ABSTRACT
Angiotensin-converting enzyme was purified 1500-fold from a homogenate of hog lungs. The purification procedure included fractionation with ammonium sulfate, inactivation of contaminating enzymes at pH 4.7, batch treatment with CM-cellulose, and chromatography on columns of DEAE-cellulose, hydroxyapatite, and Sephadex G-200. The enzyme was assayed with hippurylglycylglycine and, after pH 4.7 was established, with both hippurylglycylglycine and angiotensin I as substrates. The ratio of activity toward the two substrates remained constant throughout the later stages of purification. The final enzyme preparation, on analytical disc gel electrophoresis, showed one major protein band with the expected ratio of activities toward both substrates. The molecular weight was estimated to be approximately 300,000 by gel filtration. The pH optimums with hippurylglycylglycine and angiotensin I as substrates were 8.4 and 7.5, respectively, and chloride ion was required for optimal activity of the enzyme with both substrates. Hydrolysis of both substrates was inhibited by ethylenediaminetetraacetic acid (EDTA) or the pentapeptide pyroglutamyllysyltryptophanylalanylproline. It would, therefore, appear that a single enzyme, purified in the described manner, hydrolyzes both angiotensin I and hippurylglycylglycine.

KEY WORDS dipeptidyl carboxypeptidase carboxypeptidase chloride activation Bothrops jararaca pentapeptide
ANGIOTENSIN-CONVERTING ENZYME

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Phe, beef liver catalase, and horse spleen apoferritin were obtained from Mann Research Laboratories, Sigma Chemical Company, and Research Plus Laboratories, respectively. Hip-Gly-Gly was prepared by treating Gly-Gly-Gly (Mann Research Laboratories) with a slight excess of benzoyl chloride at pH 8.0. After acidification and washing with hot carbon tetrachloride, the product was crystallized from hot water. The melting point was 225-227°C (215-216°C [18]). There was no free glycine, Gly-Gly, or Gly-Gly-Gly in the preparation, and amino acid analysis of an acid hydrolysate showed the expected amount of glycine.

PREPARATION OF ADSORBENTS

CM-cellulose (Whatman CM-32) was treated with 0.5N NaOH and 0.5N HCl and equilibrated with 0.01M sodium acetate buffer, pH 4.7. The adsorbent was allowed to settle overnight and was resuspended in buffer to twice its settled volume. DEAE-cellulose (Whatman DE-32) was treated with 0.5N HCl and 0.5N NaOH and equilibrated with 0.01M sodium phosphate buffer, pH 7.0. Fines were removed. Hydroxyapatite (BioRad HTP) was washed several times with 0.001M sodium phosphate buffer, pH 5.7. Fines that did not settle in 3 minutes were discarded. Sephadex G-200 was allowed to swell for 5 days in 0.1M phosphate buffer, pH 7.0.

ENZYME ASSAYS

Converting enzyme assays were performed by the automated method described previously (17). This method uses the ninhydrin reaction to measure the new free amino group released when a peptide bond is hydrolyzed. Enzyme and substrate were incubated in 0.05M sodium phosphate buffer and 0.1M sodium chloride, pH 8.0, usually for 1 hour at 37°C. Substrate concentrations, unless stated otherwise, were AI 2.5 x 10^{-4}M and Hip-Gly-Gly 10^{-5}M. The analytical system was calibrated with His-Leu when the substrate was AI and with Gly-Gly when it was Hip-Gly-Gly.

One unit of converting enzyme activity was defined as that amount of enzyme which hydrolyzed 1 µmole Hip-Gly-Gly/min at 37°C under the conditions described above. The specific activity was given as the number of units per milligram of protein.

Other peptidase activities were determined by incubation of enzyme and substrate for 1 hour at 37°C in 0.05M sodium phosphate buffer and sodium chloride, pH 8.0, using AI (10^{-4}M) for angiotensinase, Cbz-Gly-Phe (2 x 10^{-4}M) for carboxypeptidase, and His-Leu, Gly-Gly, and Gly-Gly-Gly (all 5 x 10^{-6}M) for the additional peptide-hydrolyzing activities. Any increase in ninhydrin-reactive material was measured by the automated method previously described (17).

