APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 7, 195–204 (1982)

Temperature-Dependent Behavior of Immobilized Alkaline Phosphatase. I. Role of Working Conditions and pH During Coupling

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Received October 5, 1981; Accepted November 10, 1981

Abstract

Alkaline phosphatase from hog intestine was immobilized to controlled-pore glass under various conditions. The specific activity of the enzyme was not diminished by immobilization. The influence of temperature and pH on the behavior and the stability of the immobilized enzyme preparations is discussed and compared to that of the native enzyme.

Index Entries: Alkaline phosphatase, immobilization of; immobilized alkaline phosphatase, temperature-dependent behavior of; temperature-dependent behavior, of immobilized alkaline phosphatase; pH, role during immobilization of alkaline phosphatase; coupling, of alkaline phosphatase.

Introduction

Like all phosphatases from animal sources, alkaline phosphatase from hog intestine is a rather thermolabile enzyme. As shown by several authors (1–3), the pH optimum of the native enzyme depends on the nature and concentration of the phosphate ester substrate, on the amount of enzyme in the incubation mixture, on the type of enzyme preparation used, on the concentration of activators (as Mg^{2+} and Zn^{2+} in our case), and on the type of buffer employed.

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0273-2289/82/0500-0195$02.00

The aim of the studies reported in this paper was to investigate the behavior of a thermolabile enzyme at elevated temperatures when immobilized and assayed under various pH conditions and compare it to the properties of the native enzyme.

Because of the dependence of optimum activity on numerous parameters (as pointed out above), all procedures had to be carried out under carefully controlled conditions to obtain reliable results.

Materials and Methods

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

Assay of Alkaline Phosphatase

The assay was carried out according to the method of Bessey et al. (*4*) using *p*-nitrophenylphosphate as a reagent. This assay with the immobilized enzyme was performed in rotating vessels.

Preparation of the Carrier

Preparation of the carrier for the immobilization of the enzyme followed a procedure reported earlier (*5*). Controlled-pore glass was coated with 3-aminopropyltriethoxysilane according to the method of Weetall (*6*) and activated with an excess of 10% aqueous glutaraldehyde solution for 4 h at room temperature. This procedure was followed by extensive washing with ice cold water on a glass sinter funnel until the filtrate showed no reaction with 2,4-dinitrophenylhydrazine. The completion of the coupling reaction was indicated by a negative TNBS test. The quantitative *determination of the immobilized protein* was carried out according to the method of Jacobs (*7*) after acid hydrolysis with 6*M* HCl in a sealed tube for 22 h at 110°C.

Results

Influence of pH During the Coupling Procedure on the Amount, Activity, and pH Optimum of the Coupled Enzyme

For coupling, aliquots of coated glass beads activated with glutaraldehyde (see Methods section) were incubated with phosphatase (25 mg/g wet gel) in 30 mL of 0.2*M* phosphate buffer at pH values of 5, 7, and 8, respectively, each being 0.1 m*M* with respect to $MgCl_2$ and $ZnCl_2$ and 1 m*M* with respect to the substrate phosphoenolpyruvate, which was added to protect the active site. For coupling at pH 9.5 (representing a range close to the pH optimum of the native enzyme), a

0.1*M* glycine/NaOH buffer containing $MgCl_2$, $ZnCl_2$, and substrate as above was used. The suspensions were shaken at room temperature for 3 h and stored overnight in the refrigerator. Then the supernatant was removed by centrifugation for 5 min at 3000 rpm and each batch was washed first with its corresponding coupling buffer solution, and then by 0.1*M* aqueous NaCl until the supernatant showed no reaction with ninhydrin. All types of the carefully washed preparations containing the immobilized enzyme could be stored for more than 10 months at 4°C under a 1 m*M* aqueous $MgCl_2$ solution without loss of activity. Some data concerning the quality of the various gels are given in Table 1. According to this table the various preparations of immobilized phosphatase will be referred to in the following as types I–IV.

