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Preliminary communication

Affinity labelling of β-D-galactosidase from *Escherichia coli* with D-[6-^{3}H]-galactal

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As far as investigations have been made, the interaction of glycosidases with the corresponding glycals invariably leads to the formation of 2-deoxyglycoses or 2-deoxyglycosides[1]. In two instances, the β-D-galactosidase from *Escherichia coli*[2] and the β-D-glucosidase A_3 from *Aspergillus wentii*[3], the glycals cause exceptionally strong inhibition of β-D-glycoside hydrolysis with a significant retardation of its full onset. This abnormal behaviour led to the assumption of a glycosyl–enzyme intermediate that is formed in a relatively slow chemical reaction. For β-D-glucosidase from *A. wentii*, such an intermediate was identified by Legler and Roeser[3] and proved to be a covalent 2-deoxy-D-*arabino*-hexosyl–enzyme complex. Earlier attempts of Wentworth and Wolfenden to prove the existence of a covalent 2-deoxy-D-*lyxo*-hexosyl intermediate in the case of β-D-galactosidase from *E. coli* through incorporation of tritium from the incubation medium, failed[2]. The recent results of Legler and Roeser[3] prompted us to try the covalent modification of β-D-galactosidase with D-[6-^{3}H]-galactal (19.4 Ci/mol), which was prepared by oxidation of D-galactal[4] with D-galactose oxidase and reduction of the resultant 6-aldehyde with[5] NaB^3H_4.

Incubation of β-D-galactosidase with D-[6-^{3}H]-galactal and subsequent denaturation with guanidine hydrochloride gave ^{3}H-labelled protein, which could be separated from low-molecular-weight material by gel filtration (Fig. 1). The eluted protein (3rd–5th mL of eluate, Fig. 1) could be precipitated by adding 10 vol. of water. The precipitate accounted for 99% of the radioactivity in these fractions, indicating covalent labelling of the protein with D-[6-^{3}H]-galactal. Further proof for the covalent attachment of radioactivity to the protein was obtained by SDS–disc-gel electrophoresis. Radioactivity was associated only with protein (Fig. 2).

For determination of stoichiometry, a known amount of labelled protein was precipitated without preceding gel filtration, centrifuged, and washed twice with water. The radioactivity* corresponded to 3.66 mol of D-[6-^{3}H]-galactal per mol of β-D-galacto-

*Radioactivity was measured in a liquid scintillation-counter model LS 7000 (Beckman Instr. GmbH, Munich, F.R.G.). Aqueous samples were counted in Unisolve I (Werner Zinsser, Frankfurt, F.R.G.). Protein precipitates and gel discs were counted in Quickszint 501 (Werner Zinsser) after treatment with 1 mL of 13:2 Protosol (New England Nuclear, Dreieich, F.R.G.)–water.

