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Temperature-Dependent Behavior of Immobilized Alkaline Phosphatase. II. Temperature Pulse

A Tool to Enhance Enzymatic Activity for Prolonged Periods

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Abstract

The specific activity of immobilized alkaline phosphatase can be considerably increased by exposure to a brief temperature pulse. The thus-pulsed preparations show a hysteresis of higher activity at room temperature varying from 10 min to several days, depending upon their pretreatment and the conditions of the reaction mixture during the pulse. In almost all cases the pulse can be repeated many times to increase the activity.

Index Entries: Immobilized alkaline phosphatase, effect of temperature pulsing on activity; alkaline phosphatase, effect of temperature pulsing on activity of immoiblized; temperature pulsing, effect on immobilized alkaline phosphatase activity; enzymatic activity, and temperature pulsing.

Introduction

Alkaline phosphatase of animal source (E.C.3.1.3.1.) is a rather thermolabile enzyme. It could be shown (*1*) that this enzyme, covalently bound to silane-coated controlled-pore glass beads, showed an increase in specific activity (up to several

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hundred percent, in fact) when assayed at elevated temperatures. Nevertheless its stability was very weak under these conditions, and the activity declined to virtually zero after 2 h.

At room temperature, these immobilized enzyme preparations were relatively stable, but the enzyme activity was much smaller than at elevated temperatures. In this paper it is shown that, in contrast to the native enzyme, the immobilized alkaline phosphatase shows a hysteresis of increased activity at room temperature after being subjected to a pulse of heat of only several minutes duration. This allows an increase in the specific activity of the enzyme by temperature influence without obviously disturbing the active configurations of the enzyme. Optimal conditions for this treatment have been worked out and are presented and discussed in this article.

Materials and Methods

Alkaline phosphatase from hog intestine, phosphoenolpyruvate, 3-aminopropyltriethoxysilane, and 2,4,6-trinitrobenzene-sulfonic acid (TNBS) were obtained from the Sigma Chemical Co., of St. Louis; all buffer substances, glutaraldehyde, *p*-nitrophenylphosphate, and ninhydrin were purchased from Merck, Darmstadt. Controlled-pore glass (CPG-10, mesh size 120/200) was acquired from Serva.

The assays of the native and the immobilized enzymes were performed in rotating vessels according to Bessey et al. (*2*).

The preparation of the carrier for immobilization of the enzyme was carried out according to Weetall (*3*) and Pittner et al. (*4*).

The enzyme was immobilized at pH 5, pH 7, and pH 8, respectively, following the procedure described in the preceding paper (*1*). As in the foregoing article, these different immobilized enzyme preparations will be called type I, II, and III.

Results

Influence of Temperature Pulses on the Activity of the Immobilized Phosphatase

Preliminary tests showed that in contrary to the native enzyme the immobilized phosphatase had a hysteresis of increased activity at room temperature when preheated for a short time to a temperature between 40 and 55°C. This range lies below the temperature optima of the various gel types [see (*1*), Fig. 1] and was chosen to avoid the risk of overheating, since the stability decreases rapidly when the optimal temperature is exceeded.

For a further study of temperature pulse behavior, it was necessary to determine the specific activity of all three types of this immobilized enzyme at room temperature and various pH conditions because of the striking influence of the pH on the stability and the specific activity of the immobilized phosphatase (*1*). (See Table 1.)

Table 1
Activities of the Various Types of Immobilized Phosphatase at Room Temperature (20°C) When Assayed at pH 8, pH 9.5, and pH 10.5, Respectively

Type of immobilized enzyme	Activity (μkat/g bound protein) measured at 20°C		
	pH 8	pH 9.5	pH 10.5
I	13	52	16
II	15	55	15
III	9	43	18

Optimal Pulse Time

To determine the optimal pulse time, batches (20 mg of packed glass beads) of the types I, II, and III of immobilized phosphatase suspended in buffer solutions of pH 8, pH 9.5, and pH 10.5 were subjected to temperatures of 40, 50, and 55°C for 1–6 min. The change in activity with pulse time was measured at room temperature immediately after the pulse treatment. The optimal pulse times varied between 1 and 4 min (Table 2).

