NEW FLUOROGENIC SUBSTRATES FOR THE DETERMINATION OF POST-PROLINE ENDOPEPTIDASE ACTIVITY

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A new fluorogenic substrate for determination of the activity of post-proline endopeptidase (EC 3.4.21.26), Z-Cys(Bzl)-Pro-NH-Meq* has been synthesized. Affinity of this substrate to the enzyme was significantly higher than that of the hitherto employed substrates. The Michaelis constant of the post-proline endopeptidase for Z-Cys(Bzl)-Pro-NH-Meq was at the optimum pH (7·0) 1·05 . 10^{-6} mol l^{-1}. The concentration of dimethylsulfoxide used for the solubilization of Z-Cys-(Bzl)-Pro-NH-Meq (<0·4%) was outside the limits of adverse influence of the organic solvent on the enzyme activity. The fluorogenic substrate Z-Gly-Pro-NH-Meq was also synthesized for comparison (K_m = 10·35 10^{-6} mol l^{-1} at pH 7·0).

Post-proline endopeptidase (EC 3.4.21.26, PEPase), has been isolated from various mammalian tissues and some body fluids[1-7,13], from bacteria[8-10] and from sperm of *Ascidian*[11]. Recently, PEPase activities have been also detected in several plants and the enzyme has been isolated from carrot[12].

One of the proposed physiological functions of the PEPase is the specific inactivation of proline containing peptide hormones which may be of great importance in the process of the regulation of their effects[2-4,13,14]. In order to detect low levels of post-proline endopeptidase in various tissues, it is necessary to use substrates with high affinity[17]. Affinities of several substrates presented in literature[2,3,13-16] were low and often such concentrations had to be employed for determination of the enzyme activity that the organic solvent used for dissolving the substrate affected adversely the enzyme activity[13].

Among the various synthetic substrates used for the determination of the PEPase activity, the fluorogenic substrates Z-Gly-Pro-NH-Mec (ref.[17]) and Z-His-Pro-NH-

* Abbreviations are generally those recommended by IUPAC–IUB Commission on Biochemical Nomenclature (Biol. Chem. Hoppe-Seyler *366*, 3 (1985)); additional abbreviations: AMec, 7-amino-4-methylcoumarin; AMeq, 7-amino-4-methyl-2-quinolinone; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; Nap, naphth-2-yl; NMM, N-methylmorpholine; TES, N-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid; THF, tetrahydrofuran.

-Nap (ref.[18]) exhibited an increased affinity to the enzyme whereas that of the substrate Suc-Gly-Pro-NH-Mec (ref.[5,19]) was very low[5].

We have prepared the fluorogenic substrate 7-(benzyloxycarbonyl-S-benzyl--cysteinyl-prolyl-amido)-4-methyl-2-quinolinone [Z-Cys(Bzl)-Pro-NH-Meq], expecting that the presence of the cysteine residue (which is preceding the proline residue in neurohypophyseal hormones chains) at position P_2 will increase further the binding affinity enabling better substrate–enzyme secondary interactions. The fluorogenic substrate Z-Gly-Pro-NH-Meq was also synthesized for comparison. The amine 7-amino-4-methyl-2-quinolinone (AMeq) has been used as the fluorescent marker because of its high fluorescence intensity[20].

This communication deals with the synthesis and fluorescence properties of the fluorogenic substrates for post-proline endopeptidase, Z-Cys(Bzl)-Pro-NH-Meq and Z-Gly-Pro-NH-Meq and describes results of kinetic studies of their hydrolysis by porcine kidney PEPase.

EXPERIMENTAL

Synthesis of the Substrates

All asymmetric amino acid derivatives were of L-configuration and were synthesized from amino acids purchased from Fluka (Switzerland) employing the methods described in literature. AMeq was synthesized as described previously[21]. THF was passed through a column of aluminium oxide and distilled over CaH_2. DMF was distilled from ninhydrin at reduced pressure. Isobutyl chloroformate was distilled and stored over $CaCO_3$, NMM was distilled from ninhydrin. HCl was dried thoroughly prior use. DMSO for UV spectroscopy (Fluka, Switzerland) was used. All other solvents and chemicals were of reagent grade and were employed without further purification. Solvent systems for thin-layer chromatography on silica gel F-254 plates (Merck, F.R.G.) were as follows: (A) chloroform–methanol (90 : 10) or (50 : 10), (B) 1-butanol–acetic acid–water (4 : 1 : 1), (C) 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12), (D) EtOAc–-pyridine–HCOOH water (63 : 21 : 10 : 6) and (E) 1-propanol–ammonium hydroxide 25% (67 : 33). Spots were visualized by UV light, by ninhydrin and chlorine–TDM spray[22]. Melting points were determined on a Büchi apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter.

