Post-proline Cleaving Enzyme

PURIFICATION OF THIS ENDOPEPTIDASE BY AFFINITY CHROMATOGRAPHY*

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The endopeptidase, post-proline cleaving enzyme, has been purified 10,500-fold in an overall yield of 18% from lamb kidney. The enzyme possesses a specific activity of 45 μmol/mg/min as tested with the substrate Z-Gly-Pro-Leu-Gly ($K_m = 6.0 \times 10^{-5}$), has a molecular weight of 115,000, is comprised of two subunits with a molecular weight of 57,000, and exhibits maximal activity at pH 7.5 to 8.0. With the exception of the -Pro-Pro- linkage, the -Pro-X-peptide bond (X equals l- and ω-amino acid residues) located internally in the peptide sequence can be hydrolyzed (cleavage occurs faster when X = lipophilic side chain as compared to X = acidic side chain). The appropriate -Pro-X- bonds in zinc-free porcine insulin, oxytocin, arginine vasopressin, angiotensin II, bradykinin-potentiating factor were cleaved. Human gastrin, adrenocorticotropic hormone, denatured guinea pig skin collagen, and ascaris cuticle collagen were not degraded. Dipeptides with the structure Z-Pro-lm-X competitively inhibit post-proline cleaving enzyme.

* This research was supported by Research Grant AM 18399 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.
benzoyl-Gly-Phe, Z-Pro-Leu-Gly, Leu-Gly, polylisine HBr (type VII-B and hydroxyapatite; type I) (Sigma Chemical Co.), human angiogenin II, ammonium sulfate (enzyme grade) (Schwarz/Mann), and Epogel-agarose, and standard proteins for molecular weight determination (Pharmacia).

Synthesis of Peptide Tested for the Inhibition of Post-proline Cleaving Enzyme—Z-O-Pro-ONSu was synthesized as reported for the tisomer by Anderson et al. (28). The product was recrystallized from absolute ethanol, yield 55.6%; m.p. 89 to 91; [α]D +4 +82 (c 2.0, 1.4-dioxane).

C17 H36 N4 O6

Calculated: C 59.0 H 5.24 N 8.09

Found: C 59.0 H 5.47 N 7.87

All the dipeptides listed in Table 1, which summarizes yields, analytical and physical properties, were prepared using the N-hydroxysuccinimide ester method (23). The Boc group of Z-Pro-Lys(Boc) was removed with trifluoroacetic acid (25).

Methods—Protein determinations were performed according to Lowry et al. (26), using bovine serum albumin as standard. Amino acid analyses were carried out as described by Moore (27); peptides were hydrolyzed for 18 or 24 h at 110° in 6 N HCl under vacuum.

Preparation of Z-Pro-O-Ala-Poly(Lys)-Sepharose 4B Column—The method employed for the activation of Sepharose 4B was based on the procedure described by Cuatrecasas (28). A suspension of Sepharose 4B in water (50 ml of polymer plus 50 ml) was activated by adding 5 g of finely pulverized CNBr. The reaction was allowed to proceed at 20° for 12 min while the pH was maintained at 10.5 to 11 by addition of 4 M NaOH. After 30 min the product was washed with 500 ml each of 0.1 N NaOH. After 30 min the reaction was started by addition of the substrate solution and after 5 or 10 min at 37°, the reaction was stopped by addition of 0.5 ml of a ninhydrin solution (29), or in the case of [14C]AVP, by acidification with 0.005 ml of 1 N acetic acid. In controls the substrate solution was added after the ninhydrin solution.

For analysis of the [14C]AVP digest, 10- to 30-μl aliquots of the incubation mixture were subjected to high voltage electrophoresis (2500 V, 1 h, pH 3.5) on Whatman No. 3MM paper and scanned for radioactivity. When Z-Gly-Pro-Leu-Gly was used as substrate, a standard curve of Leu-Gly was determined with ninhydrin in each assay.

