

Hydrolysis of Phosphoric Acid Ester across an Enzyme Membrane: Potato Acid Phosphatase Immobilized on a Na–Perfluorocarboxylate Ionomer Membrane for Recovery of Reaction Product

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Acid phosphatase from potato was immobilized onto the surface of the external solution side of a perfluorocarboxylate ionomer membrane using a cross-linking reagent. Based on the distinct difference in membrane permeabilities to 4-nitrophenol and 4-nitrophenyl phosphate, a membrane reactor system using the immobilized enzyme has been designed for the recovery of the reaction product of enzymatic hydrolysis. To observe the recovery rate for the hydrolysis product, the flux from the external solution side to the internal solution side crossing the membrane was measured in the substrate concentration range below 1×10^{-2} mol dm^{-3}. The recovery rate was found to obey a Michaelis–Menten type equation. The Michaelis constant for the immobilized enzyme was smaller than that for the free enzyme obtained from the kinetic properties in the bulk solution. The optimum pH value for the immobilized enzyme membrane was investigated.

Different kinds of residual synthesized organic compounds in water in the environment can be decomposed by virtue of enzymes from natural sources.[1] Immobilization of an enzyme on a membrane enables its activity to be retained for a long period of time; therefore, many types of membrane reactor systems have been designed to use enzymes for the elimination and recovery of residual organic compounds in water.[2,3]

On the other hand, in the rhizosphere region of soil, organophosphates are hydrolyzed by extracellular enzymes such as urease, acid phosphatase, and alkaline phosphatase secreted from plant roots. The enzymatic activity of these extracellular soil enzymes are retained by immobilization on clay or into organic substances by adsorption or incorporation.[4–6]

In this communication, acid phosphatase from potato has been immobilized onto the surface of an artificial perfluorocarboxylate ion-exchange membrane. Potato acid phosphatase is a typical phosphatase, which catalyzes the hydrolysis of phosphoric acid monoesters. On the other hand, in perfluorinated ionomer membranes, as in inverted micellar systems, there are dispersed hydrophobic regions formed by fluorocarbon chains of the polymer backbone and hydrophilic regions formed by clustering of the ionic groups of the polymer, counter ions, and water molecules.[7–9] As described in the literature, immobilization by incorporating an enzyme into a perfluorosulfonate ionomer membrane (Nafion) has already been utilized to form the enzyme layer of biosensors.[10–12]

Based on the distinct difference in membrane permeabilities to 4-nitrophenol and 4-nitrophenyl phosphate observed in this study, a membrane reactor system using an immobilized enzyme membrane has been designed for the purpose of the recovery of the reaction product of enzymatic hydrolysis. From the substrate-concentration dependence of the recovery flux of the product from the enzyme-layer side to the other side by crossing the membrane, the Michaelis constant for the immobilized enzyme was obtained and compared with that for the free enzyme, which was obtained from the kinetic properties in bulk solution. The pH dependence of the recovery rate has also been investigated.

Experimental

Materials. Acid phosphatase (E.C.3.1.3.2) from potato with a protein concentration of 10 mg mL^{-1} was obtained as a suspension in 3.2 mol dm^{-3} $(NH_4)_2SO_4$ stabilized with bovine serum albumin (CalBiochem, U.S.A.). Disodium 4-nitrophenyl phosphate (Fluka, Switzerland) was used to prepare the substrate solution. All other reagents were of guaranteed grade and used without further purification. Water was purified by double distillation, of which one was from an alkaline potassium permanganate solution. A perfluorocarboxylate polymer membrane (Flemion 230, kindly supplied by the Asahi Glass Co., Tokyo, with an ion exchange capacity of 1.4 mmol univalent ion/g dry)[13,14] was used as the supporting material for the enzyme.

