Thermal Lability of Beta Galactosidase from Pink Salmon Liver

Abstract. *The effect of heat on the stability of a protein isolated from a cold-water fish was tested by following the decrease in enzymatic activity of beta galactosidase, purified from livers of Pacific pink salmon. The thermal stability is* p*H-dependent; it is highest at* p*H 4 and lowest at neutral* p*H, where only 30 percent of the activity remains after 10 minutes at 40°C. Comparative experiments demonstrate greater thermal stability of beta galactosidase from rat liver.*

The spawning run of salmon is an impressive and complex biological phenomenon. The fish endure an extended period of starvation and severe environmental stress accompanied by a multitude of physiological and biochemical changes. During the migration of the pink salmon from the cold water of the northern Pacific Ocean to the warmer rivers of British Columbia, the temperature of the environmental waters may increase by 10° to 15°C; this increase is, however, unlikely to exceed 20°C. We now report the effect of temperature on the activity of a purified enzyme from these fish. For this purpose we utilized β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.25), an enzyme which is stable and can be easily purified (*1*). This enzyme was purified 55-fold from the liver of pink salmon (*Oncorhynchus gorbuscha*); its thermal stability was studied and compared with that of the similar enzyme isolated from rat liver. Extensive studies have been conducted on the effect of temperature on poikilothermic animals but only relatively few on purified enzymes from fish (*2*).

The purification and properties of salmon liver β-galactosidase are as follows. Pink salmon (females) were caught in Namu on the coast of British Columbia in salt water, and were maintained for 1 week in fresh, running water, in a shallow pool, at a temperature of about 15°C. They were killed by a blow on the head, and the livers were immediately excised, chilled, and homogenized with 0.25*M* sucrose (9 ml per gram of liver). The homogenate was centrifuged for 10 minutes at 800*g*, and the supernatant was decanted and centrifuged for 15 minutes at 16,000*g*. The sediment thus obtained was dispersed in 0.25*M* sucrose (0.6 ml per gram of liver). The suspension was frozen at − 40°C and thawed, this procedure being repeated two more times. The suspension was then centrifuged for 70 minutes at 36,000*g*. The precipitate was suspended in 0.25*M* sucrose (0.6 ml per gram of liver); the suspension was frozen and thawed once, it was centrifuged for 35 minutes at 36,000*g*, and the two supernatants were combined.

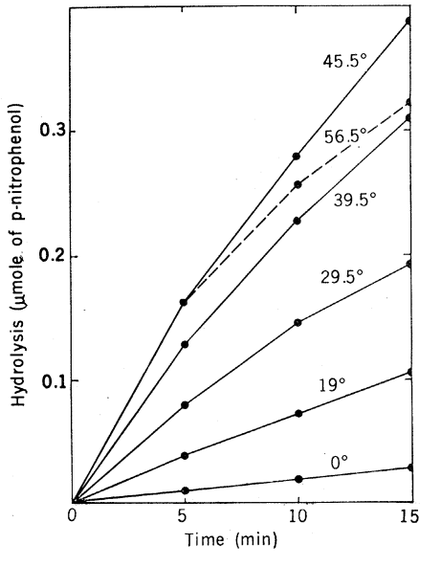

Fig. 1. Effect of temperature on salmon liver β-galactosidase at *p*H 4. Incubation mixtures, in volumes of 0.5 ml, contained 1 μmole of *p*-nitrophenyl β-D-galactoside, 80 μmole of sodium citrate buffer, *p*H 4.0, and 135 μg of salmon liver β-galactosidase (a fraction obtained at 40 to 50 percent saturation with ammonium sulfate). At the specified times, 2.5 ml of 0.25*M* glycine-carbonate buffer, *p*H 10, was added, and the color of the *p*-nitrophenol was read at 420 nm. The temperatures tested were from 0° to 56°C.

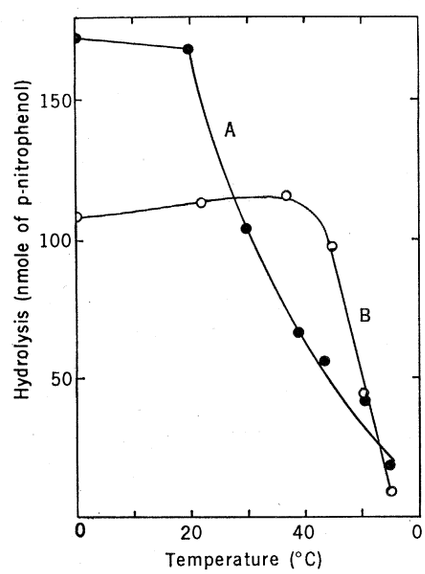

Fig. 2. Effect of temperature on β-galactosidase from salmon liver and rat liver at neutral *p*H. Mixtures containing 5 μmole of sodium phosphate, *p*H 7.0, enzyme, and water, in a volume of 0.4 ml, were incubated for 10 minutes at the specified temperature without substrate. One micromole of *p*-nitrophenyl-β-galactosidase and 80 μmole of sodium citrate buffer, *p*H 4.0, were then added, and the tubes were further incubated for 10 minutes at 37°C. After 0.2 ml of 1*N* NaOH and 2.3 ml of 0.25*M* glycine-carbonate buffer *p*H 10 were added, the color was read at 420 nm. Curve A represents 135 μg of salmon liver β-galactosidase (40 to 50 percent ammonium sulfate fraction); curve B represents 550 μg of rat liver β-galactosidase (*3*).