Types I and II preparations showed the specific activities of the native enzyme and also had the highest activities per gram of dry glass beads. Very unsatisfactory results were obtained when the enzyme was coupled in the range of its pH optimum, though at that point the highest amount of protein was bound. When coupled at pH 9.5 (type IV) 16.1 mg protein bound/g dry glass beads had a specific activity of only 0.05 μkat/g protein, resembling 0.1% activity of the native enzyme at its

Table 1
Some Data Concerning the Quality of the Various Immobilized Enzyme Preparations When Assayed at 20°C

Type of immobilized phosphatase	Protein bound, mg/g dry glass	Specific activity at 20°C and pH optimum, μkat/g protein	Activity/g dry glass beads, μkat/g glass	pH optimum	Activity,[a] %
Enzyme coupled at pH 5 (Type I)	7.9	52	0.411	9.4–9.5	96
Enzyme coupled at pH 7 (Type II)	8.3	55	0.457	9.4–9.5	102
Enzyme coupled at pH 8 (Type III)	6.7	43	0.288	9.4–9.5	80
Enzyme coupled at pH 9.5 (Type IV)	16.1	0.05	0.0008	9.4–9.5	0.1
Native enzyme		54		9.3	100

[a]The specific activity of the native enzyme at its pH optimum was set at 100%.

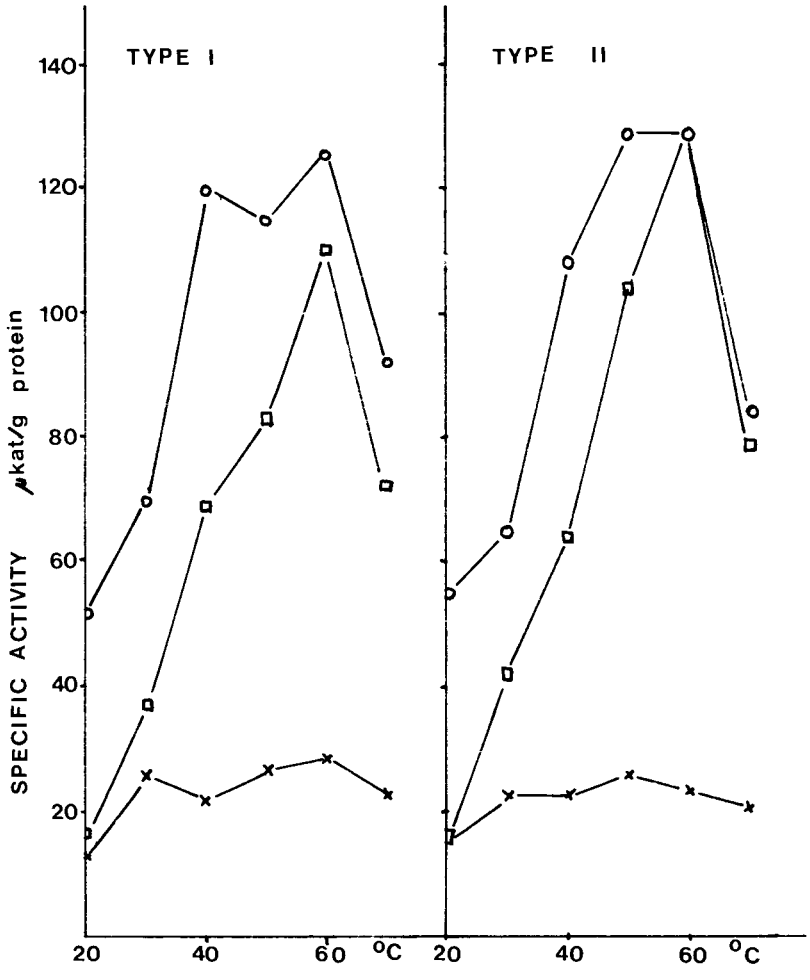

Fig. 1. Specific activity of immobilized and native phosphatase when subjected to various temperatures during the assay procedure: x, buffer conditions at pH 8; ○, buffer conditions at pH 9.5; □, buffer conditions at pH 10.5.

pH optimum. Therefore this preparation was not used for further studies. In no case could a significant shift of the pH optimum be observed as a result of the immobilization procedure.

Influence of Temperature and pH on Specific Activity During the Assay Procedure

Batches (10 mg wet gel) of immobilized enzyme (types I–III) were assayed for activity (see Methods section) at temperatures varying from 20 to 70°C and pH conditions between 8 and 10.5; the incubation time was 5 min. The results are shown in Fig. 1 and Table 2.

