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Chromogenic substrate from 4-nitro-1-naphthol for hydrolytic enzyme of neutral or slightly acidic optimum pH: 4-Nitro-1-naphthyl-β-D-galactopyranoside as an example

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ABSTRACT

At pH from 5.5 to 7.6, absorptivity of 4-nitro-1-naphthol at 450 nm is over 2.1-fold of that of *para*-nitrophenol at 405 nm and over 9.6-fold of that of *ortho*-nitrophenol at 415 nm. On 4-nitro-1-naphthyl-β-D-galactopyranoside at pH 7.4, catalytic efficiency of *Escherichia coli* β-D-galactosidase is 3-fold of that on *para*-nitrophenyl-β-D-galactopyranoside and about 40% of that on *ortho*-nitrophenyl-β-D-galactopyranoside, and produces a lower quantification limit of penicillin G by enzyme-linked-immunoabsorbent-assay. Hence, 4-nitro-1-naphthol is favorable to prepare chromogenic substrates of hydrolytic enzymes of neutral or slightly acidic optimum pH.

Hydrolytic enzymes like phosphatases and glycosidases play important roles in bio-recognition and signal transduction, and also are important tools for bio-processing and bio-analyses including enzyme-linked-immunoabsorbent-assay (ELISA).[1–9] The sensitive quantification of the activities of those hydrolytic enzymes is thus a pivotal work and the estimation of their activities from reaction curves is always favored. At pH over 8.0, *para*-nitrophenol (*p*NP) and *ortho*-nitrophenol (*o*NP) are ionized into anions possessing strong absorbance peaks at wavelengths over 400 nm where the unionized *p*NP and *o*NP have negligible absorbance. For hydrolytic enzymes, thus, chromogenic substrates from *p*NP and *o*NP are widely used to record their reaction curves by absorbance. In practice, the sensitivity to quantify the activity of a hydrolytic enzyme is proportional to the specific activity of the enzyme on a chromogenic substrate and the absorptivity of the chromogenic product. Some hydrolytic enzymes show their maximum activities at neutral or even slightly acidic pH values which greatly reduce the ionization of *p*NP and *o*NP into anions and thus their apparent absorptivity. Therefore, new chromogens are still needed to prepare chromogenic substrates of hydrolytic enzymes exhibiting the maximum activities at neutral or slightly acidic pH.

For preparing chromogenic substrates of hydrolytic enzymes with maximum activities at neutral or slightly acidic pH, 4-nitro-1-naphthol (4NNP) is a better chromogen. After ionization, the absorbance peaks of *p*NP, *o*NP and 4NNP center on 405, 415, and about 458 nm, respectively. The apparent absorptivity of 4NNP at 460 nm is 1.03–1.07 times of that at 450 nm from pH 5.5 to 9.0. At pH from 7.6 to 9.0, the apparent absorptivity of 4NNP at 450 nm is 1.7–2.1 times of that of *p*NP at 405 nm while 7.9–9.6 times of that of *o*NP at 415 nm (Fig. 1). At pH from 5.5 to 7.6, the apparent absorptivity of those three chromogens becomes smaller, but relative differences of their apparent absorptivity grow larger. Predominantly, at pH from 5.5 to 6.6, *p*NP has the apparent absorptivity below 2.7 $(\text{mM cm})^{-1}$ at 405 nm and *o*NP has the apparent absorptivity below 0.70 $(\text{mM cm})^{-1}$ at 415 nm, but 4NNP displays the apparent absorptivity over 13.0 $(\text{mM cm})^{-1}$ at 450 nm; the apparent absorptivity of 4NNP at 450 nm is 7.5–21 times of that of *p*NP at 405 nm, and 33–66 times of that of *o*NP at 415 nm. At pH from 6.6 to 7.6, the apparent absorptivity of 4NNP at 450 nm is 2.1–7.5 times of that of *p*NP at 405 nm, and is 9.6–33 times of that of *o*NP at 415 nm. From the changes of the apparent absorptivity to pH values, pK_a of 4NNP is approximated to be slightly below 6.0 while that of *p*NP is about 7.3; pK_a of 4NNP may be lower than that of methyl purple.[10] Hence, 4NNP is a chromogen better than *p*NP and *o*NP to prepare chromogenic substrates of hydrolytic enzymes with maximum activities at neutral or slightly acidic pH.

