Somatostatin Radioimmunoassay with \(^{125}\text{I-N}^\text{N}^\text{a}-\text{Tyrosyl-Somatostatin}

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Synopsis

\(\text{N}^\text{a}-\text{Tyrosyl-somatostatin was synthesized and proved to be homogeneous. Radioiodination of this tyrosine-containing somatostatin analogue by either the lactoperoxidase method or the chloramine T method led to the formation of crude iodinated compound, which was purified by ion exchange chromatography on CM-Sephadex C-25 using a linear ammonium acetate buffer gradient. This purification process was found to be satisfactorily reproducible and suitable for the preparation of }^{125}\text{I-N}^\text{a}-\text{tyrosyl-somatostatin. Using the purified }^{125}\text{I-somatostatin analogue, radioimmunoassay for somatostatin was performed and the assay system was proved to be sensitive and specific for somatostatin. Immunoassays of hot-water extracts of porcine and tupaia brain, pancreas, stomach and various regions of the intestine in the system revealed that those tissues contained immunoreactive somatostatin at various concentrations. Of the results, it was remarkable that somatostatin immunoreactivity was found in the ileum, middle colon and rectum in both animals, although the concentrations were lower when compared with those in the stomach, duodenum and jejunum.}

Development of a radioimmunoassay for somatostatin had first been reported by Arimura et al. (1975a). Since the somatostatin molecule contains neither tyrosine nor histidine residue, a somatostatin analogue, [Tyr\(^1\)]-somatostatin was used for preparation of the labelled antigen in their assay system. The radioiodinated [Tyr\(^1\)]-somatostatin was purified by ion exchange column chromatography on CM-cellulose.

We have synthesized another tyrosine-containing analogue, \(\text{N}^\text{a}-\text{tyrosyl-somatostatin, in which a tyrosine residue attaches to the N-terminus of the native hormone. N}^\text{a}-\text{Tyrosyl derivatives of human C-peptide (Melani et al., 1970; Kaneko et al., 1974), secretin (Yanaihara et al., 1976a) and substance P (Yanaihara et al., 1976b) have successfully been used for labelling in our laboratory. This communication deals with preparation of }^{125}\text{I-N}^\text{a}-\text{tyrosyl-somatostatin with high specific activity and somatostatin radioimmunoassay using the labelled antigen.}

Materials and Methods

Synthetic peptides

Synthetic somatostatin, [Leu\(^4\)]-somatostatin, [Arg\(^4\)]-somatostatin, [Leu\(^4\)]-somatostatin and [Ser\(^4,14\)]-somatostatin were prepared by the conventional method for peptide synthesis in our laboratory and purified extensively before use. The syntheses of somatostatin and these analogues will be described elsewhere. Synthesis of \(\text{N}^\text{a}-\text{tyrosyl-somatostatin was performed by the conventional method in a similar manner to that employed for synthesis of somatostatin. After construction of the whole molecule, the product was purified by ion exchange column chromatography on CM-Sephadex C-25 using a linear ammonium acetate 0.01 M-0.5 M gradient (pH 4.5) as

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eluent, and the fractions containing the desired material were pooled, desalted by gel filtration on Bio-Gel P-2 and then lyophilized to yield N²-tyrosyl-somatostatin of high purity: \( \text{Rf} (1\text{-butanol-acetic acid-H}_2\text{O} = 4 : 1 : 5) = 0.22 \), \( \text{Rf} (1\text{-butanol-pyridine-acetic acid-H}_2\text{O} = 30 : 6 : 24) = 0.68 \), \([\alpha]_D^{25} = -29.0^\circ \) (c 1.0, 1% acetic acid). \([\text{Tyr}^1]\)-Somatostatin was kindly supplied by Professor Arimura.

**Radioiodination**

Radioiodination of N²-tyrosyl-somatostatin was carried out by either the enzymic (Miyachi et al., 1972) or the chloramine T (Hunter and Greenwood, 1962) method.