Hysteresis of Increased Activity at Room Temperature

Batches of the various types of gels (10–20 mg) were subjected to temperature pulses of 40, 50, and 55°C during their respective optimal pulse times, according to Table 2. The results are shown in Figs. 1–3.

In all cases activity remained at a higher level during a certain period of time (varying from 10 min to several days), but more or less declining at the beginning before reaching a relatively constant level.

The temperature employed, the pH of the reaction mixture, as well as the type of immobilized enzyme were responsible for the level of increased activity and its conservation at room temperature for a certain period of time.

In almost all cases the pulse could be repeated many times increasing activity each time. Only the extremely high increase in activity observed after pulsing at pH 10.5, which is always followed by a rapid decline of the activity, cannot be renewed to the same level by other pulses.

Moreover, under such conditions the stability was diminished, whereas when the increased activity was lower, it remained constant for a longer time. The poorest results with respect to the increase in absolute specific activity were obtained at pH 8 with all types of immobilized enzyme (Table 1). However, under these conditions the increased activity could be maintained nearly unchanged for days, type II being the only exception.

A rather high stimulation and stabilization of activity could be observed at pH 9.5 under optimal pulse conditions. Though this increase was not more than about

Table 2
Optimal Pulse Time at Various Temperature and pH Conditions

Type of immobilized enzyme	Pulse temperature, °C	pH of the incubation mixture	Optimal pulse time, min
		8	4
I	40	9.5	3
		10.5	3
		8	3
I	50	9.5	3
		10.5	3
		8	3
I	55	9.5	3
		10.5	3
		8	4
II	40	9.5	4
		10.5	4
		8	4
II	50	9.5	4
		10.5	3
		8	4
II	55	9.5	4
		10.5	1
		8	4
III	40	9.5	2
		10.5	4
		8	3
III	50	9.5	2
		10.5	2
		8	2
III	55	9.5	2
		10.5	1

200% of the initial activity at room temperature the absolute activity was high (see Table 1). Again type II showed the poorest results.

Discussion

The studies presented show that it is possible to increase the specific activity of an immobilized enzyme—even of a rather thermolabile one—at room temperature by treatment with a temperature pulse.

It has to be pointed out that it is possible to improve the properties of the phosphatase drastically by this technique. As shown in the preceding paper, the enzyme

can be immobilized while fully preserving its activity under native conditions. As a result of temperature pulsing under optimum conditions, the activity at room temperature could be further increased and preserved for a certain time, which is not the case with the native phosphatase. So it is possible to obtain a preparation of an immobilized enzyme with an activity at room temperature higher than that of the native enzyme.

From Figs. 1–3 one can see that the difference between the various types of immobilized phsophatase is greater than one would expect from the data discussed in the preceding paper (*1*). When pulsed and assayed at pH 8, types I and III showed an increase in specific activity that could be preserved for days without significant loss of activity and stability. However, the activity of type II, although also increased by pulse treatment, declines already within an hour to the value of the unpulsed preparation. On the other hand, types I and III, which behave so similarly at pH 8, show significant differences at pH 10.5 in the percent value of excitation when pulsed with increased temperature. The dramatic increase in activity at optimum pulse conditions (400–700%) followed by a rapid decrease to about 200% activity (compared to the unpulsed preparation) could be maintained for hours without further decrease. This may result from different modifications of the immobilized enzyme, with the various immobilized states seemingly more or less stabilized by covalent bonds. More stabilized states cannot be raised to extremely high levels of activity, but they are rather stable against denaturation.

On the other hand highly activated states undergo a rapid decline in activity, followed by a considerable degree of denaturation. This behavior can be demonstrated when such immobilized enzyme preparations are pulsed again several times after having reached low values of activity. Under such conditions the high initial activity could not again be attained. From pulse to pulse the peak value of specific activity declines until the constant amount is reached that always follows the rapid decline. (See Figs. 1–3.)

