An improved spectrophotometric assay for leucine aminopeptidase

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A sensitive assay to determine the activity of leucine aminopeptidase (EC 3.4.11.1), using L-leucine thiobenzyl ester as substrate, was developed. Hydrolysis of the ester by leucine aminopeptidase can be monitored in the presence of 5,5-dithiobis-(2-nitrobenzoic acid) by continuous spectrophotometric measurement at 412 nm. Comparison with some amide substrates showed that the thiol ester provides a much more sensitive assay, its specificity constant ($V_{\max}/K_m$) being some 3000-fold higher than that of leucine *p*-nitroanilide.

INTRODUCTION

Several methods are available for assay of LAP. Most use amide or similar derivatives of leucine, which, on hydrolysis, yield a fragment that is usually detected spectrophotometrically or spectrofluorimetrically. These include leucineamide (Binkley & Torres, 1960; Hanson & Lasch, 1967), Leu-2-Nap (Bratton & Marshall, 1939; Green *et al.*, 1955; Greenberg, 1962; Lee *et al.*, 1971), Leu-*p*NA (Tuppy *et al.*, 1962) and Leu-NHMec (Saifuku *et al.* 1978). Of these, Leu-*p*NA is probably the most widely used, although, as with all amides, its rate of metabolism by the enzyme is modest. During studies of the inhibition of LAP by peptides containing sulphur, we found that Leu-*p*NA was unsuitable as a substrate because of chemical reaction with the inhibitors. In considering possible alternatives we examined thiol esters of leucine as potential chromogenic substrates for LAP. The use of thiol esters as substrates for chymotrypsin and subtilisin in the presence of Nbs₂ as chromogen was first reported by Farmer & Hageman (1975). Since then they have been used for other proteinases (Castillo *et al.*, 1979; Green & Shaw, 1979; MacCrae *et al.*, 1981). These assays are based on the formation of a stoichiometric amount of 3-carboxy-4-nitrothiophenoxide ion by reaction of Nbs₂ with the thiol ester liberated by hydrolysis of the thiol ester substrate. The present paper describes the synthesis of Leu-SBzl (I) and its use in a continuous spectrophotometric assay for LAP. A recently reported assay for LAP (Hwang *et al.*, 1986) uses as substrate the Phe-Phe analogue Phe-3-thia-Phe, which, on hydrolysis by the enzyme, yields a fragment that rapidly breaks down to produce thiophenol; this is determined by using Nbs₂.

MATERIALS AND METHODS

Cytosolic LAP from porcine kidney (Sigma type III-CP), leucineamide and Nbs₂ were from Sigma Chemical Co., Poole, Dorset, U.K. The concentration of protein in the LAP, measured by the method of Markwell *et al.* (1978), was 3 mg/ml, and its specific activity, with leucineamide as substrate, at 25 °C and pH 8.5, was 133 units/mg of protein. One unit is the amount of enzyme required to metabolize 1 μmol of substrate/min. Leu-NHMec·HCl was prepared by the method of Zimmerman *et al.* (1976). Leu-*p*NA was synthesized as described by Yasutake & Powers (1981). Leu-NHPh·HCl was prepared by a method described by Weygand & Nintz (1965). Leu-Gly-OEt·HCl was synthesized as outlined by Maher *et al.* (1972).

Leu-SBzl·HCl

Boc-Leu (3.66 g, 15.8 mmol) in dry tetrahydrofuran (60 ml) was stirred at –15 °C. *N*-Methylmorpholine (1.59 ml, 15.8 mmol) was added, followed by isobutyl chloroformate (2.16 ml, 15.8 mmol). After 2 min, benzyl mercaptan (1.85 ml, 15.8 mmol) was added. Cooling was discontinued after 30 min and stirring was then continued for a further 12 h. The solvent was removed by evaporation, and the residue was dissolved in ethyl acetate and washed successively with portions of 5% (w/v) NaHSO₄, water, 5% (w/v) NaHCO₃ and water. The organic phase was dried and evaporated to dryness. The resulting Boc-Leu-SBzl was deprotected by stirring for 30 min in a saturated solution of HCl gas in dry diethyl ether. The hydrochloride of the product, which separated out, was collected, dissolved in dry methanol and precipitated with diethyl ether. The product was homogeneous as judged by t.l.c. in methanol (R_F 0.64) or in chloroform/methanol (1:1) (R_F 0.33). Yield 1.5 g (66%). ¹H n.m.r.: δ (p.p.m.) [(²H)chloroform] 9.0 (3H, broad, NH₃⁺), 7.3 (5H, s, C₆H₅), 4.2 (2H, AB quartet, SCH₂; 1H, m, CHCO), 2.04 [2H, m, CHCH₂CH; 1H, m, CH(CH₃)₂], 1.86 [6H, d, CH₃]₂. Spectrophotometric studies were carried out on a Pye–Uvicam SP.6-450

Abbreviations used: abbreviations for amino acids, peptides and their protecting groups are as recommended in Biochem. J. (1972) 126, 773–780; chiral amino acids are all of the L configuration; LAP, leucine aminopeptidase; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); -*p*NA, leucine *p*-nitroanilide; -2-Nap, 2-naphthylamide; -NHMec, 4-methylcoumarylamide; -SBzl, thiobenzyl ester; -NHPh, anilide; -OEt, ethyl ester; Boc-, benzyloxycarbonyl-.

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spectrophotometer linked to a Philips single-pen recorder.

