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The Application of Salicylic Acid Amide for 4-Dimethylaminoaniline Determination

Oznaczanie 4-dimetyloaminoaniliny z wykorzystaniem amidu kwasu salicylowego

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In recent years the dipeptidaze enzymes are of increasing interest. The new, synthetic substrates for these enzymes are developed to find a fast, precise and cheap method of determination of their activity. The substrates are peptides, characteristic for each enzyme, and bound to the compound which is determined after releasing. Commonly a substrate contains β-naphtylamine [1–8], *p*-nitroaniline [9–14] and 4-phenylazophenylamine [15]. The concentration of the above compounds is determined by fluorometric or spectrophotometric methods, directly or after transformation to a stable coloured derivative, which should be characterized by a high molar absorptivity. The synthesis of Gly-Pro-4-dimethylaminoanilide (Gly-Pro-DMAA) and its application for diaminopeptidaze IV (DAP IV; EC 3.4.14.5) activity measurements is given in ref. [19]. The amount of dimethylaminoaniline (DMMA) released in the enzymatic reaction was determined after preparing its coloured quinonoimine derivative using the spectrophotometric method.

The main aim of our present investigations is to improve the method of 4-dimethylaminoaniline determination, and the secondary one is to find the precise and fast method of DAP IV activity measurements. In this paper the new, original method of DMAA determination is presented. The proposed method gives the coloured derivative of DMMA of higher molar absorptvity than the one presented in ref. [19].

EXPERIMENTAL

Reagents and instrumentation

Universal Britton–Robinson buffer of pH range 2.0–12.0; Tris–HCl buffer of pH = 7.8 and concentration of 0.2 mol l^{-1}; muriatic acid of concentration 1 mol l^{-1}; the DMAA solution of concentration 1 mmol l^{-1}; the salicylic acid amide of concentration 8.5 mmol l^{-1} in sodium carbonate solution of concentration 0.2 mol l^{-1}; $K_3[Fe(CN)_6]$ of concentration 2 mmol l^{-1} in the universal Britton–Robinson buffer (pH = 2.2). The solutions were prepared using twice distilled water. Muriatic acid was distilled before use. All chemicals were of analytical reagent purity.

Spectrophotometer, model Specord M40 (Carl Zeiss, Jena), with quartz trays S 10.00, was used. pH was measured with a digital pH-meter PM-1 (Polmed, Wrocław).

The spectrophotometric determination of 4-dimethylaminoaniline (DMAA)

To the mixture of 0.125 ml of DMAA solution, 0.625 ml Tris–HCl buffer and 0.05 ml HCl solution prepared as in ref. [19], the 0.8 ml of salicylic acid amide solution and 3.2 ml of $K_3[Fe(CN)]_6$ solution were added.

The $K_3[Fe(CN)_6]$ solution was prepared just before use. The absorption measurements were carried out in whole range of the visible light. The wave lenght λ_{max} = 686.5 nm which corresponds to the maximum absorption of the dye solution (Fig. 1) was chosen for the further measurements.

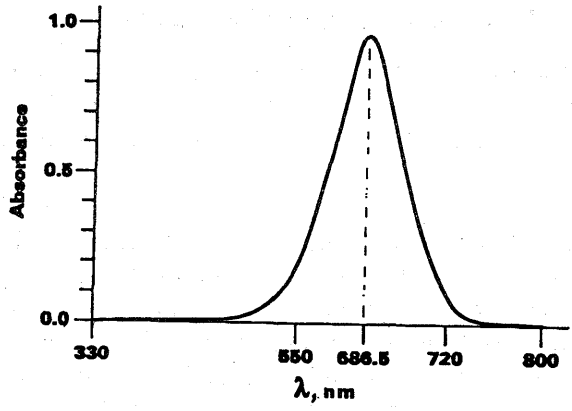

Figure 1. Absorbance of the dye solution plotted *vs.* wavelength

To find the optimum pH value for the coloured reaction a number of samples were prepared.

The investigations were carried out for pH values of the solution of $K_3[Fe(CN)_6]$ in the Britton–Robinson buffer of pH ranging from 2.1 to 4.6. Absorption of the dye solution *vs.* pH in coloured reaction is plotted in Fig. 2.

In order to find the optimal amount of salicylic acid amide to several samples prepared as above were added 0.8 ml of salicylic acid amide solutions in different (1 to 10 mmol l^{-1}) concentrations. Absorbance of the dye solution *vs.* salicylic acid amide concentration is plotted in Fig. 3.

The calibration graph for DMAA determination prepared in the above described manner is a straight line at concentration range from 0.004 to 1 mmol l^{-1}.

The investigation of stability in time of the dye proved the stability up to two hours.