The specific activity of a hydrolytic enzyme on its chromogenic substrate is also a concern for the sensitivity to quantify the enzyme, which is more pronounced for an enzyme as an ELISA label. In open databases, there are no records of glycosides of 4NNP as

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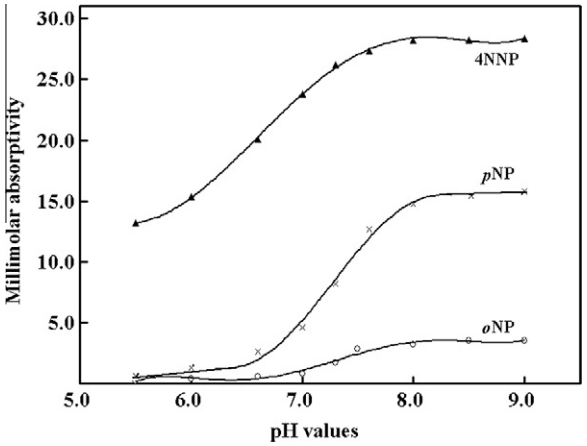
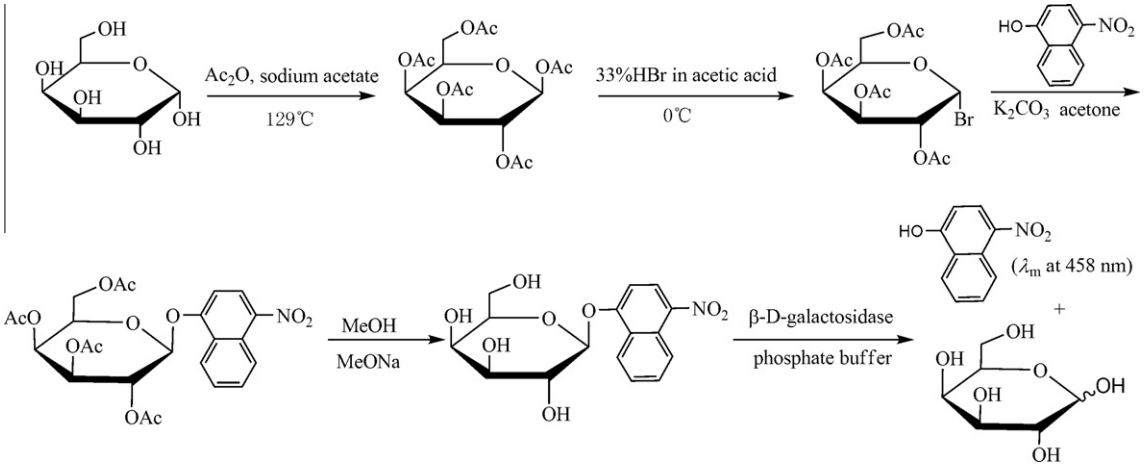


Figure 1. Changes of absorptivity of 4NNP, *p*NP and *o*NP to pH values. Buffers are sodium phosphate at 0.10 M from pH 5.5 to 7.5, and Tris–HCl at 0.10 M from pH 8.0 to 9.0.

glycosidase substrates; the sole record of a 4NNP derivative as a potential chromogenic substrate of hydrolytic enzyme is 4-nitro-1-naphthylphosphate (4NNPP), but its hydrolysis by phosphatases has not been tested yet.[11] Calf intestine alkaline phosphatase (CIAP) is an ELISA label.[5,6] At pH 8.5 in 50 mM Tris–HCl, CIAP has a Michaelis–Menten constant (K_m) of (7.7 ± 2.0) μM for 4NNPP while a K_m of (35 ± 5) μM for *para*-nitrophenylphosphate; its specific activity at 0.15 mM 4NNPP is about 40% of that on 4.0 mM *para*-nitrophenylphosphate. Thus, the sensitivity to quantify CIAP by the absorbance at 450 nm with 0.15 mM 4NNPP is comparable to that by the absorbance at 405 nm with 4.0 mM 4-nitrophenylphosphate. These results indicate that the use of 4NNPP for CIAP assay just reduces the cost of substrate and alleviates of the potential interference from contaminants in 4NNPP. CIAP has an optimum pH over 8.5 that mitigate the significance of 4NNPP, but most glycosidases have their maximum activities at neutral or slightly acidic pH. To quantify such glycosidases, their chromogenic substrates from 4NNP should be much favorable when glycosidases have sufficient specific activities. *Escherichia coli* β-D-galactosidase (BGAL) is a practical ELISA label; β-D-galactosides of methyl purple and other large chromogens are effective substrates of BGAL.[6–10,12,13] Therefore, 4-nitro-1-naphthyl-β-D-galactopyranoside (4NNPG) was prepared for the first time and compared against *para*-nitrophenyl-β-D-galactopyranoside (*p*NPG, Alfa Aesar) and *ortho*-nitrophenyl-β-D-galactopyranoside (*o*NPG, Alfa Aesar) as BGAL substrates.

