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PROPERTIES OF THE INTERNAL INVERTASE OF YEAST, *SACCHAROMYCES CEREVISIAE*

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SUMMARY

1. Internal yeast invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) has been isolated and purified from fresh, sonicated *Saccharomyces cerevisiae*. The specific activity of the pure enzyme was 3128 μmoles sucrose hydrolyzed per ml per min per mg of protein at 25°. The enzyme has an elution volume 1.6 times the void volume on Sephadex G-200 at pH 7.5.

2. A specific, reversible inhibitor effect of cations, notably NH_4^+ was found.

3. The internal yeast invertase is inhibited by iodine and the activity is regenerated by mercaptoethanol in an identical way to external invertase. The internal enzyme is also inhibited by cyanogen bromide in a two-step reaction. There is a fast, pH-dependent inhibition and a slow inhibition with a non-ionizing species; the rate of the latter reaction is affected slightly by pH. The slow reaction had a pseudo-first-order rate constant between 22 and $75 \cdot 10^{-6}$ sec^{-1} between pH 4.0 and pH 8.0, respectively. K_2PtCl_4 inhibits internal yeast invertase non-competitively with a dissociation constant of 0.025 M at pH 4.9.

4. Internal iodine-invertase has a V which is 40% the value for the native enzyme; the K_m of iodine invertase is 1.5 times the K_m of the native enzyme.

5. Internal invertase has transferring activity. Internal iodine-invertase has qualitatively the same activity. We were unable to show the formation of aniline fructosides with internal invertase, although we demonstrated such synthesis with external yeast invertase. External invertase can transfer fructose to methanol and to ethanol but not to glycollic acid. It can synthesize the *O*-fructoside with ethanol but not the analogous *S*-fructoside with ethanethiol.

6. We conclude that the inhibition by iodine is due to the reversible oxidation of a reactive methionine sulphur atom. The slow inhibition by cyanogen bromide is also due to reaction at a methionine sulphur atom; but these are different methionine residues and, consequently, we suggest that there are two methionine residues at or near the active site of yeast invertase.

7. The data presented are consistent with the mechanism of action proposed for external invertase by A. WAHEED AND S. SHALL (*Biochim. Biophys. Acta*, 242 (1971) 172) and we suggest that the external and internal yeast invertases have identical mechanisms of reaction.

INTRODUCTION

Yeast (*Saccharomyces cerevisiae*) has two invertase activities. The invertase is a β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) and the major portion of the activity is located outside the yeast cell membrane. The external yeast invertase is a glycoprotein containing about 50% (w/w) carbohydrate[1,2]. FRIIS AND OTTOLENGHI[3] and GASCÓN AND OTTOLENGHI[4] have demonstrated an invertase activity inside the yeast cell. This internal invertase has been purified and contains no carbohydrate[5]. The internal and external yeast invertases are reported to differ in amino acid composition[6]; in particular, the internal enzyme is said to be free of cysteine[6]. GASCÓN *et al.*[6] have suggested that the external and internal enzymes are composed of subunits of which at least one, the active catalytic subunit, is identical in the two enzymes while other of the subunits are different in the two forms.

WAHEED AND SHALL[7] have observed the following properties of the external invertase. Reaction of external invertase with iodine in potassium iodide gives a stable product with about half the initial enzyme activity. This inactivation is not reversed by cysteine but is reversed by mercaptoethanol or mercaptoethylamine. The pK_a value of the group involved in the maximal velocity is not altered by the iodine reaction. Iodotyrosine is not formed. Nitration of tyrosines does not reduce the activity of external invertase. CNBr inhibits external yeast invertase activity in two separate reactions. The first, fast reaction is dependent on pH. The second, slow reaction is independent of pH and is probably at a methionine sulphur atom. The iodine reaction and the inhibition by CNBr are independent reactions.