**Enzymic method:** N²-Tyroly-somatostatin was radioiodinated with \(^{125}\text{I}Na\) in the presence of lactoperoxidase according to a modification of the method described by Arimura et al. (1975a). To 25 ml of 0.4 M sodium acetate buffer (pH 5.6) were added 5 \( \mu \)g of N²-tyrosyl-somatostatin in 5 ml of 0.1 M acetate acid, 0.5–1 mCi of \(^{125}\text{I}Na\) (RCC) and 20 ng of lactoperoxidase (Calbiochem) in 10 ml of 0.1 M sodium acetate buffer (pH 5.6). A solution of 100 ng of hydrogen peroxide in 5 ml of water was then added and the mixture was allowed to stand for 10 min. The same amount of hydrogen peroxide was added and the mixture was kept to stand further 10 min and submitted to appropriate purification.

**Chloramine T method:** Five \( \mu \)g of N²-tyrosyl-somatostatin in 5 ml of 0.1 M acetic acid was mixed with 0.5–1 mCi of \(^{125}\text{I}Na\) (RCC) in 25 ml of 0.5 M phosphate buffer (pH 7.6) and 25 \( \mu \)g of chloramine T (Calbiochem) in 10 ml of 0.05 M phosphate buffer and the mixture was kept for 10 sec. The reaction was terminated by addition of 150 \( \mu \)g of sodium metabisulfite (Calbiochem) in 50 ml of 0.05 M phosphate buffer and the product was submitted to appropriate purification.

**Purification of labelled compound**

Purification of the \(^{125}\text{I}-\text{N}²\)-tyrosyl-somatostatin was performed by ion exchange column chromatography and gel filtration.

**Column chromatography on CM-Sephadex C-25:** The reaction mixture containing \(^{125}\text{I}-\text{N}²\)-tyrosylsomatostatin was applied to a CM-Sephadex column (1x2 cm) equilibrated with 0.01 M ammonium acetate buffer (pH 4.5). The column was eluted with 0.01 M (30 ml) and 0.01 M (50 ml)-0.5 M (50 ml) linear gradient ammonium acetate buffer (pH 4.5) and fractions of 3 ml each were collected in test tubes.

**Gel filtration on Sephadex G-25:** The reaction mixture was applied to Sephadex G-25 column (1x25 ml) and the column was eluted with 3 M acetic acid. Fractions of 1 ml each were collected in test tubes. Gel filtration on Bio-Gel P-6: Fraction #16 in the Sephadex G-25 gel filtration was applied to Bio-Gel P-6 column (1x35 cm) and the column was eluted with 1 M acetic acid. Fractions of 1 ml each were collected in test tubes.

**Radioimmunoassay**

The procedure of somatostatin radioimmunoassay was similar to that described by Arimura et al. (1975a). The above-described purified \(^{125}\text{I}-\text{N}²\)-tyrosyl-somatostatin was used as tracer. The antiserum #101 was a generous gift from Professor Arimura. Synthetic somatostatin prepared in this laboratory was used as standard and the purity was assessed prior to its use. The bound and free antigens were separated by the dextran-coated charcoal method and the radioactivity of the bound was determined in an autogamma counter.

**Tissue extraction**

Hot-water extraction was carried out with porcine and tupaia tissues according to the method described by Yanaihara et al. (1976b).

**Results**

Purification of synthetic N²-tyrosylsomatostatin was carried out by ion exchange column chromatography on CM-Sepahdex C-25. Fig. 1 illustrates the elution profile of the crude synthetic material from the column. Satisfactory separation of the desired N²-tyrosylsomatostatin was achieved by using a linear ammonium acetate gradient from 0.01 M to 0.5 M (pH 4.5). Fraction #56–#65 contained pure N²-tyrosyl-somatostatin. Growth hormone-release inhibiting activity of this compound was nearly to the same degree as that of synthetic somatostatin. Detailed biological properties of this compound will be described elsewhere.

