STUDIES ON A PEPTIDASE ACTING ON A SYNTHETIC COLLAGENASE SUBSTRATE

DEVELOPMENTAL PATTERN IN RAT GRANULOMA TISSUE AND DISTRIBUTION OF ENZYMES IN THE TISSUES OF VARIOUS ANIMALS

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SUMMARY

The developmental pattern in experimental rat granuloma tissue and the distribution in the tissues of a few animals (monkey, rabbit, guinea pig and rat) of a peptidase acting on a synthetic collagenase substrate, 4-phenylazobenzoylcarbonyl-L-Pro-t-Leu-Gly-t-Pro-d-Arg (Pz-peptide) has been studied. Maximum enzyme activity was found in 4-month-old rats and on the fourth day of implantation of the cotton wick. Pz-peptidase appears to have a ubiquitous distribution in animal tissues; the highest enzyme activity was generally found in liver, intestine and kidney of the animals. The total activity in other organs (spleen, heart, lungs and brain) was much less compared to that of liver, intestine or kidney.

INTRODUCTION

The enzymic cleavage of a synthetic collagenase substrate, 4-phenylazobenzoylcarbonyl-L-Pro-t-Leu-Gly-t-Pro-d-Arg (Pz-peptide) has been reported in a number of animal tissues and some of these "Pz-peptide"-hydrolyzing enzymes (hereafter designated as Pz-peptidase activity in this paper) have often been designated as "collagenases"). Unlike in the bacterial systems, the observed Pz-peptidase, at least in tadpole and rheumatoid synovial tissues, does not appear to exhibit collagenase activity. However, the rheumatoid synovial protease was shown to cleave the denatured collagen (gelatin) and collagenase breakdown fragments, TC\textsubscript{A} and TC\textsubscript{B} into smaller dialyzable fragments. Also, there are indications in earlier reports that collagen degradation or turnover was closely correlated with the Pz-peptidase activity in postpartum uterus, developing chick embryo skin, and in tumor tissue. The Pz-peptidase may, thus, have an important function in collagen breakdown especially after the initial cleavage of the molecule by collagenase. It was, therefore, considered that a study of the Pz-peptidase activity in cotton wick-induced granuloma tissue

Abbreviation: Pz-peptide, 4-phenylazobenzoylcarbonyl-L-Pro-t-Leu-Gly-t-Pro-d-Arg.
would serve a model system for studying the enzyme. The presence of this enzyme in granuloma tissue has been shown and the properties of a partially purified enzyme have been reported earlier. The development of the enzyme activity has been studied in the rat, in relation to the age of the granuloma tissue and also to the age of the animal. These results and also the data on the distribution of this enzyme in the tissues of four species of animals are given in this paper.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained commercially as indicated: Pz-peptide (Fluka, Switzerland); dithiothreitol, Tris, bovine serum albumin (Sigma Chem. Co., U.S.A.); other chemicals were commercially available reagent grade products.

Induction of granuloma tissue

Granuloma was induced by subcutaneous implantation of sterilized cotton wicks (3 cm × 0.5 cm in the case of 1-2 month-old rats and 7 cm × 0.5 cm with older rats) on either side of the abdominal region of albino rats (a local inbred strain) according to a modified procedure of the technique of Meier et al. The rats were sacrificed on the required day following the implantation and the granuloma tissue around the cotton wicks was peeled off and used as the enzyme source.

Other tissues from animals

Liver, kidney, spleen, intestine, heart, lungs and brain were removed after sacrificing the animals either by anaesthetising with Nembutal (monkey) or by a quick decapitation (rat, rabbit and guinea pig). In the case of the rat the skin was also used for determining enzyme activity. The species of animals used are as follows: monkey (Macaca radiata) and a local inbred strain of albino rat, rabbit and guinea pig. Adult animals were used in each case.

Preparation of tissue homogenates

All operations were carried out at 0-4 °C unless otherwise stated. (a) The granuloma tissue was washed with cold isotonic KCl, blotted and weighed. A 20% homogenate was prepared by initially grinding the tissue with acid-washed glass powder in a mortar and pestle at 0-4 °C and then adding isotonic KCl containing 1 mM CaCl₂. (b) Other tissues from animals were washed with cold isotonic KCl, blotted and weighed. A 20% homogenate was prepared by first cutting them into small pieces with scissors and then homogenizing with 4 vol. of cold isotonic KCl in a teflon homogenizer at 0 °C.

