Degradation of $^{125}$I-Glucagon, -Pancreatic Polypeptide and -Insulin by Acid Saline Extract of Rat Submaxillary Gland and Their Protection by Proteinase Inhibitors

YOSHIMASA TASAKA, KOJI MARUMO, YUKIKO INOUE and YUKIMASA HIRATA

Diabetes Center, Tokyo Women's Medical College
Kawada-cho 8, Shinjuku-ku, Tokyo, Japan

Abstract

The acid saline extract (ASE) of rat submaxillary gland exerts a powerful degrading effect on $^{125}$I-glucagon. In order to study the degradation of other $^{125}$I-peptides by ASE and the effects of their inhibitors, $^{125}$I-pancreatic polypeptide (PP) and $^{125}$I-insulin were used together with $^{125}$I-glucagon. The degradation studies were done by the trichloroacetic acid (TCA) method or gel filtration. Besides $^{125}$I-glucagon, $^{125}$I-PP was found to be destroyed by ASE in the ordinary immunoassay system using the TCA method, but $^{125}$I-insulin was intact in the presence of ASE. Leupeptin, and to a lesser extent p-chloromercuri phenyl-sulfonic acid (PCMS) and N-ethylmaleimide, inhibited the destruction of $^{125}$I-glucagon or -PP under the TCA method. PCMS was especially protective at high concentrations, for example 16 mM. These findings were confirmed by gel filtration of the assay mixture. In the presence of leupeptin (0.4 mM) and PCMS (16 mM), no shift in the peak of labelled glucagon or PP occurred. Thus ASE degrades not only $^{125}$I-glucagon but -PP, and thiol proteinase inhibitors have a strong inhibitory action on them.

Large amounts of glucagon-like substances have been reported in acid saline extracts of the submaxillary gland of the rat, mouse and rabbit (Bhathena et al., 1974; Silverman and Dunbar, 1974; Lawrence et al., 1976; Dunbar et al., 1976; Paulo et al., 1986). In addition, immuno-cytochemical studies have shown glucagon-like peptides in the rat salivary gland (Smith and Tomo, 1986). However, recent studies have revealed that salivary gland glucagon is a fictitious substance (Tahara et al., 1983; Tominaga et al., 1984) since tracer glucagon added to the assay system is largely degraded, even in the presence of aprotinin. These investigations prompted us to study the degradation of tracers of pancreatic hormones other than glucagon and the effects of proteinase inhibitors on them using acid saline extracts of the rat submaxillary gland, thus contributing to the clarification of the nature of proteolytic enzymes in ASE.

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Materials and Methods

Acid saline extract

Wistar albino rats weighting 200 to 300 gm were sacrificed during fasting by decapitation, and the submaxillary glands were removed. Acid saline extract (ASE) was prepared by the methods described by Lawrence et al. (1976) and Bhathena et al. (1974). The methods are as follows: 1 gm of submaxillary gland tissue was minced and homogenized with a polytron in 6 ml of normal saline adjusted to pH 2.8 with HCl to which 1.5 ml of aprotinin (10,000 IU/ml) was added. The homogenate was centrifuged at 4°C for 20 min at 7,100 xg, and the supernatant was lyophilized. The lyophilized material was dissolved in 1 ml of 0.2 M glycine buffer (pH 8.8).

Degradation study

In the degradation study, the relatively long incubation time of 48 hr was used for comparison with already reported degradation studies on 125I-glucagon (Tahara et al., 1983). Incubation of 0.1 ml of ASE and 0.4 ml of 125I-peptide solution containing 500 IU aprotinin was done at 4°C for 48 hrs (final volume 0.5 ml) in glycine buffer (pH 8.8) for 125I-glucagon (Ohtsuka Assay Lab. Tokushima, 5 pg; Unger et al., 1970), phosphate buffer (pH 7.4) for 125I-pancreatic polypeptide (PP) (Dainabott Radioisotope Lab. Tokyo, 3 pg; Schwartz et al., 1975) and borate buffer (pH 8.5) for 125I-insulin (Dainabott Radioisotope, Tokyo, 3 pg; Morgan and Lazarow, 1963).

In the study using trichloroacetic acid (TCA), cold 20% (W/V) TCA was added together with 0.1 ml sheep serum immediately after incubation (final concentration: 10%). The radioactivity of the precipitate was determined and the extent of intact 125I-peptide was expressed as a percentage of the radioactivity in a sample to which buffer was added instead of the incubation medium.

