Serum leucine aminopeptidase assay using a dialysis membrane-covered glassy-carbon electrode.

Hideaki Kinoshita Kwassui Women's College, Higashiyamate, Nagasaki850

透析膜を被覆したグラッシーカーボン電極を用いる血清中のロイシンアミノペプチダーゼアッセイ

木　下　英　明

Summary

The activity of leucine aminopeptidase in serum was determined by measuring the increase in current for a given time due to the oxidation of 5-amino salicylic acid as enzymatically released from leucine-3-carboxy-4-hydroxyanilide with a dialysis membrane-covered glassy-carbon electrode. The activities determined amperometrically for eighteen serum samples were in excellent agreement with those determined by colorimetric method using leucine-p-nitroanilide as a substrate (correlation coefficient of 0.998).

Introduction

In clinical analysis, the activity of leucine aminopeptidase (LAP) in serum has been measured by several methods based on colorimetry【1】. Among them a method monitoring p-nitroaniline formed by the enzymatic hydrolysis of leucine-p-nitroanilide has been preferred because of its simplicity and cost efficiency. Unfortunately, however, the method is sensitive to turbidity of a test solution and colored contaminants such as bilirubin in the solution. An electrochemical method is expected to be free from the influence of the turbidity and the existence of colored substances. The activity of enzyme can be easily measured by electrochemical methods when the substrate or product is electrochemically active. The time-dependent increase or decrease in the current due to the oxidation of the electrochemically active product or substrate is a direct measure of the activity of the enzyme tested. Existence of other electrochemically

active substances such as ascorbic acid and uric acid has no influence on the measurements, because the currents due to these substances remain constant during the course of the measurements. There is, however, a disadvantage that the electrode becomes foul with contaminants of proteins by the adsorption, the electrode becoming soon inactive[2,3]. We have shown that the use of a semipermiable membrane-covered electrode is effective to avoid the fouling and that the electrochemical method using this type of electrode can measure the activity of several clinically important enzyme in serum, amylase[4,5], nucleotidase [6,11], cholinesterase[7], alkaline (acid) phosphatase[8], catalase[9]and rGTP[10]. We report here a method of measuring the activity of LAP in human serum by an electrochemical method using a dialysis membrane-covered glassy-carbon electrode with leucine-3-carboxy-4-hydroxyanilide (LCH) as a substrate.

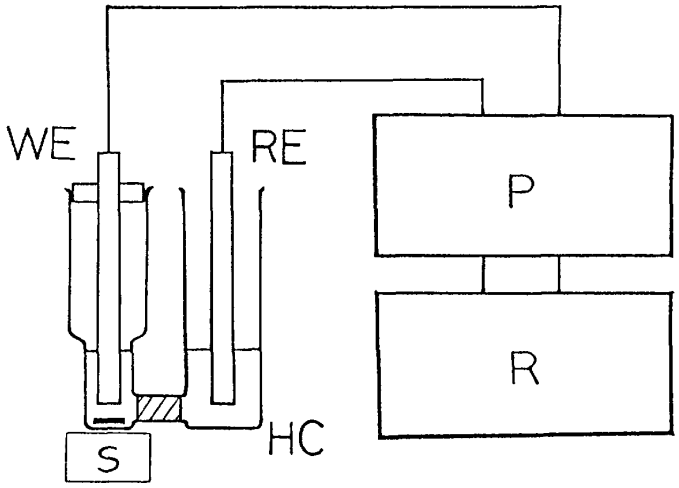
Materials and methods

Electrode and reagents

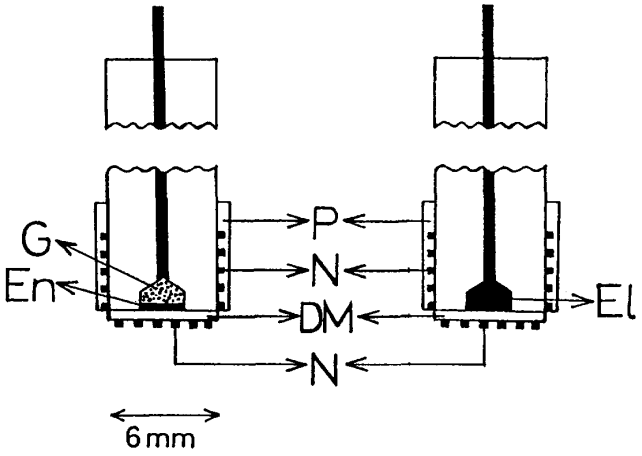
The surface of a glassy-carbon electrode (BAS Inc.; surface area, 0.07cm^2) was covered with a round-cut dialysis membrane (Union Carbide Co.; thickness, 20 μm in the dry state). The membrane was fixed with a nylon net and parafilm. Leucine-3-carboxy-4-hydroxyanilide (LCH) was purchased from Sigma Co. Leucine-p-nitroanilide, 5-aminosalicylic acid (ASA) and other chemicals were purchased from Wako Pure Chemicals Co. Samples of human serum were supplied by Nagasaki University Hospital.

