{12}\) the specificity of the chemical reaction is not important in this case. Fluorometric methods have the virtue of sensitivity, and this fluorogenic reaction, which requires no unusual reaction conditions, can easily be adapted to flow analysis.

The multi-channel flow analyzing system developed previously is useful for measurements that rely on signal subtraction. \(^{10}\) As illustrated in Fig. 1, the OPA reagent merged with the solution from the outlet of the multi-channel flow system. The merged solutions passed through the reaction coil to generate fluorescent derivatives. The difference in fluorescence obtained from the "Blank" channel and the "1-GluOD" channel (Fig. 2) was used to estimate the 1-glutamate content. The assumption included in this formula was validated by the calibration curve (Fig. 3) obtained from data retrieved from Fig. 2, and the analytical results with real samples (green tea infusions) agreed with the results obtained by HPLC (Fig. 4). The sample recovery and the reproducibility of results with this system were 96.3\% and 1.7 (c.v., \(n = 6\)), respectively.

Measurement of total amino acids

The \(1\)-amino acid oxidase purified from snake venom has been used previously to assay total amino acids in green tea infusions. \(^{11}\) The enzyme was very reactive with theanine, a tea-specific amino acid which makes a major contribution to the "Umami" taste of the infusion. \(^{12}\) The activity of this non-specific enzyme in assays of the major amino acids in tea was investigated with this system, and the results are tabulated in Table II (second column). The major amino acids in green tea infusion are theanine 54.4\%, arginine

\[
\begin{align*}
\text{Table II. Substrate Specificity of 1-Amino Acid Oxidase and the Relative Levels of the Major Amino Acids (as Percentages) in Tea Leaves} \\
\end{align*}
\]

\[
\begin{align*}
\text{Major amino} & \text{ acid in tea} & \text{Relative activity of the oxidase} & \text{Composition}\% & \text{Calculated} \\
\text{leaves} & (\times a) & (w \ w \ ^{\circ}a) & (w \ w \ ^{\circ}a) & \text{output of the system}\% \\
1-\text{Theanine} & 100.0 & 54.4 & 84.6 \\
1-\text{Glutamine} & 111.1 & 0.0 & 0.0 \\
1-\text{Arginine} & 61.1 & 13.4 & 12.7 \\
1-\text{Asparagine} & 55.6 & 0.0 & 0.0 \\
1-\text{Aspartate} & 11.3 & 9.4 & 1.7 \\
1-\text{Glutamate} & 8.3 & 8.7 & 1.1 \\
1-\text{Threonine} & 0.0 & 2.4 & 0.0 \\
1-\text{Lysine} & 0.0 & 1.0 & 0.0 \\
\end{align*}
\]

\(^{3}\) From ref. 8.

\(^{4}\) From the data of the preceding two columns.
13.4%, and asparatate 9.4% (Table II, third column). The contribution of theanine to the response of the enzyme was estimated to be 84.6%. Thus, we used theanine as the standard for assays of total amino acids in the following experiment, regarding the typical amino acid (theanine) as total amino acids in green tea infusion.

Application to the evaluation of the quality of green tea

Figure 5 shows the results of measurements of L-glutamate and total amino acids in infusions of 28 different green teas. A positive correlation is evident between the two components. Adulterations that change the amino acid profile will result in a deviation of the relationship between total amino acids and glutamate (Fig. 5). In Fig. 6, a comparison is shown of both the total amino acid and the L-glutamate contents of tea infusions with the prices of the tested tea leaves in local markets. Both components were found to be good indicators of quality. The proposed method, by virtue of its sensitivity and simplicity, seems to be useful for the evaluation of tea quality. The novel approach, using a combination of specific and nonspecific amino acid oxidases (deaminating) with the OPA-NH$_4^+$ fluorogenic reaction, holds promise for the development of other amino acid sensors for other purposes.

References