We have examined some of the properties of internal invertase to ascertain how similar it is to external invertase and to gain some insight into the active site of invertase. We have examined the reaction between iodine and internal invertase in a similar way to our earlier investigation of the reaction between iodine and external yeast invertase[7]. We have also studied the reaction of invertase with CNBr. From this and from the inhibition of internal invertase by K_2PtCl_4 we conclude that iodine reacts by reversibly oxidising a methionine residue at or near the active site. We have measured the K_m and V of internal invertase and internal iodine invertase and we have studied their transferring activity.

A portion of this work has been reported in a preliminary form[8].

MATERIALS AND METHODS

All sugars were A.R. grade from British Drug Houses Ltd.

Allyl alcohol and cinnamyl alcohol were purchased from Koch–Light Laboratories, Ltd., Colnbrook. *o*-Dinitroaniline and *p*-nitroaniline were purchased from British Drug Houses.

2-Mercaptoethanol was purchased from Sigma Chemical Co. London, and was redistilled under vacuum and stored at −20°.

CNBr was purchased from Eastman Kodak Chemicals, New York.

All other chemicals were A.R. grade.

pH was determined on a Radiometer Model 25 with a scale expander.

Enzyme activity was estimated as described by WAHEED AND SHALL[7], the substrate was 0.10 M sucrose in 0.10 M sodium acetate buffer, pH 4.9.

The kinetic parameters were estimated with the computer program kindly provided by Professor W. W. CLELAND[9,10]. The program was modified in two details. The coefficient of variance or relative standard error was calculated. The second alteration concerned the weighting factor to be used. Experiment showed that for the invertase assays used in this work the variance of the mean velocity in replicate assays varied linearly with the square of the value of the mean measured velocity. CLELAND's[10] original program assumed a constant variance at all measured velocities. In our calculations the weighting factor introduced was therefore the square of the velocity.

Purification of internal invertase

The internal yeast invertase was purified from freshly grown yeast (*S. cerevisiae*) which was kindly donated by Distillers Company (Yeast) Ltd. It was sent by air from Scotland the same day that it was grown. The enzyme was purified by the method described by GASCÓN AND LAMPEN[5], with some minor modifications. The yeast cells were disrupted in a water-jacketted continuous flow cell using a Dawe Instruments (Branson) Type 1130/A sonicator at maximum power. The yeast cell paste was suspended in an equal volume of 10 mM sodium acetate buffer, pH 4.9, and with a flow rate of 5 ml/min the yield of soluble enzyme activity was 1.2 times that of the original yeast suspension. The temperature was maintained below 10° at the sonicator tip by passing ice-cold water through the jacket. The internal enzyme was separated from the external enzyme by precipitating the former with 70% saturated $(NH_4)_2SO_4$. A pH 4.0 precipitation step followed. The next step of GASCÓN AND LAMPEN[5] was the addition of protamine sulphate. Trial experiments had shown that this step was not very helpful in our hands and it was omitted. DEAE-Sephadex A-50 was used, first for batch chromatography and then for column chromatography with a linear gradient of NaCl (0.15–0.50 M) in buffer. A sharp peak of enzyme activity and of protein was eluted at about 0.30 M NaCl. GASCÓN AND LAMPEN[5] used SE-Sephadex C-50 at pH 4.0 in a further step, although they reported no increase in specific enzyme activity as a result of this step. Accordingly we omitted this step.

The overall yield of pure internal invertase activity was 1% of the original total invertase activity in the cell suspension. However, since GASCÓN AND LAMPEN[5] have reported that the total internal invertase activity only represents 4% of the total invertase activity, we consider the yield to be about 25%, which is reasonable.

The purified internal invertase had a maximal specific activity of 3128 units/mg of protein at 25°. A unit of enzyme activity is the amount of enzyme activity which will hydrolyse 1.0 μmole of sucrose per min at the stated temperature with the conditions of the enzyme assay[7]. GASCÓN AND LAMPEN (1968)[5] reported a specific enzyme activity of 2900 units/mg of protein at 30°.