When assayed at pH 9.5 (which is the pH optimum range of both the immobilized and the native enzyme) a temperature optimum between 40 and 60°C could be

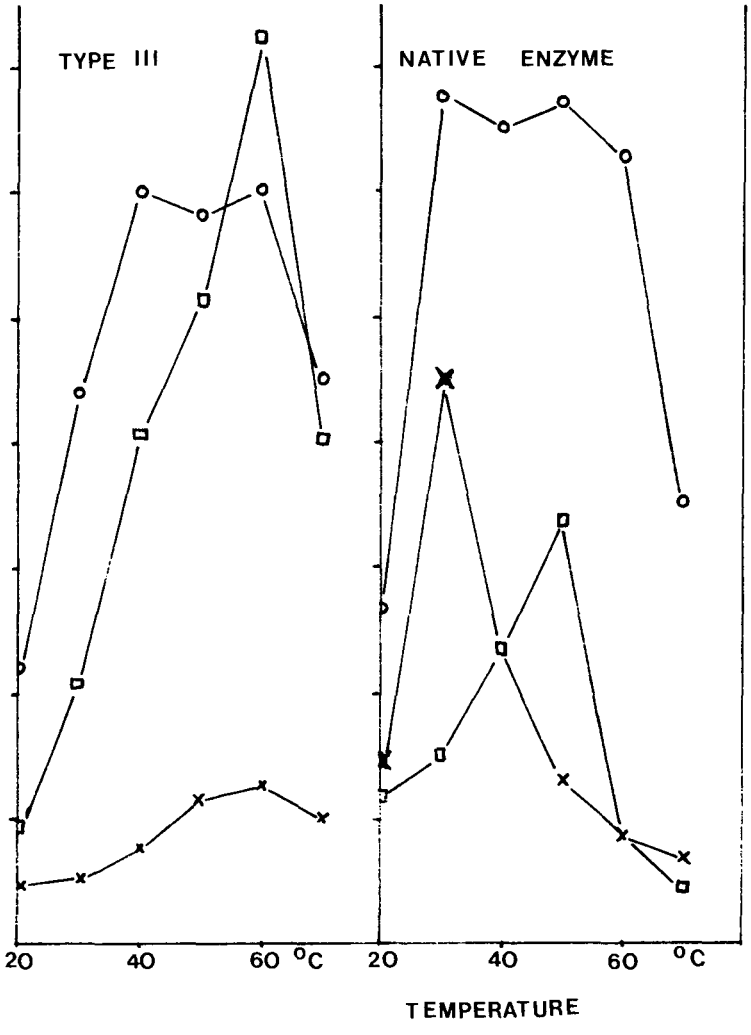

Fig. 1 (*cont.*)

observed with type I and type III immobilized phosphatase. With type II the optimum was situated between 50 and 60°C. With the native enzyme a broad temperature optimum between 30 and 60°C could be observed under the same conditions. At pH 9.5 native and immobilized enzyme (types I–III) showed quite similar specific activities at their temperature optimum, while at lower or higher pH values this was not the case.

When assayed at pH 8, all types of immobilized enzyme had rather low specific activities that remained nearly constant in the whole temperature range investigated. The activity of the native enzyme, though high at low temperatures, declined immediately after its sharp temperature optimum at 30°C.

When measured at pH 10.5, the temperature optimum shifted from 50 to 60°C and the specific activities of the immobilized enzymes were 149% (type I) to 216% (type III) higher than that of the native enzyme under the same conditions.

Table 2
Percentage Change in the Specific Activity with pH and Temperature

pH of the incubation mixture	Assay temperature, °C	% Activity			
		Type I	Type II	Type III	Native enzyme
8	20	100[a]	100[a]	100[a]	100[a]
	30	200	153	111	310
	40	169	153	167	162
	50	208	173	256	90
	60	223	160	278	59
	70	177	140	222	31
9.5	20	100[a]	100[a]	100[a]	100[a]
	30	135	118	205	250
	40	231	196	279	241
	50	221	235	270	248
	60	242	235	279	231
	70	177	153	209	130
10.5	20	100[a]	100[a]	100[a]	100[a]
	30	231	280	228	130
	40	431	427	450	204
	50	519	693	567	291
	60	625	860	806	78
	70	450	527	444	39

[a]100% always resembles the actual activity at 20°C of the various types of immobilized enzyme and the native one, respectively.

The broad temperature optimum at pH 9.5 changes into a sharp one at pH 10.5 and 60°C. In the preparation of type III, the specific activity at pH 10.5 and 60°C even exceeds that at pH 9.5 and 60°C. The native enzyme at pH 10.5 showed a temperature optimum at 50°C with much less specific activity than at pH 9.5.

Stability During Storage at Various Temperatures under Different pH Conditions

To obtain information on how long the increased specific activity of the immobilized enzyme persists at elevated temperatures, all three types of enzyme preparation were stored at 55°C. The change of activity with time was assayed at the same temperature. The data are shown in Fig. 2.

