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A Kinetic Method for Leucine Aminopeptidase Activity Assay

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A new colorimetric method for leucine aminopeptidase (LAP) activity assay with application of L-leucine-3,5-dibromo-4-hydroxyanilide (Leu-BAP) as a substrate was described. The substrate was found to have K_m value of 9×10^{-5} mol l^{-1} with a blood serum LAP. The method is several times more sensitive and quicker than the ones that were used so far. Under the defined conditions, the method is applicable to kinetic measurements of LAP activity in serum.

W pracy opisano nową kolorymetryczną metodę oznaczania aktywności aminopeptydazy leucynowej (LAP) z zastosowaniem leucylo-3,5-dibromo-4-hydroksyanilidu, w stosunku do którego enzym wykazuje K_m $9 \cdot 10^{-5}$ mol l^{-1}. Metoda jest kilkakrotnie bardziej czuła i kilkakrotnie szybsza niż dotychczas stosowane. Metodę tę zastosowano do kinetycznego pomiaru aktywności LAP w surowicy.

Leucine aminopeptidases comprise a group of enzymes which catalyze hydrolysis of peptide *N*-terminal aminoacid and aminoacid amides. Aminopeptidases of the highest activity, *i.e.* of the lowest substrate and the highest catalytic constant with respect to leucine amides are usually classified as leucine aminopeptidases (LAP). Such enzymes are found in a soluble as well as a membrane bound form. One of the membrane aminopeptidases EC 3.4.11.2 was identified as a CD13 antigen [1]. The latter is identical with melanoma cells' marker and putatively acts as an enzyme promoting invasion of tumour cells [2,3]. Physiological function of a membrane and a microsomal LAP (EC 3.4.11.1) is extensively discussed. LAP is active in immunological response [4], is supposed to be involved in a turnover of neuropeptides,

peptide neuromediators and peptide hormones as well as to play a role in an inflammatory state [5–8].

LAP activity estimation is useful for diagnosis of liver and renal disorders [9,10]. The same substrates as for LAP activity are applicable for another enzyme – oxytocinase assays. Oxytocinase activity assay with leucine amides has been applied in obstetrical diagnostics [11,12].

In our previous work we described a synthesis of the LAP chromogenic substrate, which was applied on a paper strip for enzyme activity measurement. This method is unsuitable for instrumental colorimetric measurements [13]. In this work L-leucine-3,5-dibromo-4-hydroxyanilide (Leu-BAP) was applied as a leucine aminopeptidase substrate in the colorimetric kinetic method for activity measurement in serum.

Aminophenol is released by LAP from leucylhydroxyanilides, the former giving indophenol and indoaniline dyes with phenols and with aniline derivatives in the presence of oxidative agents. The kinetics of a dye forming in the reaction of dibromoaminophenol with phenole and with aniline derivatives was investigated earlier. *N*-methylanthranilic acid was chosen as the one easily resulting in a dye formation and which is not prone to a visible oxidation by potassium hexacyanoferrate (potassium ferricyanide) or hydrogen peroxide in the presence of peroxidase. Under the reaction conditions methylanthranilic acid forms a dye of a molar absorptivity 47×10^3 l mol^{-1} cm^{-1} and absorption maximum at 702 nm [14]. With respect to leucine-3,5-dibromo-4-hydroxyanilide LAP shows a much lower Michaelis constant than with L-leucine-4-nitroanilide (Leu-pNA), whereas both catalytic constants are comparable. The method for LAP activity assay with Leu-BAP is several times quicker then the one with Leu-pNA.

EXPERIMENTAL

Umbilical and pregnant women blood serum was obtained from the Regional Hospital of Grodków, male blood serum from the Blood Bank, Wrocław. For the kinetic constant estimates an umbilical serum of the LAP activity 22×10^{-6} mol min^{-1} l^{-1} against L-leucine-4-nitroanilide (Leu-pNA) and a male serum of the LAP activity 76.6×10^{-6} mol min^{-1} l^{-1} were used.

The synthesis of 4-amino-2,6-dibromophenol (BAP), L-leucine-3,5-dibromo-4-hydroxyanilide (Leu-BAP) and *N*-methylanthranilic acid was described earlier [15]. L-leucine-4-nitroanilide (Leu-pNA), horseradish peroxidase (HRP) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Merck (Darmstadt, Germany), other reagents were from POCh (Gliwice, Poland).

Photometric data were collected using a Specord spectrophotometer. Calibration curve for 4-amino-2,6-dibromophenol (BAP) was estimated with *N*-methylanthranilic acid as a colouring component [14]. Enzyme activity assay was done as following: to 2,3 ml of 0.1 mol l^{-1} Tris–HCl buffer of pH 8.0 containing 2 millimolar methylanthranilic acid, 3.5 millimolar hydrogen peroxide and peroxidase at a concentration of 1 activity unit per ml, 0.1 ml substrate methanol solution was added at a concentration of 0.1 mol l^{-1} (Leu-pNA) or 0.02 mol l^{-1} (Leu-BAP). Enzyme activity assay was also performed in a buffer containing 2×10^{-3} and 2×10^{-4} molar tripotassium hexacyanoferrate [$K_3Fe(CN)_6$] instead of hydrogen peroxide and peroxidase. The reaction was started with an addition of 10–100 µl of a serum. Absorbance increase was recorded at 405 nm and or 702 nm for Leu-pNA and Leu-BAP as a substrate, respectively.

