# THE ACTIVITY OF YEAST INVERTASE AS A FUNCTION OF OXIDATION-REDUCTION POTENTIAL

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The activity of many enzymes is very appreciably modified by oxidizing or reducing agents. Such enzymes as urease (Hellerman, 1939; Sizer and Tytell, 1941), cathepsin and papain and papain-like enzymes (Hellerman, 1937; Greenberg and Winnick, 1940), carbonic anhydrase (Kiese and Hastings, 1940), succinic dehydrogenase (Hopkins, *et al.*, 1938), triosephosphate dehydrogenase (Rapkine, 1938), and glycerol oxidase (Barron, 1940) are activated by reducing agents and inactivated by oxidizing agents. These effects have been interpreted in terms of the configuration of thiol groups in the enzyme molecule; when the sulfur is in the reduced -SH form the enzyme is active, when in the oxidized -SS- form the enzyme is inactive. Fruton and Bergmann (1940) and Irving *et al.* (1941) believe that the activation of papain and cathepsin cannot be explained by this hypothesis, and state that activation is a two-step process involving the formation of an addition complex between the activator and the enzyme. Sizer and Tytell (1941) reported, in the case of crystalline urease, that activity is a continuous function of oxidation-reduction potential with an optimum activity at $E_h = +150$ mv. and a gradual decrease in activity at lower as well as higher potentials. It seems very important for the interpretation of enzyme action to find out whether variation of enzyme activity with oxidation-reduction potential is characteristic of other enzyme systems as well. Many enzyme systems do not appear to be sensitive to oxidants or reductants, possibly due to the presence of stabilizers accompanying the enzyme (*cf.* Irving *et al.*, 1941) or because of the greater stability of the enzyme molecule. Such an enzyme is yeast invertase which, although relatively unaffected by weak oxidants or reductants (Ito and Obo, 1939), is inactivated by a wide variety of other agents such as certain metal salts, $I_2$, $HNO_2$, phenylhydrazine (Myrbäck, 1926), by nitrophenols, quinine, and its derivatives (Rona and Bach, 1921), aniline (Euler and Svanberg, 1921), alkaloids (Rona *et al.*, 1924; Mezzadroli and Amati, 1933), alcohols (Colin and Chaudin, 1931), certain dyes and narcotics (Baur, 1939), ascorbic acid (Klodnitskaya and Strachiskii, 1938). Using

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this enzyme as an example of those not affected by mild oxidants or reductants, a systematic study of the relationship of invertase activity to oxidation-reduction potential has been made.

## *Methods*

The kinetics of the yeast invertase-sucrose system were studied by measuring the amount of invert sugar liberated during the hydrolysis, by utilizing for this purpose Sumner's dinitrosalicylic acid reagent for reducing sugars (Sumner, 1925; Sumner and Howell, 1935), which has been found satisfactory for invertase studies (Sizer, 1938 *a*, 1938 *b*). Using this technique it was found that the reaction follows a smooth curvilinear course, and that the reaction is unimolecular for the first third of the inversion (Sizer, 1938 *a*, 1938 *b*). In view of the uniform kinetics during the early portion of the inversion, the amount of invert sugar liberated in a given time can be taken as a measure of rate. In preliminary experiments the 2 minute determination of invert sugar was subtracted from the 10 minute one. If the 2 minute determination was neglected, however, the results were relatively the same, so in all subsequent experiments duplicate samples of the digest were taken for the analysis of invert sugar after the inversion had proceeded for 10 minutes.

A number of samples of commercial invertase (Digestive Ferments Co., Wallerstein Laboratories) was used as well as fresh and powdered bakers' yeast (Fleischmann) and brewers' top yeast (Haffenreffer), and several invertase preparations of varying degrees of purity prepared from bakers' yeast according to Lutz and Nelson (1934). In all cases the invertase was dissolved in distilled water and diluted to such a concentration that it would liberate 10–20 mg. invert sugar per ml. at 30 ± 0.01°C. in 10 minutes. 1 ml. of the enzyme solution was added to 1 ml. oxidant or reductant (usually 0.1 M) and incubated for 5 minutes. To this solution were then added 8 ml. 6 per cent sucrose dissolved in phthalate buffer, pH 4.6 (optimum pH for invertase). After the 10 minute inversion period duplicate samples were taken, and the amount of reducing sugars was determined colorimetrically after treatment with the dinitrosalicylic acid reagent. In all preliminary experiments the Duboscq colorimeter was used, but since more quantitative data could be obtained with the Spekker photoelectric absorptiometer, all subsequent work was done using this instrument. The number 6 light blue filter (maximum transmission at $\lambda = 4810$ Å[1]) was used. The standard solution contained 20 mg. glucose and was treated in the same manner as the experimental solutions.