Thin-layer chromatography of fructosides

The supporting medium was Silica gel G (Merck A.G., Darmstadt) made up in 0.01 M sodium acetate and were 0.25 mm thick. Staining of compounds was preceded in all cases by a visual inspection in ultraviolet light. Then the plates were sprayed with a solution of concentrated H_2SO_4–ethanol–anisaldehyde (1:18:1, by vol.) and heated at 100° for 10 min.

External invertase

External invertase was purified as described previously[7] from the sonicated cells.

RESULTS

Apparent size of internal invertase

The relative elution volume of internal invertase on Sephadex G-200 (1 cm × 100 cm) in 10 mM Tris–HCl buffer, pH 7.5, was 1.6 times (V_e = 86 ml) that of Dextran blue (V_0 = 54 ml). This is similar to the results of GASCÓN AND LAMPEN[5] and of GASCÓN *et al.*[6] and suggests a molecular weight similar to that reported, of 135 000.

Inhibition by salts

During the purification of the enzyme it was observed that the enzyme activity was inhibited by $(NH_4)_2SO_4$. Consequently, the effect of some salts on the activity of an extensively dialysed solution were examined (Table I). $(NH_4)_2SO_4$ inhibits the internal invertase activity quite strongly. The inhibition is reversible. After standing in 0.1 or 0.5 M $(NH_4)_2SO_4$ for 2 h the enzyme was diluted so that the salt concentration dropped to 5 and 24 mM respectively. The relative enzyme activities were found to be 70% and 21%. Extensive dialysis regenerated all the activity from 2 M $(NH_4)_2SO_4$.

TABLE I

EFFECT OF $(NH_4)_2SO_4$ ON THE ACTIVITY OF INTERNAL YEAST INVERTASE

The $(NH_4)_2SO_4$ was dissolved in the standard assay solution at the indicated concentrations.

$(NH_4)_2SO_4$ concn. in substrate solution (mM)	*Enzyme activity (μmoles sucrose hydrolyzed per min per ml)*	*Relative enzyme activity (%)*
0	1349	100
1	1136	84
5	973	72
25	473	35
100	0	0

Other salts also show some inhibiting effect (Table II). There is a specific effect

TABLE II

EFFECT OF DIFFERENT SALTS ON THE ACTIVITY OF INTERNAL YEAST INVERTASE

The concentration of salt was 0.1 M, and the salt was dissolved in the standard assay solution.

Salt	*Ionic strength*	*Enzyme activity (μmoles sucrose hydrolyzed per min per ml)*	*Relative enzyme activity (%)*
None	0.1	1203	100
NaCl	0.2	200	16.5
Na_2SO_4	0.3	409	34
$(NH_4)_2SO_4$	0.25	0	0

of certain ions. It is not known whether ionic strength itself has any influence on internal invertase action, but it is clear that the results shown in Table II, in part at least, are specific ion effects.

Effect of iodine on internal invertase

0.1 mM iodine in KI in pH 5.0 sodium acetate buffer inhibits the activity of internal invertase to 45% of the starting activity. The reaction is very fast and is complete within 1 min. There is no further inhibition in the following 30 min. 0.1 mM KI has no effect on internal invertase activity. The relative enzyme activity rises from 45 to 89% on treatment with 5.5 mM mercaptoethanol. The iodine-inhibited enzyme is styled iodine-invertase.

Reaction of CNBr and internal invertase

Internal invertase and internal iodine-invertase were reacted with 10 mM CNBr at pH 7.0, 25°. At intervals, aliquots were removed and the enzyme activity measured (Fig. 1). The inactivation of internal yeast invertase by CNBr was biphasic. There was a very rapid inactivation which occurred before the first reading at 1 min. This was followed by a second slow reaction, which was first-order in enzyme activity. The pseudo-first-order rate constant was 68.6 $(\pm 2.43) \cdot 10^{-6}$ sec^{-1} at 25°. The reaction