Immobilization of Enzyme. The ionomer membrane had been immersed in a 0.1 mol dm^{-3} NaCl solution for over 24 h at 25 °C before the enzyme immobilization. A mixture of 25 μL of the enzyme solution (10 mg mL^{-1} acid phosphatase) and 25 μL of the aqueous acetate buffer solution (0.1 mol dm^{-3} acetate buffer pH 4.8 with 0.1 mol dm^{-3} NaCl) was stirred and then 30 μL of it was placed on the surface of one side of the perfluorinated carboxylate ionomer membrane. After leaving it at 25 °C for one hour, the membrane with the enzyme was stored at 5 °C overnight. After the membrane covered with the enzyme suspension was returned to room temperature, 50 μL of a mixed solution of a cross-linking reagent (2.5 wt % glutaraldehyde[15–18] solution) with an equivalent volume of a phosphate buffer solution (pH 7.4) was placed into the enzyme suspension on the membrane surface. After one hour, one side of the membrane, over which the enzyme was immobilized in the micropores, was washed with a 0.1 mol dm^{-3} NaCl solution. The immobilized enzyme membrane mounted in the cell assembly was stored at 5 °C before the flux measurement.

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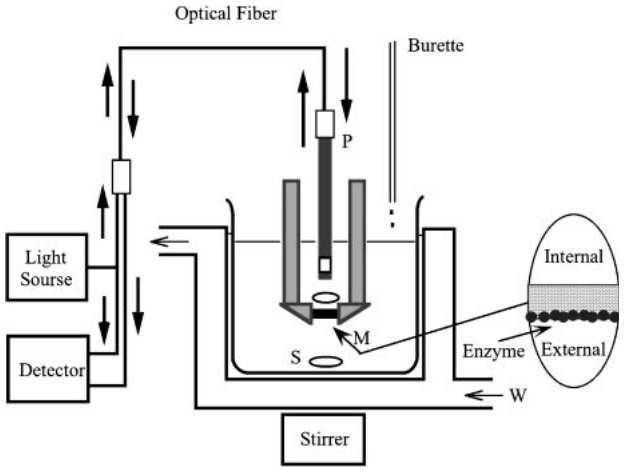

Fig. 1. Schematic diagram of assembled system for the measurement of 4-nitrophenol amount transported through the enzyme membrane. The time dependence of the amount of phenol in the internal solution was obtained through the measurement of the absorption spectrum using an immersed probe (P) connected to a fiber UV spectrophotometer. The arrow (⟶) shows the path of the ultraviolet radiation. Immobilized-enzyme membrane, magnetic spin bar, and thermostatically controlled water are designated by M, S, and W, respectively.

Measurement of Flux. The assembled measuring system for the flux of the hydrolysis product is schematically illustrated in Fig. 1. The immobilized enzyme membrane was horizontally mounted in the cell for the measurements. The area of the membrane surface adjacent to the solution (diffusion area) was 0.20 cm^2. The layer of acid phosphatase immobilized at the surface was on the external solution side of the enzyme membrane. Initially, both the external and internal solutions contained only 0.1 mol dm^{-3} NaCl. The pH's of both compartments were adjusted with a 0.1 mol dm^{-3} acetate buffer. The measurement was started by injecting the substrate (4-nitrophenyl phosphate) solution into the external solution. The hydrolysis product (4-nitrophenol) could permeate through the non-activated region of the ionomer membrane and cross the membrane–solution interface on the internal solution side. In the experiments, the amount of 4-nitrophenol in the internal buffer solution of NaCl was followed by measuring the time course of optical absorbance at 317 nm. The UV spectra for 4-nitrophenol as well as for 4-nitrophenyl phosphate at pH 4.8 are shown in Fig. 2. The linearity of the calibration curve for the optical absorbance at 317 nm vs the concentration was confirmed (correlation coefficient; R = 0.999) in an experimental concentration range (up to 2.0×10^{-4} mol dm^{-3}). A probe of the transmission type with a light-path length of 1 cm was immersed in the internal solution and connected to a dual type fiber optic spectrometer (Model SD-2000, Ocean Optics, Dunedin, Florida, U.S.A.) by an optical fiber. Throughout the measurements, the temperature of the membrane system was maintained at 25.0 °C by the circulation of thermostatted water. Each compartment was stirred by a magnetic stirrer at a speed of 650 rpm.