This decreased activity, obviously due to more stabilized states, usually remains constant for more than 10–15 additional pulses without a futher decline in activity. When treated at pH 8 and pH 9.5, such effects do not appear, though the absolute increase in activity under these conditions is sometimes rather high. (See Figs. 1–3 and Table 1). Especially after treatment at pH 9.5 the stability was much better than in all other cases and the increased activity could be entirely preserved for a longer period of time.

With thermolabile phosphatase kept at appropriate temperatures, the optimal pulse-time must be observed and must not be exceeded. Otherwise a rapid decline in activity, as well as significant denaturation, may result. This can be seen from Figs. 1b, 1c, 2b, and 2c where, in addition to optimum pulse conditions, longer pulse times were also used at 55°C.

When pulsed at lower temperature, where broad pulse-time optima occurred, this effect was not so significant, as can be seen, e.g., in Fig. 2c when batches were pulsed at 40°C.

The studies published in this paper show a new possibility for further enhancement of the activity of immobilized enzyme preparations. In previous papers (*4–6*)

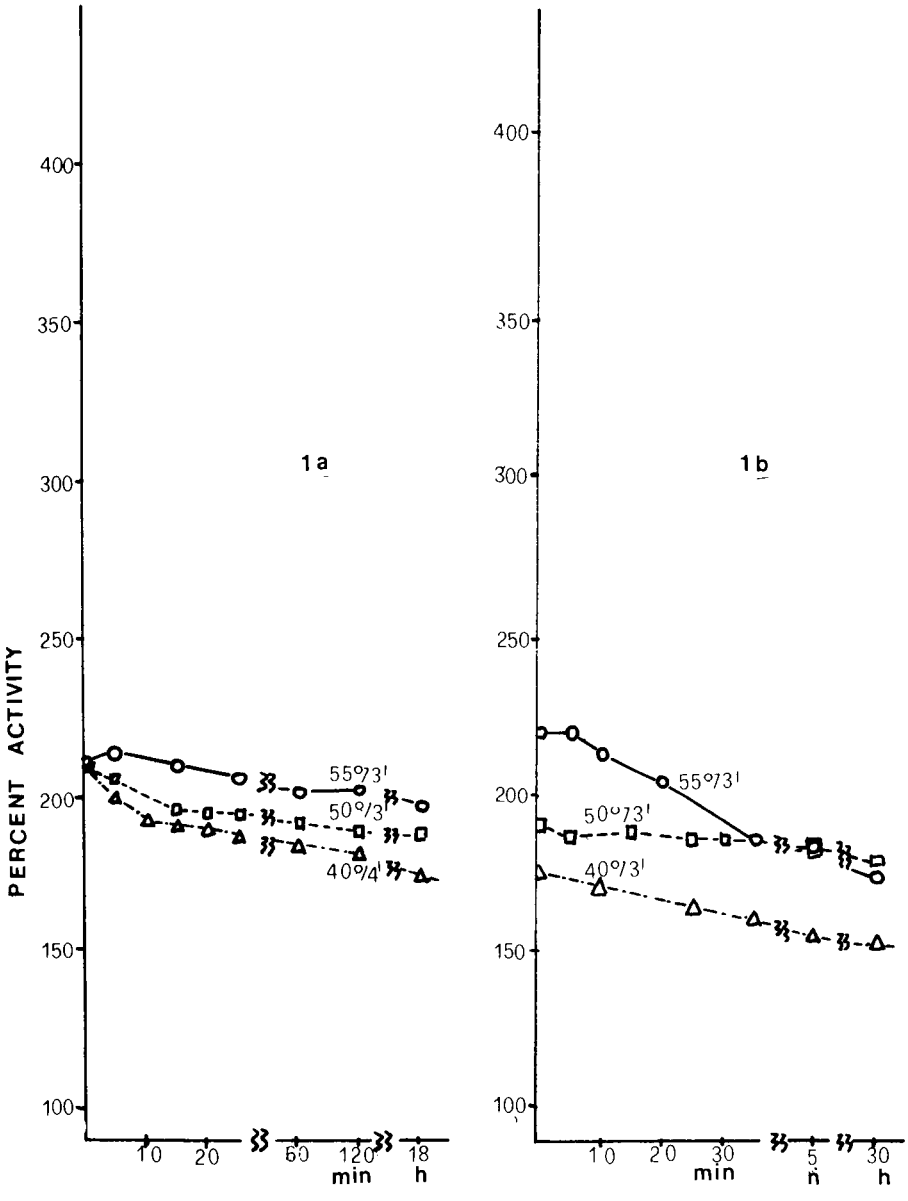

Fig. 1. Immobilized phosphatase Type I: Hysteresis of increased activity, after pulsing of immobilized phosphatase, as a function of the time when stored and assayed at room temperature. 100% Activity resembles the activity of the unpulsed immobilized enzyme at room temperature according to Table 1: (a) buffer conditions at pH 8; (b) buffer

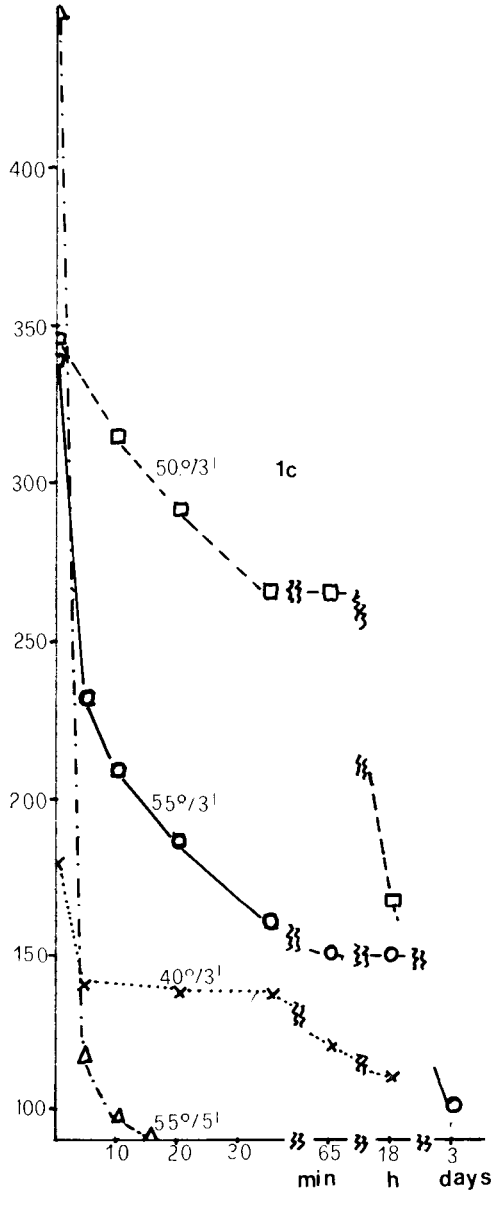

Fig. 1 (*cont.*)

conditions at pH 9.5; (c) buffer conditions at pH 10.5. The respective pulse temperatures and pulse times are added in the figures.

