Immunochemical Characterization of a Proline Endopeptidase from Rat Brain

ITS RELATIONSHIP TO PROLINE ENDOPEPTIDASE FROM OTHER TISSUES AND FROM OTHER SPECIES*

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Monospecific antiserum raised against rat brain proline endopeptidase is used to demonstrate the ubiquity of the enzyme and its unique role in the degradation of proline-containing peptides. All endopeptolytic activity directed toward proline residues in several rat tissues is shown to share one or more common antigenic determinants with rat brain proline endopeptidase. Similar activity from tissue of other species crossreacts with rat proline endopeptidase. The data presented suggest that proline endopeptidase is the sole cytoplasmic enzyme capable of degrading proline-containing peptides in every tissue examined and that previously reported proline-specific endoproteolytic activities observed in a variety of systems may be ascribed to proline endopeptidase. The putative role of proline endopeptidase in protein degradation is discussed.

Several reports of endopeptidases exhibiting peptide bond cleavage on the carboxyl side of proline using a variety of peptide substrates have appeared in recent years. Koida and Walter (1976) described the purification of a peptidase from lamb kidney which cleaves oxytocin and vasopressin on the carboxyl side of proline. These investigators subsequently referred to this enzyme as "post-proline-cleaving enzyme." This enzyme has an apparent molecular weight of 115,000 on gel filtration chromatography and was suggested to be a dimer. Further characterization of the lamb kidney enzyme has been reported by Yoshiimoto et al. (1978). Studies on a rabbit brain enzyme which hydrolyzes bradykinin at the Pro*-Phe bond have been reported (Oliviera et al., 1976; Camargo et al., 1979). In addition, the purification of an enzyme from rabbit brain which hydrolyzes several proline-containing peptide hormones including bradykinin, TRH, substance P, and neurotensin has recently appeared (Orlowski et al., 1979). Investigations of an enzyme from bovine tissue which is capable of hydrolyzing a number of peptide substrates at internal proline residues have been described (Knisatschek and Bauer, 1979; Knisatschek et al., 1980; Hersh and McKelvy, 1979; Blumberg et al., 1980; Tate, 1978; Tate, 1981).

An enzyme from rat brain responsible for deamidating thyrotropin-releasing hormone (TRH) was first purified to homogeneity by Rupnow et al. (1979). Subsequent studies of the rat brain enzyme demonstrated that the homogeneous enzyme is capable not only of deamidating TRH but also of catalyzing the cleavage of many proline-containing polypeptide hormones after internal proline residues (Taylor et al., 1980). This study and others (Taylor and Dixon, 1980; Andrews et al., 1980) have demonstrated that the TRH-deamidating enzyme from rat brain is a proline-specific serine protease capable of degrading a large number of polypeptides; thus, the enzyme has been referred to as proline endopeptidase (Taylor et al., 1980; Taylor and Dixon, 1980). Activity assays utilizing a variety of fluorogenic substrates have shown that similar enzyme activities are present in tissues other than the brain (Taylor et al., 1980).

The exact relationship between the proline endopeptidase activities found in various rat tissues is unknown. In addition, the relationship between the enzymes reported from bovine and rabbit as well as lamb kidney has been complicated by apparent differences in their physical parameters, including molecular weights and subunit composition.

This manuscript describes the production and characterization of a monospecific antiserum against rat brain proline endopeptidase and its use to examine the relationship between the many reported proline-specific endoproteolytic activities. The antiserum has been shown to completely inactivate and precipitate all proline endopeptidase activity in every rat tissue examined. In addition, the bovine hypothalamic enzyme as well as the lamb kidney enzyme is inactivated by the antiserum raised against the homogeneous rat enzyme. The relationship of the various proline endopeptidase activities to one another and the potential role of this enzyme in protein degradation will be discussed.

EXPERIMENTAL PROCEDURES

Production of Antiserum to Proline Endopeptidase—A New Zealand White rabbit was injected subcutaneously on three occasions at 2-week intervals with 200 μg of proline endopeptidase. The proline endopeptidase was injected at four sites in a 1:1 emulsion of 10 mM phosphate, pH 7.4, 0.85% NaCl (PBS), and Freund’s complete adjuvant. Whole blood was collected 10 days after the final injection. The blood was allowed to clot and the clot removed by centrifugation. The serum was subjected to precipitation with 40% saturated ammonium sulfate. The pellet was resuspended and this procedure repeated. The pellet from the second precipitation was taken up in a volume of PBS equivalent to the original serum volume. The crude antibody fraction

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1 The abbreviations used are: TRH, thyrotropin-releasing hormone; CBZ-L-Ala-L-Pro-4MNA, benzoyloxycarbonyl-L-alanyl-L-prolyl-4-methoxy-β-naphthylamide; TRH-JNA, L-prolyl-L-prolyl-L-prolyl-β-naphthylamide; L-Ala-L-Pro-pNA, L-alanyl-L-prolyl-p-nitroanilide; L-His-L-Pro-pNA, L-histidyl-L-prolyl-β-naphthylamide; PBS, phosphate-buffered saline (20 mM sodium phosphate, pH 7.4, and 0.85% sodium chloride).
was dialyzed overnight at 4 °C against PBS and stored frozen until use.