The *p*H of this solution was adjusted to 4.5 with 1*M* acetic acid; the precipitate was removed by centrifugation for 10 minutes at 13,000 rev/min, and the supernatant was adjusted to *p*H 7.2 with 1*N* NaOH. Solid ammonium sulfate was added, and the protein fractions precipitating at 40, 50, 60, and 80 percent saturation were collected by centrifugation for 10 minutes at 36,000*g*. They were each dissolved in 3 ml of 10 m*M* sodium phosphate buffer, *p*H 7.0, and dialyzed (twice) against 2 liters of the same solution. The contents of the dialysis bags were clarified by centrifugation for 10 minutes at 25,000*g* and stored frozen at − 20°C. The fraction that precipitated at 40 to 50 percent saturation had a 55-fold higher specific activity than the homogenate minus debris. The other ammonium sulfate fractions yielded enzyme that was purified 20- to 32-fold.

With *p*-nitrophenyl-β-D-galactopyranoside as substrate, the enzyme exhibited optimum activity at *p*H 3.6 at 26°C, and at *p*H 4.2 at 37°C. The latter shift is most probably due to heat inactivation of the enzyme below *p*H 4.4 (Fig. 3, curve A). It had a K_m (Michaelis constant) of 0.6 m*M* and a maximum velocity V_{max} of 7 μmole per milligram of protein per hour when measured at 30°C; the K_m value was independent of temperature between 10° and 30°C. The enzyme hydrolyzed lactose at about one-ninth of the corresponding rate of hydrolysis of *p*-nitrophenylgalactoside; it still possessed some α-galactosidase,

α-glucosidase, and phosphatase activities. The enzyme was inhibited by galactose (50 percent inhibition at 25 m*M* galactose and 2 m*M* substrate) and by *p*-hydroxymercuribenzoate (50 percent at $10^{-5}M$) but not by 1 m*M* *N*-ethylmaleimide. The inhibition by *p*-hydroxymercuribenzoate could be partly reversed by further addition of dithiothreitol.

The effect of temperature on enzymatic activity is shown in Figs. 1 to 3. In an experiment not shown in these figures, it was observed that the rate of hydrolysis of *p*-nitrophenyl-β-galactoside, when measured at 25° or 37°C in a buffer at *p*H 4, was almost linear for 90 minutes. Furthermore, only little deviation from linearity was obtained when enzyme and substrate were incubated at *p*H 4, for 10 minutes at temperatures up to 45°C (Fig. 1). It was, however, observed that at neutral *p*H, the salmon liver β-galactosidase was inactivated at temperatures above 20°C (Fig. 2, curve A). Thus, after 10 minutes at 30°C, only about two-thirds of the activity remained, and at 40°C, only one-third. This thermal inactivation at neutral *p*H was not prevented by the addition of substrates (2 m*M* *p*-nitrophenylgalactoside or 10 m*M* lactose), product (up to 50 m*M* galactose), or 1 m*M* dithiothreitol. In an experiment comparable to that shown in Fig. 2A, but in which the enzyme was first incubated without substrate for 10 minutes at *p*H 4, the respective losses at 30°C and 40°C were 10 and 25 percent of the β-galactosidase activity. For comparison, the effect of temperature on the activity of rat liver β-galactosidase (*3*) is shown in Fig. 2, curve B. Under similar experimental conditions all the activity of the latter enzyme was recovered at 40°C, but inhibition ensued at higher temperatures. [Similar results for the rat-liver enzyme have been reported (*4*).] It has also been shown that β-galactosidase from *Escherichia coli* lost no activity when heated for 30 minutes at 40°C, between *p*H 6 and 9 (*5*).

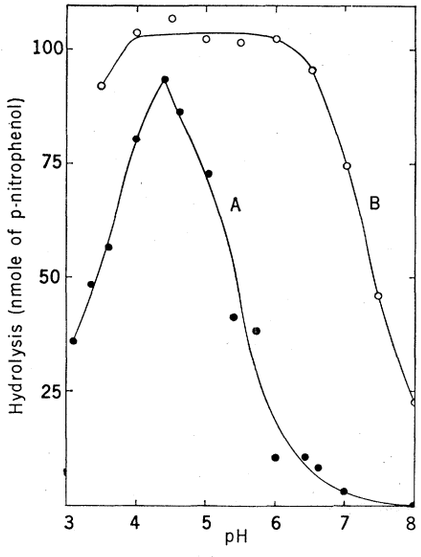

Fig. 3. Effect of *p*H on the thermal stability of β-galactosidase from salmon liver and rat liver. Mixtures containing 25 μmole of phosphate-citrate buffer at the specific *p*H values, enzyme, and water (in a volume of 0.35 ml) were incubated without substrates for 10 minutes at 48°C. Then 1 μmole of *p*-nitrophenyl-β-galactoside and 125 to 150 μmole of sodium citrate buffer, *p*H 4.0, were added. The tubes were further incubated for 10 minutes (at 28°C for the salmon liver enzyme and at 37°C for the rat liver enzyme). After addition of 0.3 ml of 1*N* NaOH and 2.2 ml of 0.25*M* glycine-carbonate buffer, *p*H 10, the color was read at 420 nm. Curve A represents 135 μg of salmon liver β-galactosidase; curve B represents 550 μg of rat liver β-galactosidase.