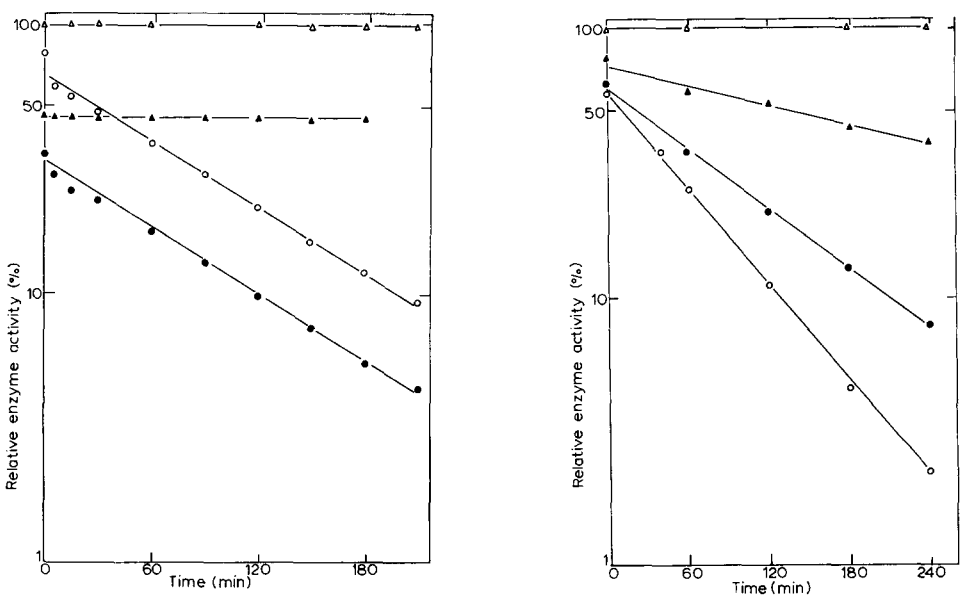

Fig. 1. The inhibition of internal yeast invertase by CNBr. Internal yeast invertase was reacted with 10 mM CNBr at pH 7.0, phosphate buffer, 25.0°. At intervals aliquots were removed from the reaction mixture and the enzyme activity was estimated in standard assay system. △, native internal invertase; ▲, internal iodine-invertase; ○, native internal invertase with CNBr; ● internal iodine-invertase with CNBr.

Fig. 2. Effect of pH on the rate of reaction between CNBr and internal yeast invertase. Invertase was incubated with 10 mM CNBr at pH 4.0, 6.0 or 8.0 and at 25.0°. △, native internal invertase at pH 8.0; ▲, invertase with 10 mM CNBr at pH 4.0; ●, invertase with 10 mM CNBr at pH 6.0; ○, invertase with 10 mM CNBr at pH 8.0.

with internal iodine-invertase was identical to that of internal invertase (Fig. 1). The slower reaction had a pseudo-first-order rate constant of 67.3 ($\pm$2.03)$\cdot 10^{-6}$ sec^{-1} at 25°. Both a rapid and a slow inhibition reaction occurred with both native internal and iodine-invertase. In the case of iodine-invertase, these two reactions occurred in addition to the inactivation already achieved by the treatment with iodine. The inhibition due to the faster reaction was similar; the activity dropped to 65% with native invertase and to 31% with iodine-invertase; but the iodine-invertase had an initial activity of 45% and therefore the cyanogen bromide caused to drop to 69% of this initial value. The rates of the slower reactions were identical.

Both native, internal invertase and iodine-invertase were stable under these conditions; the pseudo-first-order rate constants for their spontaneous inactivation were 5.02 ($\pm$2.28)$\cdot 10^{-7}$ sec^{-1} and 7.12 ($\pm$3.70)$\cdot 10^{-7}$ sec^{-1}, respectively. Both these values are about 1% of the values of the rate constants for inactivation by CNBr.

The effect of pH on the reaction between CNBr and internal invertase was examined (Fig. 2). Purified internal invertase was reacted with 10 mM cyanogen bromide at pH 4.0, 6.0 and 8.0, and 25.0°. At intervals the enzyme activity was measured by the standard assay method, and in addition the enzyme activity was determined before adding the CNBr. As a control, internal invertase was incubated at pH 8.0 at the same temperature. At all three pH values the reaction showed two phases, a rapid reaction and a slow pseudo-first-order reaction. The rate of the slower reaction was pH dependent (Fig. 3).

