DETERMINATION OF β-GLYCOSIDASES, β-GLUCURONIDASE, AND ALKALINE PHOSPHATASE BY AN ENZYME ELECTRODE SENSITIVE TO PHENOL

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A rapid and relatively simple procedure is desciibed for kinetic measurement of low activities of enzymes hydrolyzing phenol conjugates. Phenol generated by the enzyme reactions in the pH-optimum range 5·0–9·5 is continuously monitored by an oxygen membrane electrode of the Clark type. The surface of the electrode exposed to the solution is coated by a layer of chemically insolubilized polyphenol oxidase. The current response of the electrode, indicating the uptake of oxygen — diffusing from the solution together with phenol into the enzyme membrane — is proportional to the enzyme concentration (according to the kind of hydrolase in the range from 0·2 to at least 2–9 ncat). The dependences of the initial rate of enzyme reaction on enzyme and substrate concentration and the K_m- and V_{max}-values at optimal pH were measured with alkaline phosphatase, emulsin (β-glucosidase) and β-glucuronidase. The kinetic measurements showed that sweet almond emulsin contains two enzymes differing in their affinity toward phenyl -β-D-galactopyranoside.

Synthetic phenol derivatives are commonly used as substrates for activity assays of enzymes catalyzing hydrolytic cleavage of an ester or glycoside bond. Conjugates of simple phenol have been replaced in routine diagnostic tests[1-3] almost completely by chromogenic and fluorogenic substrates yielding a reaction product readily measurable by optical methods, *e.g.* phenolphthalein, nitrophenol, or naphthol. Difficulties are encountered when these methods are applied to turbid or colored solutions. These drawbacks could be circumvented by electrochemical determination of the reaction product.

We have shown in our preceding paper[4] that low phenol concentrations can readily be determined by a polyphenol oxidase electrode. Therefore it is in principle possible to measure kinetically the activity of the enzyme by analyzing the reaction mixture in which phenol is continuously generated by enzymic cleavage of its conjugate. This paper reports on an amperometric method for determination of alkaline phosphatase (EC 3.1.3.1), β-glucosidase (EC 3.2.1.21), and β-glucuronidase (EC 3.2.1.31). The optimal conditions of amperometric determination of alkaline phosphatase in serum, based on the measurement of oxygen uptake by a coupled reaction with free soluble polyphenol oxidase, were described recently by Kumar and Christian[5].

EXPERIMENTAL

Material. Disodium phenyl phosphate (hydrate) was a preparation of British Drug Houses Ltd. Phenyl-β-D-glucopyranoside, phenyl-β-D-galactopyranoside, and phenyl-β-D-glucuronide were synthesized by Dr J. Frgala, Institute of Pure Chemicals, Lachema. Brno. Calf intestinal mucosa alkaline phosphatase (type I, specific activity 200 ncat/mg with phenyl phosphate as substrate) and bacterial β-glucuronidase (specific activity 0·25 ncat/mg with phenyl-β-D-glucuronide as substrate) were preparations of Sigma Chem. Co., USA. β-Glucosidase (emulsin) was isolated from sweet almonds and purified to the degree "Reinpräparat" according to Helferich and Kleinschmidt[6]. The specific activity of the enzyme was 51 ncat/mg protein with phenyl-β-D-glucopyranoside as substrate and 10·2 ncat/mg protein with phenyl-β-D-galactopyranoside as substrate.

Buffers. 1·5M Diethanolamine–HCl buffer, pH 9·5, containing 2M-KCl and 2·5 mM-$MgCl_2$ (ref.[5]) was used with alkaline phosphatase. The β-glucosidase, β-galactosidase, and β-glucuronidase activity was measured in McIlvain buffer (0·1M citric acid–0·2M-Na_2HPO_4) at the optimal pH for the given substrate.

The electrode sensitive to phenol was obtained as described earlier[4] from the common oxygen electrode (diameter of golden disc cathode 7 mm, reference electrode Ag/AgCl, electrolyte 2M-KCl, polypropylene membrane) to which a polyphenol oxidase membrane was attached by a rubber ring. The membrane was made from a mixture of 6 μl of 10% bovine serum albumin, 6 μl (167 ncat) of champignon polyphenol oxidase, and 4 μl of 2% glutaraldehyde[4].

