ANALYTICAL SCIENCE & TECHNOLOGY
(Journal of the Korean Society of Analytical Sciences)
Vol. 8, No. 3, 1995
Printed in the Republic of Korea

Two-enzyme coupled fluorometric assay of urinary dipeptidase

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(Received Apr. 27, 1995)

이원효소 연쇄반응의 형광분석에 의한 Urinary Dipeptidase의 활성도 측정

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(1995. 4. 27. 접수)

Abstract : Urinary dipeptidase(Udpase) was assayed by fluorometric analysis of NADH which was produced from an indicator enzyme, L-alanine dehydrogenase. The reaction mixture was consisted of a dipeptide(L-ala-L-ala), β-NAD^+, L-alanine dehydrogenase in 12.5 mM sodium carbonate buffer, pH 9.0, and urinary dipeptidase which initiated the reaction. The fluorescence intensity of NADH was measured as a function of time with the excitation wavelength at 340nm and emission at 460nm. Comparison of this fluorometric method with the conventional spectrophotometric method utilizing glycyldehydrophenylalanine(Gdp) as substrate provided the correlation coefficient of 0.996 and increased the sensitivity more than ten times.

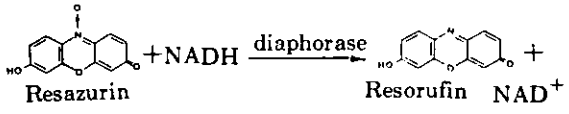
요약 : Urinary dipeptidase와 alanine dehydrogenase의 연쇄반응을 이용한 형광분석법을 개발하였다. 반응계는 기질로서 L-ala-ala, β-NAD^+, L-alanine dehydrogenase와 pH 9의 12.5mM sodium carbonate buffer를 포함하며 urinary dipeptidase를 가함으로써 반응을 시작했다. 생성된 NADH는 여기파장 340nm, 형광파장 460nm에서 측정했다. 기존의 glycyldehydrophenylalanine (Gdp)의 가수분해 방법과 형광분석법을 비교한 결과 0.996의 높은 상관계수를 나타냈으며 10배 이상의 감도 증가를 보였다.

Key words : urinary dipeptidase(Udpase), fluorometric assay, L-alanine dehydrogenase, glycyldehydrophenylalanine(Gdp)

1. INTRODUCTION

Urinary dipeptidase was identified as a Gdp-hydrolyzing entity from urine of healthy individuals.[1] The tissue origin of Udpase was suggested as kidney and the Udpase may be the released form of renal dipeptidase(EC 3.4.13.11 ; RDPase) which is relatively well characterized integral membrane protein localized on the microvilli of renal proximal tubules.[2~6] Udpase and RDPase were measured spectrophotometrically by the hydrolysis of synthetic dipeptide, Gdp. The sepctrophotometric assay was simple and convenient but its sensitivity was low. Udpase in urine sample could not be detected with Gdp without prior purification or concentration. Ito *et al.*[7] introduced three-enzyme coup-

led fluorometric assay utilizing L-ala-L-ala as a substrate of Udpase which was coupled to L-alanine dehydrogenase and diaphorase. The final reaction catalyzed by diaphorase is expressed as following chemical structures.



The current studies were undertaken to develop two-enzyme coupled fluorometric assay system for Udpase which is more sensitive than the spectrophotometric method.

2. MATERIALS AND METHODS

Udpase was purified from pooled urine of healthy volunteers, as reported previously.[1] Gdp was synthesized by the method of Campbell *et al.*[8] L-ala-L-ala, β-NAD^+, NADH, L-alanine dehydrogenase were purchased from Sigma Chemical Co., St. Louis, U.S.A. A double-beam spectrophotometer (Uvikon 930, Kontron, Switzerland) was used for measurement of Gdp hydrolysis. FP-777 fluorometer(Jasco Ltd., Japan) was used for the measurement of NADH production.

Spectrophotometric assay

Udpase was assayed spectrophotometrically by the rate of Gdp hydrolysis. The enzyme assay in total volume of 2.5 ml at 37℃ with 5×10^{-5}M Gdp was carried out as function of time at 275 nm according to the method of Campbell *et al.*[8]

Fluorometric assay

In this two-enzyme coupled fluorometric assay, Udpase was coupled to L-alanine dehydrogenase to produce fluorescent NADH. Total volume of 2.75 ml reaction mixture was consisted of 1.5 μmol L-ala-L-ala, 0.2 mg of NAD^+, 0.8 unit of L-alanine dehydrogenase, 12.5 mM sodium carbonate buffer, pH 9.0, and aliquot of Udpase. The increase of fluorescence due to NADH production was determined as a function of time for 3 min at 25℃. The excitation wavelength was 340 nm and emission wavelength was 460 nm. The amount of product was calculated from a standard curve of NADH solution. The protein concentration was determined by the method of Bradford.[9] The enzyme activity was expressed as μmol NADH produced/min or specific activity as μmol NADH produced/min/mg protein.