The difference in the response of salmon liver β-galactosidase to temperature at *p*H 4 and at *p*H 7 is amplified by the data shown in Fig. 3, curve A. The enzyme shows maximum thermal stability at about *p*H 4, but at *p*H values below or above this value the enzyme is rapidly inactivated at elevated temperature (Fig. 3, curve A). For comparison, the thermal responses of rat liver β-galactosidase, are shown in Fig. 3, curve B. Practically all the activity of the latter enzyme was recovered after 10 minutes at 48°C from *p*H 3.5 to 6.5.

The greater thermal lability of the salmon liver β-galactosidase at neutral *p*H requires special comment. Liver β-galactosidase is of lysosomal origin, with an optimum activity at about *p*H 4 (at *p*H 6 the rate of hydrolysis is 20 percent that of the optimum and at *p*H 7 about 10 percent). Considerable evidence has accumulated that the main function of the lysosomes involves the digestion of a variety of substrates of extra- or intracellular origin (*6*). Since many lysosomal enzymes have very low activities at neutral *p*H, it must be assumed that the lysosomes have an acidic microenvironment, separated from the rest of the cell. If so, the β-galactosidases of either salmon liver or rat liver exist in the lysosomes in an environment imparting to them their maximum thermal stability. If the lysosomes rupture or leak, this enzyme will spill into the cell sap, where it is a "foreign" protein which must be inactivated to prevent it from damaging the cell contents. The neutral or slightly alkaline *p*H of the cell sap will facilitate such an inactivation.

SHIMON GATT*
Scripps Institution of Oceanography, University of California San Diego, La Jolla, California

References and Notes

1. S. Gatt and M. M. Rapport, *Biochim. Biophys. Acta* **113**, 567 (1966); F. B. Jungalwala and E. Robins, *J. Biol. Chem.* **243**, 4258 (1968); A. J. Furth and D. Robinson, *Biochem. J.* **97**, 59 (1965); D. Robinson, R. G. Price, N. Dance, *Biochem. J.* **102**, 525 (1967); F. Chytil, *Biochem. Biophys. Res. Commun.* **19**, 630 (1965).
2. For reviews on the effect of temperature on protein and enzyme stability, see S. Grisolia, *Physiol. Rev.* **44**, 657 (1964); A. S. Troshin, Ed., *The Cell and Environmental Temperature* (Pergamon Press, New York, 1967); C. L. Prosser, Ed., *Molecular Mechanisms of Temperature Adaptation* (AAAS, Washington, D.C., 1967).
3. The rat liver β-galactosidase was prepared by a method similar to that used with salmon liver except that the treatment at *p*H 4.5 was omitted. Protein precipitating between 40 and 60 percent with ammonium sulfate was collected, dialyzed against 10 m*M* sodium phosphate, *p*H 7.0, and stored at —20°C.
4. T. J. Langley and F. R. Jevons, *Arch. Biochem. Biophys.* **128**, 312 (1968).
5. K. Wallenfels, M. L. Zarnitz, G. Laule, H. Bender, M. Keser, *Biochem. Z.* **331**, 459 (1959).
6. C. de Duve, in *Subcellular Particles*, T. Hayashi, Ed. (Ronald Press, New York, 1959), p. 128; ——— and R. Wattiaux, *Ann. Rev. Physiol.* **28**, 435 (1966).
7. This investigation was performed on the R.V. *Alpha Helix*, as part of its Bering Sea Expedition (supported by NSF grant GB 7173). Phase IV of this Expedition consisted of an investigation of the biochemistry and physiology of salmon during the spawning season. It was supported in part by NIH grants NB-02967 and GM-12310. I thank Dr. A. A. Benson for review of the manuscript and for discussions.
* Permanent address: Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

11 February 1969; revised 20 March 1969

Protective Effect of Antilymphocyte Serum on Mice Infected with Plasmodium berghei

Abstract. *Rabbit antiserum to mouse lymphocytes prolonged the survival of mice infected with* Plasmodium berghei. *Since antilymphocyte serum has a well-defined, potent immunosuppressive effect, the immune response of the host to the parasite actually may contribute to the death of the animal with an acute malarial infection.*

Plasmodium berghei infection in mice is fulminating (*1*), death of the animals being due to the development of high parasitemia accompanied by profound anemia (*2*). While most observers attribute the anemia to infection and subsequent destruction of