Determination of the Lysinoalanine Content in Commercial Foods by Gas Chromatography-selected Ion Monitoring

Kiyozo Hasegawa, Kaori Mukai,* Miho Gotoh, Sayoko Honjo and Teruyoshi Matoba

Department of Food Science and Nutrition, Nara Women's University, Nara 630, Japan

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A procedure for the quantitative determination of lysinoalanine (LAL) by gas chromatography-selected ion monitoring was developed. α,ε-Diaminopimelic acid was chosen as the internal standard, and the content of LAL in commercial foods was analyzed. Of the samples analyzed, the content was highest in pidan. Wheat flour-based products (Chinese noodles, pretzels and crackers) and milk products (condensed milk and lactic acid beverages) also contained a significant amount of LAL. Ordinary milk, soybean (soy milk and soy protein isolate) and meat products (ham and Hamburg steak) contained a low amount of LAL. It was confirmed that the possibility of low-level LAL formation existed in foods cooked at home without any alkaline treatment.

Lysinoalanine (LAL) is a cross-linked amino acid, which is formed during the alkaline or severe heat treatment of proteins by the reaction of the ε-amino group of the lysine residue with the double bond of the dehydroalanine residue that is produced from O-substituted serine or cystine residues.1,2) The formation of LAL in proteins is known to reduce the digestibility of proteins and to remove the nutritional availability of lysine and cystine.3~8) Moreover, LAL has been reported to cause a unique renal lesion, designated nephrocytomegaly, in rats9~11) and to inhibit the biological activity of metalloenzymes.12) This information points to the presence of LAL in foods leading to adverse effects on the food quality. Therefore, a wide screening for the LAL content in many foods is important.

There have been several reports concerning the estimation of LAL content in food proteins.13~16) In these reports, the determination of LAL was carried out by ion-exchange chromatography,13,14) gas chromatography17,18) and thin-layer chromatography.19) In the proteins of home-cooked and commercial foods, unusual amino acids other than primary protein amino acids are produced from the reaction of the proteins with other food components during processing, storage and cooking. Therefore, the determination of LAL by these chromatographic methods is liable to give erroneous results, since the peak or spot of these unusual and unidentified amino acids on these chromatograms may overlap with that of LAL. However, the determination of LAL by gas chromatography-selected ion monitoring (GC-SIM) has the ability to solve these problems.

The purpose of this study is to determine the LAL content of many foods by using GC-SIM. There have not been any previous reports concerning the estimation of LAL content in food proteins by this method.

MATERIALS AND METHODS

Materials. Commerical foods were obtained from gro-
Lysinoalanine was purchased from Miles Laboratories Inc., Indiana. z,-Diaminopimelic acid, y-amino-n-butyllic acid, trifluoroacetic anhydride, o-phthalaldehyde, n-butanol and methylene chloride were obtained from Nakarai Chemicals Ltd., Kyoto. z-Amylase (type II-A) was purchased from Sigma Chemical Company, St. Louis. A solution of 10% HCl in n-butanol was prepared by bubbling anhydrous HCl gas into n-butanol, which had been previously purified by refluxing with Mg ribbon and distilling. Methylene chloride was refluxed with anhydrous CaCl₂ and distilled.

Preparation of proteins from commercial foods. A food sample (several grams), which had been previously lyophilized, was extracted with 50 ml of a chloroform-methanol mixture (2:1, v/v) by mixing with a mortar and pestle and with a magnetic stirrer to remove the lipid component. The organic phase was removed by centrifugation, and the extraction process was repeated three times. The resulting precipitate was washed with diethyl ether and dried over P₂O₅ in vacuo. Those samples containing a high amount of carbohydrate were hydrolyzed with z-amylase to remove the carbohydrate component as follows. The sample (1 g) was incubated in 20 ml of 0.1 M phosphate buffer (pH 6.9) containing 0.025% NaN₃ and z-amylase (1 mg) at 37°C for 24 hr. The hydrolysate was thoroughly dialyzed against water, and the non-dialyzable fraction was lyophilized. The nitrogen content was determined by the micro-Kjeldahl method.