The incubation mixture (0.1 ml, ASE, 104-fold dilution) was also applied to a Sephadex G-50 superfine column (1.5×80 cm) at 4°C and eluted with 1 M acetic acid. Then 1.2 ml fractions were collected. In the study of the degradative activity of ASE by gel filtration, 0.3 ml of ASE was applied to the same column and eluted with glycine buffer (Unger et al., 1976), and the degradation of 125I-glucagon and -PP was investigated by the TCA precipitation method in each fraction.

In the degradation studies using proteinase inhibitors, the inhibitors were added in the incubation medium together with labelled hormone and ASE.

Leupeptin, p-chloromercuriphenylsulfonic acid (monosodium salt, PCMS) and N-ethylmaleimide were all obtained from Sigma Chemical Co. (U.S.A.)

Results

Degradation of 125I-glucagon, -PP and -insulin by TCA precipitation method

The destruction of 125I-glucagon, -insulin, and -PP in the incubation medium was determined by the TCA precipitation method. In a preliminary experiment, aprotinin had no effect on the protection of 125I-glucagon in this assay system. As

\[
\text{Radioactivity (TCA-precipitate, %)}
\]

\[
\text{125I-glucagon}
\]

\[
\text{125I-insulin}
\]

\[
\text{125I-PP}
\]

Fig. 1. Destruction of 125I-glucagon, -insulin and -PP by ASE (TCA method). As the concentration of ASE was increased, the percentage of radioactivity in the TCA-precipitate was decreased in 125I-glucagon and -PP experiments. The abscissa shows dilution of ASE.
shown in Fig. 1, the percentage of radioactivity of the TCA precipitate was decreased as the amount of ASE added to the incubation medium was increased in the 125I-glucagon and 125I-PP experiments, suggesting their degradation in the assay medium. In contrast, no decrease in the percentage of radioactivity in the TCA precipitate was found in the 125I-insulin experiment.

**Degradative activity of ASE by gel filtration**

Gel filtration of ASE was done using a Sephadex G-50 superfine column (1.5 × 80 cm) (Fig. 2). Active destruction of 125I-glucagon and -PP was found in the same fraction.

**Protection of 125I-glucagon and 125I-PP by proteinase inhibitors**

The effects of leupeptin, PCMS and N-ethylmaleimide as proteinase inhibitors on the destruction of 125I-glucagon and 125I-PP were studied (Tables 1 and 2). Without proteinase inhibitors, the percentage of radioactivity in the TCA precipitate was 44.0% with 0.1 ml of a 104-fold dilution of ASE in the 125I-glucagon; it was increased to 58.3% in the presence of 0.4 mM leupeptin. At a 16 mM concentration of PCMS, the degradation of 125I-glucagon was completely inhibited.

The degradation of 125I-PP by ASE was also inhibited by leupeptin and PCMS. The percentage of radioactivity of the TCA precipitate was 35.7% with 0.1 ml of a 200-fold dilution of ASE, it was increased to 63.6% and 81.5% in the presence of 0.4 mM of leupeptin and 16 mM of PCMS, respectively, and to 97.2% in the presence of both inhibitors. N-ethylmaleimide had a less inhibitory effect.

**Gel filtration of 125I-glucagon, -PP and -insulin**

As shown in Fig. 3, the peak of 125I-glucagon in the medium containing ASE was shifted to the smaller molecular weight fraction, but no shift in the peak of 125I-glucagon was found in the incubation medium containing leupeptin and PCMS. Leupeptin plus PCMS also protected against the degradation of 125I-PP by ASE (Fig. 4). No shift in 125I-insulin was found, even at high concentrations of ASE in the assay system (0.1 ml, 200-fold dilution) (Fig. 5).
Discussion

In this study, we found that not only \(^{125}\text{I}\)-glucagon but also \(^{125}\text{I}\)-PP was destroyed by ASE. In contrast, \(^{125}\text{I}\)-insulin was intact in the presence of ASE. The gel filtration study of ASE revealed that the molecular weights of the proteolytic enzymes of \(^{125}\text{I}\)-glucagon and -PP were similar. Tsubouchi et al. (1983) reported that so-called “big plasma glucagon” (BPG) might be due to

![Fig. 3. Gel filtration of \(^{125}\text{I}\)-glucagon incubated with](image)

- (a) buffer
- (b) ASE (\(\times 10^4\) dilution)
- (c) ASE, leupeptin (0.4 mM) and PCMS (16 mM)

A Sephadex G-50 superfine column (1.5\% \(\times 80\) cm) was used and the elution medium was 1 M acetic acid.