Boc-Pro-NH-Meq (*I*): To a stirred solution of Boc-Pro-OH (1·13 g, 5·25 mmol) in THF (20 ml) at −15°C, NMM (0·58 ml, 5·25 mmol) was added, followed by isobutyl chloroformate (0·69 ml, 5·25 mmol). After 7 min, a precooled suspension of AMeq (0·87 g, 5 mmol) in THF (100 ml) was added slowly. The reaction mixture was stirred for 30 min at −15°C and overnight at room temperature. The solvent was removed under reduced pressure and the resulting residue was dissolved in EtOAc. The organic phase was washed subsequently with 1M-$NaHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, dried (over Na_2SO_4) and evaporated to a small volume. The oil, formed after the addition of petroleum ether, was after decantation redissolved in EtOAc and poured into hexane under stirring to yield 1·4 g (75·5%) of white solid $[\alpha]_D^{25}$ −118·6° (*c* 1, MeOH). For $C_{20}H_{25}N_3O_4.H_2O$ (389·45) calculated: 61·68% C, 6·99% H, 10·79% N; found: 61·66% C, 7·15% H, 10·71% N.

Z-Cys(Bzl)-Pro-NH-Meq (*II*): Boc-Pro-NH-Meq (0·61 g, 1·65 mmol) was treated with 4M-HCl in THF (20·6 ml) for 30 min at room temperature. The excess acid and solvent were evaporated

and the residue was re-evaporated twice from THF, treated with dry ether, filtered and dried. The resulted hydrochloride salt was suspended in DMF (8 ml), cooled to 0°C and neutralized with NMM (0·18 ml, 1·65 mmol). Z-Cys(Bzl)-OSu (ref.[23]) (0·66 g, 1·5 mmol) was added and the reaction mixture was stirred 1 h at 0°C and 48 h at room temperature. The solvent was evaporated, the residue treated with H_2O and the product was extracted with EtOAc. The organic phase was washed subsequently with 1M-$NaHSO_4$, saturated KCl solution, 5% $NaHCO_3$ and saturated KCl, dried (over Na_2SO_4), evaporated to a small volume and poured into stirred petroleum ether. The white product (0·764 g, 85%) was re-precipitated from 2-propanol and triturated twice with 1-butanol; $[\alpha]_D^{25}$ −118° (*c* 1, MeOH). For $C_{33}H_{34}N_4O_5S.0{\cdot}5\ H_2O$ (607·73) calculated: 65·22% C, 5·80% H, 9·22% N, 5·28% S; found: 65·21% C, 5·92% H, 9·36% N, 5·30% S.

Compound *II* was also obtained by coupling of Z-Cys(Bzl)-OH with HCl.H-Pro-NH-Meq, utilizing the dicyclohexylcarbodiimide–hydroxybenzotriazole procedure[24].

Z-Gly-Pro-NH-Meq (*III*): Z-Gly-OSu (ref.[25]) (0·28 g, 0·91 mmol) and HCl.H-Pro-NH-Meq were coupled as described for compound *II*. After evaporation of the solvent, the residue was taken into an EtOAc–1-butanol mixture. The organic phase was washed consecutively with 1M-HCl, saturated KCl solution, of 5% $NaHCO_3$, saturated KCl and dried (Na_2SO_4). Evaporation and trituration with petroleum ether yielded 0·35 g (83%) of a white solid, which was triturated with EtOAc to give 0·31 g (74%) of the product $[\alpha]_D^{25}$ −172·2° (*c* 1, MeOH). For $C_{25}H_{26}N_4O_5 \cdot .H_2O$ (480·53) calculated: 62·49% C, 5·87% H, 11·66% N; found: 62·59% C, 5·67% H, 11·65% N.

Isolation of the Post-proline Endopeptidase from Porcine Kidney

The isolation was carried out according to the method published previously[2,13]. The final preparation contained about 95% of the enzyme, homogenous according to the criteria of electrophoretic and sedimentation analysis and the determination of the N-terminal amino acid. The specific activity of the preparation was 0·4 nkat per mg of protein at the temperature 30°C and pH 7·0. The protein concentration was determined according to Lowry[26].

Fluorometric Measurements

The fluorescence spectra of the compounds AMeq and Z-Cys-(Bzl)-Pro-NH-Meq were taken on a computer-controlled fluorescence spectrophotometer Fluorolog (U.S.A.) equipped with a thermostat maintained at 37°C.