Acid hydrolysis and derivatization. The samples containing both y-amino-n-butyllic acid (ABA) and z,-diaminopimelic acid (DPA) were hydrolyzed with redistilled 6 M HCl at 110°C for 24 hr in an evacuated tube. ABA and DPA were respectively used as the internal standards for high-performance liquid chromatography (HPLC) and GC-SIM analyses that are subsequently described. An aqueous aliquot of the protein hydrolysate was transferred to a screw-cap septum vial with a Teflon laminated silicon disc (Pierce Chemical Co., Illinois). After evaporating the solution to dryness, butylation and trifluoroacetylation were carried out according to the semi-micro direct esterification method of Roach and Gehrke. The N-trifluoroacetyl-O-butylerster (BTFA) derivatives obtained were dissolved in methylene chloride and used for GC-SIM.

GC-SIM analysis. GC-SIM was carried out on a Hitachi M-80 gas chromatograph-mass spectrometer, a total ion collector being used as the detector unless otherwise specified. The separation column used was of glass (1.5 m x 3 mm) packed with 2% OV-17/1% OV-210 on 100~200 mesh Supelcoport (Supelco Inc., Pennsylvania). All the mass spectra were obtained at 20 eV. Other parameters were: injection and interface ports temperature, 250°C; ion source temperature, 200°C; accelerating voltage, 1.0 ~ 1.4 kV; emission current, low. The column oven temperature was programmed from 180°C to 230°C, 5°C/min for the first 5 min, 10°C/min for the next 2.5 min, and then held constant at 230°C. The range between m/z 0 ~ 800 was scanned in 8 sec. The data from GC-SIM were recorded, calculated and graphically displayed with a Hitachi M-003 on-line computer.

HPLC analysis. The sample after 6 M HCl hydrolysis that has already been described was used for HPLC. The equipment was a Shimadzu LC-3A with a fluorescence detector (Shimadzu FLD-1B). Detection was carried out fluorometrically by the method of Benson and Hare using o-phthalaldehyde at an emission wavelength of 460 nm with an excitation wavelength of 348 nm. An integrator (Shimadzu Chromatopack C-E1B) was used for calculating the peak areas, the column (15 cm x 4.0 mm) being a Shimadzu ISC-07/S1504 (cation exchanger, sulfonic acid type). The elution buffers were 0.067 M citrate at pH 4.25 (for 6 min) and 0.1 M citrate-0.025 M borate at pH 9.0 (for 20 min). The flow rate was 0.5 ml/min, and the temperature of the column and reaction chamber was 55°C.

RESULTS AND DISCUSSION

GC-SIM analysis of LAL

The mass spectrum of BTFA-LAL has been previously reported by Hasegawa and Okamoto. The base peak at m/z 351 was used for GC-SIM, because it was characteristic of BTFA-LAL and was not found in BTFA derivatives of primary protein (normal) amino acids. Butyl behenate was found to be suitable as the internal standard for BTFA-LAL in gas chromatography (GLC) analysis, in which a flame ionization detector was used. However, butyl behenate did not give characteristic ion peaks in GC-SIM, because it was a fatty acid ester. In order to select an internal standard suitable for the present analysis from amino acid derivatives, we examined the retention times of BTFA derivatives of 2-aminoadipic acid, kainic acid and z,-diaminopimelic acid (DPA) by GLC and their mass spectra. The retention time of BTFA-DPA was close to that of BTFA-LAL, and the mass spectrum of BTFA-DPA gave a characteristic base peak at m/z 280 (Fig. 1). This peak was not found in BTFA derivatives of normal amino acids and LAL. On the other hand, the retention time of BTFA-2-aminoadipic acid was very different from that of BTFA-LAL,
and the mass spectrum of BTFA–kainic acid did not give stable ion peaks. Therefore, we selected DPA as the internal standard for the present analysis. Assignment of the fragment ions of BTFA–DPA is now in progress.