The fast reaction between CNBr and yeast invertase was too rapid to measure. The decrease in enzyme activity caused by the fast reaction was estimated by extrapolating the lines in Fig. 2 back to zero time. The inhibition due to the fast reaction

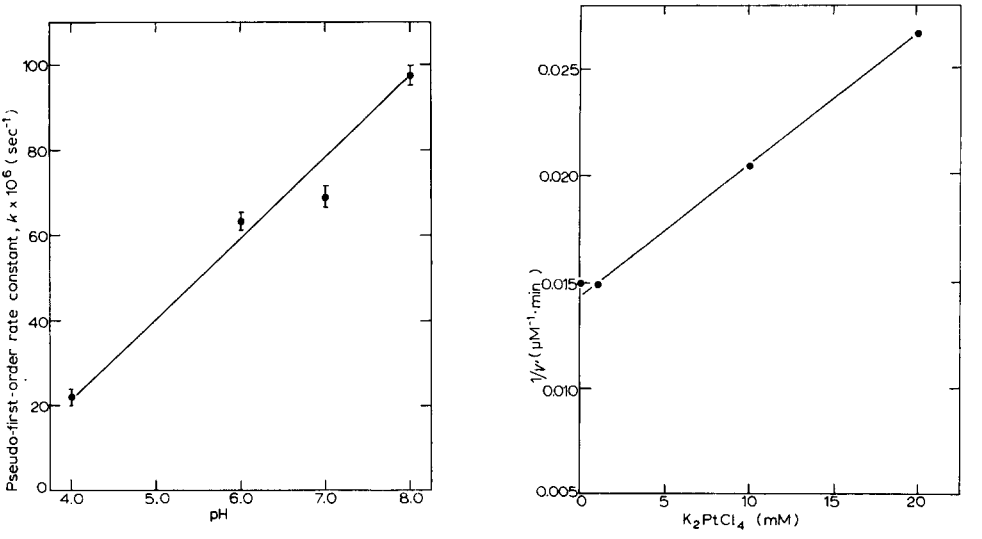

Fig. 3. Effect of pH on the pseudo-first-order rate constants for the slow reaction between CNBr and internal yeast invertase. The vertical bars indicate $\pm$1S.E. The solid line was obtained by regression from the weighted means.

Fig. 4. Plot of $1/V'$ against the concentration of inhibitor (I) (K_2PtCl_4). The inhibition constant K_i is obtained by dividing the vertical intercept by the slope.

was 25%, 40% and 44% at pH 4.0, 6.0 and 8.0, respectively. At pH 7.0 the inhibition was 35% for invertase and 34% for internal iodine-invertase.

Kinetics of internal invertase

The kinetic parameters, V and K_m, were measured with sucrose as substrate at pH 5.0, 25.0°, $I = 0.05$. The V value (131.5 ± 4.0 μmoles/ml per min) for internal iodine-invertase was 40% of the value for native, internal invertase (328.0 ± 2.4 μmoles/ml per min). The K_m value for internal iodine-invertase (48.8 ± 3.6 mM) was 1.5 times greater than the K_m value for native, internal invertase (32.3 ± 2.1 mM sucrose).

K_2PtCl_4 inhibition of internal invertase

The apparent Michaelis constant (K'_m) and the apparent maximal velocity (V') of internal invertase were determined in the presence of three different concentrations of K_2PtCl_4 at pH 4.9 and 25.0°. The platinum salt has no effect at all on the K_m' value of internal yeast invertase. The apparent maximal velocity (V') decreased with increasing platinum concentration. These observations are consistent with the standard non-competitive formulation of equation (1):

$$v_i = \frac{S \cdot V/(1 + I/K_i)}{K_m + S} \tag{1}$$

From Eqn. 1 we obtain:

$$V' = V/(1 + I/K_i) \tag{2}$$

the reciprocal of which gives:

$$\frac{1}{V'} = \frac{1}{V} + \frac{1}{V} \cdot \frac{1}{K_i} \cdot I \tag{3}$$

$1/V'$ is plotted against I in Fig. 4. A linear relationship clearly exists. We obtain the inhibition constant, K_i, by dividing the vertical intercept by the slope. The value of K_i estimated from Fig. 4 was 24.9 ± 1.16 mM K_2PtCl_4.

