HRP amplified system. The results showed that the detection limits of using amplified system were 4-40 folds increase and new enzyme amplified system (amplified system 2) gave comparable sensitivity and lower background with amplified system 1(Tab 1).

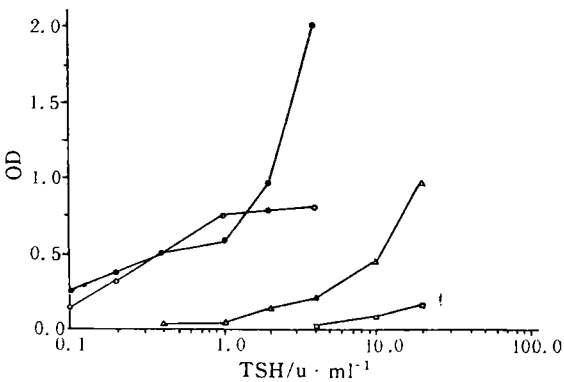


Fig 2. The signal amplification of ELISA for TSH
syst. 1(○): strep-AP coupled with alcohol dehydrogenase-diaphorase; syst. 2(●): strep-AP coupled with lactic dehydrogenase-lactate oxidase-HRP; AP(□) and HRP(△): normal EIA

Tab 1. Comparison of new amplified system(syst. 2) with other methods by using the conditions described in the methods

TSH, u/ml	AP	HRP	syst. 1	syst. 2
0	0.097	0.106	0.501	0.185
0.1	—	—	0.634	0.429
0.2	—	—	0.814	0.549
0.4	0.097	0.136	0.997	0.688
1	0.098	0.152	1.260	0.763
2	0.096	0.245	1.289	1.141
4	0.117	0.311	1.306	2.187
10	0.170	0.553		
20	0.255	0.970		

4 Discussion

We have described an enzyme amplification method and its application in TSH assay. In this method, *L*-actic dehydrogenase, lactate oxidase and HRP were used to form a cycling system to amplify the signal produced by alkaline phosphatase. The fact that the amplified signal was affected by excess NADH suggested that an additional coupled enzyme cycle to supply NADH might instantly improve the method further. Considering different optimal pHs of enzymes (lactate oxidase pH6. *L*- lactic dehydrogenase pH7.5; HRP pH6.0) and stable pH(pH3-5) of colour product, we tried two steps amplification. That was, NADH, *L*-lactic dehydrogenase and lactate oxidase were first added in pH6.5 followed by 10-20 min incubation at RT. Then, HRP and TMB were added in pH3.8. These were enable to cut enzymes down remarkably (data not showed). Three different HRP substrates[9] were compared (data not showed) and TMB gave the lowest background.

References

1 Self CH. Emzyme amplification-A general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J Immunol Methods*, 1985, **76**: 389
2 Self CH. Immunoassay with an increasing cyclic detection system. *US Patent*, no. 4,598,042, 1986
3 Bobrow MN, Harris TD, Shaughnessy KJ, *et al*. Catalyzed reporter deposition, a novel method of signal amplification. *J Immunol Methods*, 1989, **125**: 279
4 Harbron S, Eggelte HJ, Rabin BR. Amplified colorimetric assay of alkaline phosphatase using riboflavin 4′-phosphate: a simple method for measuring riboflavin and riblflavin 5′-phosphate. *Analyt Biochem*, 1991, **198**: 47
5 Wilson MR, Easterbrook-Smith SB. Emzyme complex amplification-a signal amplification mehtod for use in enzyme immunoassay. *Analyt Biochem*, 1993, **209**: 183
6 Bobrow MN, Litt GJ, Shaughnessy KJ, *et al*. The use of catalyzed reporter deposition as a means of signal amplification in variety of formats. *J Immunol Methods*, 1992, **150**: 145
7 Conyers SM, Kidwell DA. Chromogenic substrates for horseradish peroxidase. *Analyt Biochem*, 1991, **192**: 207
8 Houtz P, Tai HH. Enzyme immunoassay of thromboxane B_2 using immobilized hapten and enzyme amplification. *Adv Prostaglandin Thromboxane Res*, 1989, **19**: 678
9 Stanley CJ, Paris F, Plumb A, *et al*. Enzyme amplification. *ABL*, 1985, May/June: 48

一种新酶放大系统及其应用

王 旻 戴信雄[1]

(中国药科大学生物化学教研室,南京210009; [1]美国肯塔基大学药学院)

摘 要 建立了一种能广泛用于酶标免疫吸附分析的新酶放大系统。该系统采用 *L*-乳酸脱氢酶、乳酸氧化酶、辣根过氧化物酶、NADH 和3,3′,5,5′-四甲基联苯胺组成一个循环体系,用以放大碱性磷酸酶产生的信号。与其它酶放大系统相比,具有灵敏度高和成本低的特点。