Determination of optimal pH

Effect of pH on the fluorometric assay of Udpase was tested using purified Udpase at final concentration of 4.5 ng/ml. pH ranging from 6 to 11 were obtained with 12.5 mM sodium carbonate buffer which has pK values of 6.4 and 10.0.

Effect of buffer concentration

Effect of buffer concentration on the fluorometric assay of Udpase was tested using various concentration(6.25, 12.5, 25, 50 mM) of sodium carbonate buffer, pH 9.0. The final concentration of purified Udpase in the assay mixture was 4.5 ng/ml.

Effect of temperature on the fluorescence intensity of NADH

The 12.5 mM sodium carbonate buffer, pH 9.0 was preincubated at the indicated temperature. The reaction chamber in the fluorometer was equilibrated with circulating water system. Twenty μl of NADH stock solution(100 μg/ml) was added to 2.73 ml of above buffer solution to give final concentration of 0.73 μg/ml. The fluorescence of NADH at indicated temperature was recorded for 3 min.

3. RESULTS AND DISCUSSION

Various enzymes have been coupled to NAD^+ dependent dehydrogenase to measure NADH production at 340 nm spectrophotometrically. Following coupled assay is one of them.

$$\text{L-ala-L-ala} \xrightarrow{\text{urinary dipeptidase}} 2\text{L-ala}$$

$$\text{L-ala}+NAD^+ + H_2O \xrightarrow{\text{L-ala dehydrogenase}} \text{pyruvate}+NADH+NH_4^+$$

However, produced NADH was not measured spectrophotometrically. NADH is excited at 340 nm and emitted around 460 nm while its oxidized form, NAD^+, is not fluorescent significantly under the same condition. The spectrofluorometric assay is much more sensitive than spectrophotometric method, thereby, reproducible results may not be obtained. Slight difference in ionic strength, pH of the solution, and assay temperature may result in large difference in the fluorescence intensity.[10] To obtain reproducible results, these factors should be fixed in a narrow range when a new primary enzyme is coupled to this NAD^+ dehydrogenase system.

3. 1. Determination of optimal pH

The pH profile of Udpase as shown in *Fig.* 1 demonstrated optimal pH close to 9. Subsequent reactions were carried out at pH 9.

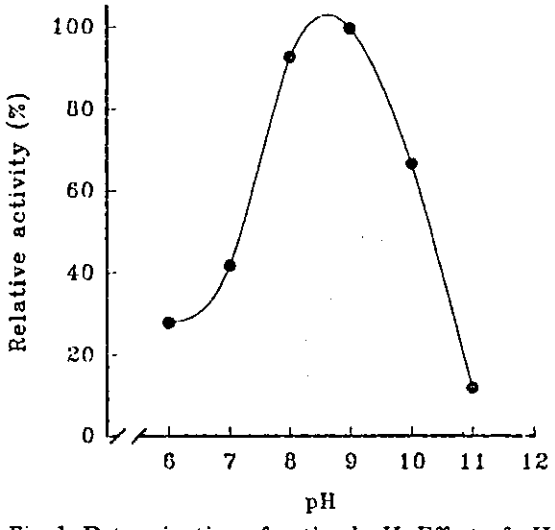


Fig. 1. Determination of optimal pH. Effect of pH on the fluorometric assay of Udpase activity was tested using Udpase at final concentration of 4.5ng/ml. Y-axis is expressed as relative activity (%) by taking the activity at pH 9 as 100%.

3. 2. Effect of buffer concentration

Udpase was assayed at different concentrations of sodium carbonate buffer, pH 9.0, as shown in *Fig.* 2. The fluorescence intensity was increased with the decreasing buffer concentration up to 12.5 mM but decreased at 6.5 mM. Based on these results, subsequent assays were carried out with 12.5 mM sodium carbonate buffer, pH 9.0.

