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Phenylalanyl-2-Sulfanilylglycine as Substrate for Leucine Aminopeptidase Assay

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A chromogenic mimic of phenlyalanyl-dipeptide, L-phenylalanyl-L-2-sulfanilylglycine (PSG), was synthesized and examined for its usability in leucine aminopeptidase (LAP) assay. The enzyme activity was easily determined by measuring the amount of diazotized adduct of sulfanilic acid released upon hydrolysis of PSG (ε_{420}=18,000/M/cm). Under the experimental conditions employed, PSG showed a Km of 0.063 mM and a Kcat of 1683/min, assessable less than 0.1 μg of LAP per milliliter. And the presence of aminopeptidase M (APM) was suggested to be negligible in LAP assay. This novel assay can circumvent the occasional yellow background in biological systems, i.e., serums, etc..

Chromogenic substrates have attracted a great interest for use in enzyme assays because of their simple and rapid capability to measure enzyme activities (14). Since they are not generally much related to corresponding substrates present in the physiological system, a reasonable compromise in terms of accessing enzyme activities can not be easily obtained (2). In this regard, natural substrates or their presumed mimicries are therefore most desirable. In recent studies, a technique based on the substitution of peptide side chains with certain nucleophilic chromogens via the α carbon of glycine residue has proved to be useful for this purpose (7, 11). Cytosolic leucine aminopeptidase (LAP; EC 3.4.11.1) is a widely distributed exoenzyme (15). Although its physiological function is still controversial, the assessment of LAP concentration in patient sera has been considered to be important for clinical diagnosis (12, 13, 16). Until L-phenylalnyl-L-3-thia-phenylalanine (PSP), a chromogenic mimic of Phe-Phe, was introduced (8), LAP was usually assayed with leucineamide as the recommended substrate (3) because chromogenic derivatives of leucine are more susceptible to attack by arylamidases (2). In a sense, PSP seems to be the most suitable substrates ever known. However, problems of the solubility and toxicity are still remained. In the course of investigating methods for sulfanilic acid transport via microbial dipeptide transport system (9, 10), we found that the peptide α-glycine residue substituted by sulfanilic acid was highly susceptible to hydrolysis by peptidases, accompanied with liberating the sulfanilic acid. Therefore, we developed a novel chromogenic substrate with sulfanilic acid as the constituent chromogen in a dipeptide and examined it for use in LAP assay.

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Key words: leucine aminopeptidase, chromogenic peptide substrate, sulfanilyl dipeptide

