SPECIFIC FLUOROGENIC SUBSTRATES FOR THE TRF-DEAMIDATING POST PROLINE CLEAVING ENZYME

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1. Introduction

The deamidation of TRF (<Glu-His-Pro-NH_2) by adenohypophyseal tissue extracts is catalyzed by an enzyme which could be characterized as a post proline cleaving endopeptidase [1,2]. From lamb kidney an enzyme with similar chemical characteristics has been isolated [4,5]. The pituitary enzyme (M_r 76 000) however, differs from the kidney enzyme (M_r 115 000) by its high sensitivity towards sulfhydryl blocking agents and other properties. Both enzymes cleave proline containing peptides specifically at the Pro-X bond. The collagenase substrate Z-Gly–Pro–Leu–Gly–Pro [6], for example, is rapidly hydrolyzed at the Pro–Leu bond. As known, however, the Pro-X bond can also be different from a peptide bond. TRF (<Glu–His–Pro–NH_2), TRF-alkylamides, and <Glu–His–Pro–β-alanylamide are also hydrolyzed by these enzymes.

Since the enzymatic liberation of β-naphthylamine can easily be determined with the highly sensitive fluorometric detection method [7], Z-His–Pro–2-NNap, Z-Gly–Pro–2-NNap and <Glu–Gly–Pro–2-NNap were synthesized. Together with <Glu–His–Pro–2-NNap these substances were tested as substrates of the post proline cleaving enzyme from pituitary extracts. It could be shown that Z-Gly–Pro–2-NNap and Z-His–Pro–2-NNap serve as enzyme specific substrates.

Abbreviations: Z, benzyloxycarbonyl; 2-NNap, β-naphthylamide; DTT, dithiothreitol; DMF, dimethylformamide; ONSu, succinimido-oxy

2. Materials and methods

Precoated silica gel plates (no. 5554), Merck AG, Darmstadt; [L-*proline*-2,3-^{3}H]TRF, New England Nuclear; <Glu–His–Pro–2-NNap, Bachem AG, Bubendorf. Buffer (A): 100 mM potassium phosphate (pH 7.4), 1mM DTT, 1 mM EDTA. Buffer (B): 20 mM potassium phosphate (pH 7.4), 1 mM DTT. Buffer (C): 100 mM potassium phosphate (pH 7.4).

2.1. *Enzyme preparation*

As described [1], the tissue extract was prepared from anterior pituitary and the post proline cleaving enzyme was purified from these extracts by $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on DEAE-cellulose and gel-filtration on Sephadex G-150.

2.2. *Enzyme assays of the post proline cleaving enzyme: TRF as substrate*

The deamidation of radiolabelled TRF was determined as in [1].

2.3. *Fluorogenic substrates*

During the chromatography fractionation procedures the enzyme activity was monitored using Z-Gly–Pro–2-NNap as substrate. Aliquots of the fractions (50–100 μl) were mixed with 2 ml substrate (10 μM in buffer A). After incubation for 15 min at 37°C the reaction was stopped with 2 ml 0.2 M glycine–NaOH buffer (pH 10.5). The enzymatically liberated β-naphthylamine was determined by fluorometric detection [7] (excitation 340 nm, emission 410 nm), and compared to a standard curve for β-naphthylamine. The measurements for K_m determinations and enzyme inhibition studies were carried out at 37°C in a water jacketed cuvette holder of a

MPF-4 Fluorometer (Perkin Elmer). Effects of inhibitors were evaluated after a 10 min incubation period. The K_m were obtained from Lineweaver-Burk plots.

2.4. *Z-Gly–Pro–Leu–Gly–Pro as substrate*

The liberation of Leu–Gly–Pro was determined by the ninhydrin method according to [3]. The ninhydrin reagent was prepared with 1 M Na-acetate buffer instead of Li-acetate buffer.