Reaction Kinetics of Free Enzyme in Bulk Solution. The reaction rate was investigated using 50 cm^3 of a 0.1 mol dm^{-3} buffer solution with 0.1 mol dm^{-3} NaCl at 25.0 °C. Five μL of the stock solution of acid phosphatase was added to this mixed

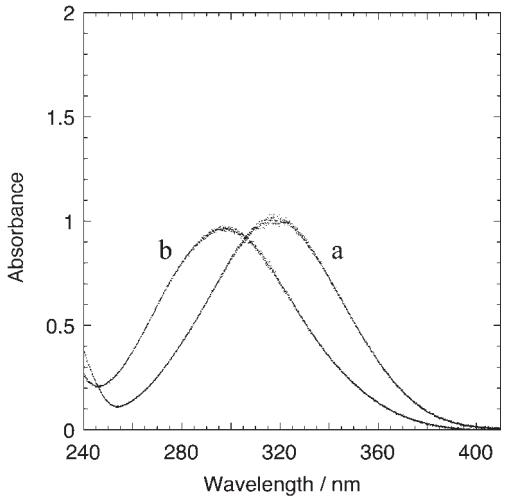

Fig. 2. Absorption spectra of 1×10^{-4} mol dm^{-3} 4-nitrophenol and 1×10^{-4} mol dm^{-3} 4-nitrophenyl phosphate in 0.1 mol dm^{-3} acetate buffer solution (pH = 4.8), which contained 0.1 mol dm^{-3} NaCl: 4-nitrophenol (a); 4-nitrophenyl phosphate (b).

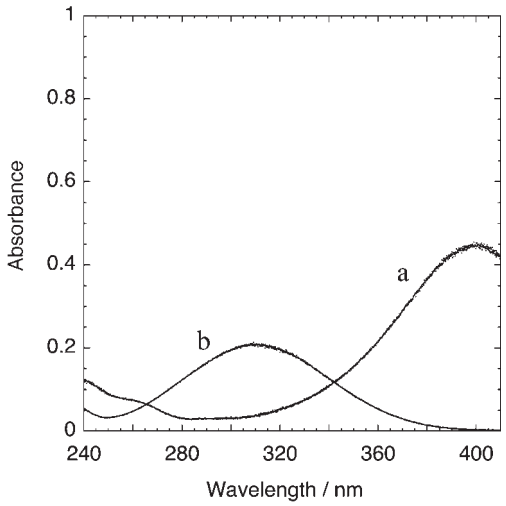

Fig. 3. Absorption spectra of 2×10^{-5} mol dm^{-3} 4-nitrophenol and 2×10^{-5} mol dm^{-3} 4-nitrophenyl phosphate in 0.1 mol dm^{-3} Tris buffer solution (pH 8.5), which contained 0.1 mol dm^{-3} NaCl: 4-nitrophenol (a); 4-nitrophenyl phosphate (b).

solution. The enzymatic reaction started with the injection of an amount of the substrate (4-nitrophenyl phosphate) solution. The temperature of the solution was maintained at 25.0 °C by circulation of the thermostatically controlled water. The solution of each compartment was stirred by a magnetic stirrer at 650 rpm. At a certain time interval, 2 mL of the reaction mixture was drawn and immediately mixed with 2 mL of 0.035 mol dm^{-3} NaOH to terminate the hydrolysis. After adjusting the pH of the solution with Tris buffer (pH = 8.5), the liberated 4-nitrophenol was determined by measuring the optical absorbance at 400 nm using the same spectrometric system used for the flux measurements. The absorption spectra for 4-nitrophenol as well as for 4-nitrophenyl phosphate in 0.1 mol dm^{-3} NaCl at pH 8.5 adjusted with Tris buffer are shown in Fig. 3. The calibration curve at pH 8.5 for the

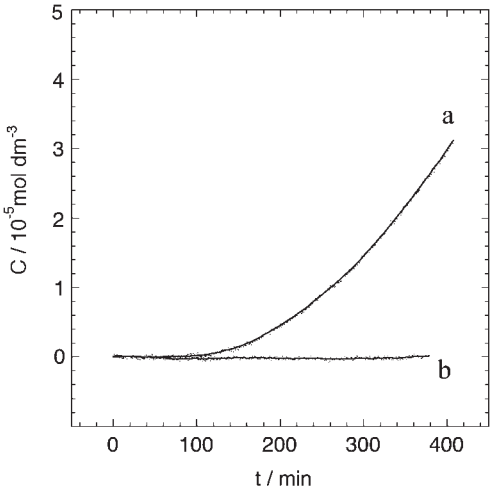

Fig. 4. Time course of the concentration in the internal solution for 4-nitrophenol (a) and that for 4-nitrophenyl phosphate (b). Two compartments are separated by the perfluorocarboxylate ionomer membrane without enzyme. Initially, both the external and the internal solutions contained 0.1 mol dm^{-3} acetate buffer (pH 4.8) and 0.1 mol dm^{-3} NaCl. Initial concentration in the external solution of 4-nitrophenol or that of 4-nitrophenyl phosphate was 0.01 mol dm^{-3}.