The addition of the enzyme plus oxidant or reductant to the sucrose solution did not appreciably modify the pH of the solution which was 4.63 ± 0.03 in all cases except for the solution containing thioglycollic acid where the pH was 4.24. A control which contained distilled water instead of the oxidant or reductant was run with every five experimental tubes, and the activity of the invertase in the presence of oxidant or reductant was expressed as a percentage of the control.

The oxidation-reduction potential of the digest was measured at room temperature after the 10 minute inversion using the Beckman pH-$E_h$ meter. The $E_h$ of the solutions containing $Na_2S_2O_4$ or $KMnO_4$ was unstable and was measured immediately

[1] Measured with the Hardy recording photoelectric spectrophotometer.

on duplicate digests instead of after the 10 minute interval. Solutions of the unstable oxidants or reductants were freshly prepared just before use.

### *Experimental and Results*

Reaction rate was measured by the amount of invert sugar formed in 10 minutes, and was expressed as a percentage of the rate of the control, which contained distilled water instead of oxidant or reductant. A search was made for oxidants or reductants suitable for poising the system at the desired oxidation-reduction potential. In most cases these compounds could be used in a final concentration of 0.01 M without exerting any effect which might be ascribed to the concentration of salt employed. More dilute solutions than this of $KMnO_4$ and I-KI had to be used in order to avoid inhibiting effects related to concentration. Preliminary experiments on commercial invertase samples using the Duboscq colorimeter indicated that there was little effect upon invertase activity of changing the $E_h$ of the digest from $-270$ to $+600$ mv., but above $+600$ mv. the activity decreased rapidly.

Quantitative studies, using the photoelectric absorptiometer for the colorimetric determination of reducing sugar, were made on Wallerstein's red label invertase scales, using fifteen different oxidizing or reducing solutions to poise the oxidation-reduction potential of the digest in the range from $-270$ to $+1{,}000$ mv. The activity of invertase in the presence of each of these solutions was meaured on 10 different days and an average of the data on each solution was taken. The average activity was then plotted against the average oxidation-reduction potential as shown in Fig. 1. Despite the appreciable scatter of the points, it is apparent that invertase activity is independent of $E_h$ up to $+400$ mv., above which the activity falls sharply reaching 0 at $E_h = 1{,}000$ mv. The points for invertase plus $H_2$-Pt asbestos or 0.01 M $Na_2S_2O_4$ are probably not significantly below the curve, since in many of the individual experiments the corresponding activities were 100 per cent. The point for invertase plus 0.00001 M I-KI, is very low; this may be related to a toxic as well as an oxidizing effect of iodine.

A number of different samples of commercial invertase were then studied to determine the relationship of the $E_h$-activity curve to the purity, source, and method of preparation of the enzyme. Difco liquid invertase, Wallerstein's blue label, green label, and liquid invertase preparations were used. The data with all four commercial samples are very similar, so only one characteristic curve is presented in Fig. 2 (curve A). The curve is very similar to that of Fig. 1, indicating a constant activity over the $E_h$ range from $-270$ to $+400$ mv. and a rapid decrease in activity above $+400$ mv.

### *Experiments with Highly Purified Invertase*

From the preceding experiments with different commercial preparations of invertase it appears that the $E_h$-activity curve is independent of the source and

type of preparation used. Since these samples of invertase may contain natural activators or stabilizers, the $E_h$-activity curve could be explained in

