Synergistic effects in enzymic reactions

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It has been shown that when two enzymes showing similar actions act in close proximity of each other they influence each other synergistically. The phenomenon of synergism is, however, not observed if the two enzymes are of dissimilar action type. The condition of closest proximity has been simulated by conducting the enzymic reactions inside the reversed micelles. In the present study we have experimented with α-amylase and invertase both hydrolysing enzymes and also with peroxidase and invertase which do not show similar actions.

It has been customary in biochemical research to isolate a particular enzyme from its source, plant or animal, and obtain its kinetic characteristic data in vitro and based on the data thus obtained draw conclusions about its behaviour in vivo. This procedure assumes that all other enzymic reactions which go on in its proximity do not influence its kinetic behaviour at all. This assumption is not entirely correct.

In nature it never happens that one particular enzyme acts in isolation, in fact several enzymic reactions go on simultaneously in the vicinity of each other and therefore should influence each other synergistically or otherwise. It appears logical to conjecture that if two enzymic reactions say 1 and 2 showing similar actions take place in close proximity of each other they are likely to influence each other synergistically. However, if the enzymes are not similar in their action one may not observe the phenomenon of synergism. In case the two reactions say 1 and 2 influence each other synergistically one may expect that the affinity of reaction 1 should influence the rate of reaction 2 and vice versa.

To test this surmise we have experimented with α-amylase and invertase both hydrolysing enzymes and also with two others of dissimilar actions namely peroxidase and invertase; peroxidase being oxidoreductase type and invertase being a hydrolysing enzyme. The optimum pH and also optimum temperature for the two enzymes in each pair are quite close.

The experiments were conducted at fixed molar ratio of water to surfactant at which enzymes were showing optimum activity. The condition of closest proximity has been simulated by performing the reactions inside reversed micelles. Chemical kinetic data have been generated to demonstrate the synergistic effect when α-amylase and invertase catalysed reactions are made to occur in close proximity of each other. It has also been shown that the rate of the α-amylase catalysed reaction is linearly related to the affinity of the invertase catalysed reaction and vice versa. In the case of peroxidase and invertase, however, no such correlation has been observed.

Materials and Methods

Materials

The α-amylase (EC 3.2.1.1), invertase (EC 3.2.1.26) and peroxidase (EC 1.11.1.7) were procured from Sigma Chemical Company USA. Bovine serum albumin (BSA), sucrose, potato starch, H2O2, guaiacol were also procured from sigma. Cetyl pyridinium chloride (CPCI) dinitro salysilic acid (DNSA) and aerosol-OT (AOT) were purchased from SD Fine Chemicals, New Delhi. The organic solvent n-hexane used was of AR grade and isooctane was obtained from Fluka (Switzerland).

Methods

Kinetic data for α-amylase and invertase were obtained in the following situations : (i), α-amylase in the usual aqueous medium; (ii), invertase in the usual aqueous medium; (iii), both α-amylase and invertase in the same aqueous medium; (iv), α-amylase in the
reversed micellar water pool; (v), invertase in the reversed micellar water pool; (vi), both α-amylase and invertase in the same reversed micellar water pool.

The situations (iii) and (vi) represent the conditions of close proximity, the situation (vi) being the condition of closest proximity. In fact it is situation (vi) which is closer to the situation in vivo. Kinetic data for peroxidase and invertase were also obtained in the above listed six situations. The above listed six situations for α-amylase and invertase pair and for peroxidase and invertase pair are diagrammatically depicted in Fig. 1 and Fig. 2 respectively.

α-amylase and invertase catalysed reactions were conducted in the reversed micelles formed by CPC1 in n-hexane. Peroxidase and invertase catalysed reactions were conducted in the reversed micelles formed by AOT in isooctane. The details of the procedures adopted are described below.