关键词 酶放大; 酶免疫分析; ELISA

A New Enzyme Amplification System and Its Application

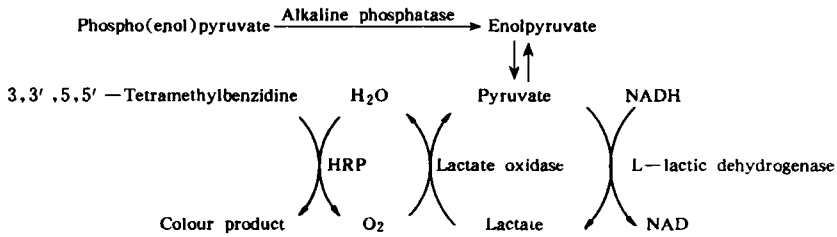
Wang Min, Tai Hsin-Hsiung[1]

Department of Biochemistry, China Pharmaceutical University, Nanjing 210009; [1]College of Pharmacy, University of Kentucky, Lexington, KY 40536-082 USA

Abstract A new enzyme amplification system which can be widely used in ELISA was developed. In this system, a complex of *L*-lactic dehydrogenase, lactate oxidase, horseradish peroxidase, NADH and 3,3',5,5'-tetramethylbenzidine were used to compose a cycling system to increase the signal for the assay of alkaline phosphatase. Comparison with other methods showed that it gave high sensitivity and low background.

Key words enzyme amplification; enzyme immunoassay; ELISA

Enzyme immunoassay is now widely used to detect antigens in biological fluids. But biological fluids are so complex that sometimes, it is impossible to detect some antigens directly without appropriate dilution. Many efforts had been made to develop more sensitive methods[1-6]. We reported in this paper a new enzyme amplification method in which alkaline phosphatase(AP) was applied to catalyze phospho(enol) pyruvate (PEP) to form pyruvate. Pyruvate initiated *L*-lactic dehydrogenase(LDH) reaction to produce lactate which was oxidized by lactate oxidase into pyruvate again and hydrogen peroxide. Then hydrogen peroxide reacted with 3, 3', 5, 5'-tetramethylbenzidine(TMB) by horseradish peroxidase(HRP) to form colour product(Scheme 1).



Scheme 1. Principle of enzyme cycling amplified system

1 Materials

Phospho(enol) pyruvate, NADP, NADH, diethanolamine, *p*-iodonitrotetrazolium violet (INT), diaphorase, *L*-actic dehydrogenase, lactate oxidase, pyruvic acid, *p*-nitrophenyl phosphate, bovine serum albumin (BSA), Trizma base(Tris), and Tween 20 were purchased from Sigma. Yeast alcohol dehydrogenase was purchased from Boehringer. Horseradish peroxidase, alkaline phosphatase, streptavidin-alkaline phosphatase conjugated (strep-AP), streptavidin-horseradish peroxidase (strep-HRP) were purchased from ZYMED. Thyroid stimulating hormone(TSH), anti-TSH monoclone IgG-biotin conjugated and anti-TSH rabbit IgG were obtained from Dr. H. H. Tai's Lab., University of Kentucky, USA. Microplate was purchased from Costar.

2 Methods

2.1 *Optimal NADH Concentration*

To 50 μl of 5 μmol/L pyruvic acid in 50 mmol/L Tris-HCl buffer containing 1 mmol/L $MgCl_2$ pH9.8, added 30 μl of 250 mmol/L phosphate buffer to adjust pH to 5.8-6.0 and added lactate oxidase, *L*-lactic dehydrogenase, HRP, TMB and NADH to a final volume of 160 μl (containing 1.25 u/ml lactate oxidase, 6.25 u/ml *L*-lactic dehydrogenase, 37.5 μg/ml HRP, 0.5 mmol/L TMB and 0.078-2.5 mmol/L NADH), followed by incubation for 10-30 min at RT. The absorbance at 650 nm recorded.

2.2 *Optimal pH*

To 50 μl of 2 μmol/L pyruvic acid in 50 mmol/L Tris-HCl buffer containing 1 mmol/L

Received May 5, 1994

$MgCl_2$ pH9.8, added 80 μl of amplification mixture and 30 μl of 250 mmol/L phosphate buffer to adjust pH to 5.8-7.3. The final reaction system contained 1.25 u/ml lactate oxidase, 6.25 u/ml *L*-lactic dehydrogenase, 37.5 μg/ml HRP, 0.5 mmol/L TMB and 0.35 mmol/L NADH. After 30 min and 60 min incubation, read the absorbance at 650 nm.