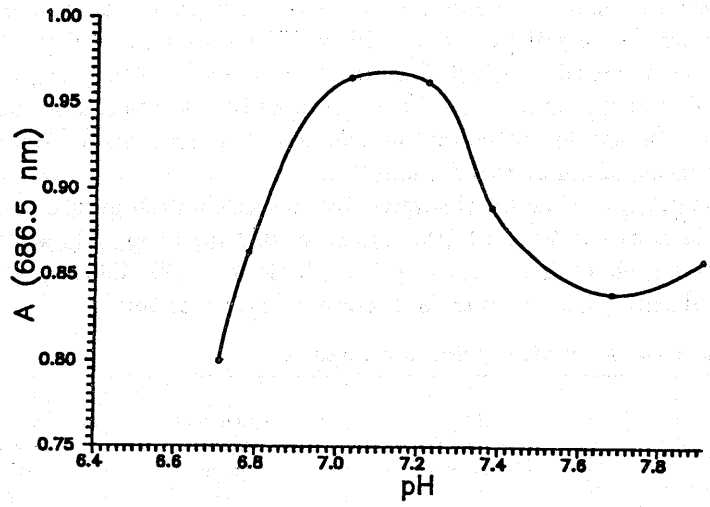

Figure 2. Influence of pH on absorbance of the dye, λ = 686.5 nm

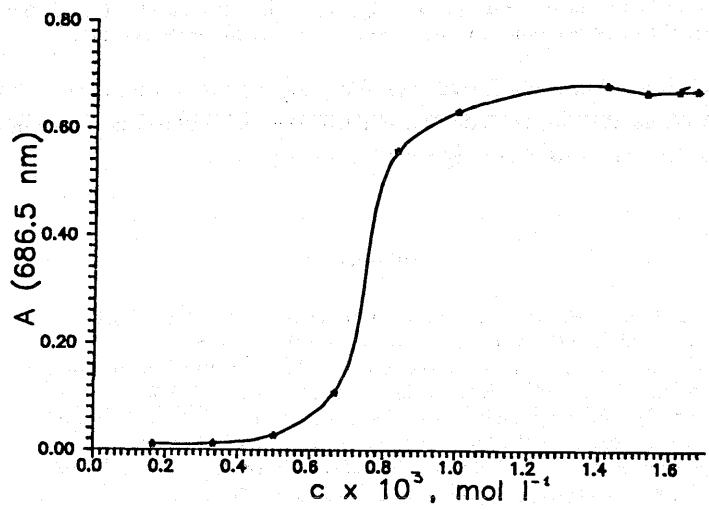

Figure 3. Absorbance of the dye solution plotted *vs.* salicylic acid amide concentration, λ =686.5 nm

RESULTS AND DISCUSSION

In the hydrolysis reaction DAP IV released 4-dimethylaniline from Gly-Pro-DMAA

$$\text{Gly-Pro-DMAA} + H_2O \xrightarrow{\text{DAP IV}} \text{Gly-Pro} + \text{DMAA}$$

The amount of released DMAA is a measure of the enzyme activity. For the purpose of determination of DMAA, we have obtained a coloured quinoimine derivative,

concentration of which can be determined by spectrophotometric method. For our reaction we have used salicylic acid amide as the substrate, and $K_3[Fe(CN)_6]$ as the oxidant. The solution of the product of this reaction was green coloured (λ_{max} = 686.5 nm).

Taking different amounts of salicylic acid amide we found that the reaction is quantitative if the amide concentration in the sample is 1.4 mmol l^{-1} (the corresponding concentration of amide is 8.5 mmol l^{-1}).

The comparison of molar absorptivities, the calibration graphs and the lowest determinable concentrations of DMAA shows that the proposed substrate for coloured reaction is better than 1-hydroxy-2-naphtoic acid [19] (Table 1). The additional advantage of salicylic acid amide is its commercial accessibility.

Table 1. The comparison of DMAA determination methods

Method	Molar absorptivity 1 $mol^{-1}cm^{-1}$	Equation of the calibration graph	Lowest determinable concentration of DMAA mol l^{-1}
A	7.0×10^3	$y = 1.11x + 1.09 \times 10^{-2}$	3×10^{-6}
B	3.7×10^4	$y = 0.56x - 1.25 \times 10^{-2}$	6×10^{-7}

A – the method of DMAA determination using 1-hydroxy-2-naphthoic acid as the substrate [19].
B – the method of DMAA determination using salicylic acid amide as the substrate.

Now we investigate *N*-(2-hydroxyphenyl)succinic acid as the substrate in coloured reaction used in DMAA determination. The first conclusion is that the product obtained in this reaction has a high molar absorptivity.

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