The enzymic activity of the hydrolases was measured in a recording analyzer, dissolved oxygen (manufactured in the Instrument Development Workshops, Czechoslovak Academy of Sciences[7]) at 30 ± 0·1°C and optimal pH and concentration of the corresponding substrate. The cathode of the enzyme electrode was polarized by a constant potential of −0·7 V *versus* a silver chloride electrode.

The reaction vessel was jacketed and contained the substrate (Table II) in 3 ml of the corresponding buffer stirred magnetically and equilibrated with atmospheric oxygen. After the electrode current had stabilized the reaction was started by the injection of 2—50 μl of the hydrolase. The initial reaction rate v_0 (in mm of 250 mm scale per min) was read from the record of the initial decrease of electrode current; this value was transformed to enzyme units (ncat) by using a calibration curve for phenol[4].

The spectrophotometric measurements were made in Cary 118 C Spectrophotometer.

RESULTS AND DISCUSSION

Evaluation of Electrode Response

The polyphenol oxidase electrode reacts to the addition of a phenol standard (as a result of oxygen uptake in the enzyme membrane) by a rapid decrease of electrode current which becomes stabilized after 30–60 s; hence, the curve of the electrode response assumed a wave-shaped form[4]. The magnitude of the steady state electrode response (wave height) is proportional to the quantity of phenol added. If this compound is continuously produced from zero initial concentration by enzymic hydrolysis of a phenol conjugate in a homogeneous reaction medium, the electrode current also starts continuously decreasing after an lag phase of 5 – 15 s. The slope of the initial linear decrease of the electrode current is proportional to the initial reaction rate (v_0). The latter can be expressed for the sake of common kinetic evaluation either in the relative manner or transformed to the weight of phenol liberated per time unit. This recalculation, however, requires that the measuring system must be calibrated by 10 – 20 mM phenol standard under identical experimental conditions as regards temperature, volume, and composition of the reaction mixture. The calibration curve obtained by plotting the steady state electrode response (wave height) *versus* the quantity of standard added (in μmol[4]) permits the slope of the electrode response to be expressed in the recommended units of enzyme activity (IU, cat).

We have checked this procedure with the cleavage of phenyl phosphate catalyzed by alkaline phosphatase at pH 9·5. Direct spectrophotometric determination of liberated phenol at 290 nm served as a reference method of determination of the hydrolysis rate. The measured changes ΔA_{290} per min were converted into enzyme activity units by calibration curve. It is clear from the results shown in Table I that the electrochemical method yields essentially the same values as direct spectrophotometry. At the same time is evident that the amperometric determination described is more sensitive. The sensitivity can be still enhanced by decreasing the volume of the reaction mixture.

Undoubtedly, the method could serve for the determination of other EC 3.1 and EC 3.2 enzymes, such as aryl esterase, aryl sulfatase, fucosidase, *etc.* It is not suitable, however, for assaying the enzymic hydrolysis of substrates derived from 2- and 4-nitrophenol, or 1-, 2-naphthol since these compounds are not oxidized by membrane-bound mushroom polyphenol oxidase.

TABLE I

Parallel Activity Assay of Alkaline Phosphatase by Polyphenol Oxidase Electrode and Optical Test at 30°C and pH 9·5

The reaction mixture contained in both cases 8 mM phenyl phosphate, 2M-KCl, and 2·5 mM-$MgCl_2$ in 1·5M diethanolamine buffer.

Quantity of enzyme μl	Amperometric determination			Optical test[a]		
	slope mm min^{-1}	activity		ΔA_{290} . min^{-1}	activity	
		mU	ncat		mU	ncat
6	25·0	46	0·77	0·009	41	0·68
10	38·5	71	1·18	0·015	73	1·22

[a] Average of two measurements, $d = 1$ cm.

Effect of pH, *Buffer Composition, and Enzyme Concentration*

The modulation of the activity of hydrolases measured as function of pH by the polyphenol oxidase electrode using the same enzyme membrane for the whole series of assays, has the usual bell-shaped form. The pH-profile of the reaction catalyzed by emulsin in McIlvain citrate-phosphate buffer shows with both substrates (phenyl-β-D-glucopyranoside and phenyl-β-D-galactopyranoside) almost the same pH-optima (about 5, Fig. 1), in accordance with data recorded in the literature[8]. The pH-optimum of cleavage of the first compound in 0·25M acetate buffer lies at pH 5·5 yet the reaction rate is lower by roughly 50%. In accordance with recorded data, two pH-optima were observed with bacterial β-glucuronidase (Fig. 1). The reported values[1] characterizing the enzyme from *E. coli* are 5·5 and 7·0. A slight shift toward the alkaline region (pH 6·1 and 7·3) of the values found by us can be ascribed to changes in the sensitivity of the polyphenol oxidase electrode to phenol at different pH (ref.[4]).