Removal of Antibodies Which Cross-React with a Serum Component—The partially purified rabbit anti-rat proline endopeptidase contained a fraction which recognized a component of rat serum. This cross-reacting material could be removed with a column prepared from cyanogen bromide-activated Sepharose 4B, by the method of Porath et al. (1973). Exactly 1.0 ml of whole rat serum was used for each 10 ml of Sepharose 4B.

In a typical purification, a 40-ml serum-agarose column was first washed with 500 ml of 0.5 M acetic acid and equilibrated with 500 ml of PBS. Then, 5.5 ml of the crude antibody fraction were applied to the column and eluted with 100 ml of PBS. The eluent was filtrated in a pressure cell to 5 ml and dialyzed against PBS at 4 °C. After dialysis, the antibody fraction was diluted to 5.5 ml with PBS and the nonspecificity of the antibody verified by double immunodiffusion and immunoelectrophoresis.

It was also possible to elute the nonspecific antibody from the column using 200 ml of 0.5 M acetic acid. The nonspecific antibody was then treated in a similar manner to that described above for the specific antibody. The nonspecific antiserum was shown to not recognize proline endopeptidase by immunotitration or immunodiffusion. Unless otherwise specified, the antibody used in these studies was the IgG fraction of a serum-absorbed antiserum to proline endopeptidase.

Immunodiffusion and Immunoelectrophoresis—Both immunodiffusion and immunoelectrophoresis were carried out in 1% agarose gels containing 0.1% Triton X-100 and 0.05% sodium azide in 20 mM sodium phosphate, pH 8.3. Immunodiffusion was carried out at 0 °C for 24-72 h. Immunoelectrophoresis was carried out at 4 °C and 3 mA/cm for 4 h. Under these conditions, hemoglobin migrated approximately 4 cm toward the anode. After the precipitin bands had developed sufficiently, both immunodiffusion and immunoelectrophoresis gels were washed in 10 mM phosphate, pH 7.4, containing 0.95% sodium chloride, 0.05% sodium azide, and 0.1% Triton X-100 at room temperature for 24 h, dried, and stained using a Stains I and II for 30 min each. The plates were destained using 10% acetic acid followed by Evans destaining solution. The precipitin bands were observed under UV light. The antiserum has no effect on enzyme activity. Both the precipitin bands indicate that for the pure enzyme from rat brain (Fig. 2A) and brain extract (Fig. 1B), both the double immunodiffusion and the immunoelectrophoresis data suggest that only one form of proline endopeptidase exists in rat brain.

Supporting this conclusion, it has been observed that the elution profiles of proline endopeptidase determined by rocket immunoelectrophoresis and by activity assay are identical throughout the course of the purification procedure described by Rupnow et al. (1979). The antiserum to proline endopeptidase will both precipitate the enzyme and inhibit its activity (Fig. 2A). Nonspecific antiserum has no effect on enzyme activity. Both the precipitation and the inhibition have been shown to be complete within 60 min at 0 °C. Immunotitration of proline endopeptidase activity in a brain extract results in a titration curve identical with that obtained using an equivalent amount of pure enzyme (Fig. 2B). This pattern was observed for all tissues examined except kidney (Taylor et al., 1980).

Titration of proline endopeptidase activity in kidney extracts does not result in complete inhibition of activity capable of releasing β-naphthylamide from TRH-PNA (Fig. 2C). Kidney extract also exhibits a pronounced lag time when TRH-PNA is used as a substrate (data not shown). None of these effects are observed using either the pure brain enzyme or extracts from other tissues. The hydrolysis of another substrate, CBZ-L-Ala-L-Pro-4-MNA, by kidney extract does not exhibit a lag time. In addition, the immunotitration of the kidney extract using CBZ-L-Ala-L-Pro-4-MNA as a substrate also results in complete loss of activity in a manner which strongly resembles that for the pure enzyme from rat brain (Fig. 2C).