Enzyme assay

Almost all of the homogenate Pz-peptidase activity was found to be associated with the soluble fraction (100 000 × g). However, for ease of operation, before the assay all the homogenates were centrifuged at 10 000 × g for 15 min to remove the fibrous tissue or the tissue debris. The enzyme was assayed in the supernatant fraction by the method of Wünsch and Heidrich containing the substrate (400 μg Pz-peptide), CaCl₂ (2.5 μmoles) and Tris–HCl buffer,
pH 7.4 (50 μmoles). After incubation for 1 h, 3 % citric acid (1 ml) was added to adjust the pH to around 3.0, and the split product, Pz-Pro-Leu was extracted into ethyl acetate (2 ml) and the absorbance measured at 320 nm and the values calculated from a standard curve for Pz-Pro-Leu. In separate experiments, the product was initially identified by thin-layer chromatography on silica gel using n-butanol-acetic acid-water (4 : 1 : 1, by vol.) system using authentic Pz-Pro-Leu.

**Enzyme unit**

1 unit of enzyme activity is defined as the quantity required to liberate 1 μmole of the product (Pz-Pro-Leu) per min. The specific activity is expressed in munits per mg protein.

Protein was determined by the method of Lowry et al.21 using crystalline bovine serum albumin as the standard.

**RESULTS**

**Variation of the enzyme level with age of granuloma tissue and with age of the rat**

The conditions of the assay were initially standardized with extracts from the granuloma tissue and the optimal conditions selected. The variation in the level of Pz-peptidase has been studied in relation to age of granuloma expressed as days after implantation and in relation to the age of the rat using the 4-day-old tissue. In both the groups, the enzyme from each rat was separately assayed and the data statistically analyzed.

The variation of Pz-peptidase activity with the age of granuloma tissue and with the age of the rat is given in Table I. It was found that the specific activity of the enzyme increased with age of the granuloma tissue, the maximum being found in the 4-day-old tissue and thereafter, the activity dropped to almost its initial value. The peak of total activity in the tissue extracts also coincided with the peak of specific activity with the 4-day-old granuloma tissue (Expt A, Table I).

As given in Expt B, Table I, both specific and total activities of the enzyme increased with the age of the rat. The maximum activity was found in 4-month-old rats, and in older animals (5-month) the specific activity decreased to the values of 1-month-old rats but the decrease in total enzyme activity was not so pronounced.

**Distribution of Pz-peptidase in animal tissues**

The enzyme activity was determined in the supernatant fractions obtained from the homogenates of liver, kidney, spleen, intestine, heart, lungs and brain of four species of animals (rat, monkey, rabbit and guinea pig). The results are given in Table II. The specific activities and the calculated values of the total activity in each organ of animals are given.

**DISCUSSION**

The results presented in this paper show that with the 4-month-old rats, the Pz-peptidase activity increased with the age of the granuloma tissue and reached a maximum value on the fourth day. This time period also corresponds to the maximum weight of the tissue. Using 4-day-old granuloma tissue, the maximal Pz-peptidase
<table>
<thead>
<tr>
<th>Expt A</th>
<th>Expt B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age of granuloma tissue (days):</strong></td>
<td><strong>Age of rat (months):</strong></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

- **Weight of granuloma tissue (g):**
  - 0.85 ± 0.11
  - 1.57 ± 0.16**
  - 1.8 ± 0.18**
  - 1.73 ± 0.14**
  - 1.34 ± 0.17*
  - 0.47 ± 0.11
  - 0.44 ± 0.07
  - 1.56 ± 0.24
  - 1.42 ± 0.19**
  - 1.16 ± 0.27**
  - N.S.
  - N.S.

- **Specific activity (munits/mg protein):**
  - 1.78 ± 0.08
  - 2.02 ± 0.08*
  - 2.92 ± 0.14**
  - 1.82 ± 0.22
  - 1.88 ± 0.02
  - 1.83 ± 0.2
  - 2.12 ± 0.35
  - 2.95 ± 0.35***
  - 1.93 ± 0.27
  - N.S.
  - N.S.
  - N.S.
  - N.S.
  - N.S.

- **Total activity (munits):**
  - 35.0 ± 13.8**
  - 97.8 ± 14.7**
  - 173.2 ± 9.1**
  - 94.0 ± 19.6*
  - 77.2 ± 3.4
  - 24.8 ± 6.3
  - 23.8 ± 20.8**
  - 103.7 ± 16.2**
  - 126.9 ± 16.6**
  - N.S.

* Significant; *P* < 0.005.
** Significant; *P* < 0.001.
N.S., not significant; *P* > 0.05.