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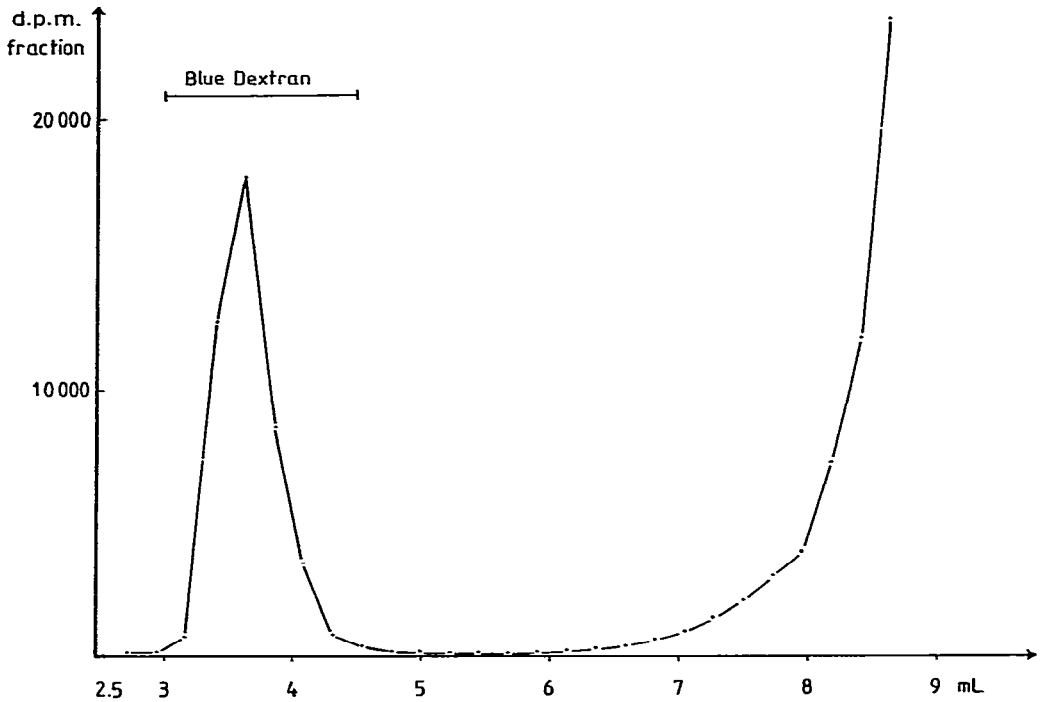


Fig. 1. Gel filtration of β-D-galactosidase after incubation with D-[6-^{3}H]-galactal and denaturation. A solution (40 μL) of β-D-galactosidase from *E. coli* K 12 strain 3300 (9 mg/mL in sodium phosphate buffer pH 6.8 (50mM) containing magnesium chloride (mM) was incubated for 30 min at 30° with D-[6-^{3}H]-galactal (5mM, 19.4 Ci/mol). The enzyme was denatured by addition of a solution (60 μL) of guanidine hydrochloride (8M) and EDTA (10mM) in sodium phosphate buffer, pH 7.0 (0.1M), and diluted with the solution (0.1 mL) used in gel filtration. Gel filtration of the whole sample (0.2 mL) was performed in potassium sodium phosphate buffer pH 7.0 (0.1M), containing guanidine hydrochloride (4M) and EDTA (mM) on Sephadex G50 superfine (column dimensions 1.5 × 6.5 cm; flow rate 0.15 mL/min).

sidase. In the same way that the enzyme catalized formation of 2-deoxy-D-*lyxo*-hexosides from D-galactal[6], the covalent modification of β-D-galactosidase with D-[6-^{3}H]-galactal could be inhibited by isopropyl 1-thio-β-D-galactopyranoside (Fig. 3).

Our investigations have proved the covalent attachment of four molecules of D-galactal per molecule of β-D-galactosidase, an enzyme composed of four subunits. The native enzyme-inhibitor intermediate disintegrates in a first-order reaction with a rate constant of 3.3×10^{-3}/s (Fig. 3). Indirect kinetic methods, by Wentworth and Wolfenden[3], and by Viratelle and Yon[7], gave at 25° and pH 7 very similar rate constants of 4.6×10^{-3}/s and 2.5×10^{-3}/s, respectively.

The spontaneous disintegration of the native enzyme-inhibitor intermediate yields the reactivated β-D-galactosidase and 2-deoxy-D-[^{3}H]-*lyxo*-hexose, identified by co-chromatography with an authentic sample. The denatured enzyme-inhibitor intermediate is reasonably stable between pH 3 and 8 (Figs. 4a and 4b).