Comparison of the transferring activity of external and internal yeast invertase

Purified internal invertase was tested for transferring activity in the following way. 0.8 ml of each alcohol was mixed with 1.2 ml of 10 mM sodium acetate buffer, pH 4.9. After dissolving sucrose to 1 M in this solution, 0.1 ml of purified internal invertase was added. The solution was then incubated for 2 h at 25° after which 1-μl samples were transferred to thin-layer plates. Internal invertase has transferring activity with methanol, ethanol, propanol, isopropanol and benzyl alcohol (Fig. 5). As the number of carbon atoms in the side chain increased so did the R_F value of the fructoside which was formed. Internal invertase did not show any transferring activity with aniline nor with *p*-nitroaniline; both of these compounds inhibited the enzyme strongly as shown by the absence of glucose and fructose on the thin-layer chromatograms.

The transferring activity of internal iodine-invertase was tested in the following way. 1 ml of each alcohol was mixed with 1 ml of 10 mM sodium acetate buffer pH 4.9 containing 1 M sucrose. 0.2 ml of a solution of internal iodine-invertase was added and the mixture was incubated for 2 h at 25°. 1-μl samples were then analyzed by

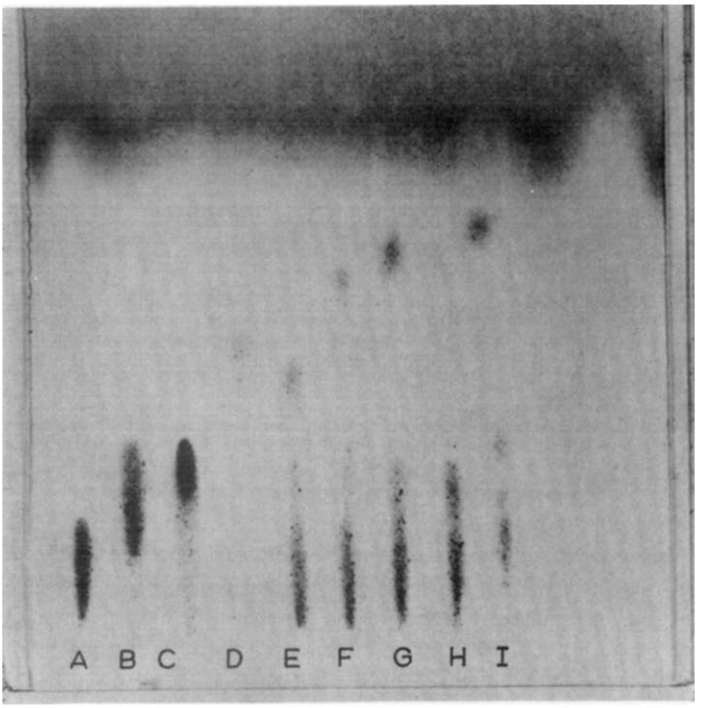

Fig. 5. Transferring activity of internal yeast invertase. The samples contained A, sucrose; B, fructose; C, glucose; D, *o*-nitrophenylgalactoside; E, methanol; F, ethanol; G, *n*-propanol; H, isopropanol; I, benzyl alcohol. A–D were controls containing only the alcohol. E–I were complete incubation mixtures as described in the text.

thin-layer chromatography. Internal iodine-invertase was able to transfer fructose to the following alcohols: methanol, ethanol, *n*-propanol, isopropanol and benzyl alcohol.