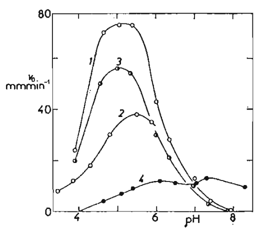

FIG. 1

Effect of pH on Rate of Enzymic Hydrolysis of Phenol Conjugates at 30°C

Reaction conditions: *1*, *2* 50 mM phenyl-β-D-glucopyranoside, 6·25 ncat of emulsin *3* 100 mM phenyl-β-D-galactopyranoside, 7·5 ncat of emulsin, *4* 10 mM phenyl-β-glucuronide, 1 ncat of bacterial β-glucuronidase. Except for *2*, where 0·25M acetate buffer was used, the medium was always McIlvain citrate–phosphate buffer. The final pH of the complete reaction mixture is shown on the abscissa.

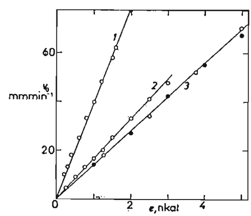

FIG. 2

Slope of Electrode Response as Function of Enzyme Quantity

The initial reaction rate v_0 was measured at 30°C in 3 ml of reaction mixture. *1* alkaline phosphatase in diethanolamine buffer, pH 9·5, 10 mM phenyl phosphate, *2* β-glucuronidase in citrate–phosphate buffer, pH 7·4, 5 mM phenyl-β-D-glucuronide, *3* emulsin in citrate–phosphate buffer, pH 5·0, 50 mM phenyl-β-D-glucopyranoside(○) or 100 mM phenyl-β-D-galactopyranoside (●) as substrate.

A medium suitable for the activity assay of alkaline phosphatase is diethanolamine buffer with the activating admixture of KCl and $MgCl_2$; it was optimized for the system phosphatase–free-soluble polyphenol oxidase by Kumar and Christian[5]. Now, it has been observed at low pH-values (0·1M citrate buffer) that the polyphenol oxidase electrode reacts to the addition of substrate even in the absence of alkaline phosphatase. The high steady state responses at pH 5·5 – 6·0 (42 mm) with a sigmoid drop of the values to both sides of the pH-scale were recorded when the dose of phenyl phosphate was 0·5 μmol. The reaction is reversibly inhibited by inorganic phosphate. These findings together with the results of other experiments indicate that the cleavage of phenyl phosphate to free phenol obviously takes place only in the enzyme membrane by the action of acid phosphatase. The latter accompanies champignon polyphenol oxidase[4] as shown by electrophoretic examination of the preparation in acrylamide gel. However, acid phosphatase does not interfere at pH 9·5 with the determination of alkaline phosphatase.

The measured rate of cleavage of the phenol conjugate is at optimal reaction conditions a linear function of the enzyme concentration for all the hydrolases tested (Fig. 2). The linearity range for emulsin with phenyl-β-glucopyranoside as substrate was confirmed up to an activity value of 9 ncat. The differences in slopes of linear plots can be ascribed to differences in pH and buffer composition. It has been shown by using emulsin as an example that the kind of the substrate is without any effect on the slope of the curve under identical reaction conditions (Fig. 2).

Effect of Substrate Concentration

To determine the kinetic parameters of the enzyme reactions examined a series of rate measurements was carried out at varying concentration of the substrate under otherwise constant reaction conditions. The usual linear relationship (Fig. 3) was obtained in double reciprocal plot of the kinetic data for alkaline phosphatase

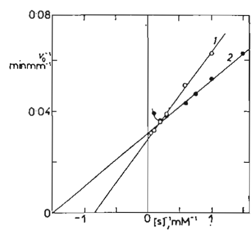

FIG. 3

Lineweaver–Burk Plot of Initial Reaction Rate *versus* Substrate Concentration for Alkaline Phosphatase and β-Glucuronidase

1 0·8 ncat of alkaline phosphatase in diethanolamine buffer, pH 9·5, *2* 1·5 ncat of bacterial β-glucuronidase in citrate–phosphate buffer, pH 7·4. The concentration of the substrate is shown on the abscissa, 30°C.

and β-glucuronidase. The values of Michaelis constant K_m and maximal reaction rate V_{max} (after recalculation of maximal slope of electrode response to µmol of phenol liberated) are given in Table II.