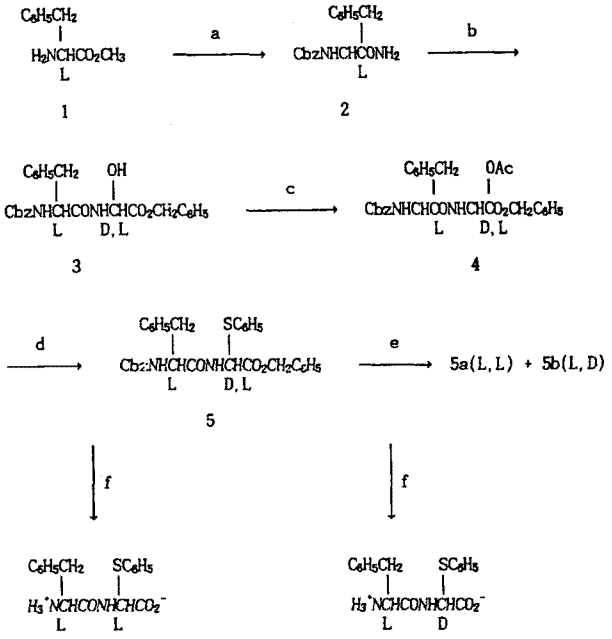
MATERIALS AND METHODS

Materials

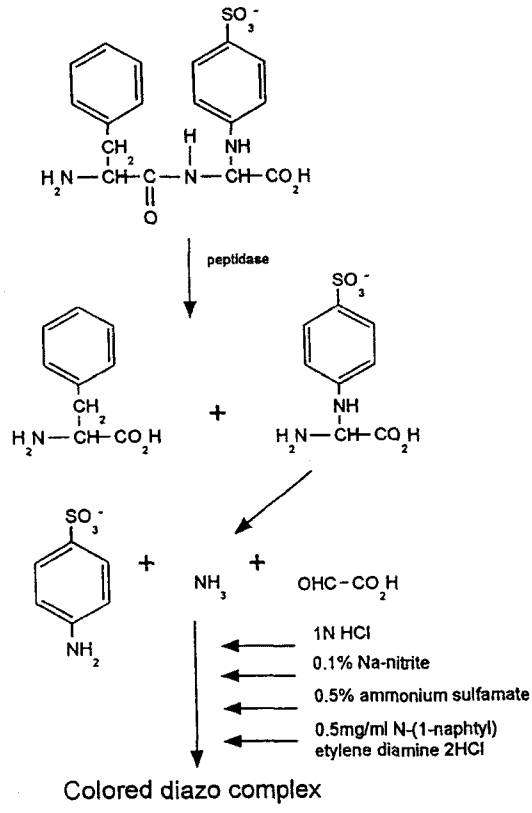
A purified preparation of LAP isolated from porcine kidney cytosol was purchased from Sigma Chem. Co., USA. The enzyme pellet was collected by centrifugation and dissolved in 50 mM Tris-HCl buffer, pH 8.2 before use. APM (microsomal; EC 3.4.11.2) was gifted from Dr. C. Gilvarg at Princeton University, NJ, USA. Leucine para-nitroanilide (Leu-p-NA) was obtained from Boehringer Mannheim GmbH, Germany; thiophenol, from Junsei, Japan; HBr in glacial acetic acid (35%), from Aldrich, USA, and N-(1-naphthyl)ethylenediamine·2HCl was from Janssen, Belgium, respectively. For other chemicals, Sigma products were used.

Synthesis

Dibenzyl(-)tartrate and benzylglyoxylate were prepared as described by Smith *et al.* and Kelley *et al.*, respectively (8). As shown in Scheme 1, PSP (6a) and PSG (6'a) were prepared by acetoxy group displacements (d,d') from the corresponding protected 2-acetoxyglycyl compounds, followed by deprotection with HBr (f) or Hydrogen gas (f'). PSP: ^{1}H NMR (D_2O/ DMSO) δ 2.8-3.1 (d, 2H), 3.9 (t, 1H), 5.3 (S, 1H), 7.2-7.4 (m, 10H). PSG: ^{1}H NMR (D_2O/ DMSO) δ 2.9-3.0 (d, 2H), 4.0-4.1 (t, 1H), 5.5 (s, 1H), 6.5-7.5 (m, 9H).



Scheme 1. Synthesis of PSP and PSG.
(a) NH_3 CH_3OH; CbzCl; (b) $HCOCO_2CH_2C_6H_5$, CH_2Cl_2, 88 h., 25°C; (c) Ac_2O, pyridine, 20 h, 5°C; (d) C_6H_5SH, DMF, Et_3N, 25°C; (e) Et_2O crystallization; (f) HBr/AcOH, 20 h., 25°C; propylene oxide, EtOH, 25°C; (d')$NH_2C_6H_4SO_3^-$, DMF, Et_3N, 25°C.



Scheme 2. The mode of breakdown and visualization of PSG hydrolysis by peptidase.

Stereochemical purities of both PSP and PSG were determined by using the method of enzymatic analysis with LAP. A value of 98% purity in LL was obtained for PSP and 95% purity in LL for PSG, quantitated from the standard curves for thiophenol (ε_{412}= 10,000/M/cm), or sulfanilic acid (ε_{560}=18,000/M/cm), respectively. An alternative attempt to estimate stereochemical purity of PSG was also done using column chromatography with DEAE-cellulose (0.8×5 cm; 5 mM phosphate buffer, pH 7.0) after its enzymatic hydrolysis. The purity of LL-PSG could be ascertained by computing the percent ratio of sulfanilic acid relative to the initial concentration of PSG from the elution profile, which was made by measuring the absorbance at 254 nm (data not shown).

Enzyme Assay

For PSP hydrolysis, 1 ml of the reaction mixture consisted of 0.1 mM PSP, 0.2 mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) (5) and 50 mM Tris-HCl buffer, pH 8.4, was placed in a 1-cm light-path cuvette and time course hydrolysis was carried out by the addition of the enzyme preparations as appropriate. The amount of yellow product formed was then continuously monitored by measuring its O.D. at 412 nm with a Shimadzu Model 2102 spectrophotometer. PSG hydrolysis was initiated by adding enzyme solution to give a final volume of 1 ml of the reaction mixture containing 0.1 mM PSG and 50 mM Tris-HCl, pH 8.4 then aliquots of 0.2 ml were taken at time intervals. The reaction was terminated by the addition of 0.2 ml of 1 N HCl and diazotized by consecutive addition of the following reagents: 0.2 ml of 0.1% Na-nitrite, 0.5% ammonium sulfamate, and 0.5 mg/ml N-(1-naphthyl)ethylenediamine·2HCl. After 20 min at room temperature, the amount of diazo-adduct formed was determined by measuring the absorbance at 560 nm (See Scheme 2) (6). For Leu-p-NA hydrolysis, 1 mM Leu-p-NA in 50 mM Tris-HCl buffer, pH 8.4 and enzyme solution were added to 1-cm light-path cuvette with a final volume of 1 ml. The enzyme activity was determined by measuring the absorbance of free nitroaniline at 405 nm.