Electrophoretic Analysis of Enzyme Preparations—The purity of the enzyme preparation was examined by disc gel electrophoresis based on the method of Ornstein (30) and Davies (31). A 7% acrylamide gel in 0.37 M Tris/HCl (pH 8.9) was prepared using ammonium persulfate as the catalyst. For pre-electrophoresis a current of 2 mA/tube was applied to the gels for 1 h in 0.37 M Tris/HCl (pH 8.9) containing 1 mM diethiothreitol and 1 mM EDTA. This activity preparation was dissolved in 62 mM Tris/HCl (pH 6.7) containing 1 mM diethiothreitol and 1 mM EDTA at 10% sucrose (w/v). Matched pairs of gels were prepared. One of the gels was stained for protein in 0.1% Coomassie brilliant blue in 10% trichloroacetic acid, the other sliced into 1-mm sections. The enzymatic activity of each disc, which was suspended in 50 μl of 0.2 M phosphate buffer, pH 6.8, and was then analyzed under standard conditions using [14C]AVP (diluted 1:1) as substrate. Incubation periods were 15, 30, or 60 min depending on enzyme activity and 20-μl aliquots of the mixture were spotted on a Whatman No. 3MM paper and scanned for radioactivity.

Purification Procedures of Post-proline Cleaving Enzyme—The method given below is designed for purification of the enzyme from 6 to 8 lamb kidneys having a total weight of 250 to 350 g. All steps were carried out at 4° unless otherwise described and all centrifugations carried out at 24,500 × g for 15 min. During purification four kinds of buffers were used. Buffer A: 2 mM diethiothreitol and 2 mM EDTA in 50 mM sodium phosphate buffer (pH 6.8); Buffer B: 1 mM diethiothreitol and 1 mM EDTA in 25 mM sodium phosphate (pH 6.8); Buffer C: 1 mM diethiothreitol and 1 mM EDTA in 55 mM sodium phosphate (pH 6.8); and Buffer D: 1 mM diethiothreitol and 1 mM EDTA in 50 mM sodium phosphate (pH 6.8).

TABLE 1

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>Crystallization solvent</th>
<th>Yield</th>
<th>Melting point (not corrected)</th>
<th>Formula (molecular weight)</th>
<th>Calculated</th>
<th>Found</th>
<th>O.R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Pro-Glu(ObBu)</td>
<td>EtOAc</td>
<td>%</td>
<td>133-136°</td>
<td>C16H22N6O6 (488.51)</td>
<td>64.1</td>
<td>6.02</td>
<td>5.98</td>
</tr>
<tr>
<td>Z-Pro-Glu</td>
<td>EtOAc/EtOH</td>
<td>39</td>
<td>134-136°</td>
<td>C16H22N6O6 (378.36)</td>
<td>57.1</td>
<td>5.90</td>
<td>7.40</td>
</tr>
<tr>
<td>Z-Pro-o-Ala</td>
<td>EtOH/EtOH</td>
<td>46</td>
<td>168-170°</td>
<td>C16H22N6O6 (401.67)</td>
<td>47.0</td>
<td>8.64</td>
<td>8.88</td>
</tr>
<tr>
<td>Z-Pro-o-Ala</td>
<td>EtOH/EtOH</td>
<td>46</td>
<td>168-170°</td>
<td>C16H22N6O6 (320.35)</td>
<td>60.0</td>
<td>6.29</td>
<td>8.74</td>
</tr>
<tr>
<td>Z-Pro-o-Ala</td>
<td>EtOH/EtOH</td>
<td>46</td>
<td>168-170°</td>
<td>C16H22N6O6 (396.45)</td>
<td>66.6</td>
<td>6.10</td>
<td>7.07</td>
</tr>
<tr>
<td>Z-Pro-o-Ala</td>
<td>EtOH/EtOH</td>
<td>46</td>
<td>168-170°</td>
<td>C16H22N6O6 (417.42)</td>
<td>56.3</td>
<td>5.67</td>
<td>9.85</td>
</tr>
</tbody>
</table>

* Analysis performed by Robertson Laboratories, Florham Park, N.J.

* DCHA, dicyclohexylamine.
of Buffer A in a Vir-Tis model 45 homogenizer for four 30-min periods, stirred at 22°C for 1 h, and centrifuged. The pellet was washed with 300 ml of Buffer A and the combined supernatant fraction, solid ammonium sulfate was slowly added with constant stirring until 50% saturation (313 g/liter at room temperature) was reached. After stirring at 22°C for 2 h, the precipitate which formed was separated by centrifugation, washed with 200 ml of Buffer A saturated to 50% with ammonium sulfate and the ammonium sulfate concentration in the combined supernatant fraction was raised to 65% by gradual addition of solid ammonium sulfate (101 g/ml) while being stirred at 22°C for 1 h. The suspension was kept overnight and centrifuged. The supernatant fraction was combined and the enzyme precipitate was dissolved in 3 ml of Buffer B and stirred at 22°C for 1 h. The suspension was kept overnight and precipitate formed by addition of 0.5 ml of nihydrin reagent.