Following iodination of N²-tyrosyl-
somatostatin by either the enzymic method or the chloramine T method, the labelled compound was separated by ion exchange chromatography or gel filtration. Fig. 2-a shows the elution pattern of the reaction mixture obtained by the lactoperoxidase method from a CM-Sephadex C-25 column. The distribution pattern of the radioactivity was identical with the elution pattern (Fig. 1) of the cold compound from the same kind of column which was monitored by UV absorption at 278 nm, with the exception of peak I mainly for free $^{125}$I. Peak II contained little immunoreactive material, which was assumed to be polymerized compounds of the somatostatin analogue. Peak III contained the desired labelled product with the highest binding ability to the antiserum and the location of this peak corresponded to that for N*-tyrosyl-somatostatin (Fig. 1). Some of the fractions corresponding to main peaks were examined with respect to binding ability (B/T %) and non-specific binding. The results are presented in Table 1. The material obtained from peak III was rechromatographed, on the same CM-Sephadex column and the elution profile is shown in

![Diagram](image1)

![Diagram](image2)

Fig. 1. Chromatographic purification of synthetic N*-tyrosyl-somatostatin on CM-Sephadex C-25. Column: $1.5 \times 7$ cm, Eluent: 0.01 M (100 ml) and 0.01 M (500 ml)-0.5 M (500 ml) ammonium acetate buffer (pH 4.5), Fraction: 10 ml each.

Fig. 2. (a) Purification of $^{125}$I-N*-tyrosyl-somatostatin by CM-Sephadex C-25 column chromatography. Column: $1 \times 2$ cm, Eluent: 0.01 M (30 ml) and 0.01 M (50 ml)-0.5 M (50 ml) ammonium acetate buffer (pH 4.5), Fraction: 3 ml each. (b) Rechromatography on Sephadex C-25 column of Fractions #35 and #36 corresponding to peak III in (a). Column: $1 \times 2$ cm, Eluent: 0.01 M (30 ml) and 0.01 M (50 ml)-0.5 M (50 ml) ammonium acetate buffer (pH 4.5), Fraction: 3 ml each.

Table 1. Binding activity and non-specific binding of fractions corresponding to major peaks in CM-Sephadex C-25 column chromatographic purification of $^{125}$I-N*-tyrosyl-somatostatin

<table>
<thead>
<tr>
<th>Fraction number (see Figure 2-a)</th>
<th>2</th>
<th>22</th>
<th>35</th>
<th>36</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding activity (%)</td>
<td>72.2</td>
<td>35.7</td>
<td>24.3</td>
<td>32.0</td>
<td>44.9</td>
</tr>
<tr>
<td>Non-specific activity (%)</td>
<td>71.9</td>
<td>29.5</td>
<td>6.3</td>
<td>3.3</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Binding activity and non-specific binding are B/T% in assay systems with and without anti-somatostatin serum, respectively.
Fig. 2-b, which proved efficient purification of the labelled compound by the procedure. Fig. 3 illustrates the distributions of radioactivities in electropherogram of the enzymic iodination mixture and fractions #2, #22 and #36 which correspond to peaks I, II and III, respectively. The results indicate clearly complete removal of free $^{125}\text{I}$ from fraction #36. Specific activity of the purified $^{125}\text{I}-\text{N}^{\alpha}\text{-tyrosyl-somatostatin}$ was 200–250 $\mu\text{Ci}/\mu\text{g}$. The same result was also obtained using a CM-Sephadex C-25 column chromatography of the crude mixture of iodination by the chloramine T method.

Using a linear ammonium acetate gradient, ion exchange chromatography on CM-cellulose also led to satisfactory separation of $^{125}\text{I}-\text{N}^{\alpha}\text{-tyrosyl-somatostatin}$ from the reaction mixture. As shown in Fig. 4, the elution profile of the radioactivity from this column was similar to that in the case of CM-Sephadex C-25 column chromatography. The binding ability and non-specific binding of fractions corresponding to the major peaks are shown in Table 2.