Apparatus and electrochemical measurements

A Yanagimoto P1100 Polarographic analyzer was used for electrochemical measurements in two-electrode mode, and the currents were recorded by using a TOA EPR151A recorder. Tris buffer of pH 7.5 containing 0.1% Triton X-100 was used as a base solution for determining the activity of leucine aminopeptidase (LAP). A 2.0ml volume of the base solution was taken into an H-type cell and the solution was stirred at 600rpm with a magnetic stirrer during the electrochemical measurements. The potential was measured against a saturated calomel electrode. All measurements were done at 25℃. The blockdiagram for this measurement and the structure of electrodes are shown below.



Blockdiagram for Electrochemical Measurement
P: potentiostat
R: recorder (t-Y or X-Y)
WE: working electrode
RE: reference electrode
HC: H-type cell
S: magnetic stirrer



Structures of a membrane-covered membrane-covered electrode (right)
and an enzyme-immobilized graphite paste electrode containing mediator(left)
G: mediator-mixed graphite paste
El: platinum, glassy-carbon, or graphite paste electrode
En: immobilized enzyme layer
DM: semipermeable membrane
N: nylon net
P: parafilm

Results and discussion

The dialysis membrane-covered glassy-carbon electrode was immersed in the basal solution or the solution containing LCH or ASA, and currents were measured after applying given potentials to the electrode. A large anodic current appeared with the solution containing ASA and the anodic current reached a steady state about 3min after applying the potential. On the other hand, only very small current was obtained with the solution containing LCH. Figure 1 shows the potential dependence of the current at a steady state, I , obtained with the basal solution (A), the solution containing 300 μM LCH (B), the solutions containing 100 μM and 200 μM ASA, respectively (C and D). The currents obtained with the ASA solutions started to appear at 0.2V and increased with increasing positive potential to attain limiting currents at 0.3V (Fig.1C and D). The magnitude of the limiting current was proportional to the concentration of ASA in the range between 1 to 500 μM when the correction was made for the base current. The current with the LCH solution started to appear at 0.25 V and increased gradually with increasing positive potential. However, the current magnitude was rather small compared with that obtained with the ASA solution. ASA is the product of the LAP reaction with LCH as a substrate. The big difference in the current magnitude between ASA and LCH assures that the LAP reaction can be followed by recording a current at a fixed potential more positive than 0.3V. The current at 0.4V was measured in the following.

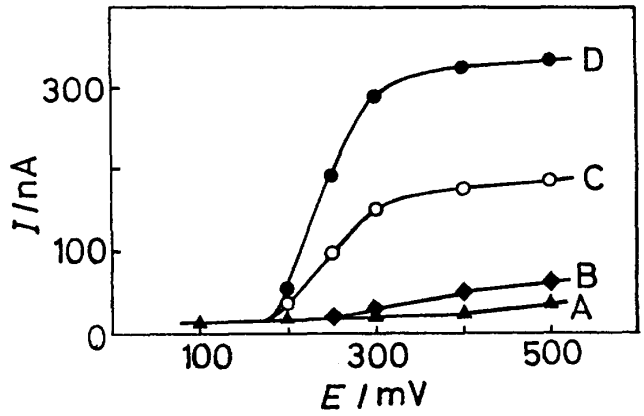


Fig.1 Current at a steady state, I , vs. potential, E, curves for the tris buffer solution containg 0.1% Triton x-100 of pH 7.5(A), and the buffer solutions containing 300 μM leucine-3-carboxy-4-hydroxyanilide (B), 100 μM 5-amino salicylic acid (C) and 200 μM 5-amino salicylic acid (D) at 25 ℃ with a membrane-covered glassy-carbon electrode.

Figure 2 shows the current-time curves obtained with the addition of 300 μM LCH to 2ml of the base solution, then 20, 40, and 80 μl of human serum, respectively (A-C), with the addition of 40 μl of human serum and then 300 μM LCH (D), and with the addition of 40 μl of human serum, 4 μM uric acid, and then 300 μM LCH (E). When 300 μM LCH was first added to the base solution, the current due to LCH increased to reaach a steady-state (A-C). Addition of human serum to this solution resulted in the current increase. A linear increase with time was attained about 4min after the addition of human serum. The slope of the current-time curves in the linearly increasing portions was proportional to the amount of serum added. The results indicate that the current increase is due to the production of ASA by the LAP contained in serum. Thus, the enzyme activity can be measured from the slope of the curves. The same slope was obtained when human serum was added first (B and D in Fig.2). D and E in Fig.2 show that human serum itself also produces small steady-state current as uric acid does. This indicates that the oxidizable compounds are contained in the human serum. The presence of the oxidizable compounds has no influence on the measurement of LAP activity. This is confirmed by the