4NNPG is prepared via the following four reactions (Scheme 1).[10,14] (a) Full acetylation of β-D-galactose. In detail, 5.0 g D-galactose, 2.4 g anhydrous sodium acetate and 24 mL acetic anhydride are mixed in a flask under room temperature. After reaction for 2.5 h under refluxing, the solution is diluted with 200 mL ice-water and stirred for 2 h; the precipitates are collected via filtration and are extracted with 100 mL water under stirring for 1 h. Via filtration, the insoluble precipitates are finally collected and re-crystallized with ethanol to give the white solids of β-D-galactose-1,2,3,4,6-*O*-pentaacetate with a melting point of 145 °C. (b) Bromination of the acetylated β-D-galactose. To 2.0 g β-D-galactose-1,2,3,4,6-*O*-pentaacetate in 7.0 mL acetic acid in ice-water bath, 33% HBr in 2 mL acetic acid was added drop-by-drop. After reaction for 2 h, the solution is neutralized with saturated sodium bicarbonate solution and then extracted with CH_2Cl_2. The organic layer is washed twice with saturated sodium bicarbonate solution and then with water, and dried over anhydrous sodium sulfate. The removal of CH_2Cl_2 yields solid residuals that are re-crystallized with ether/petroleum ether (5:1) to give white solids with a melting point of about 80 °C. (c) Displacement of bromide. In detail, 0.46 g 2,3,4,6-*O*-tetraacetyl-α-D-galactopyranosyl bromide, 0.22 g 4NNP (Alfa Aesar), 0.156 g anhydrous potassium carbonate, and 10.0 mL acetone are mixed and refluxed for 5 h. After the removal of solvent, the residuals are dissolved in CH_2Cl_2 and extracted twice with 10 mL solution of 1.0 M NaOH. After being dried, CH_2Cl_2 is removed to give solid residuals with a melting point of about 138 °C. (d) Deacetylation. To 0.56 g 1-(4-nitro-1-naphthyl)-2,3,4,6-*O*-tetraacetyl-β-D-galactopyranoside in 3 mL methanol, 200 μL sodium methoxide at 1.0 M in methanol is added for reaction at 25 °C for 30 min. The solution was kept at <−10 °C in a refrigerator for 10 h; the precipitates are collected and re-crystallized trice with methanol–water (3:1), yielding 4NNPG with a melting point of about 228 °C. The molar yield from D-galactose is slightly over 3%. The resulting 4NNPG has the purity about 93% based on the quantity of 4NNP released by the action of a purified BGAL (Sigma–Aldrich, G4155).

4NNPG structure is confirmed by NMR and element formulation. ^{1}H NMR data are (Brucker Avance 500 MHz, DMSO): δ 8.578 (d, 1H, *J* = 9.0 Hz, ArH), δ 8.517 (d, 1H, *J* = 8.5 Hz, ArH), δ 8.432 (d, 1H, *J* = 9 Hz, ArH), δ 7.868 (t, 1H, *J* = 7.5 Hz, ArH), δ 7.742 (t, 1H,*J* = 8.0 Hz, ArH), δ 7.3105 (d, 1H, *J* = 8.5 Hz, ArH), δ 5.22 (d, 1H, *J* = 7.5 Hz, GAl-CH), δ 3.84 (d, 1H, *J* = 7.5 Hz, GAl-CH), δ 3.79 (s, 1H, GAl-CH), δ 3.75 (t, 1H, *J* = 6.0 Hz, GAl-CH), δ 3.60 (t, 1H, *J* = 5–6 Hz, GAl-CH), 3.53 (t, 2H, *J* = 8.0 Hz, GAl-CH), δ 4.7–5.7 (m, 4H, GAl-OH). ^{13}C NMR data are: δ 157.968 (s, 1C, Ar–C), δ 139.249 (s, 1C, Ar–C), δ 130.261(s, 1C, Ar–C), δ 127.1 (s, 1C, Ar–C), δ 126.8 (s, 1C, Ar–C), δ 125.8 (s, 1C, Ar–C), δ 125.0 (s, 1C, Ar–C), δ 123.1 (s,



Scheme 1. Synthesis of 4-nitro-1-naphthyl-β-D-galactopyranoside and its hydrolysis by BGAL.