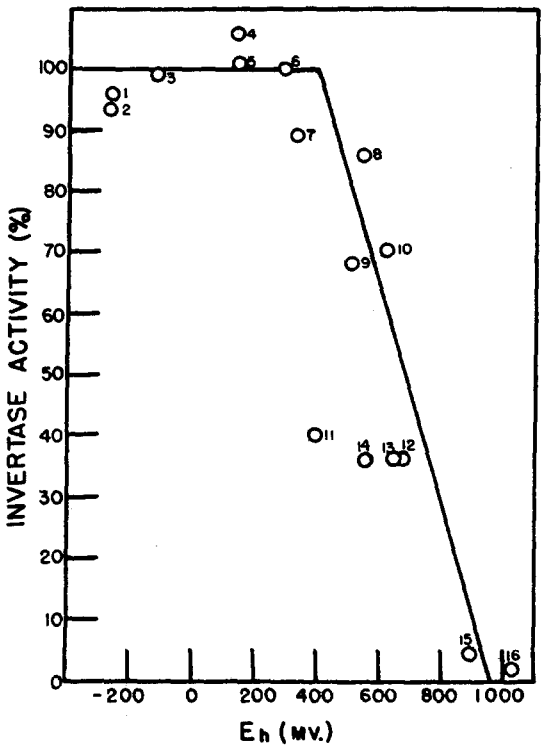


FIG. 1. Per cent invertase activity is plotted as a function of the oxidation-reduction potential of the digest. The digest contained 0.005 per cent Wallerstein's red label invertase, 5 per cent sucrose, phthalate buffer pH 4.6, and one of the following:

1. $H_2$ + Pt asbestos
2. 0.01 M $Na_2S_2O_4$
3. 0.001 M $Na_2S_2O_4$
4. 0.01 M cysteine
5. 0.01 M thioglycollic acid
6. Water
7. 0.01 M $K_4Fe(CN)_6$
8. 0.01 M $K_3Fe(CN)_6$
9. 0.01 M $K_2Cr_2O_7$
10. 0.01 M $H_2O_2$
11. 0.00001 M I-KI
12. 0.0001 M I-KI
13. 0.001 M I-KI
14. 0.00001 M $KMnO_4$
15. 0.0001 M $KMnO_4$
16. 0.001 M $KMnO_4$

terms of the effects of the oxidants or reductants on such impurities rather than on the invertase itself. With impure papain (Irving, *et al.*, 1941) and impure urease (Sizer and Tytell, 1941) such contaminants have been shown to be important in activation-inactivation phenomena. In view of this evidence it