The widespread tissue distribution and the fact that the rat proline endopeptidase from a number of tissues shares one or more common antigenic determinants suggested the possibility that the antiserum raised against the rat brain enzyme might be useful in exploring the relationship between the rat brain enzyme and similar enzyme activities reported in bovine brain and rabbit brain as well as lamb kidney. The molecular weight and subunit composition are among the reported physical differences between rat proline endopeptidase and lamb kidney “post-proline-cleaving enzyme.” The enzyme isolated from rat brain has been reported to be a monomer of 73,000 daltons, while the enzyme isolated from lamb kidney was originally reported to be a dimer of 115,000 daltons. In an effort to resolve this apparent difference, the elution pattern of lamb

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5. Evans, J. E. Dixon, unpublished observations.

kidney extract on a gel permeation column was determined as shown in Fig. 3. The enzymatic activities toward TRH-βNA and CBZ-L-Ala-L-Pro-4MNA co-elute. The latter substrate has been shown to be a substrate for the lamb kidney enzyme by Yoshimoto et al., 1978. It is also a substrate for rat proline endopeptidase. Both activities elute at a volume corresponding to $M_r = 70,000$. In addition, it was possible to show that another enzyme known to be present in lamb kidney, post-proline dipeptidyl aminopeptidase, eluted with the anticipated molecular weight of 220,000. No evidence was observed for a $M_r = 115,000$ enzyme from lamb kidney capable of hydrolyzing any of these substrates. These observations are supported by the recent report by Walter et al. (1980) that the lamb-kidney “post-proline-cleaving enzyme” has an apparent molecular weight of 70,000 rather than 115,000 as originally reported (Yoshimoto et al. 1978).

The immunochemical cross-reactivity of the rat brain and the lamb kidney enzymes is noted by the observation that the hydrolytic activity of CBZ-L-Ala-L-Pro-4MNA may be immunoprecipitated from lamb kidney extract using rabbit anti-rat proline endopeptidase antiserum (Fig. 4A). The nonidentity of the two titration curves indicates that the two enzymes do not share identical antigenic determinants.

An immunotitration was also performed on proline endopeptidase activity in bovine and rabbit brain extracts (Fig. 4B). The activity in bovine brain extracts is completely inhibited by rabbit anti-rat proline endopeptidase. Proline endopeptidase from rabbit brain is not inhibited by antisera raised against rat proline endopeptidase, although the rabbit brain enzyme has many similar physical properties in common with the rat enzyme (Table I). It is possible that using the

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1This observation was reported as an unpublished result in a review by Walter et al. (1980) which appeared while this work was in progress.
rabbit for production of the antiserum precludes isolation of antibodies which recognize rabbit proline endopeptidase.

**DISCUSSION**

Both the immunotitration and the immunodiffusion data demonstrate that a proline endopeptidase is found in many rat tissues. This result is consistent with the observations of Taylor et al. (1980) who showed that an enzyme activity capable of hydrolyzing synthetic substrates on the carbonyl side of proline was also present in a wide number of tissues from the rat. These data collectively suggest that the proline endopeptidase is the sole soluble proline-specific endopeptidase in all rat tissues examined. The kidney is the only rat tissue in which a significant non-proline endopeptidase activity capable of hydrolyzing TRH-BNA is observed. In this case, the lag time and the substrate specificity suggest that the nonimmunoprecipitable activity in the rat kidney capable of hydrolyzing TRH-BNA may be due to the action of multiple enzymes. For example, a pyroglutamyl aminopeptidase might degrade TRH-BNA to L-His-L-Pro-BNA and pyroglutamate. Neither of these compounds fluoresces at the excitation wavelength used. However, L-His-L-Pro-BNA may be degraded by postproline dipeptidyl aminopeptidase, resulting in release of the fluorescent compound β-naphthylamine. Post-proline dipeptidyl aminopeptidase activity has been observed in kidney (Yoshimoto and Walter, 1977) and pyroglutamyl aminopeptidase activity has been reported from mammalian sources (Armentrout, 1968; Taylor and Dixon, 1978). The observation that a lag time is not observed when the proline endopeptidase substrate CBZ-L-Ala-L-Pro-4MNA is used and that all enzyme activity capable of hydrolyzing this substrate is immunoprecipitated from the kidney supports the contention that even in kidney the only proline endoproteolytic activity can be assigned to proline endopeptidase.

The similarity observed on immunotitration of pure proline endopeptidase and the activity in crude rat brain extracts suggest that they share one or more antigenic determinants. The quantitative titrations with antiserum indicate that the activity measured using TRH-BNA as a substrate is an accurate gauge of the amount of enzyme present in brain extracts. The quantitative nature of the titration also suggests that no appreciable concentration of immunologically recognizable inactive enzyme is present in the brain extract and that inhibitors of the proline endopeptidase, which would not substantially alter immunoreactivity of the enzyme, are not present in significant concentration in brain extracts.

The immunotitration data demonstrate that proline endopeptidase from lamb kidney and bovine brain cross-react with antiserum prepared against the enzyme isolated from rat brain.