Figures 5-a and 5-b illustrate gel filtration patterns of the iodination products on Sephadex G-25 and Bio-Gel P-6, in which

![Fig. 3](image1.png)

![Fig. 4](image2.png)

**Table 2.** Binding activity and non-specific binding of fractions corresponding to major peaks in CM-cellulose column chromatographic purification of $^{125}\text{I}-\text{N}^{\alpha}\text{-tyrosyl-somatostatin}$

<table>
<thead>
<tr>
<th>Fraction number (see Figure 4)</th>
<th>#3</th>
<th>#19</th>
<th>#26</th>
<th>#28</th>
<th>#29</th>
<th>#30</th>
<th>#31</th>
<th>#32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding activity (%)</td>
<td>65.8</td>
<td>32.0</td>
<td>11.2</td>
<td>8.3</td>
<td>16.6</td>
<td>37.4</td>
<td>38.4</td>
<td>37.4</td>
</tr>
<tr>
<td>Non-specific activity (%)</td>
<td>63.9</td>
<td>30.1</td>
<td>11.2</td>
<td>6.5</td>
<td>4.9</td>
<td>5.1</td>
<td>6.0</td>
<td>5.4</td>
</tr>
</tbody>
</table>
diluted acetic acid was used as eluent. Immunological activities of fractions of the main peaks were as shown in Table 3. Sephadex G-25 followed by Bio-Gel P-6 gel filtration did not give successful separation of the desired product with high immunoreactivity.

The $^{125}$I-N°-tyrosyl-somatostatin, which was prepared by the enzymic iodination followed by CM-Sephadex purification, was lyophilized and kept at $-20^\circ$C. After 4- or 7-day storage under the condition, the material was rechromatographed on CM-Sephadex C-25 with the same eluent system as before. In the elution patterns, there were detected reappearance of peaks corresponding to peaks I and II in Figure 2-a even after 4-day storage, and the peaks became larger and the peak of $^{125}$I-N°-tyrosyl-somatostatin smaller with prolonged period of storage.

Fig. 6 represents a standard curve for somatostatin radioimmunoassay. The tracer used was $^{125}$I-N°-tyrosyl-somatostatin prepared by the enzymic radiiodiation and CM-Sephadex purification. The logit plot of this curve gave a straight line over a range from 2 pg to 256 pg of standard somatostatin per tube. In the present assay system, $^{125}$I-N°-tyrosyl-somatostatin was found to be slightly more sensitive to displacement with standard somatostatin as compared with $^{125}$I-[^Tyr$^1$]-somatostatin which was also prepared by the enzymic radiiodination of [Tyr$^1$]-somatostatin followed by CM-Sephadex purification.

Crossreactivities of various somatostatin-

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Table 3. Binding activity and non-specific binding of some major fractions from Sephadex G-25 and Bio-Gel P-6 gel filtration used for purification of $^{125}$I-N°-tyrosyl-somatostatin

<table>
<thead>
<tr>
<th>Fraction number (see Figure 5)</th>
<th>Sephadex G-25</th>
<th>Bio-Gel P-6*</th>
</tr>
</thead>
<tbody>
<tr>
<td>#14</td>
<td>#16</td>
<td>#18</td>
</tr>
<tr>
<td>Binding activity (%)</td>
<td>8.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Non-specific binding (%)</td>
<td>4.7</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Fraction #16 of Sephadex G-25 gel filtration was submitted to the Bio-Gel P-6 column.
Fig. 6. A standard curve for somatostatin radioimmunoassay. Antiserum: #101 (rabbit antiserum elicited against somatostatin-human serum $\alpha$-globulin conjugate, final dilution 1 : 21,000). Tracer: $^{125}$I-N$^{\beta}$-tyrosyl-somatostatin, Standard: synthetic somatostatin, Separation: dextran-coated charcoal method.