RESULTS AND DISCUSSION

PSG Hydrolysis by LAP

A time course determination of PSG hydrolysis was carried out under different concentrations of LAP. Fig. 1 shows a linear increase in the product formation with

time. The time course also reveals that the assay can quantitate 0.05 μg/ml of the enzyme concentration. The rate of enzymatic hydrolysis was directly proportional to the enzyme concentration in the range examined (Fig. 2). Accessing the LAP concentration, PSG would be efficient substrate as that covers the range of enzyme concentration over 3 times than PSP.

LAP-Catalyzed Kinetics of Peptide Mimicries and Amides

The kinetic parameters for the LAP-catalyzed hydrolysis of PSG were determined using Michaelis-Menten kinetic and were compared to those values of PSP. The kinetic function of LAP catalysis for these two substrates appeared to obey Michaelis-Menten kinetics, and the resulting kinetic parameters were evaluated from the Lineweaver-Burk plot. As can be seen in Fig. 3, a higher binding affinity was observed for PSG with a Km of 0.063 mM (0.083 mM for PSP), but with a lower V_{max} value (0.007 μmole/μg protein/min). These kinetic constants are contrasted with those non-peptide substrates in Table 1. In terms of the kinetic power (K_{cat}/K_m), peptide mimicries are more suitable substrates for LAP than amides. PSP appeared to possess a higher kinetic power having 61, 470/min/mM, supposed to be 2 fold better than PSG as substrate. Nevertheless, since enzyme inhibition encountered in the presence of DTNB may cause undesirable problems (8), accurate LAP-catalyzed kinetics for PSP should be carried out discontinuously. However, although PSG hydrolysis could not be monitored continuously in this experiment, a well known

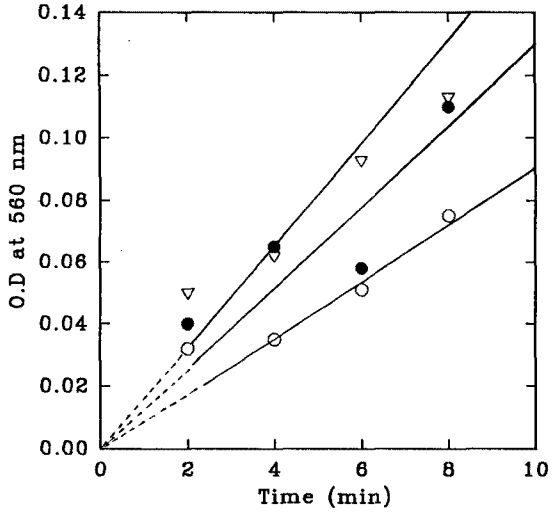


Fig. 1. Time course hydrolysis of PSG by LAP.
Enzyme reaction was carried out at room temperature. Enzyme concentrations (μg/ml): 0.05 (○), 0.15(●), 0.25(▽). See Materials and Methods for conditions.

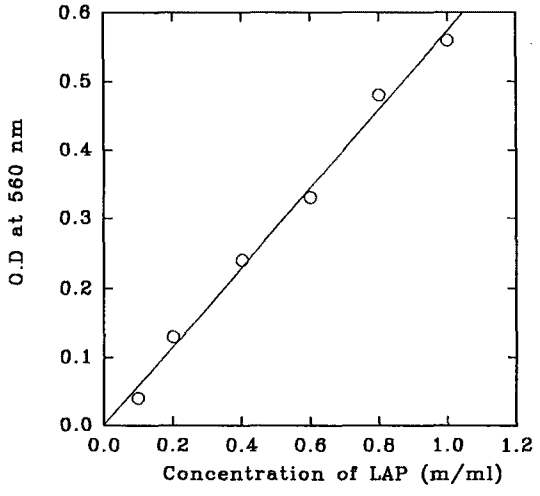


Fig. 2. Dependence of the rate of hydrolysis of PSG on LAP concentration.
O.D. indicates the mean value per minute after 10 min incubation. See Fig. 1 for conditions.