Identification of Cleavage Products of Naturally Occurring Peptides by Post-proline Cleaving Enzyme—Peptide (0.5 to 1.0 μmol) was dissolved in 200 ml of 0.1 M ammonium bicarbonate (pH 7.8) containing 1 mM dithiothreitol and 1 mM EDTA. The reaction was carried out for 2 min at 37°C and terminated by addition of 0.5 ml of nihydrin reagent.

Results

As can be seen in Fig. 1, gradient elution from DEAE-Sephadex A-50 (36) resulted in two peaks capable of inactivating [14C]AVP. While the first peak released labeled Arg-Gly-NAH and Gly-NAH from [14C]AVP, the second produced only Arg-Gly-NAH. Moreover, only fractions of the latter peak released Leu-Gly from Z-Gly-Pro-Leu-Gly. Therefore, the second peak contained the post-proline cleaving enzyme. The yield of the enzyme at this step was consistently greater than 100% (>g) in one experiment 160% and 140% in terms of cleavage of Z-Gly-Pro-Leu-Gly and [14C]AVP, respectively, possibly revealing the presence of enzyme inhibitor or activation of a precursor of the enzyme.

Further Purification of Post-proline Cleaving Enzyme by Affinity Chromatography—Affinity chromatography using Sepharose as the carrier for the column, poly(Lys) as the bridge, and the inhibitor Z-Pro-n-Ala as the ligand, was most successful among a number of combinations of spacer groups and ligands tested. A major increase in the specific activity of the post-proline cleaving enzyme preparation was obtained (Fig. 2). Z-Pro-Phc or Z-Pro-Glu (5 mM) were found to be most successful in eluting the enzyme. The final step, a rechromatography on the DEAE-Sephadex A-50 column, removed the dipeptide used for enzyme elution and to a certain extent other proteins absorbed on the affinity column.

Criteria for the Purity of Post-proline Cleaving Enzyme—Progress in the purification of the enzyme was followed by analytical disc gel electrophoresis (Fig. 3). The post-proline cleaving enzyme preparation after the affinity chromatography step had an average specific activity of 40 units/mg. The preparations revealed two sharp minor bands with slightly lower migration rates than a third band (Fig. 3). The latter is the major band and a scan of the gel is shown in Fig. 3. Analysis of 1-mm gel slices using [14C]AVP as substrate showed that enzymatic activity was associated only with the major band.

Molecular Weight Estimate—The molecular weight of the

Post-proline Cleaving Enzyme 7595

Effect of pH on Enzyme Activity—For kinetic studies the reaction mixture (200 μl) contained 1 to 4 milliunits of enzyme activity and 6.4 to 10 × 10⁻⁶ nmol of peptide(s) (the substrate alone or substrate plus inhibitor) in 25 mM sodium phosphate buffer (pH 7.7) containing 1 mM dithiothreitol and 1 mM EDTA. The reaction was carried out for 2 min at 37°C and terminated by addition of 0.5 ml of nihydrin reagent.

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Molecular Weight Estimate—The molecular weight of the
Post-proline Cleaving Enzyme

Fig. 1. First DEAE-Sephadex A-50 column chromatography of post-proline cleaving enzyme.

Fig. 2. Affinity column chromatography of post-proline cleaving enzyme by using Z-Pro-β-Ala-poly(Lys)-Sepharose 4B. The arrow indicates start of elution of the enzyme with 5 mM Z-Pro-Phe.

Fig. 3. Scan of polyacrylamide gel electrophoretic pattern of post-proline cleaving enzyme at the final stage of purification. The inset shows the progress of purification and migration pattern of post-proline cleaving enzyme determined by analytical gel electrophoresis. A, sample after first DEAE-Sephadex A-50 chromatography; B, after hydroxyapatite step; C, after DEAE-Sephadex A-50 chromatography following affinity chromatography. Enzyme activity is only associated with the major band in C. The scan corresponds to Sample C.

enzyme at the final stage of purification was estimated to be 115,000 by gel filtration on a Sephadex G-150 column. Gel electrophoresis in the presence of sodium dodecyl sulfate indicates an average molecular weight of 57,000 for post-proline cleaving enzyme monomer.