1C, Ar–C), δ 122.6 (s, 1C, Ar–C), δ 106.8 (s, 1C, Ar–C), δ 101.1 (s, 1C, GAl-C), δ 76.0 (s, 1C, GAl-C), δ 73.0 (s, 1C, GAl-C), δ 70.2 (s, 1C, GAl-C), δ 67.9 (s, 1C, GAl-C), δ 60.2 (s, 1C, GAl-C). By ESI-HRMS, *m*/*z* calculated for $[M+Na]^+$ of 4NNPG is 374.0846 while that determined is 374.0848.

BGAL activity is measured in 0.10 M sodium phosphate buffer at pH 7.4 and 25 °C, unless stated otherwise, using Shanghai Mapada UV-1600 PC ultraviolet spectrophotometer. One unit of BGAL activity is defined as that to release one micromole chromogen per min. Recorded with Shimadzu UV 2550, 4NNPG displays an absorbance peak around 375 nm; BGAL hydrolyzes 4NNPG to produce 4NNP, with clear increases in absorbance around 458 nm (Fig. 2). After the correction of the effects of pH on absorptivity, the optimum pH for the actions of BGAL on 4NNPG, *p*NPG and *o*NPG is consistently about 7.0; there is about 30% increase in the specific activity of BGAL on 4NNPG after temperature is increased from 25 to 37 °C; the optimum temperature of BGAL on 4NNPG is about 50 °C, consistent with those on *o*NPG and *p*NPG.

By Lineweaver–Burk plot analysis of the response of initial rates to substrate concentrations, BGAL possess K_m of (66 ± 5) μM for 4NNPG ($n = 3$), about one-third of that for *p*NPG and less than one-fifth of that for *o*NPG, but comparable to that for β-D-galactoside of methyl purple.[10] The specific activity of BGAL on 0.20 mM 4NNPG is about 140 U/mg at 25 °C, corresponding to a maximum catalytic activity of about 175 U/mg and a turnover number of about 2.0×10^4 min^{-1}. This turnover number of BGAL on 4NNPG is about twice of that on β-D-galactoside of methyl purple,[10] about three times of that on *p*NPG and about 40% of that on *o*NPG. However, considering the absorptivity, the sensitivity to quantify BGAL activities on 0.20 mM 4NNPG by the absorbance change rates at 450 nm is about six times of that on 6.0 mM *p*NPG, about three times of that on 6.0 mM *o*NPG. Conventional microplate readers provide filters for light sources at 405 nm. If the absorbance change rate of BGAL on 6.0 mM *o*NPG is quantified at 405 nm, the sensitivity to quantify BGAL on 0.20 mM 4NNPG by absorbance change rate at 450 nm is four times higher. Hence, the use of 4NNPG to quantify BGAL is advantageous over the uses of *p*NPG and *o*NPG.

To test the potential application of 4NNPG for quantifying BGAL as an ELISA label, BGAL (Sigma–Aldrich, G4155) is conjugated to penicillin G for competitive ELISA of penicillin G. In brief, 0.73 g penicillin G is activated with 0.40 g 1-ethyl-(3-dimethylaminopropanyl)-carbadiimide for 30 min at 25 °C in 2.0 mL *N*,*N*′-dimethylformamide containing 0.30 g 1-hydroxyl-benzotriazole; the resulting yellow solution is diluted by 1:14 with 0.10 M sodium phosphate buffer at pH 7.4. The activated penicillin G solution of 50 μL is then mixed with 0.44 mg BGAL in 250 μL of the sodium phosphate buffer at pH 7.4 for reaction at 4 °C for 120 min. Modified BGAL is then passed through a Sephadex G25 column (10 mm × 150 mm) equilibrated and eluted with the sodium phosphate buffer at pH 7.4; the first peak in a total of 2.0 mL is collected based on the absorbance at 280 nm. After chromatographic purification, the penicillin G-modified BGAL acts as the tracer; it has a specific activity of about 100 U/mg on 0.20 mM 4NNPG at 25 °C and a concentration of about 11 U/mL in the elution. Competitive ELISA of penicillin G is performed as follows (Scheme 2).