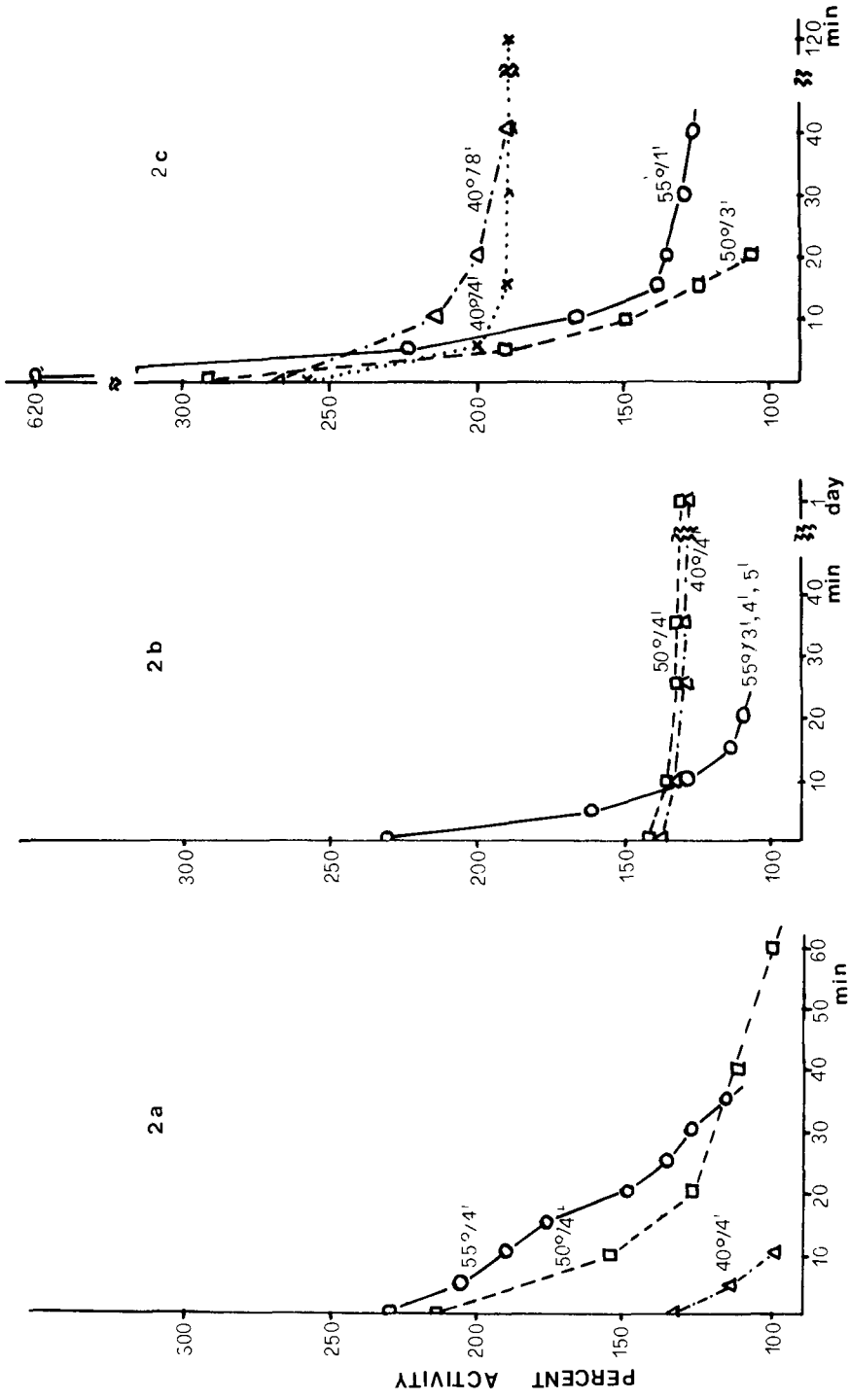

Fig. 2. Immobilized phosphatase Type II. (For details, see Fig. 1 caption).