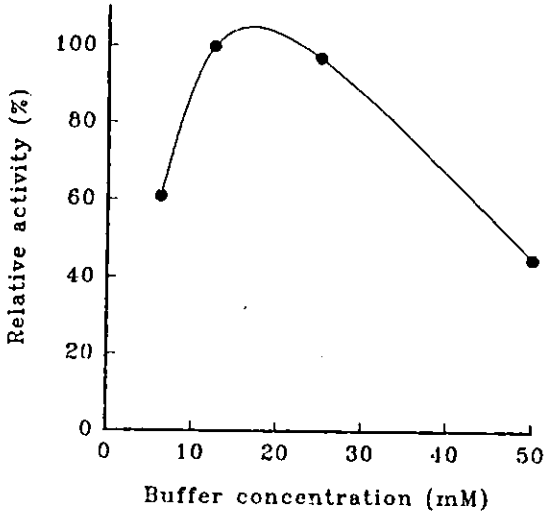


Fig. 2. Effect of buffer concentration. Effect of buffer concentration on the fluorometric assay of Udpase was tested using 4.5 ng/ml of Udpase at various concentrations(6.25, 12.5, 25, 50 mM) of sodium carbonate buffer, pH 9.0. Y-axis is expressed as relative activity (%) by taking the highest activity(12.5 mM) as 100%.

3. 3. Effect of temperature on the fluorescence intensity of NADH

The effect of temperature on the fluorescence intensity of NADH was examined in *Fig.* 3a and 3b. Generally, enzyme activity is increased with increasing temperature until denaturation point of enzyme protein but it may not apply to this system because NADH is not stable above 25℃. The stability of standard NADH expressed as slope was different with increasing temperature as shown in *Fig.* 3a. The slope was increasing below 25℃ but decreasing above 25℃. In *Fig.* 3b, the slope of

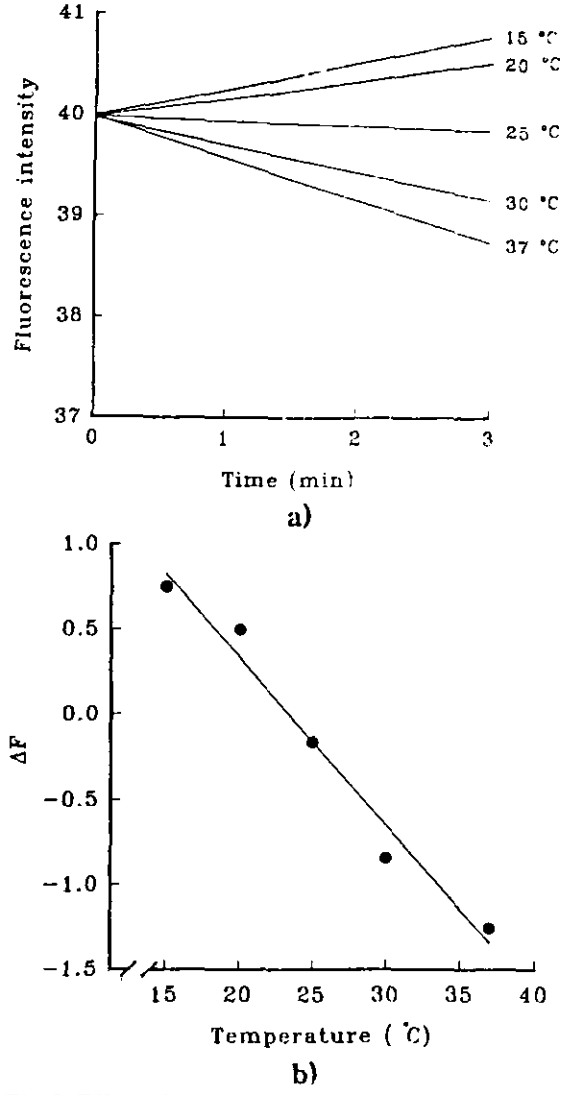


Fig. 3. Effect of temperature on the fluorescence intensity of NADH. The fluorescence of NADH at different temperature was measured as described nder "MATERIALS AND METHODS". a) The fluorescence of each standard solution at different temperature was recorded for 3 min. b) The slope(△F for 3 min) of standard NADH was plotted at different temperature.

NADH with increasing temperature demonstrated reciprocal relationship. The most stable temperature of NADH was 23℃. Generally enzyme assays are performed at 30 or 37℃, but this experiment clearly demonstrated the importance of carrying out the reaction between 20~25℃ when NADH is involved.

3. 4. Effect of Udpase amount on NADH production

In *Fig.* 4, The relationship between enzyme concentration and enzyme activity exhibited linear relationship over the range of enzyme concentration employed with the correlation coefficient(r) of 0.955. The controls obtained without Udpase exhibited insignificant amount of NADH produced under the assay condition. The average specific activity calculated from the slope was 7.38 μmol NADH produced/min/mg protein.