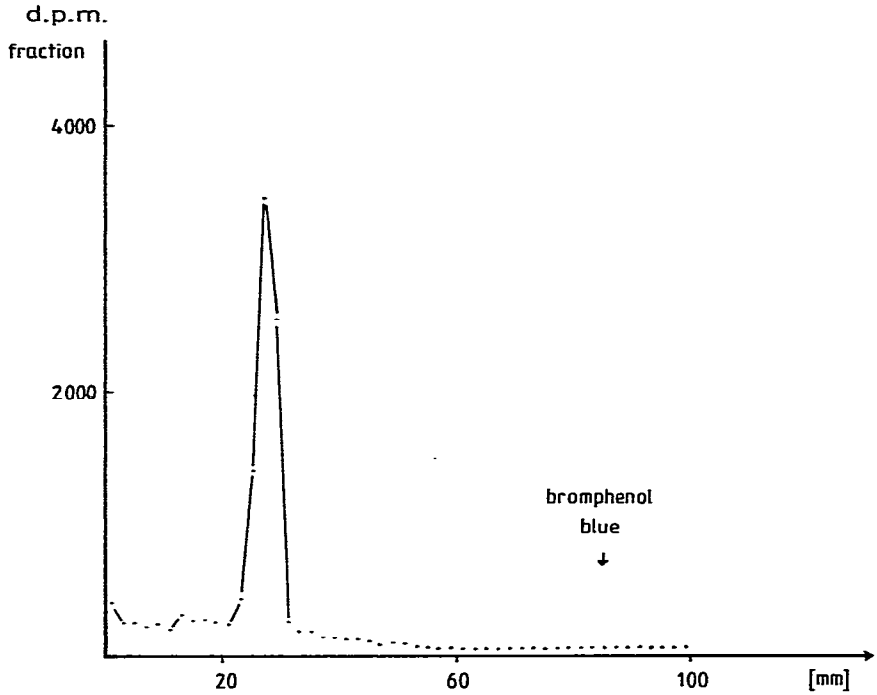


Fig. 2. Distribution of radioactivity after dodecylsulfate–poly(acrylamide) gel electrophoresis of β-D-galactosidase labelled by D-[6-^{3}H]-galactal. The discontinuous dodecylsulfate–polyacrylamide gel electrophoresis[8] was performed in tubes (6 mm dia. × 170 mm) according to O'Farrell[9], after precipitation of incubated enzyme (see legend to Fig. 1). The total acrylamide concentration of the gels was 7.5% at a ratio of acrylamide to bisacrylamide of 97.3:2.7. The length of the separation gels was 110 mm. Samples (90 μg) of protein were applied after heating for 5 min to 95°. The temperature during electrophoresis was maintained at 10° and the current was kept constant at 1 mA/gel tube. After electrophoresis, one gel was cut into 2-mm thick slices in which the radioactivity was determined. A second gel was fixed with 12.5% trichloroacetic acid and stained with Coomassie Brilliant Blue R 250.

However, it suffers rapid hydrolysis under more-acidic as well as more-basic conditions. These properties correspond well with the formation of a covalent 2-deoxy-D-*lyxo*-hexosyl–enzyme intermediate, where the glycosidic attachment to the protein is mediated through an ester bond.

The results prove the existence of a covalent 2-deoxy-D-*lyxo*-hexosyl–protein in the denatured state. The question as to whether such an intermediate actually exists in the native state and whether as such it is obligatory for the production of 2-deoxy-D-*lyxo*-hexose from D-galactose cannot be answered. It could well be that the native 2-deoxy-D-*lyxo*-hexosyl–enzyme intermediate exists as an ion pair that collapses to the covalent complex on denaturation.