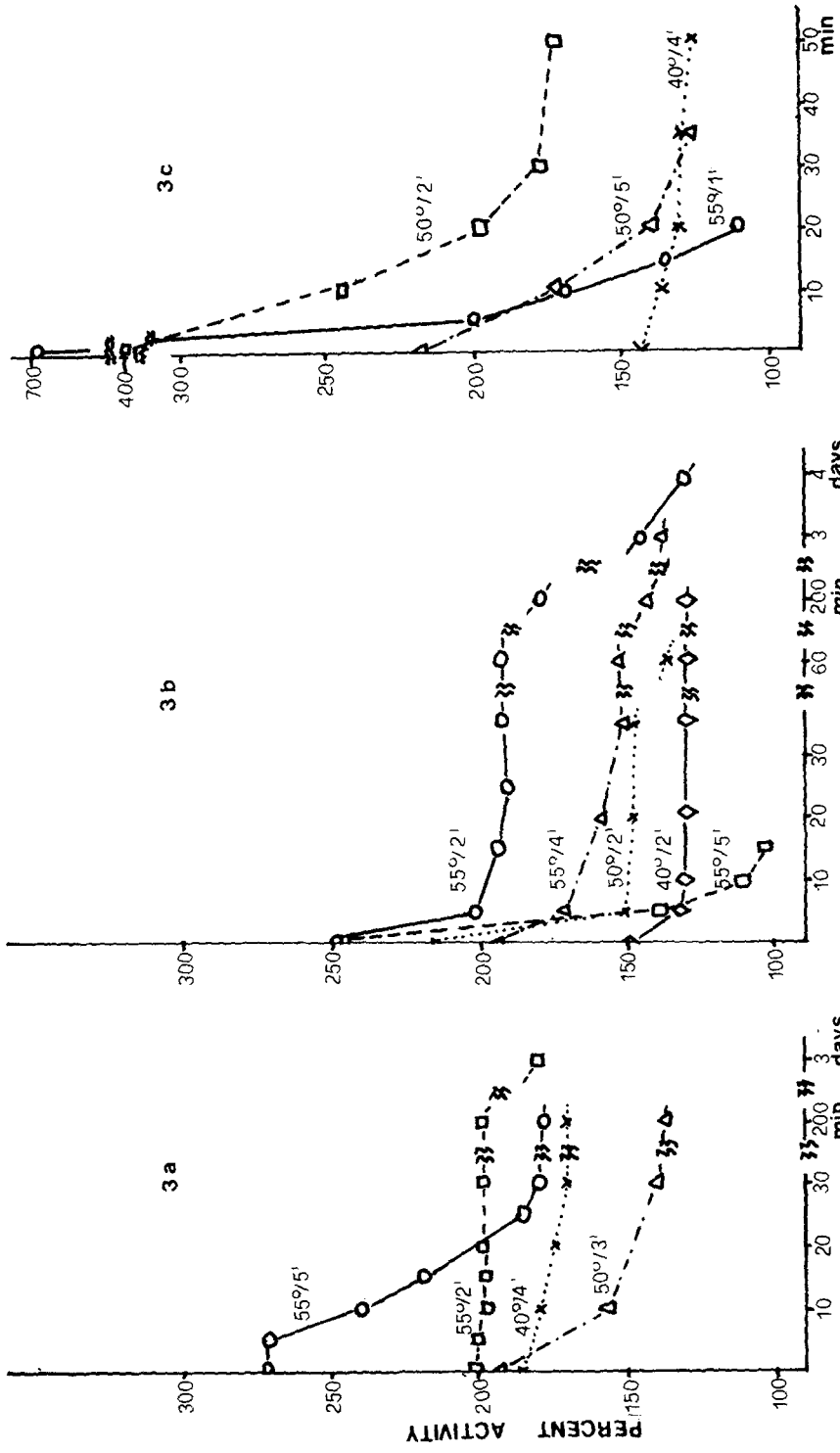

Fig. 3. Immobilized phosphatase Type III. (For details, see Fig. 1 caption).

the influence of the matrix or the presence (or pretreatment) of solvents and organic compounds on the further increase of immobilized enzyme activity was demonstrated. This study shows once again that, from the point of view of economics, it is always necessary to study how further to improve the properties of immobilized enzyme preparations by appropriate after-treatments.

The temperature pulse method offers a cheap and quick auxiliary means for this purpose as it can be easily carried out with rather simple and inexpensive equipment.

Studies on the applicability of the temperature pulse method to immobilized enzyme preparations of other specificities are in progress.

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