The complexes of the tracer and mouse anti-penicillin antibody (Abcam ab15070) are captured by goat anti-mouse IgG polyclonal antibodies immobilized on wells (Pierce 15134). The anti-penicillin antibody is diluted by 1:500 and 20 μL of the diluted antibody solution is employed in each well to saturate the capture antibodies. The quantity of the tracer is optimized via step-by-step increase till the change of absorbance by the action of the tracer bound in the absence of penicillin G is over 0.080 after reaction for 40 min at 25 °C. Finally, 20 μL of the elution of the tracer is utilized with 4NNPG, which basically saturate anti-penicillin antibody; 50 μL of the same elution of the tracer with a total activity of 0.32 U on 6.0 mM *p*NPG is used with *p*NPG, which completely saturate anti-penicillin antibody. The binding ratio is calculated as the activity of the tracer bound in the presence of penicillin G at a given level divided by the activity of the tracer bound in the absence of penicillin G. The detection limit is the lowest quantity of penicillin G to produce a change of absorbance over trice the random variation of absorbance by the microplate reader within 40 min. The response of binding ratios to logarithmic quantities of penicillin G in wells is plotted. The lower limit and upper limit of quantification are the smallest and largest quantity of penicillin G in wells within the linear part of such a response, respectively.

The low K_m of BGAL for 4NNPG makes it reasonable to compare the use of 0.20 mM 4NNPG against the use of 6.0 mM *p*NPG. Competitive ELISA indicates the effectiveness of 4NNPG as a chromogenic substrate of BGAL. The uses of 4NNPG and *p*NPG to quantify the tracer yield similar response curves of binding ratios to logarithmic quantities of penicillin G in wells and consistent recoveries of penicillin G (Fig. 3). The estimation of the binding ratios over 30% displays the coefficients of variation below 3% ($n > 5$). Clearly, there is more rapid decrease of the binding ratios to logarithmic quantities of penicillin G with 4NNPG, which should be due primarily to the use of a smaller quantity of the tracer. The random

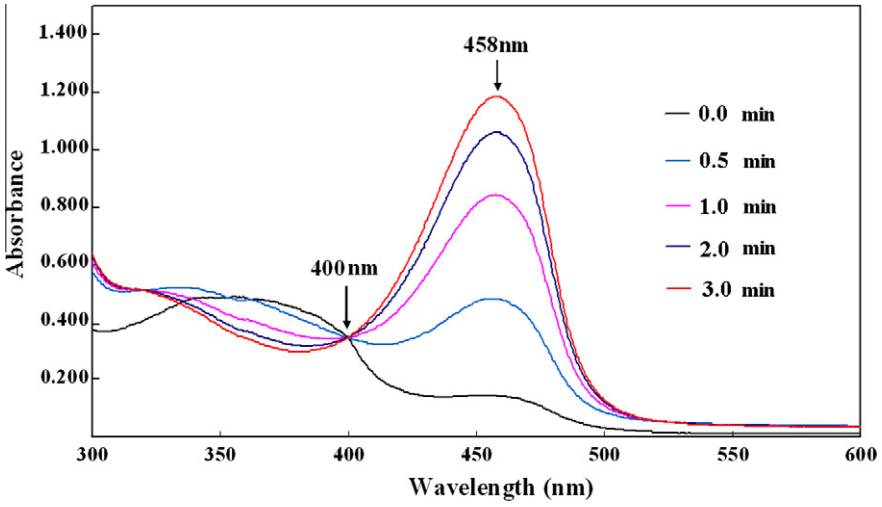
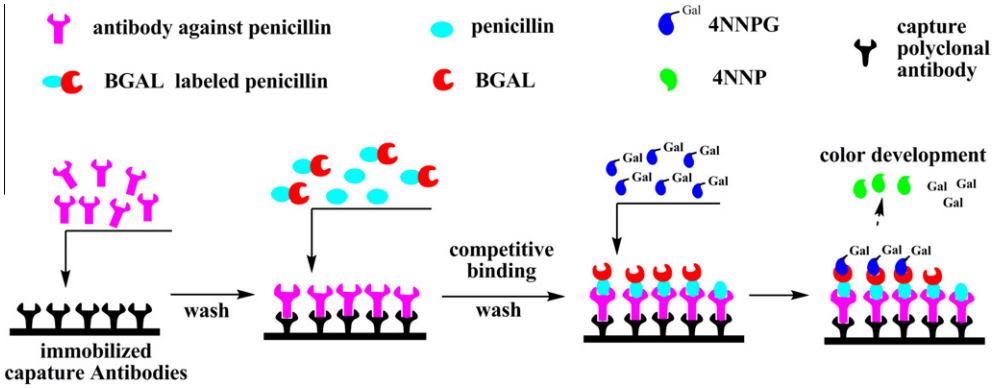


Figure 2. Absorbance spectra during BGAL action on 4NNPG in 0.10 M sodium phosphate at pH 7.4.