Fig. 7. Dose-response curves of various somatostatin-related peptides: (●) somatostatin, (○) N$^{\beta}$-tyrosyl-somatostatin, (▲) [Arg$^4$]-somatostatin, (△) [Leu$^4$]-somatostatin, (□) polymerized forms of somatostatin, (◆) [Leu$^9$]-somatostatin, (◇) [Ser$^{314}$]-somatostatin and (×) H-Lys-Asn-Phe-Phe-Trp-Lys-OH, H-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser-OH and secretin.

related peptides were examined in the system (Fig. 7). N$^{\beta}$-Tyrosyl-somatostatin, [Leu$^4$]-somatostatin and [Arg$^4$]-somatostatin showed crossreactivities nearly identical with that of somatostatin itself. On the other hand, [Leu$^9$]-somatostatin, [Ser$^{314}$]-somatostatin and somatostatin-related peptide fragments such as H-Lys-Asn-Phe-Phe-Trp-Lys-OH and H-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser-OH displaced the tracer very weakly or not at all. Secretin embodying a partial structure of somatostatin, -Thr-Phe-Thr-Ser-, did not compete with the tracer at concentration up to 50 ng per tube.

Crude extracts from porcine and tupaia tissues were assayed for immunoreactive somatostatin. Fig. 8 shows the dose-response curves of the immunoreactivities in a variety of the extracts. Somatostatin-equivalent concentrations per mg of tissues in various
Table 4. Somatostatin-like immunoreactivity in porcine and tupaia tissue extracts

<table>
<thead>
<tr>
<th>Porcine (pg somatostatin per mg wet weight of tissue) (n=1)</th>
<th>Tupaia (pg somatostatin, mean±S.E., per mg wet weight of tissue) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach (antrum)</td>
<td>Stomach 1</td>
</tr>
<tr>
<td>Duodenum</td>
<td>2</td>
</tr>
<tr>
<td>Jejunum 1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Duodenum 1</td>
</tr>
<tr>
<td>Ileum</td>
<td>2</td>
</tr>
<tr>
<td>Cecum</td>
<td>3</td>
</tr>
<tr>
<td>Middle colon</td>
<td>4</td>
</tr>
<tr>
<td>Rectum</td>
<td>Jejunum 1</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2</td>
</tr>
<tr>
<td>Pituitary</td>
<td>3</td>
</tr>
</tbody>
</table>

Each of the stomach, duodenum and jejunum was equally divided into 3 or 4 portions, which were numbered consecutively from the upper portion.

Immunoreactivities are expressed by weight equivalent of somatostatin.

organs are summarized in Table 4. Relatively high concentrations of somatostatin-like immunoreactivity were detected throughout the gastrointestinal tracts.

Discussion

We have prepared highly purified N'-tyrosyl-somatostatin and the analogue was found to possess a biological potency comparable to that of the authentic synthetic somatostatin in respect to growth hormone-release inhibiting activity. Recently, Ohashi et al. (1976) reported some biological activities of N'-tyrosyl-somatostatin.

Efficient purification of N'-tyrosyl-somatostatin was achieved by ion exchange column chromatography with a linear ammonium acetate gradient from 0.01 M to 0.5 M on CM-Sephadex C-25. This purification method was successfully utilized for the separation of \(^{125}\)I-N'-tyrosyl-somatostatin with high specific activity from crude radioiodination product of N'-tyrosyl-somatostatin. The fractions corresponding to the peak III in Fig. 2-a contained satisfactorily purified labelled compound and the peak II was assumed to represent mainly polymerized forms of the compound, since CM-Sephadex chromatography of the crude cold analogue showed the same elution profile and the fractions corresponding to the peak II were found to contain polymerized forms of the analogue. The iodinated compound could also be purified by CM-cellulose chromatography with the same gradient elution system as used for CM-Sephadex, and the result was almost identical. Arimura et al., (1975a) employed CM-cellulose chromatography with a stepwise elution for purification of \(^{125}\)I-[Tyr\(^1\)]-somatostatin.