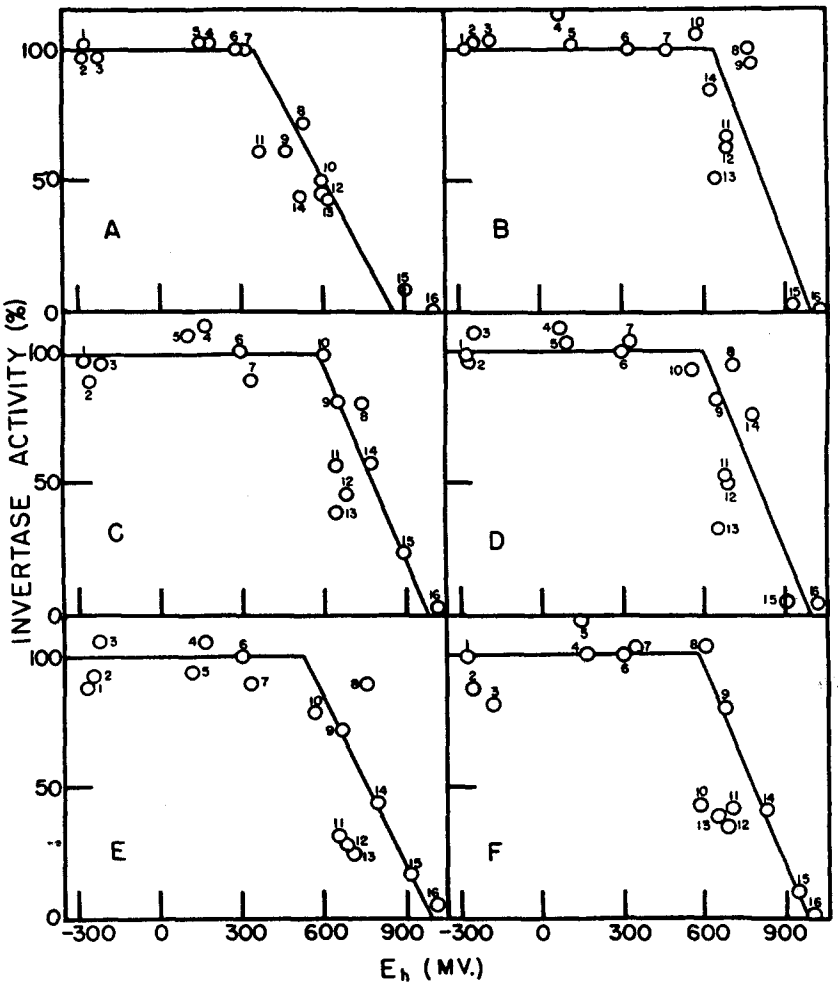


FIG. 2. Per cent invertase activity is plotted against the oxidation-reduction potential of the digest. The numbers refer to the same oxidants or reductants as in Fig. 1. Different invertase preparations were used for each curve. A, Wallerstein green label invertase. B, fresh Fleischmann's bakers' yeast. C, dried, powdered Fleischmann's bakers' yeast. D, filtrate of toluene autolysate of yeast. E, kaolin eluate of invertase prepared according to Lutz and Nelson. F, dialyzed $(NH_4)_2SO_4$ filtrate of highly purified invertase prepared according to Lutz and Nelson.

seemed advisable to determine the possible rôle of contaminants by studying the $E_h$-activity curve of invertase as a function of enzyme purity.

For impure invertase use was made of untreated fresh and powdered bakers' yeast. In the former practically 100 per cent of the yeast cells were alive (as

determined by the differential methylene blue staining technique, Nelson, *et al.*, 1932), while in the dried yeast about 2/3 of the cells were dead. The corresponding $E_h$-activity curves for these two sources of invertase are shown in Fig. 2, curves B and C. The curves are similar to those for commercial invertase, although the break seems to occur at a somewhat higher $E_h$. The question arises in the case of the fresh yeast as to whether or not the action of the reagent on the enzyme inside the cell is preceded by the death of the cell. Experiments on this problem indicated that only in the presence of $KMnO_4$ and I-KI, is the mortality of the cells appreciable in a period of 15 minutes. Experiments were also performed on brewers' yeast and it was found that the $E_h$-activity curve was very similar to that for bakers' yeast.

Invertase was prepared from bakers' yeast[2] by the method of Lutz and Nelson (1934). By this method a 500-fold increase in activity above that of the original toluene autolysate was obtained. At each step in the purification samples of the invertase were taken in order to determine how the $E_h$-activity curve changed as the purity of the enzyme increased. Only three typical curves for yeast invertase in three different stages of purification are shown in Fig. 2; curve D is for the filtrate obtained after autolysis of the yeast with toluene, curve E for the eluate after kaolin absorption, curve F for the filtrate after saturation with $(NH_4)_2SO_4$ and dialysis. All the curves are very similar with no change in activity between $-270$ and $+600$ mv.; above the latter the activity falls rapidly becoming 0 at $E_h = +1{,}000$ mv. The most highly purified preparations seemed somewhat less stable, and were slightly inactivated by the 0.01 M $Na_2S_2O_4$. The fact that the $E_h$-activity curve is essentially independent of invertase purity makes it seem unlikely that the curve can be explained in terms of effects on natural activators, stabilizers, or inhibitors which accompany the invertase. This conclusion must be accepted with caution since pure crystalline invertase was not used, and since it is not impossible that during the purification of invertase these substances possibly concerned with the $E_h$-activity curve maintained their same relative concentrations.

### *Other Oxidizing or Reducing Compounds*

Oxidizing or reducing agents cannot be used indiscriminately to poise the invertase system, since some of them have a specific toxic action in addition to $E_h$ effects. In this category are such substances as $Br_2$, $Na_2S$, $FeCl_3$, ascorbic acid, and iodoacetic acid. On the other hand many other compounds prove quite satisfactory and the typical $E_h$-activity curve can be obtained when they are used to poise the digest (Fig. 3, curve A).

[2] The author wishes to acknowledge the assistance of Mr. Herbert Jaffe in the purification of the invertase.