The transferring activity of external, yeast invertase was examined in the following way. 0.8 ml of each alcohol was mixed with 1.2 ml of 10 mM sodium acetate buffer, pH 4.9. After dissolving sucrose to 1 M in this solution, 0.04 ml of enzyme solution (1 mg/ml) was added. The solution was then incubated at 25° for 2 h after which time 1-μl samples were analyzed by thin-layer chromatography. Those alcohols which were either immiscible or insoluble in the buffer, were tested as 0.20 M solutions in 50% (v/v) dimethyl formamide and buffer, containing 1 M sucrose. External invertase showed transferring activity with the following compounds: methanol, ethanol, *n*-propanol, isopropanol, benzyl alcohol, aniline, nitroaniline, 2-mercaptoethanol, allyl alcohol, 2-chloroethanol. We were not able to detect any fructoside formation with: phenol, *p*-nitrophenol, cinnamyl alcohol, ethanethiol, 2,3-dinitronaphthol, 2,3-dinitrophenylhydrazine, *p*-nitrobenzyl alcohol, *tert.*-propanol, 6-bromo-2-naphthol, cyclohexanol or glycollic acid.

Isopropanol was exceptional because it was the only secondary alcohol that showed transferring activity; this activity was found with both external and internal invertases. Although Analar Grade (May and Baker) isopropanol was used, this

observation was checked after redistillation of the alcohol at 82°. The redistilled isopropanol produced fructoside with an R_F value of 0.80.

DISCUSSION

Internal invertase was purified directly from freshly grown yeast cells in order to avoid autolysis which may be responsible for the heterogeneity reported in external invertase[7]. Yeast cells could be satisfactorily disrupted in a flow cell of a small, laboratory sonicator. With a flow rate of 5 ml/min complete disruption was achieved as measured by the release of bound invertase. This method can cope with 300 ml of yeast suspension an hour when the suspension is made by adding equal volumes of packed cells and buffer. This will permit a useful laboratory scale preparation of enzyme.

The inhibition of internal invertase by NH_4^+ is of practical importance, although it is completely reversible. This inhibition recalls the inhibition of external invertase by other amines such as 2-amino-2-hydroxymethyl propan-1,3-diol (Tris)[11] and by anilines[12–15]. MYRBÄCK[11,13–15] has shown that the inhibition is due to the cationic form of all these amines. He has shown in the case of Tris (ref. 11) that the enzyme–inhibitor complex has an acid group with a pK_a of 2.2 which we would assign to a carboxylate group. Thus, the evidence is that cationic amines interact with a carboxylate anion in the enzyme and inhibit it. This conclusion is consistent with the hypothesis advanced by SHALL AND WAHEED (1968)[16] and by WAHEED AND SHALL (1971)[7] that there is an anionic group at or near the active site of invertase.

The inhibition of internal invertase by iodine is apparently identical to the inhibition of external invertase by iodine (WAHEED AND SHALL, 1971)[7]. This establishes that the inhibition results from reaction with the protein fraction since internal invertase has little or no carbohydrate associated with it[5,6]. The inhibition reaction was very rapid indeed and was complete within 1 min with an iodine concentration of 0.10 mM. The reversal of the inhibition by mercaptoethanol was observed; this is identical to the behaviour of external invertase[7,8,16]. The behaviour of internal invertase did not differ in any respect from external invertase. The activity of external iodine-invertase is not regenerated by cysteine, glutathione, ascorbic acid or sodium amalgam[17]. However, mercaptoethanol can regenerate the activity of both external iodine-invertase[7,8,16] and internal iodine invertase. We suggest that the iodine reaction is the reversible oxidation of a methionine sulphur atom and that this reaction leads to an approximate halving of the number of effective active sites. Since the reaction is reversible by mercaptoethanol the inhibition is probably not due to iodination of an aromatic ring. This view is supported by the evidence that in external invertase neither the active site imidazole nor the tyrosines are iodinated; also nitration does not affect enzyme activity[7]. The iodine reaction is independent of pH[18] and there is no cysteine detectable in internal yeast invertase[6] which, as we have shown above, can nonetheless be inactivated and reactivated in the same way as external invertase[7,8,16]; from this we conclude that iodine is oxidising a particularly reactive methionine sulphur atom.