Oxytocinase (methionine insensitive leucine aminopeptidase – MILAP) was assayed in the same conditions as LAP in a buffer containing additionally 10^{-2} molar concentration of L-methionine.

A substrate constant and a maximal velocity of leucine aminopeptidase LAP and MILAP were estimated at 37°C and 25°C. Molar absorptivity of 9.8×10^{3} l mol^{-1} for nitroaniline and 4.7×10^{4} l mol^{-1} cm^{-1} for the dye resulting from the reaction of aminodibromophenol with methylanthranilic acid was considered in the estimations. The estimations were done with EnzFitter PC program.

RESULTS AND DISCUSSION

3,5-Dibromo-4-hydroxyanilides were used ealier as chromogenic substrates for hydrolases [14–16]. Under the present reaction conditions the same substrate was adopted to kinetic measurements of LAP activity. At a methylanthranilic acid and potassium hexacyanoferrate concentration applied, an initial nonlinear lag phase is almost unrecordable (Fig. 1), absorbance increase is linear, and the reaction rate depends on the amount of serum in a sample. According to an equation $y = a + bx$ (y – absorbance; x – μl of serum) the dependence is characterized by a very good correlation coefficient $R = 0.9981$; $a = 5.3\times10^{-3}$, std. error 5.6×10^{-3}; $b = 6.4\times10^{-3}$, std. error 1.05×10^{-4} or according to an equation $y = bx$ $R = 0.9979$; $b = 6.51\times10^{-3}$, std. error 6.1×10^{-5}.

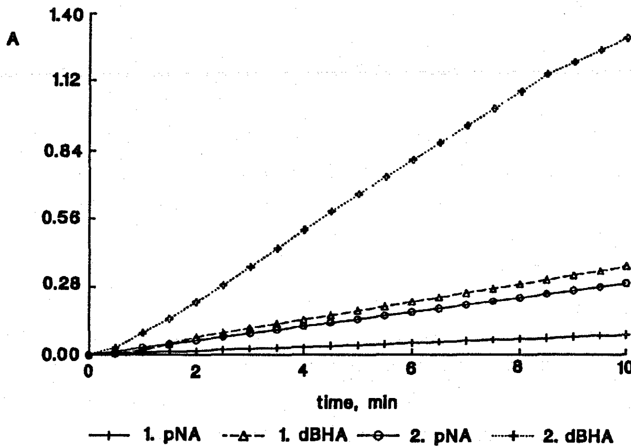

Figure 1. Absorbance increase at 405 nm for Leu-pNA and 702 nm for Leu-BAP as a substrate. Umbilical human blood serum of LAP activity of 22×10^{-6} mol min^{-1} l^{-1} (No 1) and male blood serum of LAP activity of 76.6×10^{-6} mol min^{-1} l^{-1} (No 2). $R = 0.999$ (2–10 min period)

In the presence of hydrogen peroxide and peroxidase in the sample, longer lag phase is observed, absorbance increase is linear after the first two to five minutes of the reaction. For the Michaelis constant as well as maximum velocity measurements a linear fragment of absorbance increase function of time was considered. An initial, nonlinear fragment was omitted (Fig. 2). For comparison, K_m and V_{max} were estimated in the solution of the same composition and with Leu-pNA as a substrate (Fig. 3,

Table 1.). K_m value for Leu-pNA was very similar to the result which was found by others [5,11].

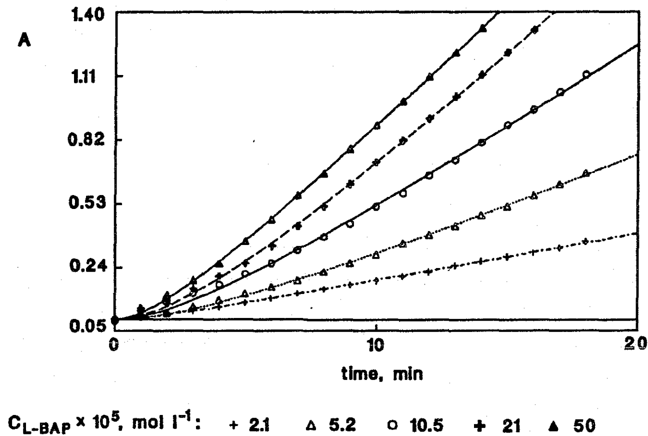

Figure 2. Absorbance increase at 702nm for Leu-BAP as a substrate. Serum No 2 was used and hydrogen peroxide as an oxidant [2.3 ml of 0.1 mol l^{-1} Tris–HCl buffer of pH 8.0 containing 2 millimolar methylanthranilic acid, 3.5 millimolar hydrogen peroxide and peroxidase at a concentration of 1 activity unit per ml, 0.1 ml substrate (Leu-BAP) methanol solution was added to a final concentration 2.1; 5.2; 10; 21; 50 x 10^{-5} mol l^{-1}]