Under these conditions the immobilized enzymes behaved similarly to the native material, which under all conditions studied was denatured after 90–100 min.

At room temperature (20°C), both the native and the immobilized enzyme were rather stable, keeping their initial activities for at least 5–6 days; at 4°C both preparations were stable for more than 10 months.

Discussion

From the data given in Table 1 the conclusion may be drawn that coupling in slightly acidic or neutral media at room temperature does not much influence the enzymatic activity and the amount of protein bound. Types I and II show almost equivalent properties. Slightly alkaline coupling conditions led to some decrease in specific activity and in the amount of protein bound. These two parameters led to a distinct deterioration of the activity per gram of dry glass beads. With enzyme coupled in the range of the optimum pH, considerable differences were observed.

This rather unexpected effect may be explained by several facts: (1) The extremely high protein concentration on the glass beads may cause steric hindrance of the enzyme molecules, resulting in less active conformations and in active sites that are less accessible to the substrate. (2) Type IV immobilized phosphatase was the only one that could not be coupled in phosphate buffer because of its pH of 9.5. Phosphate is a well known competitive inhibitor of the enzyme, and perhaps had greater ability to protect the active site than did the substrate phosphoenolpyruvate (which was added to all incubation mixtures during the coupling procedure; see Methods section).

At room temperature and optimum pH, the specific activities of all types of immobilized phosphatase studied were nearly identical with that of the soluble enzyme. This is not surprising for it is well known (*8, 9*) that native alkaline phosphatase from animal tissues is intimately associated with insoluble cellular particles. So artificial immobilizates can be considered to be quasi-native preparations of this enzyme. The fact that, in the optimum pH range, types I–III preparations, as well as the native enzyme, show nearly identical behavior (Fig. 1) further strengthens this assumption.

The data of the same figure further show that pH conditions during the coupling procedure have no significant influence on the specific activity of the immobilized enzyme preparations. The shapes of the activity curves under comparable pH conditions plotted against temperature were all comparable. However, the force of the covalent linkages to the rigid matrix led to some differences compared to the soluble enzyme. When kept and measured at pH 8, the native enzyme showed a distinct maximum in activity, whereas the activities of the various types of immobilized enzyme remained lower and more constant over the temperature range under investigation.

Another stabilizing effect could be detected. When assayed at pH 10.5, the activities of the immobilized enzymes increased with higher temperatures and reached the range of the pH optimum at the temperature maximum. On the other hand, under the same conditions the activity of the native enzyme was considerably lower (Fig. 1).

Without regard to the assay conditions at pH 8 (where the activities of the immobilized enzymes were rather low compared to the native enzyme), the maximum activity was reached at higher temperatures as a result of immobilization (Fig. 1), which also accounts for the preservation of a stable conformation. However, stability decreased rapidly when the temperature optimum was exceeded.