MISCELLANEOUS METHODS

Protein concentrations were determined by an automated modification of the method of Lowry et al. (19). Enzyme solutions at various stages of purification were concentrated with an Amicon ultrafiltration apparatus using a PM-10 membrane. Analytical disc gel electrophoresis (20) was performed with a Canaleco apparatus model 1200 at pH 9.5 with a 7% gel. Electrophoresis was conducted for 2 hours at room temperature with 2 mA/tube. Gels were stained with 0.05% Coomassie Blue in 12.5% trichloroacetic acid.

Results

PREPARATION OF CONVERTING ENZYME

Fresh whole hog lungs were frozen and thawed twice and cut into small pieces. After draining away excess blood, the tissue was homogenized with cold 0.05M sodium phosphate buffer, pH 7.0 (2 liters/kg tissue) in a Waring blender for 2 minutes. The homogenate was strained through gauze, frozen and thawed twice, and centrifuged at 2,000 g for 2 hours. All subsequent operations were performed at 0-5°C. The supernatant solution (fraction A, Table I) was fractionated with ammonium sulfate between the limits of 1.2-3.0M, and the resulting precipitate was dissolved in and dialyzed against 0.001M sodium phosphate buffer, pH 7.0 (fraction B, Table 1). All dialyses were carried out against at least 50 volumes of buffer for 18 hours with one change of buffer after 4 hours. The enzyme preparation was refractionated with ammonium sulfate between the limits of 1.9-2.5M. The precipitate was dissolved in and dialyzed against 0.001M sodium phosphate buffer, pH 7.0 (fraction C, Table 1), titrated to pH 4.7 with 0.1M phosphoric acid, centrifuged to remove the precipitate which formed, and dialyzed overnight against 0.01M sodium acetate buffer, pH 4.7, to yield fraction D (Table 1).

A CM-cellulose suspension (60 ml/g protein) was added to the enzyme solution, and the mixture was stirred for 30 minutes. After filtration through Whatman no. 54 paper on a Buchner funnel, the enzyme was concentrated...
**TABLE 1**

**Purification of Converting Enzyme**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Enzyme* (units)</th>
<th>Specific activity (units/mg)</th>
<th>A1/HGG†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Lung extract‡</td>
<td>2,180</td>
<td>42,500</td>
<td>283</td>
<td>0.0067</td>
<td></td>
</tr>
<tr>
<td>B 1.2-3.0M (NH₄)₂SO₄</td>
<td>455</td>
<td>13,400</td>
<td>146</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>C 1.9-2.5M (NH₄)₂SO₄</td>
<td>152</td>
<td>3,040</td>
<td>91</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>D pH 4.7, supernatant solution</td>
<td>188</td>
<td>2,290</td>
<td>88</td>
<td>0.038</td>
<td>0.37</td>
</tr>
<tr>
<td>E Batch CM-cellulose filtrate</td>
<td>19</td>
<td>53</td>
<td>40</td>
<td>0.75</td>
<td>0.40</td>
</tr>
<tr>
<td>F DEAE-cellulose column pool</td>
<td>10</td>
<td>5.9</td>
<td>18</td>
<td>3.1</td>
<td>0.38</td>
</tr>
<tr>
<td>G Hydroxyapatite column pool</td>
<td>2.5</td>
<td>1.2</td>
<td>6.1</td>
<td>5.1</td>
<td>0.41</td>
</tr>
<tr>
<td>H Sephadex G-200 column pool</td>
<td>2.6</td>
<td>0.4</td>
<td>4.0</td>
<td>10</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*One unit = 1 μmole Hip-Gly-Gly hydrolyzed/min under standard assay conditions (see text).
‡Calculated on the basis of 1 kg (wet weight) of fresh hog lungs. Actual weight of lungs for this preparation was 2.5 kg.

Almost to dryness by ultrafiltration, taken up in 0.01M sodium phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer (fraction E, Table 1).

At this stage of purity, the converting enzyme activity was stable for at least 6 months at —20°C and could withstand repeated freezing and thawing. Enzyme diluted to 0.1 mg protein/ml was stable for 4 days at 5°C at pH values between 4.5 and 9.0. Below pH 4.5, enzyme activity was lost. The preparation exhibited no hydrolysis of His-Leu, Gly-Gly, Gly-Gly-Gly, or Cbz-Gly-Phe (carboxypeptidase activity). However, traces of angiotensinase activity remained.