Kinetic constants for LAP were measured for the enzyme contained in a serum. Umbilical and a male serum were used to avoid oxytocinase influence on the estimates. In the first experiments potassium hexacyanoferrate was applied as an oxidizing agent, which at a 10^{-3} mol l^{-1} concentration partially inactivated leucine aminopeptidase. Partial LAP of umbilical serum inactivation with hexacyanoferrate was observed during measurements undependently on weather Leu-BAP or Leu-pNA as a substrate were used. At a potassium ferricyanide concentration of 10^{-4} mol l^{-1} inactivation was not observed. Its inactivation was putatively a result of zinc and manganese cations precipitation by hexacyanoferrate ions giving hardly soluble salts. It seems logical that leucine aminopeptidase as an enzyme requiring zinc and manganese cations for its activity is inactivated by potassium hexacyanoferrate and similarly by chelating agents. Consequently, hexacyanoferrate ion was replaced by hydrogen peroxide and peroxidase. With hydrogen peroxide and HRP at a low BAP concentration, an initial colour reaction is dependent on methylanthranilic acid concentration and peroxidase activity in a solution. At a lower methylanthranilic acid concentration and a lower peroxidase activity, longer retardation of absorbance increase (lag phase) in the first minutes of reaction was observed. During oxytocinase measurements with the hydrogen peroxide apparent time dependent inactivation was observed. Presumably, decreasing of the hydrogen peroxide concentration as a result of methionine oxidation seems as the oxytocinase time dependent inactivation, and so oxytocinase as a MILAP with potassium hexacyanoferrate as an oxidant was estimated.

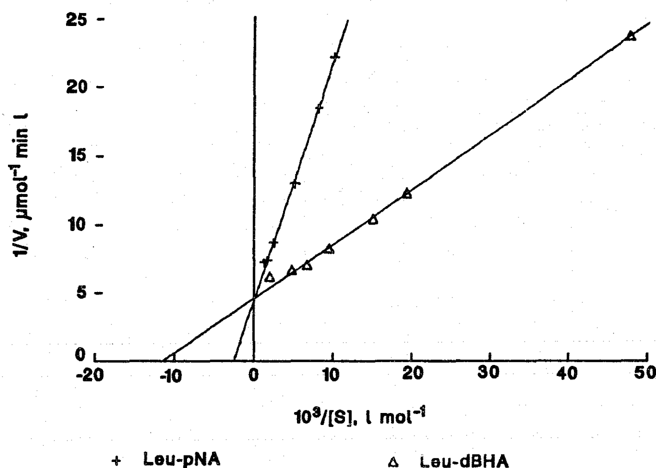

Figure 3. Lineweaver–Burk plot, LAP in a serum No 2. The linear fragment of absorbance increase as a function of time from plots in the Fig. 3 was considered

Table 1. Kinetic parameters of LAP and MILAP with Leu-BAP and Leu-pNA estimated from the Lineweaver–Burk equation

Temperature °C	Leu-BAP		Leu-pNA
	K_m mol l^{-1}	V_{max} mol l^{-1} s^{-1}	K_m mol l^{-1}
37	$6.8 \times 10^{-5} \pm 0.5$ $*7.1 \times 10^{-5} \pm 0.4$	7.2×10^{-7} 75% Leu-pNA	6.6×10^{-4}
25	$9.1 \times 10^{-5} \pm 0.6$ $*1.05 \times 10^{-4} \pm 0.1$	4×10^{-7} 90% Leu-pNA	4.5×10^{-4}
MILAP (with methionine 10^{-2} mol l^{-1})			
25	$4.1 \times 10^{-4} \pm 0.3$	7.6×10^{-7} 37% Leu-pNA	$4.3 \times 10^{-4} \pm 0.4$

* Estimated by a kinetic method using hydrogen peroxide.

Leucine-3,5-dibromo-4-hydroxyanilide appears to be a good substrate for leucine aminopeptidase. LAP shows similar V_{max} value against the one and leucine-4-nitroanilide (Leu-pNA). Michaelis constant is more than five times lower for the studied compound than for nitroanilide and its hydrolysis product forms a dye of a five times higher molar absorption than for nitroanilide. As a consequence, activity measurement with this substrate takes only a few minutes by a kinetic method. Absorbance increase is about 4.3 times higher than with Leu-pNA for LAP and nearly the same as with Leu-pNA for oxytocinase assay (Table 1).

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