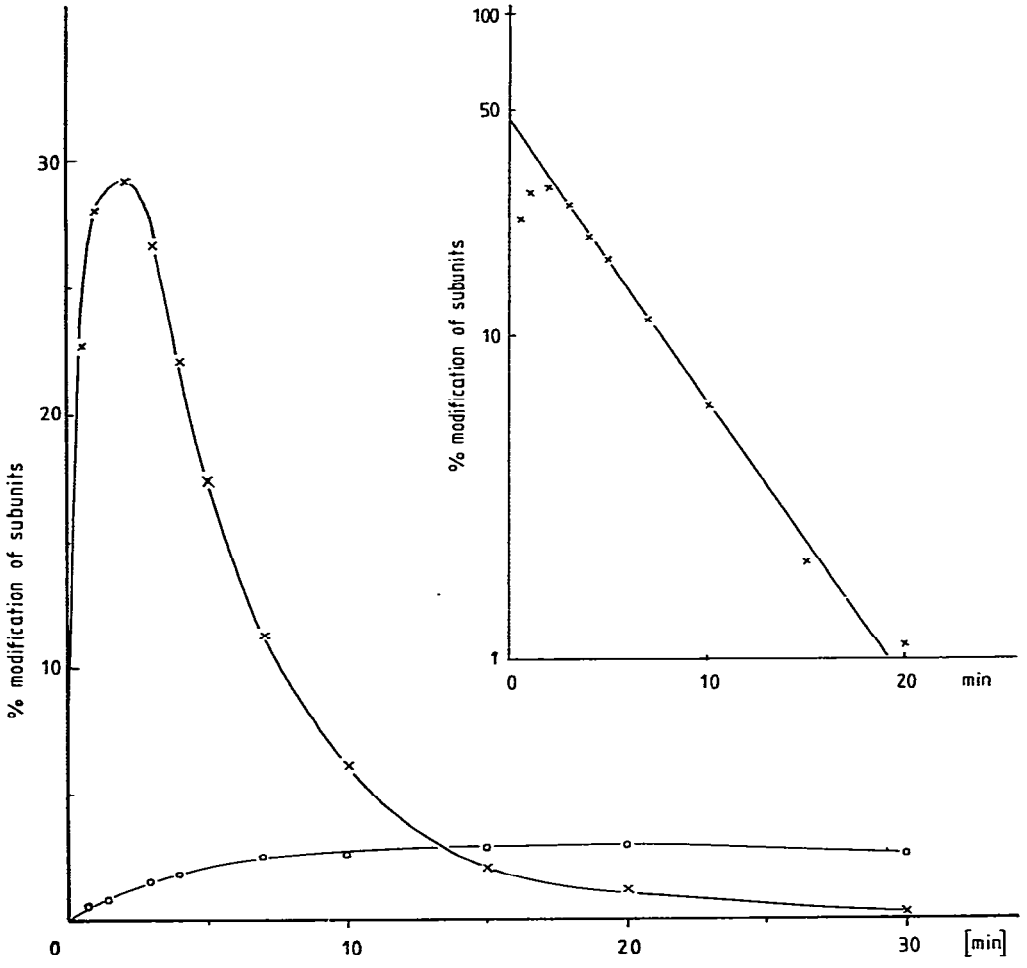


Fig. 3. Kinetics of β-D-galactosidase modification by D-[6-^{3}H]-galactal and its inhibition by isopropyl 1-thio-β-D-galactopyranoside. (a) (X—X). β-D-Galactosidase (7.9 mg/mL) was incubated with 30 μM D-[6-^{3}H]-galactal as indicated in Fig. 1 (modification under these conditions cannot be >50%). Aliquots were taken after time intervals, and the protein was denatured and precipitated as described in the legend of Fig. 1. The radioactivity of the pellet was determined by liquid-scintillation counting. Under these conditions, the rate of the onset of modification (first 3 min) is too high to be measured accurately.
(b) (O—O). Conditions for incubation, denaturation, and precipitation of protein were the same as described under (a), except that 42.4mM of isoproyl 1-thio-β-D-galactopyranoside was added.