A GLC analysis of normal amino acid mixture (17 amino acids) containing LAL and DPA was carried out, the GLC chromatogram being shown in Fig. 2. The retention times of BTFA–DPA and BTFA–LAL were 5 min and 9 min, respectively, indicating a good separation of these two derivatives from the normal amino acid derivatives. Next, the selected ion recording of GC-SIM between 4 min and 10 min of retention time is shown in Fig. 3, the ion peaks at m/z 280 and m/z 351 being the base peaks for BTFA–DPA and BTFA–LAL, respectively.

The results just described indicate that DPA is a suitable internal standard for the de-
Table I. Lysinoalanine Content in Foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Heating process</th>
<th>LAL content (mg/16gN)</th>
<th>Food</th>
<th>Heating process</th>
<th>LAL content (mg/16gN)</th>
</tr>
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<tr>
<td></td>
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<td>GC-SIM</td>
<td>HPLC</td>
<td></td>
<td>GC-SIM</td>
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<td>Chinese noodle</td>
<td>n.c.&quot;</td>
<td>390</td>
<td>480</td>
<td>skim milk powder</td>
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<tr>
<td>raw, wet form</td>
<td>boiled for 3 min</td>
<td>330</td>
<td>380</td>
<td>whipping agent</td>
<td>17</td>
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<td>precooked</td>
<td></td>
<td></td>
<td></td>
<td>lactic acid</td>
<td>52</td>
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<td>fried A(a)</td>
<td>n.c.</td>
<td>22</td>
<td>25</td>
<td>beverage</td>
<td>8</td>
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<tr>
<td>fried B</td>
<td>n.c.</td>
<td>24</td>
<td>30</td>
<td>yogurt</td>
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<td>fried C</td>
<td>n.c.</td>
<td>16</td>
<td>20</td>
<td>Meat products</td>
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<td>fried D</td>
<td>n.c.</td>
<td>13</td>
<td>18</td>
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<tr>
<td>fried E</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>non-fried</td>
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<td>52</td>
<td>96</td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
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<td>0</td>
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<td></td>
<td>boiled for 30 min</td>
<td>31</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>white</td>
<td>fresh</td>
<td>9</td>
<td>t'</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>boiled for 3 min</td>
<td>33</td>
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<td>boiled for 10 min</td>
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<td>scrambled egg</td>
<td>cooked</td>
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<td>810</td>
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<td></td>
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<td>white</td>
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<td>Dairy products</td>
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<td>0</td>
<td>pretzel A</td>
<td>16</td>
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<td>heated for 1 min</td>
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<td>0</td>
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<td>39</td>
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<tr>
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<td>n.c.</td>
<td>0</td>
<td>0</td>
<td>cracker A</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>heated for 1 min</td>
<td>6</td>
<td>t</td>
<td>cracker B</td>
<td>5</td>
</tr>
<tr>
<td>ordinary milk C</td>
<td>n.c.</td>
<td>6</td>
<td>t</td>
<td>cake</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>heated for 1 min</td>
<td>14</td>
<td>t</td>
<td>Others</td>
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<tr>
<td>UHT-milk</td>
<td>n.c.</td>
<td>4</td>
<td>t</td>
<td>smoked salmon</td>
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<td></td>
<td>heated for 1 min</td>
<td>10</td>
<td>t</td>
<td>dried bonito A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23</td>
<td>50</td>
<td>dried bonito B</td>
<td>t</td>
</tr>
</tbody>
</table>

a n.c., not cooked.
\(a\) Alphabetic characters indicate the difference of manufactures in each food group.
\(c\) t, trace.
\(d\) Retort pouch food.