Stability and Storage of Enzyme – The purified enzyme dissolved in 1 mM EDTA and dithiothreitol at pH 7.0 may be stored frozen at −20 to −70° for several months without any detectable loss of activity. Lyophilized material had lost about 10% of its activity after 1 day and 50% after 1 week when kept at room temperature.

Profile of Enzyme Activity as Function of pH – The activity of the enzyme was examined in citrate, phosphate, and borate buffers covering a pH range of 3.5 to 9.5. Maximal activity was observed in the range of pH 7.5 to 8.0 with Z-Gly-Pro-Leu-Gly as substrate, identical to earlier observations (14). Thus, all experiments described below were carried out at pH 7.7 to 7.8.

Interaction of Oligopeptides with Post-proline Cleaving Enzyme – All N-blocked dipeptides used with the general formula Z-Pro-X were not cleaved by post-proline cleaving enzyme, i.e., Z-Pro-β-Ala, Glu, Leu, Lys, Phe. Even when the incubation time was extended from 4 to 50 min no detectable cleavage occurred. However, these Z-Pro-X peptides were found to competitively inhibit the cleavage of Z-Gly-Pro-Leu-Gly (Table II).
Z-Pro-Glu was the strongest inhibitor with a $K_i$ value of $1.3 \times 10^{-5}$. Z-Pro-D-Ala and Bz-Gly-Phe were found to be neither substrates nor inhibitors. Other amino acids and peptides which were neither cleaved nor inhibited the enzyme included proline, Z-Pro, Ala, Pro-Gly, Pro-Leu-Gly, Gly-Pro-Hyp, Leu-Gly-NH$_2$, formyl-Pro-Leu-Gly-NH$_2$, and Pro-Leu-Gly-NH$_2$. Moreover, while Z-Pro-Leu-Gly was found to be an inhibitor, Z-Gly-Pro-Leu was a good substrate. Z-Gly-Pro-Leu-Gly-Pro was an even better substrate and the best was Z-Gly-Pro-Leu-Gly with a $K_i$ of $6.0 \times 10^{-5}$.

### TABLE II

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>Per cent inhibition</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Pro-Leu</td>
<td>4.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>Z-Pro-Ala</td>
<td>7.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>Z-Pro-Phe</td>
<td>21</td>
<td>$3.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>Z-Pro-Glu</td>
<td>20</td>
<td>$3.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Z-Pro-Glu(OBz)</td>
<td>22</td>
<td>n.d.</td>
</tr>
<tr>
<td>Z-Pro-Gly</td>
<td>11</td>
<td>n.d.</td>
</tr>
<tr>
<td>Z-Pro-Lys(Boc)</td>
<td>22</td>
<td>n.d.</td>
</tr>
<tr>
<td>Z-Pro-D-Ala</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bz-Gly-Phe</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The incubation mixture (100 µl, final volume) contained 6.4 milliunits of enzyme activity, 100 nmol of Z-Gly-Pro-Leu-Pro in the presence or absence of 120 nmol of N-blocked dipeptide. Incubation was at 37°C for 5 min. The reaction was stopped by addition of 0.5 ml of ninhydrin reagent.

* n.d., not determined.

### TABLE III

**Sites of hydrolysis of naturally occurring peptides by post-proline cleaving enzyme**

<table>
<thead>
<tr>
<th>Substrate and site of cleavage</th>
<th>Identified product(s) and amino acid composition</th>
<th>Electrophoretic mobility ($R_f$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (Zn$^{2+}$-free)</td>
<td>Lys-Ala</td>
<td>0.61*</td>
</tr>
<tr>
<td>Oxytocin, Cys-Tyr-Ile-Gln-Aas-Cys-Pro-Leu-Gly-NH$_2$</td>
<td>Leu-Gly-NH$_2$</td>
<td>0.55*</td>
</tr>
<tr>
<td>Angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>NH$_2$-terminal heptapeptide</td>
<td>0.19*</td>
</tr>
<tr>
<td>Bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Plw-Arg</td>
<td>Phe</td>
<td>0.13*</td>
</tr>
<tr>
<td>Bradykinin, Gln-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Gly-Pro-Gly-Ile-Pro-Pro</td>
<td>Asp-Arg, Val-Tyr-Ile-His-Pro-Pro</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Arg-Pro-Pro</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Gly-Phe-Ser-Pro</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Phe-Arg</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Arg-Pro</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Gly-Pro (trace)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Glu-Ile-Pro-Pro (trace)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Gly-Pro-Glu-Ile-Pro-Pro</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* $R_f$ is expressed in relation to the rate of migration of Gly-NH$_2$. For experimental detail, see text.