![Fig. 4. Gel filtration of \(^{125}\text{I}\)-PP incubated for 2 days at 4°C with](image)

- (a) buffer
- (b) ASE (\(\times 200\) dilution)
- (c) ASE, leupeptin (0.4 mM) and PCMS (16 mM)
Table 1.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (mM)</th>
<th>TCA-precipitate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid saline extract (×10^4, 0.1 ml)</td>
<td>44.0%</td>
<td></td>
</tr>
<tr>
<td>Buffer + ASE</td>
<td>0.05</td>
<td>47.5</td>
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<td></td>
<td>0.1</td>
<td>48.2</td>
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<td></td>
<td>0.2</td>
<td>52.0</td>
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<tr>
<td>Leupeptin</td>
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<td>58.2</td>
</tr>
<tr>
<td>ASE + PCMS</td>
<td>0.5</td>
<td>42.2</td>
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<td></td>
<td>2</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>77.0</td>
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<tr>
<td></td>
<td>16</td>
<td>99.2</td>
</tr>
<tr>
<td>Leupeptin + PCMS</td>
<td>0.2 + 4</td>
<td>82.7</td>
</tr>
<tr>
<td>Leupeptin + PCMS</td>
<td>0.4 + 16</td>
<td>97.2</td>
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Protective effect of leupeptin, PCMS and A-ethylmaleimide on destruction of 125I-glucagon caused by its incubation with ASE. Various amounts of the agents indicated in the second column were added to a mixture of 125I-glucagon and ASE. Intact 125I-glucagon was expressed as a percent of total radioactivity in the precipitate (TCA method).
Table 2.

<table>
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<th>Chemical</th>
<th>Concentration (mM)</th>
<th>TCA-precipitate %</th>
</tr>
</thead>
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<tr>
<td>Buffer</td>
<td>Acid saline extract ($\times 2 \times 10^3$, 0.1 ml)</td>
<td>35.7%</td>
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<td>ASE+</td>
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<td></td>
<td>0.1</td>
<td>47.6</td>
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<tr>
<td></td>
<td>0.2</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>63.6</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.5</td>
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</tr>
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<td></td>
<td>2</td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
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</tr>
<tr>
<td></td>
<td>16</td>
<td>81.5</td>
</tr>
<tr>
<td>PCMS</td>
<td>0.2 + 4</td>
<td>73.6</td>
</tr>
<tr>
<td></td>
<td>0.4 + 16</td>
<td>97.2</td>
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<tr>
<td>N-ethylmaleimide</td>
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</tr>
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<td>38.2</td>
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Protective effect of leupeptin, PCMS and N-ethylmaleimide on destruction of $^{125}$I-PP caused by its incubation with ASE. Various amounts of the agents indicated in the second column were added to a mixture of $^{125}$I-PP and ASE. Intact $^{125}$I-PP was expressed as a percent of total radioactivity in the precipitate (TCA method).

The degradation of $^{125}$I-glucagon during the incubation period of radioimmunoassay, and the destruction of labelled glucagon would give rise to the presence of false BPG. Although gel filtration of such degraded $^{125}$I-glucagon has not been done in the above paper, it is possible that a similar gel filtration pattern would be obtained.

Aprotinin, a serine proteinase inhibitor, had no inhibitory effect. Hatton et al. (1982) reported the marked degradation of $^{125}$I-glucagon by ASE from rat brain in spite of the presence of aprotinin, pepstatin, EDTA or phenylmethysulfonylfluoride. In our experiment, aprotinin had no inhibitory effect on either $^{125}$I-glucagon or -PP degradation (unpublished results), whereas thiol proteinase inhibitors such as PCMS or N-ethylmaleimide had a strong effect on them, just as on the proteolytic activity in the BPG fraction of serum (Tsubouchi et al., 1983).

The degradation of $^{125}$I-glucagon by ASE is thought to occur at the arg-arg bond of the 17th and 18th of 29 peptides (Tahara et al., 1983). In this experiment bovine $^{125}$I-PP was used, and bovine PP has an arg-arg bond at the 25th and 26th of 35 peptides. From the position of $^{125}$I-material in gel filtration, the destruction of $^{125}$I-PP seemed to occur at a position different from the arg-arg bond.

Although the chemical nature and mechanism of action of proteolytic enzymes in ASE are still unknown, it is interesting that the $^{125}$I-peptide-degrading enzyme in
the ASE or rat submaxillary gland is inhibited by thiol proteinase inhibitors similarly to that in the BPG fraction of human serum. Thus a similar $^{125}$I-peptide-degrading enzyme might be present in various tissues or body fluids. Further studies will be necessary for the elucidation of the mechanism of action of proteolytic enzymes on these hormones.

Acknowledgements

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References


