Some Enzymic Properties of a Microbial β-Galactosidase of Human Whole Saliva

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The β-galactosidase of *E. coli*, acting on *o*-nitrophenyl-β-D-galactoside is usually considered to be a Na^+-activated enzyme. When lactose is the substrate, K^+ ions have been found to be the best activator.[1-5] Other monovalent cations also play a role in determining the affinity and activity.[2] β-Galactosidases of other organisms, *Saccharomyces*, *Lactobacillus*, and *Clostridium*,[3] are also activated by monovalent cations. It is stated that these enzymes also show divalent ion (Mg^{2+}, Mn^{2+} and Fe^{2+}) activation. This activation is masked in the *E. coli* enzyme until it is treated with complexing agents.[3]

The aim of this communication is in the first instance to provide information about the metal ion activation of a β-galactosidase of human whole saliva. This enzyme was found to originate from bacterial coverings of the teeth, the bacterial plaque. There may be several enzymes with β-galactosidase activity in the human whole saliva or bacterial plaque. The enzyme studied here formed most of the enzyme activity towards *o*-nitrophenyl-β-D-galactoside in the material investigated. Its origin in the bacterial plaque was ascertained by experiments (not given here in more detail) similar to those used earlier to show the identity of salivary and plaque proline iminopeptidases.[6] The β-galactosidase studied was shown to be Mg^{2+}-dependent (when *o*-nitrophenyl-β-D-galactoside was the substrate). A lower activation by K^+, Na^+, Li^+, and NH_4^+ ions was observed.

The substrate, *o*-nitrophenyl-β-D-galactoside, was obtained from Mann Research Laboratories, Inc. (New York, N.Y., U.S.A.). The estimation of the β-galactosidase activity was carried out in reaction mixtures consisting of the following ingredients: 0.3 ml of 0.05 M phosphate buffer (usually pH 7.0), 0.1 ml of 10^{-3} M substrate solution, 0.1 of water or aqueous solutions of compounds to be tested, and 0.1 ml of enzyme solution. The mixtures were incubated for various periods of time (usually 180 min) and the enzymic reactions were stopped by adding 1 ml of 0.2 N NaOH-solution (this amount was found to cause a sufficient change in the pH of the mixture to stop the enzymic hydrolysis of the substrate). The amount of liberated *o*-nitrophenol was determined on a Hitachi Perkin 139 Spectrophotometer at 410 mμ.

Human whole saliva and bacterial plaque were obtained and treated and the enzyme preparations made as described earlier.[6] In principle the enzyme preparations were fractionated on DEAE-cellulose columns as described previously with the exception that now the crude protein solutions were passed through Sephadex® G-25 column before the ion exchange chromatography. A typical result from such a fractionation is shown in Fig. 1. Only one highly active enzyme peak was observed. The infrequently occurring peaks with lower activity were not studied more closely. The resultant enzyme preparations did not hydrolyze *p*-nitrophenyl-α-D-glucoside. When the active fractions were pooled and the resultant enzyme preparations were

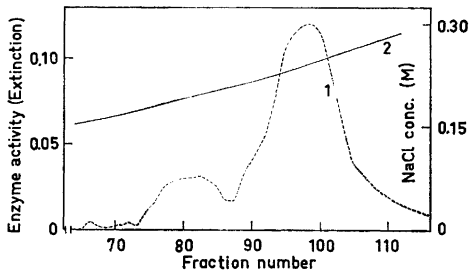

Fig. 1. Fractionation of β-galactosidase from human whole saliva supernatant fluid on DEAE cellulose. Column: 1.4 × 15.6 cm (Schleicher & Schüll, 230–270 mesh); elution buffer: 0.01 M tris-HCl buffer, pH 7.3, containing a sodium chloride gradient from 0 to 1.0 M; mixing volume: 150 ml + 150 ml. The crude enzyme preparation was passed before the DEAE cellulose chromatography through a Sephadex G-25 column with the above buffer. Enzyme activity and sodium concentration was estimated for each fraction (for estimating the sodium content, see Ref. 6). The enzyme peak possessing the highest enzyme activity was studied. No $MgCl_2$ was added to the reaction mixtures when the enzyme activity of the fractions was determined. 1 = enzyme activity; 2 = sodium chloride concentration.

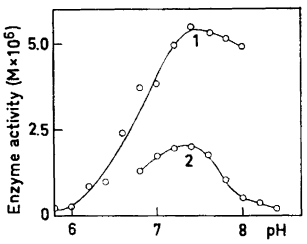

Fig. 2. Plot of pH against v (rate of the hydrolysis of *o*-nitrophenyl-β-D-galactoside, expressed as M min^{-1}) in the β-galactosidase-catalyzed hydrolysis of *o*-nitrophenyl-β-D-galactoside. 1=in 0.025 M phosphate buffer; 2=in 0.05 M tris-HCl buffer. No $MgCl_2$ was added.

tested for their enzymic properties, the following results were obtained.