HPLC analysis of LAL

LAL was also determined by ion exchange chromatography according to a common amino acid analysis technique with some modifications, in which the time program for each elution step was changed to accomodate the

termination of LAL by GC-SIM.
Lysinoalanine Content in Foods

LAL analysis (see Materials and Methods). Fig. 4 shows the elution pattern of the 17 normal amino acids containing LAL and γ-aminoo-n-butylic acid (ABA), indicating a good separation of LAL from the normal amino acids. ABA, which was eluted at the position between phenylalanine and LAL, was used as the internal standard.

**Standard curve for the quantitative determination of LAL**

A quantitative determination of LAL was carried out by GC-SIM and HPLC method using DPA and ABA as the respective standards. The standard curves obtained ranging in an amount from 0.5 to 200 μg of LAL (Fig. 5). The close linear relationship between the response ratio and weight ratio of LAL to each internal standard can be seen. The regression coefficients of the curves were 0.9997 and 0.9898 for the GC-SIM and HPLC analyses, respectively, the respective standard deviations being estimated to be 0.067 and 0.009. These results indicate that the quantitative determination of LAL by both methods is highly reproducible and reliable.

**LAL content in commercial foods**

The LAL content in commercial foods determined by the GC-SIM and HPLC methods is shown in Table I. On the whole, the values obtained by the HPLC method were slightly higher than those by the GC-SIM method. This can be explained from Fig. 4, in which the peak position of LAL on the chromatogram from HPLC is very close to that of histidine. In the present analysis, a very small amount of LAL had to be analyzed, and the histidine content was usually fairly high compared to the LAL content in all the samples analyzed. The presence of excessive histidine at the position close to LAL elution was the cause of the overestimation of LAL.

Of all the samples analyzed, the LAL content was highest in pidan. Chinese noodles, crackers and pretzels, which are wheat flour-based products, and milk products also contained a significant amount of LAL, the content being especially high in raw Chinese noodle. In the case of egg, fresh egg contained a trace amount of LAL, but the content in the egg white was increased by boiling. On the other hand, ordinary milks, soybean products and meat products contained a low level of LAL.

In general, it is known that the alkaline treatment of proteins forms LAL.\(^1,2,7\) The formation of LAL by heating in the acidic and neutral pH regions has also been reported.\(^14\) As shown in Table I, the finding of significant amounts of LAL in pidan and raw Chinese noodles seems to be based on alkaline exposure of these foods during processing and storage. In the foods which were not exposed to an alkaline medium (egg, soybean products, meat products), the LAL content was slightly increased by heating. This indicates that home-cooking processes (heat treatment) without any alkaline treatment also produce LAL in foods, although the level formed is low.

Sternberg's group has reported the LAL content of foods and food ingredients as the results on ion-exchange chromatographic and thin-layer chromatographic analyses.\(^13,14\) In most of the samples, the data obtained in the present study were similar to those by Sternberg’s group, although a significant difference in milk and whipping agents was observed. This may be due to different processing procedures and storage conditions for the foods between the United States and Japan.

Whether LAL would cause renal lesions in the human being or not is obscure. A renal lesion, designated nephrocytomegaly, has been observed in rats fed on diets containing alkali-treated proteins that provided about a 3,000 ppm level of dietary protein bound LAL, while a dietary level of 1,000 ppm was without effect.\(^7,9\) The latter level corresponds to about 3 g of LAL intake per day in the human being (50 kg of body weight), calculated on the basis of data obtained in rats by Struthers et al.\(^11\) (body weight of rat, 250 g; feed consumption, 15 g per day; LAL level of diet, 1,000 ppm). In order to ingest 3 g of LAL, for
example, from Chinese noodle (LAL: 300 ~ 390 mg/16 gN) containing a high amount
of LAL as shown in Table I, about 12 ~ 20 kg
of the noodle would be required. It is im-
possible for the human being to ingest such a
quantity of noodle per day. To predict the level
of LAL in foods that could affect human
health on the basis of information available for
rats is inadequate. However, as long as the
information for rats is applied to human health,
the level of LAL in commercial foods and
home-cooked foods is significantly low.

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