2.3 *Application in TSH Assay and Comparison with Other Methods*

Microplates were coated with appropriately diluted anti-TSH rabbit IgG in 0.1 mol/L carbonate buffer pH9.6, overnight at 4℃ and blocked with 0.1%BSA, 10 mmol/L phosphate buffer saline, 0.05% Tween 20 pH7.4 (EIA buffer) for 2 h at RT. The wells were washed with 10 mmol/L phosphate buffer, 0.05% Tween 20 pH7.4 (wash buffer) 3 times. TSH standard(0.1-20 u/ml) and TSH monoclone IgG-biotin conjugated both in EIA buffer were added to each well. After 1 h incubation, added strep-HRP or strep-AP and allowed to incubate for 30 min at RT. Then washed 3 times with washing buffer for following uses.

In cases of direct determination, added the substrates for HRP (TMB and hydrogen peroxidase) or for AP (17 mmol/L *p*-nitrophenol phosphate and 5 mmol/L $MgCl_2$ in 50 mmol/L diethanolamine buffer pH9.8) and read absorbance at 650 nm or 405 nm.

For strep-AP coupled with alcohol dehydrogenase-diaphorase amplified system (amplified system 1)[2,7,8], 0.2 mmol/L NADP containing 1 mmol/L $MgCl_2$ in 50 mmol/L diethanolamine buffer pH9.8 was used as the substrate of AP. After 3 h incubation at RT, an amplified mixture was added (containing 0.55 mmol/L INT, 0.4 mg/ml alcohol dehydrogenase, 0.4 mg/ml diaphorase and 4% alcohol in 25 mmol/L phosphate buffer pH7.2). Absorbance at 490 nm was measured after 15 min to 1 h incubation.

For strep-AP coupled with lactic dehydrogenase-lactate oxidase-HRP amplified system (amplified system 2), 5 mmol/L PEP containing 1 mmol/L $MgCl_2$ in 50 mmol/L Tris-HCl buffer pH9.8 was supplied as the substrate of AP and incubated for 3 h. Put in 30 μl of 250 mmol/L phosphate buffer to adjust pH to 5.8-6.0 and added 80 μl of amplified mixture. Final reaction system contained 1.25 u/ml lactate oxidase, 6.25 u/ml lactic dehydrogenase, 37.5 μg/ml HRP, 0.5 mmol/L TMB and 0.35 mmol/L NADH. After 15 to 30 min incubation at RT, was added 20 μl of 2 mol/L H_2SO_4 to stop reaction and recorded the absorbance at 450 nm.

3 Results

3.1 *Optimal Conditions for Amplified System*

Amplified signals were markedly influenced by the concentration of NADH and pH. The optimal NADH concentration was about 0.325 mmol/L (Fig 1-a). The effect of pH was more complicated because of different optimal pHs of three enzymes involved in cycling system. Strong signal was given in pH6.5, while the reaction colour product was more stable in acid surrounding (Fig 1-b). It was suggested that it was better used the pH between 5.8-6.0.

3.2 *Application and Comparison*

Fig 2 showed a comparison of the results generated in TSH assay by using strep-AP, strep-HRP, strep-AP coupled with alcohol dehydrogenase-diaphorase amplified system and strepp coupled with lactic dehydrogenase-lactate oxidase-

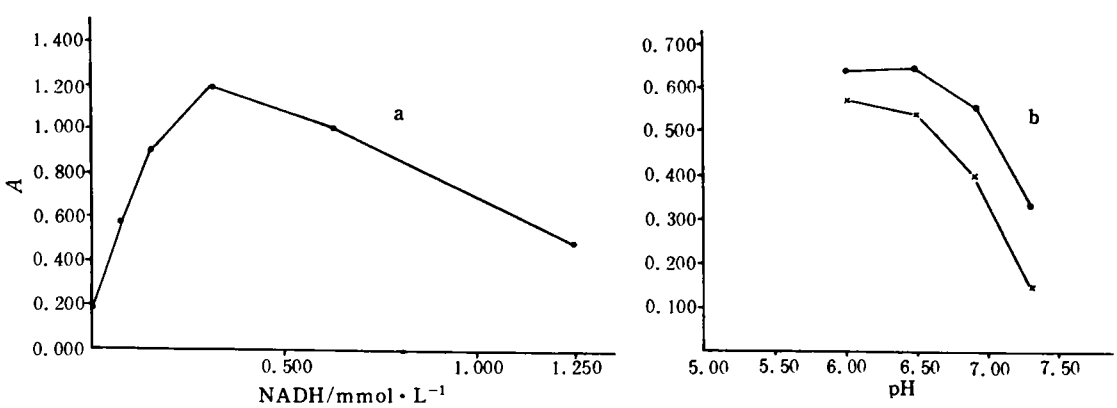


Fig 1. Effect of NADH(a) and pH(b) by using the conditions described in the methods
Fig 1-b; —●—after 30 min incubation, —×—after 1 h incubation

HRP amplified system. The results showed that the detection limits of using amplified system were 4-40 folds increase and new enzyme amplified system (amplified system 2) gave comparable sensitivity and lower background with amplified system 1(Tab 1).