Assay

The enzyme as purchased contains an excess of Mg^{2+} ions. To test whether these affected the hydrolysis of the thiol ester, the assay was conducted both in the presence of these excess ions and also after their removal by gel filtration as described by Van Wart & Lin (1981). Assays were carried out at 25 °C in 0.2 M-Tris/HCl buffer, pH 8.5, containing 0.2 M-NaCl. The buffer was prepared by adjusting an approx. 0.2 M solution of Tris/NaCl to pH 8.5 with HCl and diluting to 0.2 M. Substrate concentrations were in the range 400–25 μ M and all assay mixtures contained Nbs₂ (0.5 mM) as chromogen. All runs were carried out in duplicate with a total assay volume of 1 ml in a cuvette of path length 1 cm. Stock solutions of substrate (10 mM) in HCl (1 mM) and of Nbs₂ (50 mM) in methanol were prepared. Aliquots of substrate and of Nbs₂ (10 μ l) were diluted into buffer in the cuvette, and, when thermal equilibrium had been established, the reaction was started by adding enzyme solution (10 μ l; 30 μ g of protein). The rate of hydrolysis of the thiol ester was monitored by observing the increase in A_{412} due to formation of 3-carboxy-4-nitrothiophenoxide ion ($\epsilon_{412} = 13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$; Ellman, 1959; Green & Shaw, 1979). For comparison the enzyme was also assayed with leucineamide (Binkley & Torres, 1960), Leu-pNA (Tuppy *et al.*, 1972), Leu-NHMec, Leu-Gly-OEt and Leu-NHPh (R. E. B. Beattie, D. T. Elmore & C. H. Williams, unpublished work). In order to show that reaction of the liberated thiol with Nbs₂ was not rate-limiting, the enzymic reaction was measured at six different concentrations of the reagent (0.5–3 mM) with each of two substrate concentrations (0.3 and 0.4 mM).

The method of Elmore *et al.* (1963) was used to compute initial velocities. Kinetic constants were calculated by two methods: (a) from a double-reciprocal plot based on measurements at a minimum of eight concentrations of substrate, and (b) using the method of Roberts (1977) to calculate K_m values by applying a least-squares method directly to the Michaelis–Menten equation.

RESULTS AND DISCUSSION

Kinetic constants for hydrolysis of the thiol ester were the same in both the presence and absence of excess Mg^{2+} ions. Experiments in which the concentration of Nbs₂ was varied over the range 0.5–3 mM at each of two fixed

substrate concentrations (0.35 and 0.4 mM) gave constant rates of formation of chromophore (44 and 46 μ mol/min), indicating that, under steady-state conditions, the rate of formation of the thiophenoxide ion was not rate-limiting. Non-enzymic hydrolysis of the thiol ester at pH 7.5 was approx. 1% of the enzymic rate; at pH 8.5 it approached 2%. This was easily corrected for by appropriate subtraction. The K_m for the thiol ester at pH 8.5 was found to be 0.21 mM, lower than those found for a number of amide substrates (Table 1), and this, coupled with its much higher rate of hydrolysis of LAP (60 μ mol/min compared with a value of 0.29 for Leu-pNA), yielded a rapid assay of high sensitivity. The assay described by Hwang *et al.* (1986) was reported to be more sensitive ($k_{cat.}/K_m = 1780\text{ mM}^{-1}\cdot\text{s}^{-1}$) than those using other amide substrates ($k_{cat.}/K_m$ values in the range 0.68–190 $\text{mM}^{-1}\cdot\text{s}^{-1}$), partly accounted for by the low K_m value (0.054 mM) typical of dipeptides unsubstituted on the $-\text{CO}_2\text{H}$ group (Hanson & Frohne, 1976), although the $k_{cat.}$ value was higher than those reported for several other substrates, with the exception of leucineamide and leucine hydrazide. In the absence of details of the operational molarity of the enzyme, comparisons of $k_{cat.}$ values from different sources can only be approximate. In the present study we have compared the substrates listed in Table 1, assayed under the same conditions, using the concentration of protein to calculate a value of $k_{cat.}$, and assuming the M_r of LAP to be 320 000 (Shen & Melius, 1977). The final column shows relative values for specificity constants ($V_{max.}/K_m$) based on a value of 1 for leucineamide. It can thus be seen that Leu-SBzl is an outstandingly good substrate for assay of LAP. Finally, Hwang *et al.* (1986) report that, when used at concentrations greater than 0.2 mM, Nbs₂ inhibits the hydrolysis of Phe-3-thia-Phe. We have not observed such inhibition during the present study with Leu-SBzl. When hydrolysis of the thiol ester by LAP was allowed to proceed for 10 min before addition of Nbs₂ (0.5 mM) the absorbance produced was the same as that which developed during a similar time in the normal assay, indicating that the rate of formation of product (benzyl thiol) was unchanged by addition of Nbs₂.

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Table 1. Kinetic data for substrates of LAP measured at pH 8.5 and 25 °C

Substrate	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($\text{mM}^{-1}\cdot\text{s}^{-1}$)	Relative specificity* constant ($V_{max.}/K_m$)
Leu-pNA	3.5	49	14	1
Leucineamide	5	765	154	11
Leu-NHMec	0.072	32	440	31.3
Leu-NHPh	1.1	87	77	4.9
Leu-Gly-OEt	2.04	383	190	13.5
Leu-SBzl	0.2	10430	52200	3756

* Values relative to Leu-pNA = 1.

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