2.5. *Syntheses*

<Glu–Gly–Pro–2-NNap: <Glu (40 mg, 304 μmol), Gly–Pro–2-NNap (90 mg, 304 μmol) and *N*-hydroxybenzotriazole (51 mg, 344 μmol) were dissolved at 0°C in 20 ml DMF. At −18°C *N,N'*-dicyclohexylcarbodiimide (69 mg, 344 μmol) was added. After stirring for 4 h at −18°C and 24 h at 4°C the precipitated *N,N'*-dicyclohexylurea was removed by filtration. After evaporation the product was purified by chromatography on a silica gel column (2 × 20 cm) using chloroform/methanol (9/1, v/v) as eluent. Crystallization from chloroform/ether gave 45 mg (40%). R_F 0.26 (chloroform/methanol, 9/1, v/v), R_F 0.61 (chloroform/methanol, 7/3, v/v). Amino acid analysis gave the following molar ratios: Glu, 0.95; Gly, 0.98; Pro, 1.0

Z-Gly–Pro–2-NNap: This was prepared from Z-Gly–ONSu (0.28 mmol) and Pro–2-NNap · HCl (0.28 mmol) dissolved in 8 ml acetonitrile containing 0.56 mmol triethylamine. After stirring at room temperature for 24 h the solvent was evaporated. The product was purified by chromatography on a silica gel column (2 × 15 cm) using 2-butanone/chloroform/petroleum ether/acetic acid, (10/5/5/0.5, by vol.) for elution. Crystallization from chloroform/petroleum ether gave 61 mg (52%), m.p. 139–140°C; R_F 0.19 (2 butanone/chloroform/petroleum ether, 2/1/1, by vol.). Amino acid analysis gave the following molar ratios: Gly, 1.0; Pro, 1.0. MS fragments (intensity): M^+ 431 (1), 323 (0.9), 153, (2.5), 149 (1.4), 143 (1)

Z-His–Pro–2-NNap: Z-His (0.5 mmol) and Pro–2-NNap · HCl (0.5 mmol) were dissolved at 0°C in 20 ml DMF containing 0.5 mmol triethylamine. Then, *N,N'*-dicyclohexylcarbodiimide (0.5 mmol) was added. After standing at room temperature for 12 h the solution was filtered and evaporated. The product was purified by preparative layer chromatography on silica gel plates (Merck no. 5717) using the solvent system chloroform/methanol (9/1, v/v) (R_F 0.44). Amino acid analysis gave the following molar ratios: His, 0.91; Pro, 1.0.

2.6. *Amino acid analysis*

The peptides were hydrolyzed under vacuo for 18 h at 110°C in 6 N HCl. Amino acid analysis was carried out on a Durrum D-500 analyzer using the standard program of the 'one column' method.

3. Results and discussion

All fluorogenic substrates tested, exhibit a higher affinity (K_m) towards the enzyme than TRF (table 1). When the glycine moiety in the two substrates Z-Gly–Pro–2-NNap [8,9] and <Glu–Gly–Pro–2-NNap was substituted by histidine, an increase in enzyme affinity and a decrease in the relative V_{max} could be observed. Since <Glu containing peptides are known to be hydrolyzed by pyroglutamate aminopeptidases, <Glu–His–Pro–2-NNap and <Glu–Gly–Pro–2-NNap were not further investigated. In contrast, Z-Gly–Pro–2-NNap and Z-His–Pro–2-NNap are not degraded by the known aminopeptidases. Unfortunately, Z-Gly–Pro–2-NNap, which exhibits a higher V_{max} than Z-His–Pro–2-NNap, has only a low solubility in aqueous buffer (10 μM) which is below the K_m of 14 μM. Z-His–Pro–2-NNap, however, is soluble at 50 μM (5-times K_m). In order to increase the solubility of these substrates, agents such as methanol, ethanol, *n*-butanol, DMF, dioxane, acetonitrile, Triton X-100 or Tween 80 can not be used since they cause rapid inactivation of the enzyme activity. An easily soluble substrate could be obtained by reacting *p*-sulfonyl-phenylisothiocyanat [10] with Gly–Pro–2-NNap. This thiocarbamate derivative exhibits, however, a low affinity (app. K_m = 1 mM) to the post proline cleaving enzyme, whereby significant substrate inhibition is already observed at 0.28 mM. Further attempts to obtain substrates of higher solubility were not made, since product inhibition has to be expected for all substrates [1,9]. Under this aspect Z-His–Pro–2-NNap appears to be a suitable substrate of the post proline cleaving enzyme. With appropriately adjusted enzyme concentrations, catalyzing the liberation of 74 pmol β-naphthylamine/

Table 1

Substrates	K_m (μM)	V_{max} (nmol . min^{-1} . mg^{-1})	Solubility (μM)
<Glu–His–Pro–2-NNap	15	71.5	>100
<Glu–Gly–Pro–2-NNap	160	82	>100
Z-Gly–Pro–2-NNap	14	132	10
Z-His–Pro–2-NNap	9.5	62.5	50