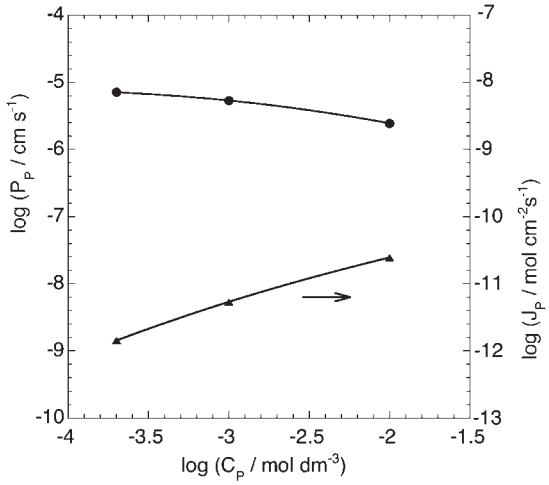

Fig. 5. The membrane permeability of the perfluorocarboxylate ionomer membrane in the absence of the enzyme. The membrane permeability to 4-nitrophenol, P_P, and the flux of 4-nitrophenol, J_P, are shown as a function of the initial concentration of 4-nitrophenol in the external solution.

absorbance at 400 nm vs the concentration was linear in an experimental concentration range up to 4×10^{-5} mol dm^{-3} (correlation coefficient; R = 0.999).

Results and Discussion

Membrane Transport across the Ionomer Membrane without Enzyme. The sample of the ionomer membrane was immersed in 0.1 mol dm^{-3} NaCl for over 24 h at 25 °C before the flux measurement. Under the same conditions as for the experiments of the enzyme membrane system, the flux measurements were carried out for 4-nitrophenol and 4-nitrophenyl phosphate across the perfluorocarboxylate ionomer membrane of the sodium form that was used as the supporting membrane for the enzyme immobilization. Initially, both the external and internal solutions contained only 0.1 mol dm^{-3} NaCl. The pH's of both compartments were adjusted with acetate buffer (pH = 4.8). Flux measurement was started by injecting a 4-nitrophenyl phosphate solution or 4-nitrophenol solution into the external solution. The time course of the concentration was followed by the setup of the fiber optic spectrometer shown in Fig. 1 at 25 °C. Each compartment was stirred at a speed of 650 rpm. An example of the time course for the amount of each compound transported across the membrane without the enzyme is shown in Fig. 4. A higher permeability has been observed for 4-nitrophenol in contrast to 4-nitrophenyl phosphate, to which this membrane exhibits no significant permeability under the conditions of the experiments. The flux of 4-nitrophenol, J_P, was obtained from the slope of the linear portion after a transient region in the time course curve. The concentration dependence of J_P is shown in Fig. 5, together with that of the membrane permeability to 4-nitrophenol, P_P, which is calculated according to the following equation:

$$J_P = -P_P \Delta C_P, \tag{1}$$

where ΔC_P is the concentration difference of 4-nitrophenol between the external and internal solutions.

Hydrolysis of 4-Nitrophenyl Phosphate across Immobilized Enzyme Membrane. In this study, the enzyme was immobilized at the surface region on the external solution side of the substrate (4-nitrophenyl phosphate). Because the layer of the ion-exchange polymer membrane exhibits a high permeability only to the reaction product (4-nitrophenol) as already mentioned, the liberation of 4-nitrophenol in the enzyme layer induces flux of the product to the internal solution for recovery. After the flux measurement, the pH of the internal solution was adjusted to pH 8.5. Then, the UV spectrum was measured to confirm that the absorbance of 4-nitrophenyl phosphate near 310 nm was not observed. Although diffusional flow in the opposite direction occurs simultaneously, this flow was not followed in this study. The flux of the product, J_P, was obtained from the slope of the linear portion of the time-course curve for the amount of the reaction product recovered in the internal solution. The results are plotted in Fig. 6 as a function of the initial concentration of the substrate in the external solution. The concentration dependence displays a saturation curvature similar to the Michaelis–Menten kinetics for a free enzyme reaction.