Experiment with α-amylase and invertase

Experiments with α-amylase and invertase in the usual aqueous medium were conducted as described earlier. Reducing sugars produced by invertase catalyzed reaction were estimated by Nelson Somogyi method as described by Oser. Unhydrolyzed starch was estimated using KI-I2 reagent by the method of Shain and Mayer or the maltose formed by the breakdown of starch due to the action of α-amylase was estimated by DNSA method as described in literature. Specific activity of invertase is expressed as mg of reducing sugars produced min⁻¹ mg⁻¹ protein and that of α-amylase is expressed as mg starch hydrolyzed min⁻¹ mg⁻¹ protein.

To perform the reactions inside the reversed micelles the procedure similar to the one described in the earlier publications was adopted. The enzyme α-amylase was entrapped inside the reversed micelles formed by CPC1 (0.0193 g) in 20 ml of n-hexane. The assay of α-amylase was done by entrapping 0.2 ml of different strengths of starch solution (15 mg ml⁻¹ to 150 mg ml⁻¹) and 0.2 ml of the enzyme (10 units ml⁻¹) inside the reversed micelles. Similarly when activity of invertase was to be assayed 0.2 ml of sucrose solutions of known strengths (0.05 M to 0.7 M) prepared in appropriate buffer and 0.2 ml of the enzyme (22.7 units ml⁻¹) was solubilized inside the reversed micelles. Sodium acetate buffer (6 mM, pH = 4.7) was used for the assay of the enzymes.

The activities of the two enzymes were also estimated when they were made to act in proximity of each other in the aqueous medium and also in the reversed micellar medium i.e. situation (iii) and (vi). The procedure adopted was the same as described in our earlier publication.

Experiments with peroxidase and invertase

Peroxidase activity was assayed according to the method of Egley et al. Assay mixture in a total vol-
ume of 3 ml contained 10 mM sodium acetate (pH 5.5), 2 mM H₂O₂ and 9 mM guaiacol. Enzyme (5 μl, 11.5 units ml⁻¹) was added and the increase in absorbance was measured at 420 nm at interval of 30 sec up to 2 min using a UV-visible Elico spectrophotometer. Activities of peroxidase was expressed as ΔA₄₂₀ min⁻¹ mg⁻¹ protein because the products have unknown extinction coefficients. The assay of invertase activity in aqueous medium when it acted in isolation has already been described above with the only modification that the buffer used was 10 mM sodium acetate (pH 5.5) for the preparation of sucrose and enzyme solutions. The activities of invertase and peroxidase were also estimated when they acted together in the same aqueous medium.

To estimate the activities of peroxidase inside the reversed micelles the following procedure was adopted. AOT (0.13 g) was dissolved in 3 ml of isooctane. The desired concentrations (0.5 to 5 mM) of H₂O₂ and 9 mM guaiacol were entrapped alongwith 5μl of peroxidase (11.5 units ml⁻¹) inside the reversed micelles formed by AOT in isooctane. Similarly, when activity of invertase was to be assayed desired strengths of sucrose (0.05 to 0.7 M) and invertase were entrapped inside the reversed micelles. The activities of the two enzymes were also estimated using the methods described above when they acted inside the same reversed micelle.

**Protein estimation**

In all enzyme preparations protein was estimated by the method of Lowry et al.⁹.

**Results and Discussion**

The values of specific activity and Kₘ values for situations (i) to (vi) for both α-amylase and invertase are recorded in Table 1. Similar data for peroxidase and invertase are recorded in Table 2. To get the statistically significant values of specific activity and Kₘ the method of direct linear plot developed by Eisenthal and Cornish-Bowden⁶ was used. Deficiencies in the method of direct linear plot and the advantages of the direct linear plot developed by Eisenthal and Cornish-Bowden over these have been discussed by several authors. One typical plot using the method of Eisenthal and Cornish-Bowden is shown in Fig. 3.