The optimum pH for the hydrolysis of *o*-nitrophenyl-β-D-galactoside was 7.2—7.4, when tested in two different buffers. The pattern of the effect of pH on the rate of the hydrolysis is shown in Fig. 2 which also reveals that $tris^+$ ions inhibited the enzymic hydrolysis. The action of the enzyme preparations on the substrate was slightly (by appr. 10 %) activated by dithiothreitol (the best activation was obtained at 1.66×10^{-5} M activator). L-Cysteine at the same concentration inhibited the hydrolysis by appr. 20 %. *p*-Chloromercuribenzoate also caused inhibition: K_i values of 1.8×10^{-4} M were obtained from plots of $1/v_i$ against [I], where v_i is the rate of reaction in the presence of the inhibitor and [I] is the concentration of the inhibitor. On the other hand, *N*-ethylmaleimide did not inhibit to any large extent, by appr. 20—30 % only. L-1-Tosylamido-2-phenylethylchloromethyl ketone had no inhibitory effect, nor did phenylmethanesulphonyl fluoride inhibit the reaction. Guanidine hydrochloride, α-D-glucose, β-D-galactose, and saccharose were not inhibitory when used at the same concentrations as the substrate (1.66×10^{-4} M). The strongest inhibitor was EDTA which inactivated the enzyme completely at the concentrations tested (from 1.66×10^{-3} to 0.83×10^{-5} M). When the effects of various mono- and divalent metal cations on the reaction were tested, it was found that Mg^{2+} ions caused an appr. 80 % activation at 1.0×10^{-3} M. The other divalent metal cations tested (Fe^{2+}, Sn^{2+}, Co^{2+}, Pb^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}, Zn^{2+}, all as chlorides, except Pb^{2+} which was used as nitrate; all salts at concentrations ranging from 1.66×10^{-3} M to 8.3×10^{-4} M) inhibited the enzymic reaction. The stannous ions were most potent inhibitors. Of monovalent cations tested (Li^+, Na^+, K^+, NH_4^+) none caused activation when used as chloride at the same concentration as the divalent metal ions. As the enzyme preparations used contained monovalent $tris^+$ cations (due to the performing of the fractionations on DEAE cellulose with 0.01 M tris-HCl buffer, pH 7.3), it could be assumed that these cations could have acted antagonistically, masking the possible activating effect of Na^+ ions, for example. When the tris-HCl buffer was replaced by 0.05 M phosphate buffer, pH 7.0 (by passing the protein solutions in question through a Sephadex G-25 column), a slight activating effect by the monovalent cations mentioned was observed when they were tested in reaction mixtures buffered by phosphate buffer but Mg^{2+} ions again caused markedly higher activation.

When the apparent Michaelis constant, K_m, was determined by various graphical methods of plotting at four different $MgCl_2$ concentrations (*i.e.* without added $MgCl_2$, at 0.1×10^{-3} M, 0.3×10^{-3} M, and 1.0×10^{-3} M), the results shown in Table 1 were obtained, *i.e.* the value of the apparent K_m seemed not to be dependent on the concentration of Mg^{2+} ions present in the reaction mixture and, therefore, Mg^{2+} ions evidently had no effect on the affinity of the enzyme for its substrate. Plots of $v/[S]$ against v yielded essentially similar re-

Table 1. Effect of $MgCl_2$ on K_m. The values of the constant were determined graphically from plots of $1/v$ againts 1/[S] and $[S]/v$ against [S].

Concentration of $MgCl_2$ (M)	$K_m \times 10^4$	
	$1/v$ against 1/[S]	$[S]/v$ against [S]
Without added $MgCl_2$	0.75	0.80
0.10×10^{-3}	0.60	0.70
0.33×10^{-3}	0.75	0.80
1.00×10^{-3}	0.85	0.70

sults. The data obtained also showed that the kinetics of the hydrolysis of *o*-nitrophenyl-β-D-galactoside seemed fairly closely to follow the Michaelis-Menten behaviour under the restricted conditions employed.

The results obtained showed that the β-galactosidase briefly described in this communication resembles in many respects other microbial β-galactosidases. For example, one of the latest β-galactosidases studied, that of *Saccharomyces lactis*, acting on *o*-nitrophenyl-β-D-galactopyranoside, possesses a pH optimum at pH 7.2, and is activated by Mg^{2+} ions.[7] The organisms responsible for its production in the human oral cavity are unknown, although at least oral lactobacilli have been shown to be capable of forming this kind of enzyme. The best source of the enzyme studied seemed to be the soft bacterial covering of oral surfaces from which it can be obtained by extraction with buffers, or in greater amounts by disintegrating the microbial cells of the plaque with an ultrasonic disintegrator.

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Coordination Compounds of Phenylazothioformamides

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A characteristic difference between nickel(II) compounds of 2-alkylthiosemicarbazides and 1-alkylthiosemicarbazides is that the latter are easily oxidized in air, forming intensely coloured complexes. When sodium hydroxide is added to a solution of nickel(II) chloride and 1-methylthiosemicarbazide the usual brown innercomplex compound precipitates first but dissolves again in excess sodium hydroxide. This solution rapidly develops an intense blue colour, which originates at the phase boundary between air and solution and the colour development is accelerated by shaking the solution. The blue compounds formed from 1-methylthiosemicarbazide and other alkylthiosemicarbazides (except 1,4-di-*tert*-butylthiosemicarbazide) were too unstable for isolation. The 1-phenyl derivatives are more stable, however, but their stability is dependent upon the nature of the substituents in the 4-position. Very stable compounds were prepared from 1-phenyl-4-*tert*-butylthiosemicarbazide and from 1-phenyl-4,4-dialkylthiosemicarbazides (in the following discussion these ligands will be designated by LH_2). Oxidation of the innercomplex nickel(II) compounds, $Ni(LH)_2$, with iodine gave strongly coloured, high-melting crystalline compounds of the composition NiL_2 of high purity.

These compounds may be formulated as compounds with quadrivalent nickel — as was done for a corresponding derivative of thiobenzhydrazide [1] — but they may also be derived from the oxidized ligand, a phenylazothioformamide. It was found that phenylazothioformamides could be prepared in good yields by oxidation of thiosemicarbazides with benzoquinone (most other oxidants attack the sulfur atom). A few azothioformamides had been prepared earlier by essentially the same method [2] and after the completion of our work Pluijgers *et al.*[3] have prepared 1-