For determining the K_m and relative V_{max} values of the fluorogenic substrates purified enzyme [1] was used. (For comparison: TRF, K_m = 410 μM, see [1]; V_{max} = 82 nmol . min^{-1} . mg^{-1}). All compounds were predissolved in methanol and then injected into buffer (C). Substrate solutions with the following concentrations were prepared. <Glu–Gly–Pro–2-NNap (0.1 mM); <Glu–His–Pro–2-NNap (0.1 mM); Z-His–Pro–2-NNap (0.05 mM); Z-Gly–Pro–2-NNap (0.01 mM). Methanol was always <0.1% final conc. These stock solutions were stable for ≥2 weeks at 4°C. Prior to use, DTT and EDTA were added to give 1 mM final conc. each.

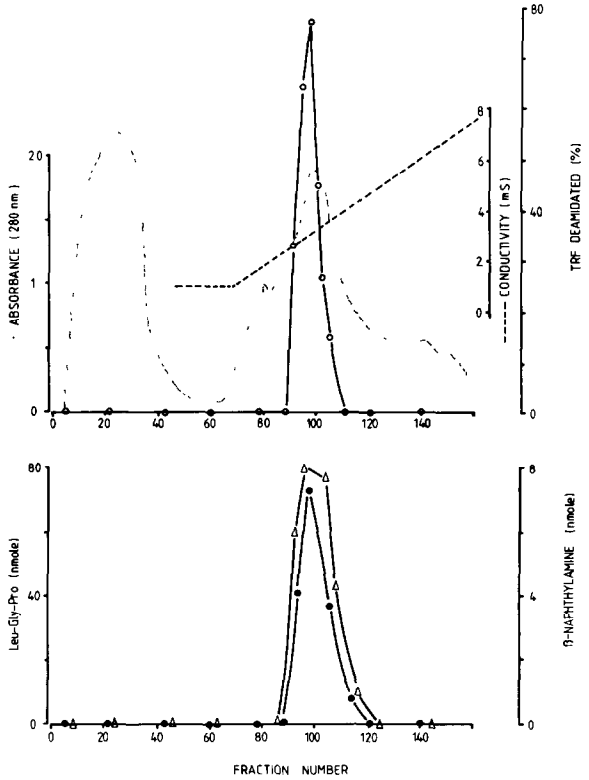

Fig.1. Adsorption chromatography of anterior pituitary extract on hydroxylapatite C (Clarkson Chem. Co.). Equilibration buffer (1 mM potassium phosphate buffer (pH 7.4), 1 mM DTT) was passed over the column (3 × 15 cm) at 49 ml/h flowrate. Bound protein was eluted with a phosphate gradient (1–150 mM each 100 ml). The deamidation of [^{3}H]TRF (○——○), the β-naphthylamine liberation from Z-Gly–Pro–2-NNap (△——△) and the cleavage of Z-Gly–Pro–Leu–Gly–Pro (●——●) were determined as in section 2.

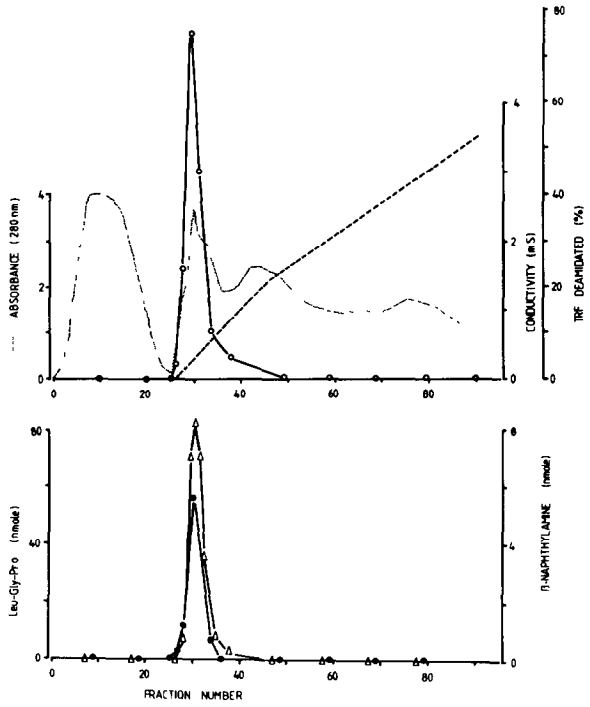

Fig.2. Ion-exchange chromatography of anterior pituitary extract on DE-52 cellulose (Whatman Biochemicals). The column (3 × 30 cm) was equilibrated with buffer B and the protein eluted with a linear KCl gradient (0–300 mM KCl in 350 ml buffer B). Flowrate, 42 ml/h. Cleavage of the various substrates is indicated by the same symbols as in fig.1.