The enzyme was pumped (1.0 ml/min) onto a 2.5 X 45-cm column of DEAE-cellulose and eluted with a linear gradient of sodium chloride (0-0.15M) in 0.01M sodium phosphate buffer, pH 7.0 (initial volume of buffer in

**FIGURE 1**

Purification of converting enzyme (130 mg of fraction E) on a column of DEAE-cellulose. Converting enzyme (●), protein (○), angiotensinase (△).

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gradient mixing chamber was 1 liter). The elution position of the enzyme nearly coincided with that of a major protein peak (Fig. 1); however, the final traces of angiotensinase activity in the preparation were completely removed. Fractions 76 through 107 were pooled, concentrated almost to dryness, and taken up in 0.01M sodium phosphate buffer, pH 7.0 (fraction F, Table 1). Purification of this step was about 4-fold with a recovery of 45% of the converting enzyme activity.

Following dialysis overnight against 0.001M sodium phosphate buffer, pH 5.7, the enzyme was pumped (0.5 ml/min) onto a 1.2 X 22-cm column of hydroxyapatite. Elution was performed with a linear gradient of sodium phosphate buffer from 0.001M, pH 5.7, to 0.1M, pH 6.8 (initial volume of buffer in gradient mixing chamber was 400 ml). The elution pattern is shown in Figure 2. Fractions 20 through 30 were pooled, concentrated almost to dryness by ultrafiltration, and taken up in 0.1M sodium phosphate buffer, pH 7.0 (fraction G, Table 1). The purification of this step was 1.6-fold with a recovery of 34%. The converting enzyme at this stage of purity tended to lose activity slowly even when stored at -20°C.

The enzyme preparation was applied to the top of a 2.5 X 102-cm column of Sephadex G-200, and 0.1M sodium phosphate buffer, pH 7.0, was pumped through the column at a flow rate of 0.23 ml/min. As shown in Figure 3, the converting enzyme activity coincided with one of two protein peaks. The fractions containing the enzyme activity were pooled and concentrated to small volume by ultrafiltration (fraction H, Table 1). With calibration of the column with catalase and apoferritin (21), the estimation of the molecular weight of lung converting enzyme was approximately 300,000.

The preceding purification sequence resulted in a 1500-fold purification with recovery of 1.4% of the enzyme activity present in the initial lung extract. A subsequent preparation, during which care was taken to concentrate the enzyme immediately after each chromatography step, yielded 3% of the initial activity. Most of the improvement in yield took place during the hydroxyapatite chromatography step.
ALTERNATE ATTEMPTS TO EXTRACT ENZYME

The procedure described above was repeated with 0.4% Triton X-100 included in the 0.05M sodium phosphate buffer used for homogenization of the lung tissue. There was no increase in the amount of converting enzyme activity extracted as determined by assay after the first ammonium sulfate step (fraction B).

An attempt was made to isolate lung converting enzyme by solubilization of a particulate fraction. A kilogram of fresh hog lungs was cut into small pieces and homogenized for 1 minute in 0.05M sodium phosphate buffer, pH 7.0. The homogenate was centrifuged for 1 hour at 2,000 g to remove debris and then at 25,000 g for 5 hours. The precipitate was homogenized for 2 minutes in a Waring blender in 0.01M sodium phosphate buffer, pH 7.0, containing 0.4% Triton X-100 and then stirred for 2 hours. Centrifugation at 120,000 g for 2 hours yielded a clear supernatant solution which contained 23 units of converting enzyme activity. This was further purified by fractionation with ammonium sulfate and treatment with CM-cellulose as described above. The resulting preparation had 4.6 units of converting enzyme activity with a specific activity of 0.64 and a ratio of the hydrolysis of AI to that of Hip-Gly-Gly of 0.38 compared with fraction E which had a yield of 40 units, specific activity of 0.75, and substrate-activity ratio of 0.40.

IDENTIFICATION OF REACTION PRODUCTS

Hip-Gly-Gly (0.5 μmole) was incubated with enzyme from fraction E for 1 hour at 37°C in 0.05M sodium phosphate buffer and 0.1M sodium chloride, pH 8.0. The reaction was stopped by boiling, and a sample was applied to the amino acid analyzer (Technicon single-column analysis). Gly-Gly (0.35 μmole) was identified as one of the reaction products. There were no traces of Gly-Gly-Gly or free glycine.