Presuming a mode analogous to the Michaelis–Menten type, we plotted the inverse of the flux versus the inverse of the substrate concentration. It can be seen that the linear relation holds between these two quantities:

$$J_P = J_P^{max} \frac{C_S}{K_M^* + C_S}, \tag{2}$$

where C_S denotes the initial concentration of the substrate in the external solution. J_P^{max} is the maximum rate of recovery and K_M^* is the Michaelis constant for the immobilized acid phosphatase from potato. K_M^* and J_P^{max} could be obtained

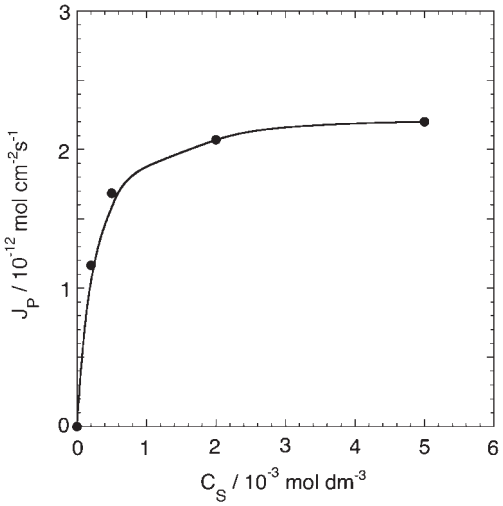

Fig. 6. Concentration dependence of flux across the enzyme membrane at pH 4.8. The flux of 4-nitrophenol produced during the hydrolysis of 4-nitrophenyl phosphate using immobilized enzyme was determined by measuring the absorption spectra of the internal solution. The initial concentration of 4-nitrophenyl phosphate was in the range of 2×10^{-4} mol dm^{-3}–5×10^{-3} mol dm^{-3}. 0.1 mol dm^{-3} acetate buffer (pH 4.8) and 0.1 mol dm^{-3} NaCl were included in the substrate solution.

according to the following equation for an enzyme membrane system:

$$\frac{1}{J_P} = \frac{1}{J_P^{max}} + \frac{K_M^*}{J_P^{max}} \frac{1}{C_S}. \quad (3)$$

From the double reciprocal plot of $1/J_P$ vs $1/C_S$ (correlation coefficient; R = 0.999), we obtained K_M^* and J_P^{max} to be 1.92×10^{-4} mol dm^{-3} and 2.29×10^{-12} mol cm^{-2} s^{-1}, respectively. The Michaelis constant for the immobilized enzyme was greater than that for the free enzyme (described in next section).

The pH dependence of the performance of the enzyme immobilized membrane reactor was studied. In the pH range from pH = 3.7 to 6.2, the pH dependence of the recovery flux of 4-nitrophenol through the enzyme membrane was investigated. The initial concentration of the substrate in the external solution was 5×10^{-3} mol dm^{-3}. The pH was adjusted using different buffers (acetate buffer, pH range: 3.7–5.5; citrate buffer, pH range: 5.5–6.2). The results are shown in Fig. 7. The optimum pH for the immobilized enzyme is in the range 4.7–5.0, which is near the optimum pH for the activity of the free enzyme reported in the literature.[4]

A perfluorocarboxylate membrane with the immobilized acid phosphatase was stored at 5 °C after each series of flux measurements in this study. During the 40-day test, a decrease in the performance of the recovery rate (flux of the reaction product) was not entirely observed.

Reaction Kinetics of Free Enzyme in Bulk Solution. Examples for the time course of the product are shown in Fig. 8. The initial velocity, V_0, was obtained from the initial slope of these time course curves. The dependence of V_0 on the substrate concentration is shown in Fig. 9. From the Lineweaver–Burk plot (correlation coefficient; R = 0.991) for the free acid phosphatase in bulk solution at pH 4.8, the Michaelis constant, K_M, and the maximum velocity, V_{max}, are obtained to be 8.1×10^{-5} mol dm^{-3} and 3.7×10^{-8} mol dm^{-3} s^{-1}, respectively. For the potato acid phosphatase, K_M values have been reported in the literature[4,18,19] as summarized in Table 1. In this study, a smaller value has been obtained for K_M at 25 °C under the condition of pH 4.8, adjusted by the 0.1 mol dm^{-3} acetate buffer in the presence of 0.1 mol dm^{-3} NaCl. Because the enzyme is present in the reaction mixture after the hydrolysis, it is necessary to separate it for reuse.