The data summarized in Table 1 and Table 2 reveal the following facts:

(i) All three enzymes presently studied show enhanced activity when entrapped inside reversed micelles (Table 1 and Table 2). The activity of enzymes invertase and peroxidase in the reversed micelles formed by AOT/isoctane shows a remarkably high enhancement ~ 5.87 times for invertase and ~ 5.47 times for peroxidase. The observation on enhanced activity of enzymes when entrapped inside reversed micelles is consistent with the well documented literature reports.²⁶

(ii) In the case of α-amylase and invertase one notices the phenomenon of synergism. The activities of both α-amylase and invertase are enhanced when they act in close proximity of each other in comparison to when they act in isolation; the synergism is maximum when they act inside the reversed micelles which rep-

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**Table 1—** Specific activity and Kₘ values for purified invertase and α-amylase when they acted in isolation as well as in close proximities in aqueous system and within the reverse micelles of CPC1 in n-hexane. [Kₘ for invertase is expressed in M and for α-amylase as mg starch ml⁻¹. Data represent mean ± s.d.]

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<tr>
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<th>In aqueous system</th>
<th>In reversed micelles</th>
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<tbody>
<tr>
<td></td>
<td>α-Amylase (ii)</td>
<td>Invertase (i)</td>
</tr>
<tr>
<td></td>
<td>α-Amylase (iii)</td>
<td>Invertase (iv)</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>5.263 ± 0.320</td>
<td>0.235 x 10⁻⁴ ± 0.012 x 10⁻⁴</td>
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<td></td>
<td>8.330 ± 0.458</td>
<td>0.371 x 10⁻⁵ ± 0.026 x 10⁻⁵</td>
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<tr>
<td>Kₘ</td>
<td>0.227 ± 0.034</td>
<td>0.204 ± 0.031</td>
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<td>0.182 ± 0.025</td>
<td>0.120 ± 0.017</td>
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<th>In isolation</th>
<th>In close proximity</th>
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<tr>
<td></td>
<td>(iv)</td>
<td>(v)</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>7.140 ± 0.464</td>
<td>0.333 x 10⁻⁴ ± 0.019 x 10⁻⁴</td>
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<tr>
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<td>16.671 ± 0.993</td>
<td>0.588 x 10⁻⁵ ± 0.035 x 10⁻⁵</td>
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<td>Kₘ</td>
<td>0.200 ± 0.003</td>
<td>0.143 ± 0.020</td>
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<td>0.154 ± 0.022</td>
<td>0.083 ± 0.012</td>
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Note: Roman numerals within the parentheses correspond to the different situations depicted in Fig. 1.
Table 2—Specific activity and $K_m$ values for peroxidase and invertase when the two enzymes acted in isolation as well as in close proximities in aqueous system and entrapped in the reverse micelles of AOT in isooctane. [$K_m$ for peroxidase is expressed in mM and for invertase in M. Data represent mean ± s.d.]

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<td>Peroxidase</td>
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<td>(ii)</td>
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<td>48.564 ± 2.718</td>
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<td>$K_m$</td>
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|                         | (iv)         | (v)               | (vi)      | (vi)       |
| Sp. activity            | 133.329 ± 7.466 | 265.424 ± 15.925 | 213.330 ± 12.373 | 197.048 ± 11.822 |
| $K_m$                   | 0.110 ± 0.015   | 0.800 ± 0.110 | 0.103 ± 0.010   | 0.989 ± 0.085   |

Note: Roman numerals within the parenthesis correspond to the different situations depicted in Fig. 2.

Fig. 3—A typical Cornish Bowden plot for peroxidase catalysed reaction inside the reversed micelles formed by AOT in isooctane.

Fig. 2—A typical Cornish Bowden plot for peroxidase catalysed reaction inside the reversed micelles formed by AOT in isooctane.

Table 2—Specific activity and $K_m$ values for peroxidase and invertase when the two enzymes acted in isolation as well as in close proximities in aqueous system and entrapped in the reverse micelles of AOT in isooctane. [$K_m$ for peroxidase is expressed in mM and for invertase in M. Data represent mean ± s.d.]

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Note: Roman numerals within the parenthesis correspond to the different situations depicted in Fig. 2.