* The electrophoretic mobility of this peptide was also compared with authentic material.
Post-proline Cleaving Enzyme

Summary of purification of post-proline cleaving enzyme

Enzyme activity was measured in 25 mM sodium phosphate buffer, pH 7.7, containing 1 mM diisothiocyanat and EDTA using the substrate Z-Gly-Pro-Leu-Gly in a final concentration of 1.6 mM.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Yield</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue extract (23,500 × g; super)</td>
<td>38</td>
<td>4.3 × 10^3</td>
<td>163</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation (50–70%)</td>
<td>4.0</td>
<td>1.0 × 10^4</td>
<td>52</td>
<td>92</td>
<td>9</td>
</tr>
<tr>
<td>DEAE-Sepharose A-50</td>
<td>0.25</td>
<td>0.33</td>
<td>82</td>
<td>50</td>
<td>76</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>2.5 × 10⁻⁴</td>
<td>1.9</td>
<td>47</td>
<td>29</td>
<td>4.4 × 10⁻⁴</td>
</tr>
<tr>
<td>Affinity and DEAE-Sepharose A-50</td>
<td>6.4 × 10⁻⁴</td>
<td>45</td>
<td>29</td>
<td>18</td>
<td>1.05 × 10⁴</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A (37), and Leu-Gly-NH₂ demonstrates the absence of carboxypeptidase, aminopeptidase, and aminopeptidase activity. Therefore, it is concluded that the release of the COOH-terminal phenilalanine moiety from angiotensin II, which was also observed with the partially purified preparation (14), was caused by the post-proline cleaving enzyme and not by a contaminating activity. Endopeptidases are known to be capable of releasing terminal amino acid residues, although generally the rate of cleavage is considerably reduced.

The collagenase substrate, Z-Gly-Pro-Leu-Gly-Pro, is degraded by post-proline cleaving enzyme, however, the products formed are Z-Gly-Pro and Leu-Gly-Pro, which are different from those produced by collagenase (38). Z-Gly-Pro-Leu-Gly, which has been used as a substrate throughout the purification, has an even higher affinity than the collagenase substrate for post-proline cleaving enzyme, with an apparent Kₘ of 6.0 × 10⁻⁵. An interesting observation is that Z-Gly-Pro-Leu acts as a good substrate while Z-Pro-Leu-Gly is found to be an extremely poor substrate and inhibits the enzymatic cleavage of Z-Gly-Pro-Leu-Gly. This finding suggests that the presence of the urethane-type protecting group at the NH₂-terminal side of the proline residue is an important structural requirement for a peptide to be an inhibitor. An additional requirement for inhibition seems to be the l configuration of the proline since Z-Pro-d-Ala failed to inhibit the enzyme.

Finally, a series of naturally occurring peptides were studied as possible substrates of post-proline cleaving enzyme (Table III). Whenever cleavage did occur it was without exception by hydrolysis of the peptide bond at the carboxyl side of proline residues. Thus, in case of zinc-free porcine insulin only the Pro-Lys bond was cleaved. Earlier results with oxytocin (13, 14), arginine vasopressin (39), and angiotensin II (39) were confirmed and extended. The results with bradykinin reveal that the Pro Pho peptide bond is more rapidly cleaved than the Pro-Gly bond. Judging from the rate of appearance of products in the case of bradykinin-potentiating factor, the Pro-Arg and Pro-Gly bonds are cleaved fairly rapidly and at about the same rate, while scission of the Pro-Glu peptide linkage proceeds at an exceedingly slow rate. The Pro-Pro bonds in the two peptides are not attacked. The enzyme seems to degrade low molecular weight peptides faster than the large molecules where Pro-X bonds located toward the end of the COOH-terminal of the sequence are preferred for cleavage. This is for example the case with insulin and it may also explain the resistance of the Pro-X bonds in gastrin, adrenocorticotropic hormone, and denatured collagen to enzymatic attack.

The data presented, along with those obtained previously, reveal that the post-proline cleaving enzyme is an endopeptidase which exhibits a strict substrate specificity for the Pro-X-peptide bond while appearing to be sensitive to the tertiary structure of the substrate. The species, organ and cellular distribution, and possible physiological role of this enzyme is currently under investigation.

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