A similar experiment was carried out using 1 μmole of AI and enzyme from fraction G.
Biological activity of converting enzyme-AI reaction product as shown by countercurrent distribution and pressor assay in the rat. At zero time (substrate added after boiling enzyme for 10 minutes) (○); values were calculated as AI. After incubation for 1 hour at 37°C (●); values were calculated as AII.

Analysis showed 0.43 µmole of His-Leu with no traces of free amino acids.

To demonstrate AII as a product, 0.2 µmole of AI was incubated with enzyme from fraction G as above. A sample was subjected to ten-tube countercurrent distribution and bioassay in the rat as described previously (17). The distribution pattern shown in Figure 4 demonstrates conversion of AI to AII.

PROPERTIES OF THE LUNG CONVERTING ENZYME

Analytical disc gel electrophoresis of enzyme preparations. Left: 37 µg of fraction E. Right: 8 µg of fraction H.
Hydrolysis of Hip-Gly-Gly (●) and AI (○) by converting enzyme (fraction H) as a function of enzyme concentration. Incubation conditions are described in the text.

Figure 6 shows the converting enzyme activity as a function of enzyme concentration. The data were obtained using enzyme from fraction H after it had been stored in the freezer for 2 months, during which period it had been frozen and thawed four times. Calculation of the specific activity from Figure 6 (6 units/mg) showed that the enzyme had lost 40% of its initial specific activity (Table 1, 10 units/mg). However, in spite of this loss of activity, the ratio of hydrolysis of the two substrates was not significantly altered (Fig. 6 0.41, Table 1 0.39).

The pH optimum for lung converting enzyme was about 7.5 for AI and 8.4 for Hip-Gly-Gly (Fig. 7).

The effect of increasing sodium chloride concentration on converting enzyme activity is shown in Figure 8. The biphasic curve obtained with Hip-Gly-Gly as substrate was observed with enzyme from both fractions G and H and appeared to be a valid description of the activation by chloride. The high activity at 1M chloride cannot be explained in terms of an altered response of the analytical system at this high salt concentration. The rate of hydrolysis of Hip-Gly-Gly in the absence of chloride was less than 2% of the rate with 0.1M chloride. With AI as substrate, this value was about 6%. It is not known whether the enzyme has residual activity in the absence of chloride or whether the observed activity is due to contamination by trace amounts of chloride. Concentrations of sodium chloride below $10^{-4}$M did not increase the lung converting enzyme activity toward either substrate.

The activity of lung converting enzyme toward both AI and Hip-Gly-Gly was decreased by about 50% by incubation of the enzyme (fraction E) for 10 minutes at 37°C with $5 \times 10^{-4}$M Pyr-Lys-Trp-Ala-Pro, a peptide isolated from the venom of Bothrops jararaca (22). Incubation with $10^{-5}$M ethylenediaminetetraacetic acid (EDTA) resulted in greater than 90% inhibition with both substrates.

**Discussion**

The extraction of converting enzyme from lung tissue in a soluble rather than an
FIGURE 7
Converting enzyme activity as a function of pH. Hydrolysis of Hip-Gly-Gly by 0.25 µg of fraction G (•); hydrolysis of Al by 1.0 µg of fraction G (o). Incubation conditions as described in text except that sodium phosphate buffers of varying pH were used.

FIGURE 8
Converting enzyme activity as a function of sodium chloride concentration. Hydrolysis of Hip-Gly-Gly by 0.125 µg of fraction G (•); hydrolysis of Al by 1.0 µg of fraction G (o). Incubation conditions as described in the text except for variation in sodium chloride concentration. The change in the pH of incubation mixtures (pH 8.0) at high sodium chloride concentration was not significant. Values shown are corrected for a decrease in the observed ninhydrin reactivity of Gly-Gly and His-Leu at sodium chloride concentrations above 0.2M.
The insoluble fraction represents a compromise. Because of the particulate nature of the enzyme (7, 8, 14), our initial attempts at its isolation involved sedimentation at 25,000 or 120,000 g, followed by solubilization with Triton X-100, as reported by Cushman and Cheung (8). However, yields of enzyme activity obtained by this means were low and not reproducible. Furthermore, the processing of large amounts of lung tissue with the available centrifuge equipment was impractical. On the other hand, the method we described in this paper gave a much improved and a reproducible yield of extracted enzyme. It is probable that the freezing and thawing steps, along with homogenization in a Waring blender, effected considerable solubilization of the enzyme. It is noteworthy that the ratio of activities toward the two substrates, AI and Hip-Gly-Gly, was similar with enzyme prepared by the two different methods of extraction.