(iii) In the case of peroxidase and invertase which do not represent the enzymes of similar action no synergistic effects are observed (Table 2) when the two enzymes act in close proximity of each other, peroxidase activity is inhibited whereas the invertase activity is enhanced.

The qualitative observations on the synergistic effect imply that the rate $J_1$ of the invertase catalyzed reaction say reaction 1 should be influenced by the affinity $A_2$ of the $\alpha$-amylase catalysed reaction say reaction 2 and vice versa. The variation of $J_1$ with $A_2$ and $J_2$ with $A_1$ are shown in Fig. 4a, b. The affinities were calculated using the well known relationship

$$A = RT \ln K - RT \ln K'$$

where $K$ is the equilibrium constant of the reaction and $K'$ is the non-equilibrium value of $K$ i.e. the ratios of the product’s concentrations to the reactant concentrations at any time $t$. The rates $J$ at different times were estimated from the slopes at different times $t$ of the curve obtained by plotting product concentration $[P]$ against time:

$$J = \frac{d[P]}{dt}$$

The invertase catalysed reaction and the $\alpha$-amylase catalysed reaction which have been designated as reaction 1 and 2 respectively were written as follows for the purpose of calculations:

**Reaction 1**

$$\text{Sucrose} \xrightarrow{\text{invertase}} \text{Glucose} + \text{fructose} \quad \ldots (3)$$

**Reaction 2**

$$\text{Starch} \xrightarrow{\alpha \text{- amy lase}} \text{Maltose} \quad \ldots (4)$$

In writing reaction 2 only the major product maltose has been considered; dextrins which are the minor products have been ignored.
Fig. 4—(a), (i): Variation of reaction rate \( J_2 \) of the \( \alpha \)-amylase catalysed reaction with the affinity \( A_1 \) of the invertase catalysed reaction when the two reactions occurred in the same aqueous medium in the absence of reversed micelles.

(ii): Variation of reaction rate \( J_1 \) of the invertase catalysed reaction with the affinity \( A_2 \) of the \( \alpha \)-amylase catalysed reaction when the two reactions occurred in the same aqueous medium in the absence of reversed micelles.

(b), (i): Variation of reaction rate \( J_2 \) of the \( \alpha \)-amylase catalysed reaction with the affinity \( A_1 \) of the invertase catalysed reaction when the two reactions occurred inside the reversed micelles.

(ii): Variation of reaction rate \( J_1 \) of the invertase catalysed reaction with the affinity \( A_2 \) of the \( \alpha \)-amylase catalysed reaction when the two reactions occurred inside the reversed micelles.

As can be seen from Fig. 4a, b the rate \( J_1 \) of reaction 1 varies linearly with the affinity \( A_2 \) of reaction 2 and similarly \( J_2 \) varies linearly with \( A_1 \). However, no such variation was observed in case of peroxidase and invertase acting in close proximity of each other.

It appears logical to guess on \textit{a priori} grounds that when two reactions occur in very close proximity of each other the phenomenon of synergism should become visible only after the affinities of the two reactions attain a certain threshold value. This guess is corroborated by the extrapolated positive intercept of the \( J_2 \) against \( A_1/RT \) and \( J_1 \) against \( A_2/RT \) straight lines on the affinity axis when the two reactions 1 and 2 occur inside the reversed micelles [Fig. 4b plots (i) and (ii)]. The extrapolated positive intercept on the affinity axis is not conspicuous in the case when the two reactions occur in the same aqueous medium in the absence of reversed micelles [Fig. 4a plot (i)]. This observation can be rationalized in view of the fact that reactions occurring inside the reversed micelles represents the condition of closest proximity for the two reactions; a situation much closer to the \textit{in vivo} situation which certainly is not the case when the two reactions occur in the same aqueous medium in the absence of reversed micelles.

The observations reported in this communication appear interesting and call for further investigations to discover the detailed rules that govern the phenomenon of synergism in enzymic reaction. Once this is achieved, \textit{in vitro} kinetic data on enzymic reactions can be safely used to predict their \textit{in vivo} behaviour.

**Acknowledgement**

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