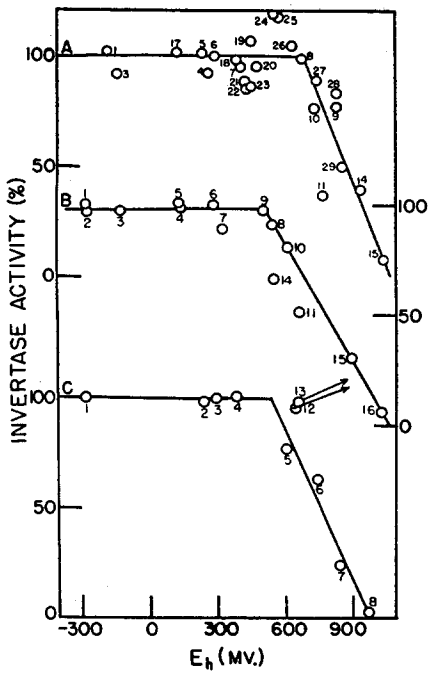


FIG. 3. Per cent invertase activity is plotted against the oxidation-reduction potential of the digest.

Curve A. Invertase purified according to Lutz and Nelson. The oxidants or reductants are the same as in Fig. 1 plus the following:

17. 0.1 saturated $H_2S$
18. 0.01 M methionine
19. 0.01 M glutathione
20. 0.01 M $CeCl_3$
21. 0.01 M $FeCl_2$
22. 0.01 M $Na_2SeO_3$
23. 0.01 M KCNS
24. 0.01 M KCN
25. Saturated quinone
26. Saturated cystine
27. 0.01 M $Na_2SeO_4$
28. 0.01 M $CrCl_3$
29. 0.00001 M $Ba(MnO_4)_2$

Curve B. 0.008 per cent Wallerstein red label invertase. The oxidant or reductant was added to the enzyme, then removed by dialysis before the sucrose solution was added. Symbols are the same as in Fig. 1; the ordinate scale is on the right.

Curve C. 0.008 per cent Wallerstein red label invertase, 5 per cent sucrose, plus one of the following:

1. $H_2$-Pt asbestos
2. 0.01 M thioglycollic acid
3. Water
4. 0.01 M $K_4Fe(CN)_6$
5. 0.0001 M $KMnO_4$ + 0.0009 $K_4Fe(CN)_6$
6. 0.0001 M $KMnO_4$ + 0.0007 $K_4Fe(CN)_6$
7. 0.0001 M $KMnO_4$ + 0.0004 $K_4Fe(CN)_6$
8. 0.0001 M $KMnO_4$

### *Do $E_h$ Changes Act upon the Enzyme or Substrate?*

The oxidant or reductant was incubated with the invertase for 5 minutes, the solution then placed in a cellophane sausage casing (Central Scientific Co.), and the oxidant or reductant removed by dialyzing for 1 hour under turbulent tap water (chemical tests showed this time to be sufficient for the removal of these substances). The invertase activity of the solution was then measured in the usual manner (Fig. 3, curve B). This curve is very similar to the others, and indicates that the oxidant or reductant acts solely on the enzyme, not on its substrate.

TABLE I

Activity of invertase after treatment with oxidizing agents, followed by dialysis. Platinized asbestos was then added to all the enzyme solutions and the invertase activity was determined with and without the addition of hydrogen.

| Oxidant | Hydrogen added | Invertase activity |
|---|---|---|
| | | *per cent* |
| Water, control | No | 100 |
| 0.0001 M $KMnO_4$ | No | 6 |
| " " " | Yes | 6 |
| 0.0001 M I-KI | No | 0 |
| " " " | Yes | 0 |
| 0.01 M $K_3Fe(CN)_6$ | No | 84 |
| " " " | Yes | 62 |
| 0.01 M $K_2Cr_2O_7$ | No | 84 |
| " " " | Yes | 91 |
| 0.01 M $H_2O_2$ | No | 84 |
| " " " | Yes | 85 |

### *Reversibility of the Inhibiting Action of Oxidants*

It is apparent from Fig. 3, curve B, that the effects of oxidizing agents are not readily reversible, since the invertase did not regain its activity after these inhibitors were removed by dialysis. The problem of reactivation was also studied by bubbling $H_2$ (activated by platinized asbestos) into the invertase solution freed of oxidants by dialysis. By reducing the $E_h$ to a low value it seemed possible that reactivation might be effected. Examination of Table I, however, reveals that no reactivation was obtained by this method. Reactivation was also attempted by adding the reducing agents, $Na_2S_2O_4$, $Na_2S_2O_3$, and $K_4Fe(CN)_6$, to the invertase which had been incubated for 5 minutes with the oxidant. Although the oxidant was not previously removed, the addition of the reducing agent brought about a marked fall in $E_h$. In the series of studies made on the reversal by reductants of invertase inactivation by each of the oxidants there was little or no reactivation. It appears from this in-

vestigation that the inactivation of invertase by oxidants is essentially irreversible.