Hydrolysis of the Substrates

Kinetics of the Z-Cys(Bzl)-Pro-NH-Meq and Z-Gly-Pro-NH-Meq hydrolysis with post-proline endopeptidase was followed at 37°C in a reaction mixture containing in the total volume 2·8 ml: 500 μl of TES buffer ($5 \cdot 10^{-2}$ mol l^{-1}) of the appropriate pH, 100 μl enzyme (1 mg) and various concentrations of the substrate (in the range of $0{\cdot}2-1{\cdot}0 \cdot 10^{-6}$ mol l^{-1}; total volume adjusted with water). The concentration of DMSO was in the range of 0·1—0·4%. The measurements were performed within the pH range of 5·5—8·0.

The reaction mixture was excited at 340 nm and the fluorescence was recorded at 430 nm. The concentrations of the liberated AMeq determined from the calibration curve were used for the calculation of initial reaction rates and for the determination of the corresponding K_m by means of the Lineweaver–Burk plot. The dependence of K_m and V_{max} values on pH was plotted according to Dixon[27].

RESULTS

The substrates Z-Cys(Bzl)-Pro-NH-Meq (*II*) and Z-Gly-Pro-NH-Meq (*III*) were prepared in good yield and purity by the reaction of Z-Cys(Bzl)OSu (ref.[23]) and Z-Gly-OSu (ref.[25]), respectively, with HCl. H-Pro-NH-Meq, which was obtained from Boc-Pro-NH-Meq (*I*) by treatment with 4M-HCl in THF (compound *I* was prepared by coupling of the fluorescent amine AMeq with Boc-Pro-OH, utilizing the mixed anhydride procedure).

The fluorescence spectra of the compounds AMeq and Z-Cys(Bzl)-Pro-NH-Meq are shown in Fig. 1 and their fluorescence properties are summarized in Table I. Both the amine and the substrate are fluorescent compounds having emission maxima at 420 nm and 370 nm, when excited at 330 nm and 340 nm, respectively. The enzymic release of AMeq can be followed at $\lambda_{ex} = 340$ nm and $\lambda_{em} = 430$ nm, where the relative fluorescence intensity of AMeq is more than 150-fold higher than that of an equimolar solution of the substrate.

The Michaelis constant (K_m) of post-proline endopeptidase for the hydrolysis of Z-Cys(Bzl)-Pro-NH-Meq has been determined in the pH range of 5·5–8·0 and the results obtained are summarized in Table II. The optimum pH for the hydrolysis of the substrate was 7·0. From Table III presenting Michaelis constants of post-proline endopeptidase for various substrates at optimum pH, it follows that the affinity of Z-Cys(Bzl)-Pro-NH-Meq to the enzyme ($K_m = 1{\cdot}05 \cdot 10^{-6}$ mol l^{-1} at pH 7·0) is 10 times higher than that of Z-Gly-Pro-NH-Meq.

DISCUSSION

In the early studies on post-proline endopeptidase, radiolabelled peptide hormones were used as very sensitive substrates[1,28]. However, for practical reasons, a tetrapeptide Z-Gly-Pro-Leu-Gly-OH was adopted later as a substrate for the general assay method[2,3,13–16].

Yoshimoto et al.[16,17] proposed new specific substrates for post-proline endopeptidase, Z-Gly-Pro-NH-Nap, Z-Gly-Pro-ONp and Z-Gly-Pro-NH-Mec and determined with their aid distribution of the enzyme in a variety of rat tissues. The fluorogenic substrate, Z-Gly-Pro-NH-Mec showed the highest affinity for post-proline endopeptidase ($K_m = 2{\cdot}0 \cdot 10^{-5}$ mol l^{-1}). Knisatchek et al.[18] introduced the substrate Z-His-Pro-NH-Nap which exhibited an approximately 2-fold higher affinity to this enzyme. The affinity of the new substrate Z-Gly-Pro-NH-Meq ($K_m = 10{\cdot}35 \cdot 10^{-6}$ mol l^{-1}) is the same as that of Z-His-Pro-NH-Nap and 2-fold higher than that of Z-Gly-Pro-NH-Mec. However, the affinity of the substrate, Z-Cys(Bzl)-Pro-NH-Meq is even higher than those of all the former ($K_m = 1{\cdot}05 \cdot 10^{-6}$ mol l^{-1}). It is evident that the increase of affinity is mainly due to the presence of cysteine at P_2 position and partly to the fluorescent amine AMeq. Thus, our new

TABLE I

Fluorescence properties of Z-Cys(Bzl)-Pro-NH-Meq and AMeq. The reagents ($0{\cdot}85 \cdot 10^{-6}$ mol . $.1^{-1}$) were dissolved in TES buffer ($5 \cdot 10^{-2}$ mol l^{-1}, pH 7·0), in the presence of 0·2% DMSO and the measurements were carried out at 37°C