**TABLE 1**

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Soluble</th>
<th>Molecular weight</th>
<th>Number of subunits</th>
<th>pI</th>
<th>pH optimum</th>
<th>Protease class</th>
<th>Primary specificity</th>
<th>Alanyl residues cleaved</th>
<th>Inactivation by sulfhydryl directed reagents</th>
<th>Stability in urea</th>
<th>Thermal stability</th>
<th>Inhibition by anti-rat proline endopeptidase</th>
</tr>
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<tbody>
<tr>
<td>Rat brain proline endopeptidase</td>
<td>All tissues</td>
<td>Yes</td>
<td>73,000</td>
<td>1</td>
<td>4.5</td>
<td>7-8.5</td>
<td>Serine</td>
<td>Proline</td>
<td>Yes*</td>
<td>Yes</td>
<td>Yes</td>
<td>1.4</td>
</tr>
<tr>
<td>Lamb kidney post-proline cleaving enzyme</td>
<td>All tissues</td>
<td>Yes</td>
<td>74,000</td>
<td>1</td>
<td>4.8</td>
<td>7.8</td>
<td>Serine</td>
<td>Proline</td>
<td>Yes</td>
<td>Partial</td>
<td>3.5</td>
<td>43</td>
</tr>
<tr>
<td>Rabbit brain prophyl endopeptidase</td>
<td>N.D.*</td>
<td>Yes</td>
<td>66,000</td>
<td>N.D.</td>
<td>N.D.</td>
<td>8.3</td>
<td>Serine*</td>
<td>Proline</td>
<td>N.D.</td>
<td>Yes</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Bovine pituitary proline endopeptidase</td>
<td>N.D.</td>
<td>Yes</td>
<td>76,000</td>
<td>N.D.</td>
<td>N.D.</td>
<td>7.4-7.6</td>
<td>Serine*</td>
<td>Proline</td>
<td>N.D.</td>
<td>Yes</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Bovine brain proline endopeptidase</td>
<td>N.D.</td>
<td>Yes</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>7.4</td>
<td>Serine*</td>
<td>Proline</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Cleavage after alanyl residues represents a minor portion of the hydrolytic activity.
* Concentration at which 50% of the activity is lost in 30 min at 25°C, pH 7.0.
* Temperature at which 50% of the activity is lost in 15 min at pH 7.0.
* Rupnow et al., 1979; Andrews et al., 1980; Taylor et al., 1980.
* Yoshimoto et al., 1978; Walter and Yoshimoto, 1978; Walter et al., 1980.
* Orlovski et al., 1979.
* Not determined.
* Inactivation by diisopropyl fluorophosphate is the criterion by which classification as a serine protease was made.
* Knisatschek and Bauer, 1979; Knisatschek et al., 1980.
* Hersh and McKelvey, 1979; Blumberg et al., 1980.
The properties of proline endopeptidase and similar peptidases from various sources are summarized in Table I. The similar molecular weights obtained by gel permeation chromatography and similarities in substrate specificities of the activity from several sources suggest that these enzymes are most likely physically and functionally similar. The recent correction of the molecular weight of "post-proline-cleaving" enzyme to 73,000 from 115,000 (Walter et al., 1980) agrees with our observations and supports the conclusion that the enzymes are similar. Consistent with these molecular weights, "post-proline-cleaving" enzyme purified from lamb brain has been recently reported to have a molecular weight of 74,000 (Yoshimoto et al., 1981). There are few remaining differences in physical properties reported for the enzymes from the various sources.

Proline endopeptidase from rabbit, rat, and bovine brain and lamb kidney has been shown to be capable of hydrolyzing a number of proline-containing polypeptide hormones. Based upon the substrate specificity and immunochemical properties of the enzyme, we suggest that proline endopeptidase is the only soluble proline-specific endopeptidase in all of the tissues examined. We suggest that many of the endoproteolytic activities reported to degrade specific peptide hormones via cleavage after a proline residue may be attributed to this enzyme. Because proline endopeptidase exhibits a wide substrate specificity, the degradation of proline-containing polypeptide hormones most likely represents only one potential role of proline endopeptidase. It may also function in the overall process of protein degradation.

The catabolism of proline-containing polypeptides is a unique process in intracellular protein degradation. The conformation and steric character of the imino acid proline disallow hydrolysis on either the carboxyl or the amino side of the residue by a number of proteases. Indeed, cells have a complement of proline-directed proteases, including aminopeptidase P (Dehm and Nordwig, 1970a), carboxypeptidase P (Dehm and Nordwig, 1970b), dipetidyl aminopeptidase IV (Yoshimoto and Walter, 1977), and the proline endopeptidase described here. It is, therefore, interesting to note that the proline endopeptidase is the only soluble proline-specific endopeptidase found in every rat tissue examined and may play a singular role in cytoplasmic degradation of proline-containing polypeptides.

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