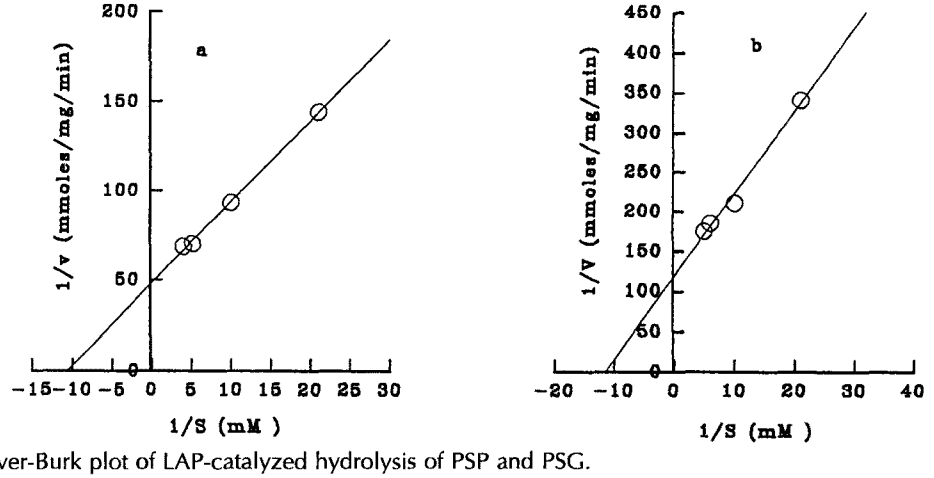


Fig. 3. Lineweaver-Burk plot of LAP-catalyzed hydrolysis of PSP and PSG.
0.1 μg of enzyme was used. a, PSP. b, PSG.

Table 1. Comparison of the kinetic properties for substrates of LAP.

Substrate	K_m (mM)	K_{cat} (min^{-1})	K_{cat} (min^{-1} mM^{-1})
PSG	0.063	1,683	26,714
PSP	0.083	5,102	61,470
*Leu-β-naphthylamide	0.15	1,720	11,500
*7-Leu-4-methyl-conumarinylamide	0.16	1,300	8,130
*Leucinamide	28	110,000	3,930
*Leucinehydrazide	23	80,000	3,480
*Leu-p-NA	1.0	42	42

*Ref. (8).

continuous flow system (4) or an automated continuous assay (1) can be applied to circumvent the time consuming acid aided single point assay as noted in "Materials and Methods".

Substrate-Based Discrimination Between APM and LAP

As presented in Table 2, Leu-p-NA seems to be a suitable substrate for APM, having more than 20 times of kinetic power than that for LAP. Conversely, the difference in kinetic powers between PSG and Leu-p-NA for LAP appeared to be over 600 times. This, in turn, implies the possibility of selective determination of LAP activity in the presence of arylamidases such as APM. Therefore, the combined use of an arylamide and a peptide mimicry is thought to be an ideal combination for measuring aminopeptidase levels (LAP and APM) in patient sera. In fact, we found recently some consistency in the relationship between the serum ratio of LAP activity to APM activity and the type of diseases (unpublished data).

Potency of Sulfanilic Acid as a Peptidyl Chromogen

Our studies strongly recommended the use of peptides bearing sulfanilic acid as a chromogenic substituent in following aspects: 1) Synthesis; Sulfanilyl substitution of peptide α glycine residues can be easily accomplished, whereas that of thiophenol should be carried out with special caution. 2) Solubility; PSG is readily dissolved in aqueous solutions but PSP is virtually insoluble in water. 3) Stability; Reduction rate of PSG is less than 1% per hour at room temperature (PSP≥5%). 4) Spectrochemistry; the extinction coefficient number of pink-colored diazo-adducts is near 2 fold extensive than that of yellow-colored thiophenol.

Chromogenic substrates for LAP were originally devised because of the need for a histochemical method to investigate the location of the enzyme in tissues. These substrates are based on the aromatic nature of chromogens, i.e., nitroaniline, naphthylamine or phenylazophenylamide, etc. (2). Unfortunately,

Table 2. Substrate-based discrimination between APM and LAP.

Substrate	APM		LAP	
	K_m (mM)	K_{cat} (min^{-1})	K_m (mM)	K_{cat} (min^{-1})
PSG	0.12	65	0.063	26,714
Leu-p-NA	0.20	2600	1.0	42

these arylamides are all highly susceptible to recognition by arylamidases (8). Moreover, the cytosolic LAP employed showed little specificity against Leu-p-NA (data not shown). In this regard, PSG may be useful when one needs to distinguish LAP from the above enzymes. It should also be considered in relation to the issue of diazotized color, which is avoidable from the occasional disturbance attendant to the high backgrounds of yellow color in biological systems, i.e., sera.

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