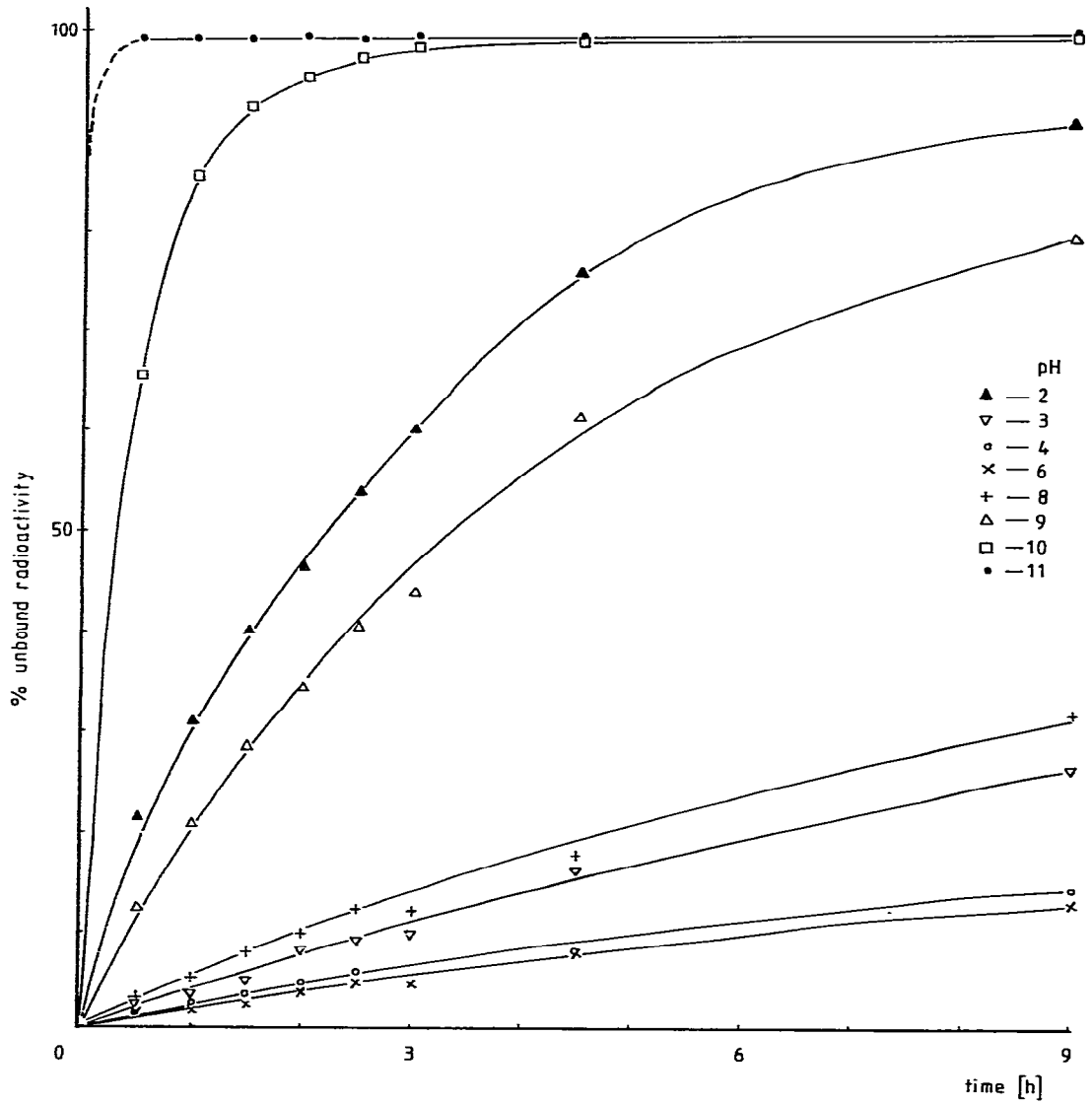
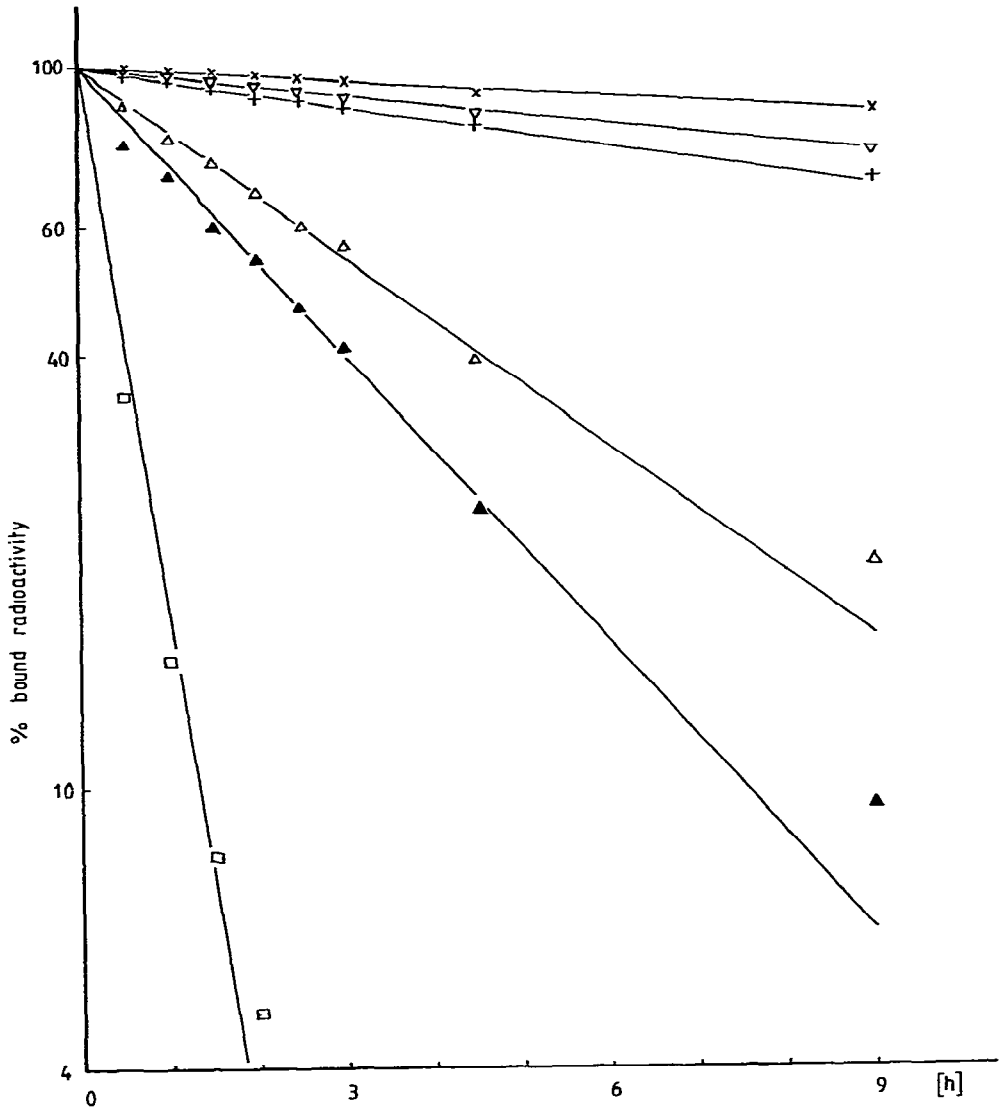


Fig. 4. (above and facing page). pH Stability of the denatured enzyme-inhibitor intermediate, β-D-Galactosidase (14.5 mg/mL) was incubated, denatured, and chromatographed as described in Fig. 1. Aliquots of the column eluate containing the radioactive protein were adjusted to pH 2–11 with 2M hydrochloric acid or 2M sodium hydroxide, respectively, and stored at 30°. After time intervals, aliquots of each of the solutions were diluted with 50 vol. of 0.1M potassium sodium phosphate buffer pH 6.8, centrifuged, and washed. Radioactivity in the pellet and supernatant solution was determined by liquid-scintillation counting.



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