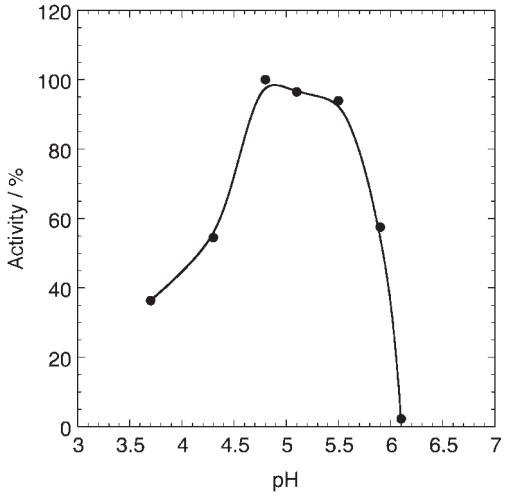

Fig. 7. The pH dependence of the flux of 4-nitrophenol produced during the hydrolysis of 4-nitrophenyl phosphate using the immobilized enzyme. Activities (%) relative to the flux at pH 4.8 are plotted as a function of the pH in the solution. The 0.1 mol dm^{-3} buffer and 0.1 mol dm^{-3} NaCl were included in the substrate solution. The initial concentration of 4-nitrophenyl phosphate in the external solution was 5×10^{-3} mol dm^{-3}.

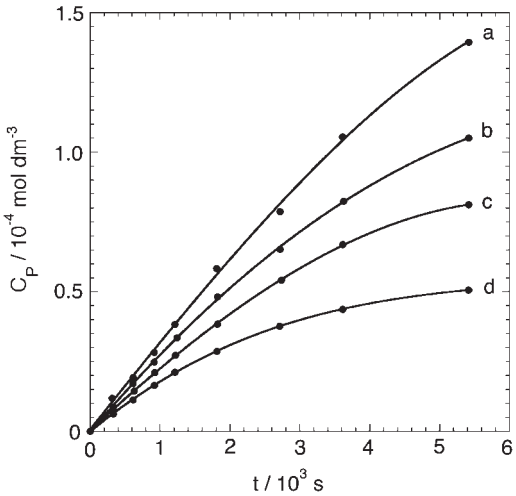

Fig. 8. Time dependence of concentration of produced 4-nitrophenol catalyzed by free phosphatase for the initial substrate concentration of 5×10^{-4} mol dm^{-3} (a), 2×10^{-4} mol dm^{-3} (b), 1×10^{-4} mol dm^{-3} (c), and 8×10^{-5} mol dm^{-3} (d). The pH of the solution was 4.8.

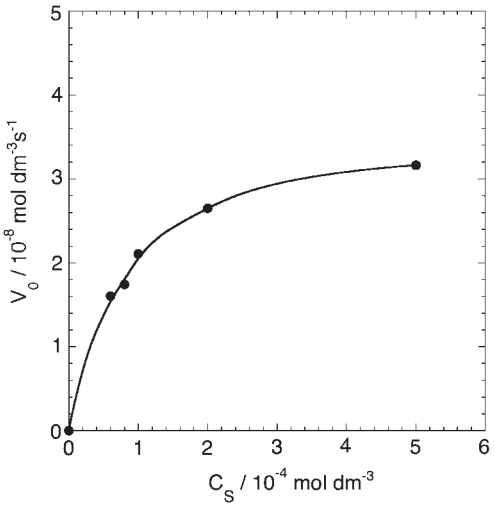

Fig. 9. Substrate-concentration dependence of the initial reaction rate for free acid phosphatase.

Table 1. Michaelis Constant for Free Enzyme

	(i)[a)]	(ii)[b)]	(iii)[c)]
K_M/mol dm^{-3}	4.9×10^{-4}	4.3×10^{-4}	6.2×10^{-4}
Reference	[4]	[18]	[19]

a) pH 6.0 (maleate buffer), 37 °C. b) pH 4.9 (acetate buffer), 38 °C. c) pH 5.5 (acetate buffer), 38 °C.

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