Scheme 2. Competitive ELISA of penicillin G with BGAL and 4NNPG.

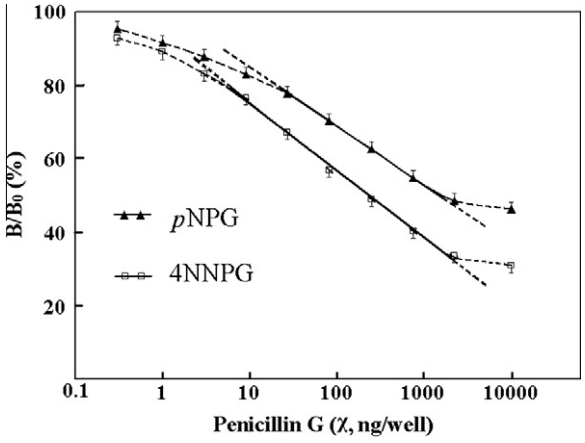


Figure 3. Responses of binding ratios to logarithmic quantities of penicillin G. Milk (drink) is centrifuged at 12,000 rpm for 15 min and filtered through 0.50 μm membrane to remove insoluble substances. Penicillin G is added to the filtrate for known final levels. Other operations follow conventional guides. For competitive binding, reaction buffer is 0.10 M sodium phosphate at pH 7.4. Biotek ELX 800 is used to record absorbance at 405 nm with *p*NPG and at 450 nm with 4NNPG. The binding ratios are determined at least in triplicate with coefficients of variation <3%. With 4NNPG, the linear part gives $B/B_0 = 93 - 18 \times \log(\chi)$, R^2 >0.994; with *p*NPG, the linear part gives $B/B_0 = 100 - 15 \times \log(\chi)$, R^2 >0.999.

variation of absorbance for continuous record of BGAL reaction curve within 40 min with Biotek ELX800 is about 0.002. The detection limit of penicillin G with 4NNPG corresponds to a binding ratio of 95% for less than 0.3 ng/well while that with *p*NPG corresponds to a binding ratio of 92% for about 1.0 ng/well. The linear response of binding ratios to logarithmic quantities of penicillin G gives the upper limit of quantification of about 2.2 μg/well with either substrate, but the lower limits of 9 ng/well and 27 ng/well with 4NNPG and *p*NPG, respectively. Based on the detection limit and quantification limit of penicillin G by ELISA, the use of 4NNPG as BGAL substrate should be advantageous over the use of *o*NPG. Therefore, for quantifying BGAL as an ELISA label, 4NNPG is a better chromogenic substrate.

In practice, the toughest challenge to ELISA is how to enhance its efficiency. Conventional microplate readers already can swiftly alter detection wavelengths to quantify the absorbance of one solution simultaneously at multiple wavelengths. Dual-enzyme simultaneous assay in one reaction solution via concurrent quantification of the absorbance of two chromogens at two wavelengths may be feasible and applicable to ELISA for much higher efficiency. This new format of ELISA requires a pair of enzyme labels, a pair of chromogenic substrates with special spectral properties and particular methods to process data. A chromogenic substrate of a hydrolytic enzyme will produce an isoabsorbance wavelength where the chromogenic substrate and product have the same absorptivity. For the new format of ELISA of much higher efficiency, the action of one enzyme label on its chromogenic substrate should produce the longest isoabsorbance wavelength where the chromogenic product of the other enzyme can be quantified with sufficient sensitivity. The action of BGAL on 4NNPG produces the longest isoabsorbance wavelength of 400 nm where *p*NP can be quantified by absorbance (Fig. 2); the combination of BGAL on 4NNPG and α-D-glucosidase on *para*-nitrophenyl-α-D-glucoside is effective for this new format of ELISA (data unpublished). 4NNP has excellent stability, small size, large apparent absorptivity at pH below 7.6 and simple structure for preparing chromogenic substrates of hydrolytic enzymes. Taken together, 4NNP is a better chromogen to prepare chromogenic substrates of hydrolytic enzymes with maximum activities at neutral or slightly acidic pH; the combination of a chromogenic substrate of 4NNP and that of *p*NP for two enzyme labels may facilitate the application of dual-enzyme simultaneous assay in one reaction solution to ELISA for much higher efficiency.

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