According to the results of the chromatographic purification of the radiiodination mixtures, it may be concluded that either the lactoperoxidase or the chloramine T method could be used for labelling safely. However, we chose the enzymic method for our practical purpose because of the structural feature of somatostatin.

Consecutive use of gel filtrations on Sephadex G-25 and Bio-Gel P-6 did not give satisfactory separation from radiiodination mixture of the desired labelled compound with high specific activity.

Stability of the purified labelled compound was found to be rather poor. Namely, the \(^{125}\)I-N'-tyrosyl-somatostatin was stored at \(-20^\circ\text{C}\) for 4 days in lyophilized form and submitted to rechromatography on CM-Sephadex C-25. The result revealed
already reappearance of the peak I, and with prolonged period of storage under such condition, the peak I became larger. Radioimmunoassay for somatostatin was performed using $^{125}$I-N$^\alpha$-tyrosyl-somatostatin as tracer and antiserum # 101. The specificity of the antiserum had been examined by Arimura et al. (1975a). The standard curve shown in Fig. 6 assessed high sensitivity of the present assay system. N$^\alpha$-Tyrosyl-somatostatin used for labelling crossreacted in the same manner as did somatostatin itself in this system. Synthetic analogues such as [Leu$^3$]-somatostatin and [Arg$^4$]-somatostatin showed the complete crossreaction. On the other hand, as reported by Vale et al. (1976), 8-substituted analogue, [Leu$^8$]-somatostatin displaced the tracer very weakly. Although linear somatostatin has been known to yield 15–17% crossreactivity in the assay system using the antiserum # 101, [Ser$^{b14}$]-somatostatin, which does not contain disulfide bridge, showed little crossreaction, suggesting the importance of the Cys residues or disulfide in the antigen-antibody interaction. In addition, any synthetic somatostatin-related peptide fragments examined did not displace the tracer. A gastrointestinal hormone, secretin consisting of 27 amino acid residues, contains a tetrapeptide sequence corresponding to somatostatin (10–13) fragment in its N-terminal region from positions 5 to 8. Robberecht et al. (1975) suggested that the activation of the rat exocrine pancreas with somatostatin depends on the interaction of somatostatin with secretin receptors. In the present assay system, however, synthetic porcine secretin did not compete with the tracer at concentration up to 50 ng per tube.

It has been known that somatostatin-like immunoreactivity is present in the central nervous system (Hökfelt et al., 1975a and 1975b; Pelletier et al., 1975), pancreas and gastrointestinal tracts (Arimura et al., 1975b; Polak et al., 1975; Brownstein et al., 1975; Rufener et al., 1975a and 1975b; Helmstaedter et al., 1976 and 1977). Using the present somatostatin immunoassay system, we have examined for immunoreactive somatostatin in extracts from porcine and tupaia brain, pancreas and various parts of gastrointestinal tracts. Dose-response curves of the immunoreactivities in these extracts were parallel to the standard curve. Table 4 lists pg somatostatin-equivalent of the immunoreactivity per mg wet weight of tissue. In the intestinal tracts, relatively high concentration of the immunoreactivity was found in the duodenum and jejenum of both animals. Although the concentrations were lower than those in the duodenum and jejenum, the immunoreactivity was also detected in other parts of the intestines such as ileum, middle colon and rectum. In tupaia, the concentrations of somatostatin immunoreactivity in lower portions of the duodenum and jejenum were much lower than in the pyloric region of the stomach and the upper portion of the duodenum. On the other hand, porcine jejenum contained the immunoreactivity greater than that in the duodenum and stomach. These results may suggest difference between animals in distribution patterns of immunoreactive somatostatin in the intestine. In addition, immunoassay of the pituitary extracts in both animals showed high contents of the immunoreactivity in the organ.

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References