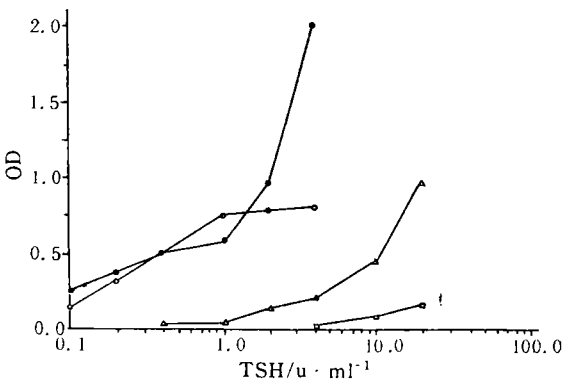


Fig 2. The signal amplification of ELISA for TSH
syst. 1(○): strep-AP coupled with alcohol dehydrogenase-diaphorase; syst. 2(●): strep-AP coupled with lactic dehydrogenase-lactate oxidase-HRP; AP(□) and HRP(△): normal EIA

Tab 1. Comparison of new amplified system(syst. 2) with other methods by using the conditions described in the methods

TSH, u/ml	AP	HRP	syst. 1	syst. 2
0	0.097	0.106	0.501	0.185
0.1	—	—	0.634	0.429
0.2	—	—	0.814	0.549
0.4	0.097	0.136	0.997	0.688
1	0.098	0.152	1.260	0.763
2	0.096	0.245	1.289	1.141
4	0.117	0.311	1.306	2.187
10	0.170	0.553		
20	0.255	0.970		

4 Discussion

We have described an enzyme amplification method and its application in TSH assay. In this method, *L*-actic dehydrogenase, lactate oxidase and HRP were used to form a cycling system to amplify the signal produced by alkaline phosphatase. The fact that the amplified signal was affected by excess NADH suggested that an additional coupled enzyme cycle to supply NADH might instantly improve the method further. Considering different optimal pHs of enzymes (lactate oxidase pH6. *L*- lactic dehydrogenase pH7.5; HRP pH6.0) and stable pH(pH3-5) of colour product, we tried two steps amplification. That was, NADH, *L*-lactic dehydrogenase and lactate oxidase were first added in pH6.5 followed by 10-20 min incubation at RT. Then, HRP and TMB were added in pH3.8. These were enable to cut enzymes down remarkably (data not showed). Three different HRP substrates[9] were compared (data not showed) and TMB gave the lowest background.

References

1 Self CH. Emzyme amplification-A general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J Immunol Methods*, 1985, **76**: 389
2 Self CH. Immunoassay with an increasing cyclic detection system. *US Patent*, no. 4,598,042, 1986
3 Bobrow MN, Harris TD, Shaughnessy KJ, *et al*. Catalyzed reporter deposition, a novel method of signal amplification. *J Immunol Methods*, 1989, **125**: 279
4 Harbron S, Eggelte HJ, Rabin BR. Amplified colorimetric assay of alkaline phosphatase using riboflavin 4′-phosphate: a simple method for measuring riboflavin and riblflavin 5′-phosphate. *Analyt Biochem*, 1991, **198**: 47
5 Wilson MR, Easterbrook-Smith SB. Emzyme complex amplification-a signal amplification mehtod for use in enzyme immunoassay. *Analyt Biochem*, 1993, **209**: 183
6 Bobrow MN, Litt GJ, Shaughnessy KJ, *et al*. The use of catalyzed reporter deposition as a means of signal amplification in variety of formats. *J Immunol Methods*, 1992, **150**: 145
7 Conyers SM, Kidwell DA. Chromogenic substrates for horseradish peroxidase. *Analyt Biochem*, 1991, **192**: 207
8 Houtz P, Tai HH. Enzyme immunoassay of thromboxane B_2 using immobilized hapten and enzyme amplification. *Adv Prostaglandin Thromboxane Res*, 1989, **19**: 678
9 Stanley CJ, Paris F, Plumb A, *et al*. Enzyme amplification. *ABL*, 1985, May/June: 48

一种新酶放大系统及其应用

王 旻 戴信雄[1]

(中国药科大学生物化学教研室,南京210009; [1]美国肯塔基大学药学院)

摘 要 建立了一种能广泛用于酶标免疫吸附分析的新酶放大系统。该系统采用 *L*-乳酸脱氢酶、乳酸氧化酶、辣根过氧化物酶、NADH 和3,3′,5,5′-四甲基联苯胺组成一个循环体系,用以放大碱性磷酸酶产生的信号。与其它酶放大系统相比,具有灵敏度高和成本低的特点。

关键词 酶放大; 酶免疫分析; ELISA