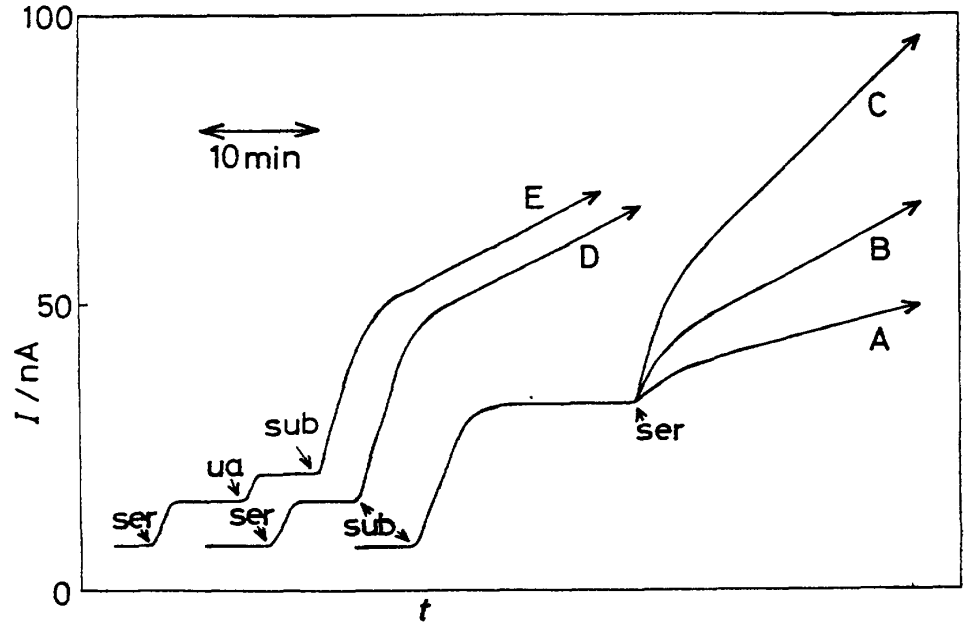


Fig.2 Anodic current at 0.4V vs. time curves for the tris buffer solutions of pH 7.5, in which 300 μM leucine-3-carboxy-4-hydroxyanilide, serum and 4 μM uric acid were added at the points indicated by "sub", "ser" and "ua" at 25℃. Sera were diluted to give concentrations of 1/80(A), 1/40(B, D and E) and 1/20(C) of the serum samples.

observation that the same slope of the current-time curve in the linearly increasing portions was obtained with the test solution (D) and with the solution in which uric acid was added (E). The slope was also independent of the presence of ascorbic acid in the test solution.

Activity of LAP in human serum samples was measured from the slope of the current-time curvess; a 50 μl portion of human serum was added to 2ml of the base solution containing 300 μM LCH, then the linearly increasing current was measured as the difference in the current at 10min and at 20min after the addition of human serum, ΔI. The relative standard deviation (RSD) of this difference in current was 3.2% for six runs. Figure 3 presents a comparison of the results of monitoring of LAP activity obtained by the present method and by a colorimetric method using leucine-p-nitroanilide as a substrate for eighteen samples. The results obtained with two methods are in excellent agreement with a correlation coefficient of 0.998. A dialysis membrane-covered glassy-carbon electrode could be used repeatedly if the electrode was rinsed in water for 30s after each measurement. The RSD for the measurement of ASA taken dayly over one week period was 3.6%.

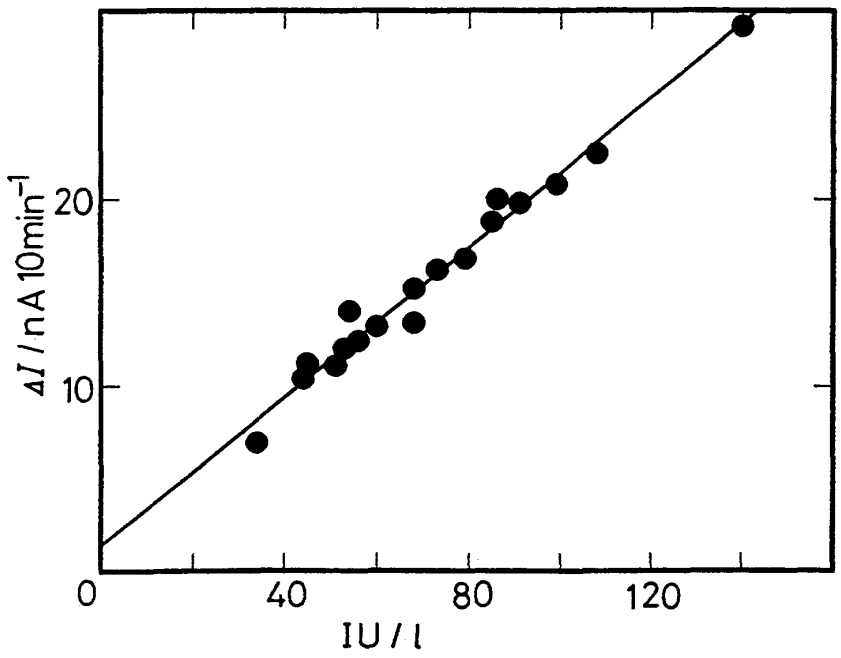


Fig.3 Comparison of the activities of leucine aminopeptidase in serum samples determined by the present method and by a colorimetric method using leucine-p-nitroanilide as a substrate.

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