TABLE I

RELATIONSHIP OF P2-PEPTIDASE ACTIVITY WITH AGE OF GRANULOMA TISSUE AND AGE OF RATS

In Expt A, five animals (4-month-old) in each group and in Expt B, ten animals in each age group were used. Enzyme determinations were made on the tissue from each animal, 2nd-6th day in Expt A and 4-day-old tissue in Expt B. All the results are expressed as mean ± S.D. Statistical analysis was done by the Student's 't' test. *P* denotes the statistical significance of the difference between 2-day-old granuloma and the tissue at the other periods in Expt A, and in Expt B it denotes the difference between 1-month-old rats and the animals in the other age groups.
### TABLE II

**DISTRIBUTION OF Pe-PEPTIDASE IN ANIMAL TISSUES**

The results are expressed as mean ± S.D. Five animals were used in each group and determinations were made on the tissues from each animal separately.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Monkey</th>
<th>Rabbit</th>
<th>Guinea pig</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue wt (g)</td>
<td>Spec. act.*</td>
<td>Total act.**</td>
<td>Tissue wt (g)</td>
</tr>
<tr>
<td>Liver</td>
<td>72.5 ± 30.2</td>
<td>2.18 ± 0.09</td>
<td>9.84 ± 5.55</td>
<td>48.0 ± 27.0</td>
</tr>
<tr>
<td>Intestine</td>
<td>30.7 ± 3.6</td>
<td>1.42 ± 0.07</td>
<td>5.58 ± 0.48</td>
<td>23.0 ± 2.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.8 ± 3.8</td>
<td>1.82 ± 0.1</td>
<td>1.85 ± 0.52</td>
<td>8.42 ± 1.26</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.19 ± 2.17</td>
<td>1.78 ± 0.08</td>
<td>0.96 ± 0.31</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>Heart</td>
<td>8.98 ± 3.8</td>
<td>1.12 ± 0.13</td>
<td>0.49 ± 0.11</td>
<td>4.46 ± 3.3</td>
</tr>
<tr>
<td>Lungs</td>
<td>14.2 ± 1.47</td>
<td>0.77 ± 0.11</td>
<td>0.57 ± 0.06</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>Brain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.16 ± 1.04</td>
</tr>
</tbody>
</table>

* Specific enzyme activity is expressed as munits/mg protein of the tissue extract.
** Total enzyme activity is expressed as international units/organ.
activity was observed in the tissue from 4-month-old rats although the maximum tissue weight was seen in the 3-month-old rats.

Pz-peptidase activity appears to be widely distributed in animal tissues (Table II). From a study of its distribution in the tissue of various animals, it is seen that the highest specific activity of the enzyme was found in rabbit heart and guinea pig kidney and the lowest in monkey and rabbit lungs. In general, in each animal species, the liver contains the highest total peptidase activity followed by intestine and kidney. The total activity in other organs (spleen, heart, lungs and brain) is much less compared to that of liver and kidney in each animal. No uniform pattern of distribution of Pz-peptidase was found in different tissues of one animal. The following general observations can be made. The highest specific activity of the enzyme in each animal was found in the liver (monkey), heart (rabbit), brain (rat), and kidney (guinea pig), respectively. Similarly, the lowest activity was found in each animal in lungs (monkey and rabbit) and in heart (rat and guinea pig), respectively. A comparison of the specific enzyme activity of different animals of the same tissue shows that the highest activities were present in monkey liver, in guinea pig kidney and in rabbit heart. The spleen, intestine, lungs and brain of the guinea pig showed the highest specific activity, and in general its tissues exhibited higher specific activities than in other animals. The skin enzyme was studied only in the case of the rat (not shown in Table II) and the specific activity (3.8 ± 0.08) was the highest observed so far in any of the tissues.

The physiological significance of the Pz-peptidase is not clear. While studying the peptidase and collagenase activities in the invasion zones of tumours of the breast, Kendttsch and Strauch found that the pattern of distribution of Pz-peptidase and collagenolytic activity was similar. With a growth culture of primary mouse fibroblasts and in the rat uterus during post-partum involution, a close correlation between Pz-peptidase and collagen metabolism was noted. In a study of collagen development in the chick embryo skin, Woessner reported that the peak of this peptidase activity coincided with that of free hydroxyproline. Since the synthetic substrate is not susceptible to the action of a number of proteases and peptidases but only to bacterial collagenase and the specific Pz-peptidase from animal tissues reported here and elsewhere, the results would indicate a close relationship between the Pz-peptidase and collagen metabolism. In fact, some evidence has recently been reported for its possible role in collagen degradation in vivo; Harris and Krahe have shown that a rheumatoid synovial culture fluid protease acting on gelatin and the Pz-peptidase but not on native collagen, has the capacity to cleave collagenase breakdown products, and after denaturation, into smaller dialyzable fragments. The relationship between this protease and the Pz-peptidase purified from rat granuloma extracts and the Pz-peptidase reported here in the tissues of various animals is not clear at present. A definitive conclusion on the possible role of the Pz-peptidase present in different tissues in the physiological degradation of collagen or its fragments must await further experimental evidence.

ACKNOWLEDGEMENTS

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REFERENCES