Parameters	Z-Cys(Bzl)-Pro-NH-Meq	AMeq
Excitation maximum (nm)	330	340
Emission maximum (nm)	370	420
Relative fluorescence at 430 nm, excited at 340 nm	1	180
Upper limit of the concentration without self-quenching (. 10^{-6} mol l^{-1})	3	4

TABLE II

Michaelis constants of post-proline endopeptidase for Z-Cys(Bzl)-Pro-NH-Meq at different pH

pH	K_m, 10^{-6} mol l^{-1}
5·5	3·89
6·0	2·85
6·5	2·85
7·0	1·05
7·5	1·35
8·0	1·85
8·5	5·70

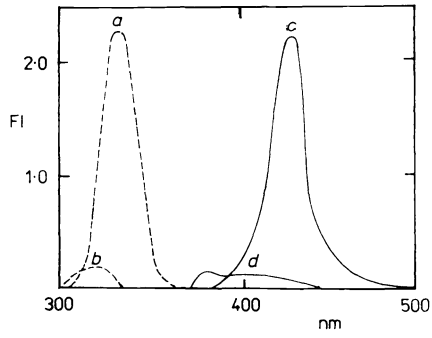

FIG. 1

Fluorescence spectra of Z-Cys(Bzl)-Pro-NH, -Meq and AMeq at pH 7·0 and 37°C. FI; fluorescence intensity in arbitrary units-wavelength in nm. The reagents were dissolved in TES buffer ($5 \cdot 10^{-2}$ mol l^{-1}) to the same concentration ($0{\cdot}85 \cdot 10^{-6}$ mol . $.1^{-1}$); 0·2% DMSO. Excitation spectrum of AMeq (*a*) or Z-Cys(Bzl)-Pro-NH-Meq (*b*); emission spectrum of AMeq (*c*) or Z-Cys-(Bzl)-Pro-NH-Meq (*d*)

fluorogenic substrate is suitable for the detection of low levels of the post-proline endopeptidase. Moreover, as in the concentration region of the K_m of the enzyme for this substrate the concentration of the organic solvent used for the solubilization of Z-Cys(Bzl)-Pro-NH-Meq is lower than 0·4%, it does not influence the course of the enzyme reaction as in the case of substrates with lower affinity[13].

TABLE III

Michaelis constants of post-proline endopeptidase for various substrates at optimum pH

Substrates	Assay conditions		K_m, 10^{-6} mol^{-1} l	References
	pH	temp., °C		
Z-Gly-Pro-NH-Meq	7·0	37	10·35	this paper
Z-Cys(Bzl)-Pro-NH-Meq	7·0	37	1·05	this paper
Z-Gly-Pro-NH-Mec	7·0	37	20	17
Suc-Gly-Pro-NH-Mec	5·9	37	430	5
Z-Gly-Pro-ONp	7·0	37	80	16
Z-Gly-Pro-Leu-Gly-OH	7·8	37	60	15, 16
	7·8	25	75	2, 13
Z-Gly-Pro-NH-Nap	7·0	37	135	17
Z-Gly-Pro-NH-Np	7·8	37	100	13, 16
Z-His-Pro-NH-Nap	7·4	37	9·5	18

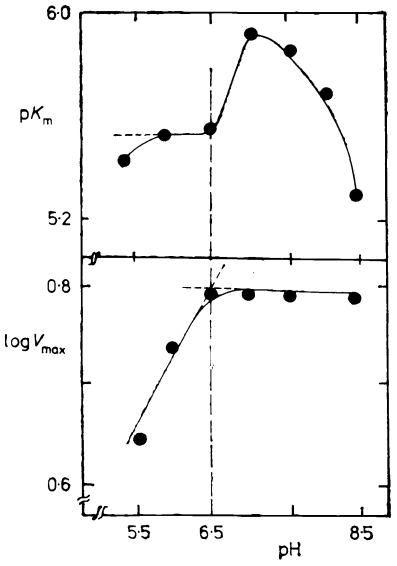

FIG. 2

Dixon–Webb's plot of the dependence of pK_m (log K_m) and log V_{max} on pH for the hydrolysis of Z-Cys(Bzl)-Pro-NH-Meq with post-proline endopeptidase

The pH-optimum determined for the hydrolysis of Z-Cys(Bzl)-Pro-NH-Meq was 7·0, as in the case of the hydrolysis of Z-Gly-Pro-NH-Mec (ref.[17]). However, maximum activity of the post-proline endopeptidase was observed also at higher pH values in assays with other substrates[2,13,15,16,18].

The dependence of the K_m and V_{max} on pH for the enzyme hydrolysis of Z-Cys(Bzl)--Pro-NH-Meq plotted according to Dixon[27] (Fig. 2) reveals a value 6·5 for the molecular ionization constant. The values pK_1 and pK_2 could not be estimated exactly due to the complex shape of the curves.

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