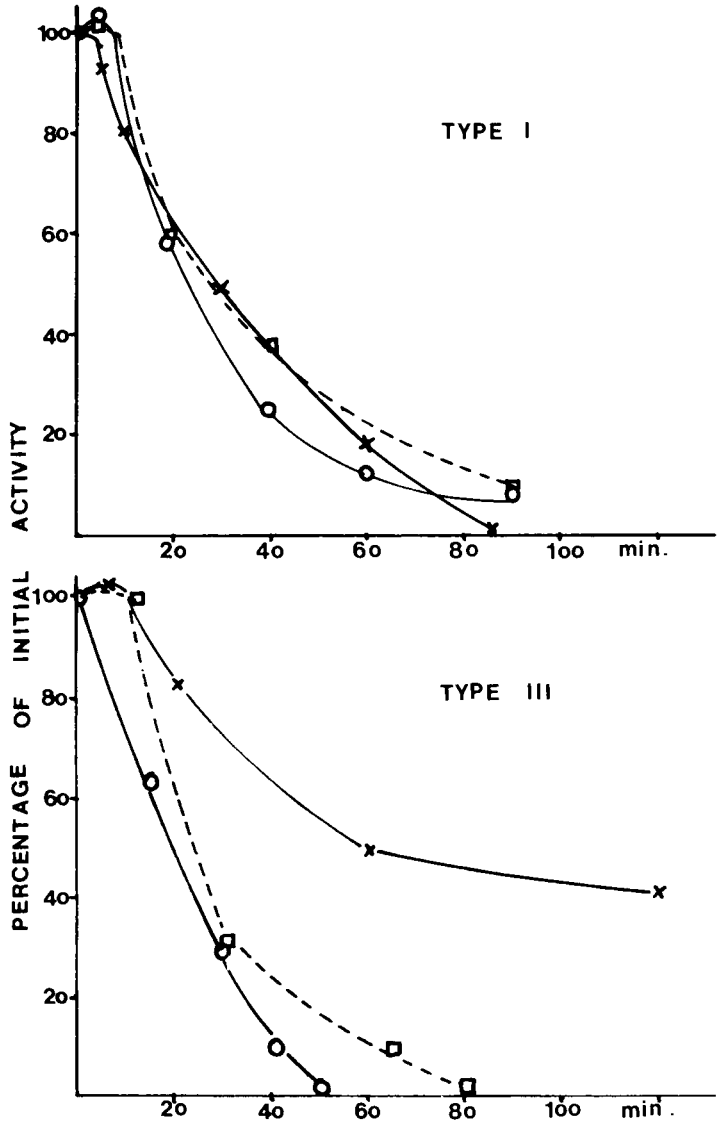

Fig. 2. Stability of the various gels when stored at 55°C for 0–120 min and assayed at the same temperature: x, buffer conditions at pH 8; □, buffer conditions at pH 9.5; ○, buffer conditions at pH 10.5.

Figure 2 shows that despite the stabilization effects mentioned, the immobilized phosphatase remains rather thermolabile, similar to the native enzyme because it is irreversibly inactivated after a comparatively short period of time when kept at higher temperatures. At room temperature both the immobilized enzyme, as well as the native one, are rather stable.

In spite of all the denaturation effects observed, it must be remembered that, under some of the conditions mentioned above and by comparison to the native material, there was a significant increase in activity and stabilization as a result of im-

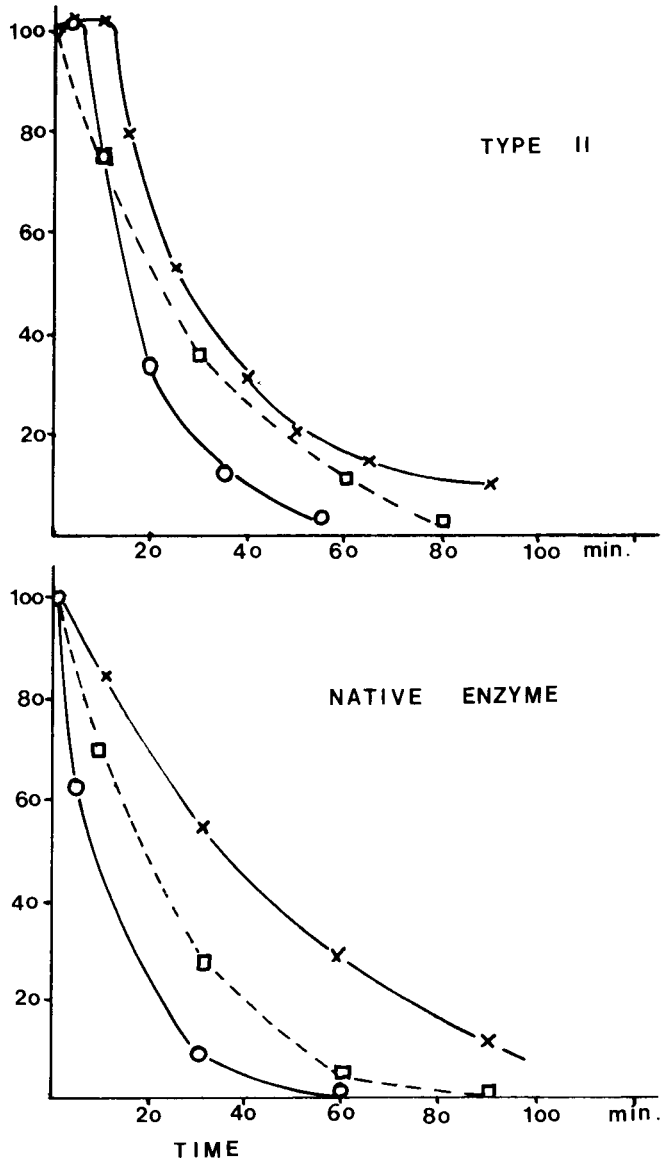

Fig. 2 (*cont.*)

mobilization, but only for a short period of time. How it is nevertheless possible to increase the activity of such a thermolabile enzyme over a prolonged period of time by temperature treatment will be shown in the following paper.

Acknowledgment

The author thanks Prof. O. Hoffmann-Ostenhof for critically reviewing the manuscript.

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