### *Toxicity of Oxidants*

The inactivation of invertase by oxidants may be due to their high oxidation-reduction potential or may be caused by a specific toxicity of the oxidant unrelated to $E_h$. The fit of points obtained with different oxidants to a single smooth curve (Figs. 1–3) may be taken as evidence of the former hypothesis. Additional evidence on this problem can be obtained by adding a reductant ($K_4Fe(CN)_6$, $Na_2S_2O_3$, and $Na_2S_2O_4$ were used) to the various oxidants before adding the enzyme, thereby bringing about a corresponding fall in $E_h$. Since the concentration of the oxidant is held constant in all these experiments, it may be argued that any observed increase in activity (as compared with the activity in the presence of oxidant alone) must be due to the fall in potential. From this it follows that the original effect of the oxidant could be attributed to the high $E_h$, and not to a specific toxic action unrelated to $E_h$. A typical experiment to illustrate this point is shown in Fig. 3, curve C, where adding increasing amounts of ferrocyanide to a permanganate solution effects a corresponding decrease in $E_h$, and an increase in the activity of the invertase subsequently added to the mixture. These results cannot be explained on the basis that the addition of ferrocyanide has caused the disappearance of the "toxic" $MnO_4^-$ ions, because chemical analysis indicates that the majority of the $MnO_4^-$ ions are still present in the solution.

Essentially similar results to those with $KMnO_4$ were obtained for I-KI, $K_2Cr_2O_7$, $H_2O_2$, and $K_3Fe(CN)_6$, for when $K_4Fe(CN)_6$, $Na_2S_2O_3$, or $Na_2S_2O_4$ were added to the oxidant before the invertase was added, the expected increased activity was obtained in most cases. These results suggest that the inhibiting action of oxidants on invertase is primarily related to their high oxidation-reduction potentials rather than to a specific toxicity unrelated to their oxidizing powers.

## DISCUSSION

It is apparent from this investigation that invertase is a stable enzyme over a wide range of oxidation-reduction potential, but loses its activity in strong oxidizing solutions. It might be thought that the inactivating action of such oxidizing solutions is analogous to the more violent conditions characteristic of the oxidative degradation of proteins used in total nitrogen determinations. Such is apparently not the case, however, since the dilute 0.01 M solutions of oxidants acting for only 15 minutes at 25°C. brought about no apparent protein degradation. While this oxidative degradation of invertase by oxidants seems to be ruled out, other explanations of their action can be suggested. An explanation in terms of changes in substituent sulfur groupings in the

enzyme is unlikely since free thiol groups can usually be oxidized at lower potentials than those required to inactivate invertase (Hellerman, 1939; Sizer and Tytell, 1941). Strong oxidants might react with constituent amino acids of invertase such as tyrosine, tryptophane, or histidine and in this way inactivate the enzyme. This would be analogous to pepsin inactivation by the iodination of tryrosine in the pepsin molecule (Herriott, 1937). Another plausible explanation for invertase inactivation by oxidants is that these agents act by denaturing the protein enzyme. Northrop (1939) has shown that denaturation of certain proteases is always accompanied by loss in enzyme activity. Reducing agents apparently do not denature proteins (Mirsky, 1938), but the denaturation of proteins by oxidants has not yet been sufficiently studied to state whether or not this mechanism is likely.

## SUMMARY

The activity of yeast invertase as a function of oxidation-reduction potential has been investigated using a large number of oxidants and reductants. The activity is constant over the range of $E_h$ from $-270$ to $+600$ mv., but above $E_h = +600$ mv. there is a sharp decrease in activity reaching 0 at $E_h = +1{,}000$ mv. The inhibiting action of strong oxidants is upon the enzyme rather than on the substrate and appears to be essentially irreversible Experiments indicate that the inhibiting action of strong oxidants on invertase is primarily related to their high oxidation-reduction potential rather than to a specific toxic action unrelated to $E_h$. The effects of oxidation-reduction potential upon invertase activity are independent of the purity of the enzyme, since they are the same for commercial invertases, fresh bakers' yeast, powdered bakers' yeast, brewers' yeast, and highly purified invertase. Possible mechanisms involved in the inactivation of invertase by oxidants are discussed.

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