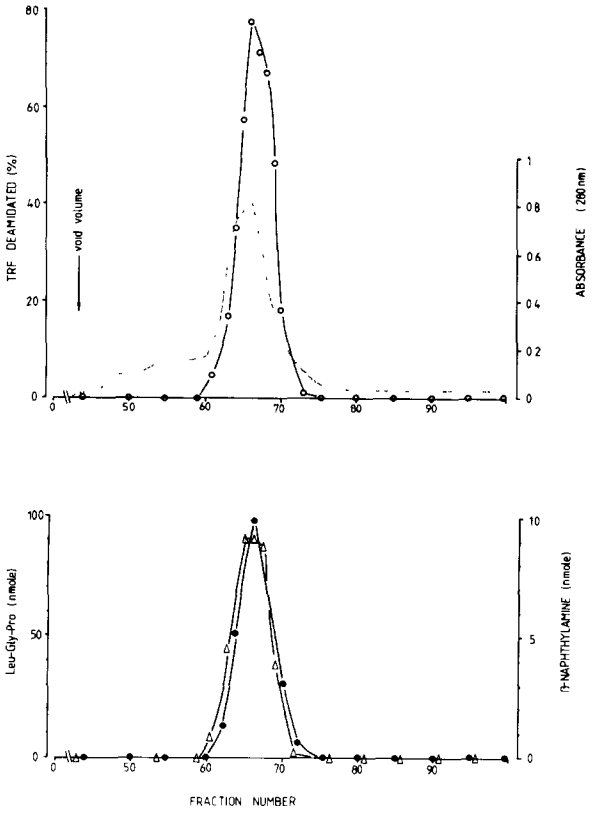

Fig.3. Gel-filtration of the active fractions from the ion-exchange chromatography on Sephadex G-150 (Pharmacia Fine Chem.). The column (2.5 × 100 cm) was equilibrated with buffer A. Flowrate 20 ml/h. Cleavage of the various substrates is indicated by the same symbols as in fig.1.

min, a linear relationship with time could be observed over a 5 h incubation period (compared to 30 min for Z-Gly–Pro–2-NNap under the same conditions).

Finally we examined whether Z-Gly–Pro–2-NNap and Z-His–Pro–2-NNap can be used as enzyme specific substrates. The extract of bovine anterior pituitary was fractionated by various chromatographic methods. The column eluate was tested for enzyme activity with 3 different substrates (TRF, Z-Gly–Pro–2-NNap, Z-Gly–Pro–Leu–Gly–Pro). We always observed one single peak of enzyme activity only (fig.1–3).The notion that these substrates are hydrolyzed by one enzyme, is further supported by the findings that the deamidation of TRF and the β-naphthylamine liberation from Z-Gly–Pro–2-NNap are comparably affected by various enzyme inhibitors (table 2). Therefore Z-Gly–Pro–2-NNap and Z-His–Pro–2-NNap can be used as enzyme specific substrates. Thus, a highly sensitive, rapid and convenient test is available for determining specifically the activity of the post proline cleaving enzyme. In addition, these substrates should also be suitable for histochemical studies.

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Table 2
Effect of functional reagents

Test substance	Conc. (mM)	Inhibition (%)	
		[^{3}H]TRF	Z-Gly–Pro–2-NNap
a			
Hg^{2+}	0.001	65–80	80
N-Ethyl maleimide	0.005	40–55	53
p-Chloromercuribenzoate	0.005	100	100
b			
Diisopropyl fluorophosphate	0.01	90–100	93
Phenylmethanesulfonyl fluoride	0.1	0	0
c			
EDTA	5.0	0	0
Bacitracin	0.01	55–70	70

The deamidation of TRF and the cleavage of Z-Gly–Pro–2-NNap were determined after a 10 min preincubation period as in section 2. (a) Buffer C with 1 μM DTT, no EDTA; (b) buffer A with 0.5% methanol; (c) buffer A

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