TABLE II

Kinetic Parameters of Hydrolases Measured by Polyphenol Oxidase Electrode at 30°C

Enzyme (source)	Substrate	pH optimum	K_m mM	V_{max} µmol/mg min
Alkaline phosphatase (calf intestinal mucosa)	phenyl phosphate	9·5[a]	1·2	12·50
β-Glucuronidase (bacteria)	phenyl-β-D--glucuronide	7·4[b]	0·67	0·019
Emulsin[c] (sweet almonds)	phenyl-β-D--glucopyranoside	5·1[b]	57 (57·5)	6·87 (6·81)
	phenyl-β-D--galactopyranoside[d]	5·0[b]	116 (81)	0·823 (1·22)
			3·8 (12·5)	0·232 (0·316)

[a] Diethanolamine–HCl buffer containing KCl and $MgCl_2$; [b] McIlvain citrate–phosphate buffer; [c] the data in parentheses were read from the graph in Fig. 4*b*; [d] parameters of two β-galactosidase components of emulsin. V_{max} is in all cases related to mg of protein.

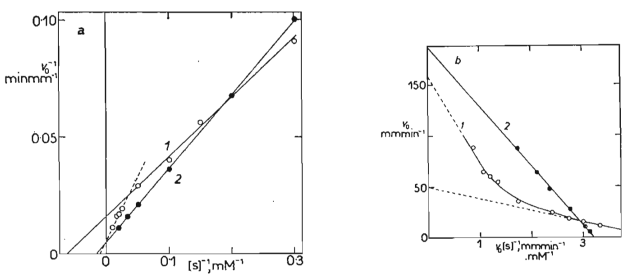

FIG. 4

Lineweaver–Burk (*a*) and Woolf–Augustinsson–Hofstee (*b*) plot of Initial Rate *versus* Substrate Concentration for Emulsin

1 7·5 ncat of emulsin with phenyl-β-D-galactopyranoside, *2* 6·25 ncat of emulsin with phenyl-β-D-glucopyranoside as substrate. In both cases citrate–phosphate buffer, pH 5·0 (3 ml, 30°C) was used.

A straight line was obtained when the data on emulsin were treated by the double reciprocal plot only when phenyl-β-D-glucopyranoside was used as substrate. With phenyl-β-D-galactopyranoside as substrate the experimental points corresponding to the highest concentrations used (40–100 mM) declined toward the abscissa (Fig. 4*a*). This showed that the enzyme preparation used contained two enzymes differing in affinity for this substrate. The corresponding kinetic constants of both enzyme components were calculated according to Neal[9] after extrapolation of both shoulders of the curve (Fig. 4*a*). The presence of two enzymes was confirmed by another diagnostic graph, *i.e.* by plotting initial rate v_0 *versus* $v_0/[s]$. A linear plot was again obtained only with phenyl-β-D-glucopyranoside as substrate. By contrast, the plot obtained with phenyl-β-D-galactopyranoside was concave (Fig. 4*b*) and the approximate K_m- and V_{max}-values for both enzyme components were calculated from the extrapolation of the linear parts of the curve. It can be concluded from a comparison of the K_m-values (Table II) that emulsin contains in addition to β-glucosidase itself (characterized by a higher K_m-value and thus a lower affinity toward phenyl-β-D-galactopyranoside) still another enzyme showing merely a marked β-galactosidase activity (lower K_m-value for phenyl-β-D-galactopyranoside). The ratio of glucosidase to galactosidase activity, calculated for this emulsin preparation, was 5, as follows from the specific activities measured (*cf.* Material). Helferich and Kleinschmidt[6], who obtained a value of 7·7 with their preparation, were not able to resolve the two activities on a preparative scale[8,10] even when using ion-exchange Sephadex columns.

A direct comparison of the values of kinetic constants, obtained with the hydrolases examined, with recorded data can be carried out yet only inexactly since the data presented in literature, if any, were mostly determined under different experimental conditions. The reported value for calf intestinal mucosa alkaline phosphatase[11] is K_m = 0·86 mM (38°C, 0·04M ethanolamine–HCl buffer, pH 10); this value is very close to that indicated in Table II.

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