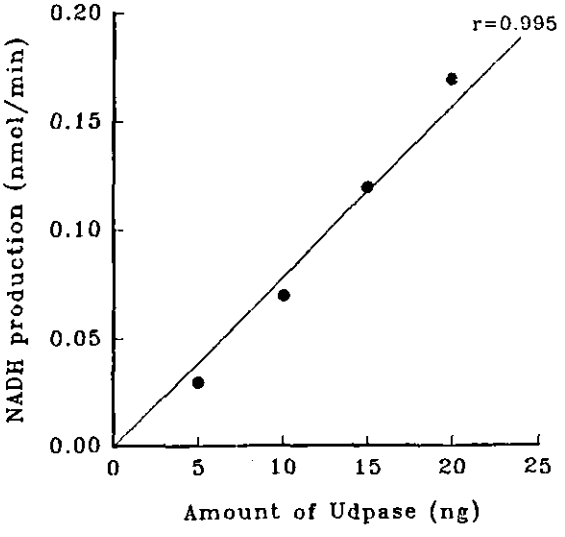


Fig. 4. Effect of Udpase amount on NADH production. The fluorescence intensity of NADH was measured with increasing amount of Udpase. Production of NADH was calculated from NADH standard curve. Correlation coefficient(r) was found to be 0.995.

3. 5. *Correlation coefficient*

Sensitivity and validity of this two-enzyme fluorometric assay of Udpase were evaluated by comparing it with the conventional spectrophotometric assay as shown in *Fig.* 5. The details of assay and calculation were reported in previous paper.[5] The amount of NADH production(fluorometric met-

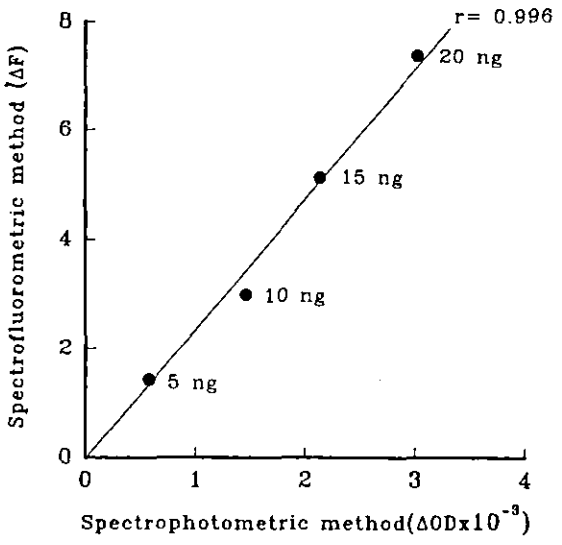


Fig. 5. Correlation Coefficient. Increasing amounts of Udpase were used for both methods. In spectrophotometric assay, 5×10^{-5}M Gdp was used as dipeptide substrate. △F and △OD are slopes for 3 min for spectrofluorometric and spectrophotometric method, respectively. Correlation coefficient (r) was found to be 0.996.

hod) agreed well with the amount of Gdp hydrolyzed(spectrophotometric method) with the correlation coefficient of 0.996. When same amount of enzyme were used for both methods, readings of spectrophotometric method were reduced by over three orders of magnitude. However, the actual sensitivity increase may be ten times or more. Udpase of 5 ng could be assayed by spectrofluorometric method but more than 50 ng is recommended by spectrophotometric method.

In the three-enzyme coupled system of Ito *et al*,[7] L-ala produced by Udpase was converted to pyruvate and ammonia by L-alanine dehydrogenase which reduce NAD^+ to NADH at the same time. The third enzyme, diaphorase oxidized NADH back to NAD^+ with concomittant production of fluorescent resorufin. The reaction was carried out for 30 min at 37℃ and stopped with NaOH solution. The sensitivity of two-enzyme coupled assay and three-enzyme coupled assay were similar although direct comparison between the two methods were not possible.

In this two-enzyme coupled method, the fluorescence intensity of NADH was measured from a continuous slope, the initial velocity of first 3 min. It can offer several advantages over the method which measures the difference of fluorescence between the sample and control after 30 min of reaction time. First, the initial velocity of enzyme catalyzed reaction is measured while the slope is linear which is essential for accurate measurement of enzyme activity. The linearity of each assay is checked while measuring the slope of the reaction. Second, it is not necessary to carry out controls to subtract them from the sample values to obtain the difference. Third, it is obvious that the initial velocity measurement is time saving compared with the longer incubation time to see the significant dif ference between sample and control. Fourth, a nonspecific increase of fluorescence could be avoided after NaOH addition. NaOH solution could not stop the reaction completely(data is not presented). Finally, the two-enzyme coupled system was less expensive than the three-enzyme coupled system because the most expensive diaphorase and its susbstrate(resazurin) were omitted.

ACKNOWLEDGMENTS

This study was supported by Chonnam National University Research Fund, 1993.

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