The concentrations of converting enzyme in lung and plasma of the hog were 283 nmoles/min g⁻¹ tissue and 18 nmoles/min ml⁻¹, respectively, as measured by our assay method (10⁻⁴M Hip-Gly-Gly, 0.05M sodium phosphate buffer and 0.1M sodium chloride, pH 8.0, 1 hour at 37°C). The ratio of specific activities calculated from these values was 19 to 1 (lung to plasma). This may be compared with a ratio of 30 to 1 in the rat (5). Lee et al. (13) reported lung-plasma ratios of 3.2 and 1.1 for guinea pig and hog when calculated on the basis of units per gram tissue or milliliter plasma.

Hip-Gly-Gly is a very useful substrate for the assay of converting enzyme activity by the automated ninhydrin method (17). The compound itself gives a negligible blank and is hydrolyzed very slowly, even by crude extracts, in the absence of added sodium chloride. The product, Gly-Gly, is not hydrolyzed by crude lung preparations (fraction A) at dilutions of enzyme used for assay with Hip-Gly-Gly. In contrast, AI is attacked rapidly by angiotensinases present in lung extracts. The product of the hydrolysis of AI by converting enzyme, His-Leu, is also hydrolyzed rapidly by lung preparations prior to the pH adjustment step (fraction D). The lowering of the pH to 4.7 inactivated the bulk of the His-Leu-hydrolyzing activity; the remaining traces were removed during the batch CM-cellulose step.

We have demonstrated that the products of lung converting enzyme with AI are All and His-Leu. No other amino acid or peptide fragments were found. Thus, the only bond in AI hydrolyzed by this enzyme is the phenylalanyl-histidyl bond. This is an important demonstration since lung extracts contain a very potent His-Leu-hydrolyzing activity (13), and it has been shown that under certain conditions leucine rather than His-Leu can be the major C-terminal fragment formed from angiotensin I (23).

Huggins et al. (9) and Bakhle and Reynard (10) reported pH optima of 7.25 and 7.0, respectively, for dog lung converting enzyme acting on AI, compared with our value of 7.5 for the enzyme from hog lung. The pH optimum of 8.0-8.5 found by Cushman and Cheung (11) for rabbit lung enzyme acting on Hip-His-Leu is similar to our value of 8.4 for Hip-Gly-Gly.

The chloride concentration curve (Fig. 8) shows a striking difference between the two substrates. A somewhat similar situation has been observed for leucine aminopeptidase, where increasing potassium chloride concentration inhibits hydrolysis of leucine hydrazide but enhances (biphasic curve) hydrolysis of leucine p-nitroanilide 100-fold up to 4.6M potassium chloride (24).

The molecular weight of hog lung converting enzyme of approximately 300,000 is considerably larger than the value of 150,000 reported by Lee et al. (13). The molecular weights of converting enzyme from other sources are hog plasma 155,000 (25), human plasma 150,000 (26), and human lung 480,000 (12). Lung and plasma converting enzymes have similar enzymological properties (9).
however, their identity has not been established.

Yang et al. (15) reported that converting enzyme ("dipeptide hydrolase" or "dipeptidyl carboxypeptidase") from hog plasma hydrolyzed several tri- and tetrapeptide derivatives with a constant ratio of hydrolysis. Eliseeva et al. (27) showed that a converting enzyme preparation purified from hog kidney ("carboxycathepsin") hydrolyzed many peptides with at least three amino acid residues in addition to AI and bradykinin. However, these authors presented no quantitative data. In the present work, the constant ratio of the rate of hydrolysis of AI to that of Hip-Gly-Gly throughout the later stages of purification (Table 1) is taken as evidence that both substrates are hydrolyzed by the same enzyme protein.

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Purification and Properties of Angiotensin-Converting Enzyme from Hog Lung
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