CNBr reacts with a number of amino acids and the reaction depends on the pH. In alkaline solution CNBr reacts with a variety of nucleophilic groups in proteins[19]. In acid solution, only two amino acids react with CNBr: methionine and cysteine.

WAHEED AND SHALL[7,8,16] have shown that CNBr inactivated external invertase and that two separate reactions occurred; a very rapid pH-dependent reaction and a slow pH-independent reaction.

The inhibition of internal yeast invertase by CNBr also consisted of two reactions; a very fast and a slow reaction (Figs. 1 and 2). The rate of the fast reaction was too great to measure, but the extent of this reaction was clearly pH dependent. This may be explained as being due to a relatively slow, pH-dependent conformational equilibrium with only one of the two forms reacting with the reagent.

The slower reaction with CNBr was first-order in enzyme activity (Figs. 1 and 2). The pseudo-first-order rate constants (Fig. 3) range from $22 \cdot 10^{-6}$ to $75 \cdot 10^{-6}$ sec^{-1}. The concentration of CNBr was 0.01 M. WAHEED AND SHALL[7] measured the analogous rates with external invertase and found a mean rate constant of $36.5 \cdot 10^{-6}$ sec^{-1}, with a CNBr concentration of 0.1 M. It seems that the concentration of CNBr does not affect the rate of the reaction.

The increase of the pseudo-first-order rate constant for the slow reaction (Fig. 3) was 4.4 times between pH 4.0 and 8.0. If the rate constant were dependent on the ionization of an acid over this pH range an increase in rate constant of 10^4 would be anticipated. From this consideration we conclude that in this reaction CNBr is not reacting with an ionizing group but is reacting with a non-acid species. However, the rate constant is affected slightly by the state of ionization of another group on the protein. The slow reaction of CNBr with internal invertase is probably with a methionine residue because it is slow and independent of pH and because GASCÓN *et al.*[6] report that they were unable to detect cysteine in internal invertase. We do not know why external invertase showed no pH effect when reacting with CNBr[7].

The reactions with iodine and with CNBr are independent reactions, although we have concluded that they are both reacting at a methionine sulphur atom. This leads to the conclusion that there are at least two methionine groups at or near the active site of yeast invertase.

Further evidence for the presence of a methionine residue at or near the active site of invertase was obtained from the inhibition of invertase by K_2PtCl_4 since it inhibits invertase non-competitively with a dissociation constant of 0.025 M (Fig. 4). DICKERSON *et al.*[20] observed that K_2PtCl_4 bound to a methionine sulphur atom in every case in six examples of five different proteins. In addition, there were two examples of binding to one sulphur of an exposed disulphide bond and three examples of weak binding to histidine. K_2PtCl_4 can therefore be used, with caution, as an amino acid specific reagent, binding preferentially to methionine and occasionally to exposed disulphide links and to a much lesser extent to histidine.

It has been clearly established that internal yeast invertase has transferring ability for a variety of primary aliphatic alcohols as well as for isopropanol and benzyl alcohol. To this extent it is not different from the external enzyme. It is clear, therefore, that the carbohydrate plays no part in the transferring activity. However, some difference between the external and internal enzyme was observed. The external enzyme will use aniline and nitroaniline while the internal enzyme did not seem to synthesise fructosides from these compounds. However, since the anilines are very potent inhibitors of the internal enzyme, it may be that we have not found the best conditions for demonstrating the formation of the fructosides of these compounds.

There was no detectable qualitative difference between the transferring ability of internal invertase and internal iodine-invertase.

Although external invertase can use ethanol to form fructosides, it does not use glycollic acid. Once again, we have an example of a negatively charged reagent which is unacceptable to yeast invertase. The external enzyme can use 2-mercaptoethanol, but presumably synthesises only the *O*-fructoside and not the *S*-fructoside because it does not synthesise a fructoside with ethanethiol.

All the information presented in this paper on internal invertase is consistent with the mechanism of action earlier proposed by WAHEED AND SHALL[7] for external yeast invertase. The essence of the proposal is acid catalysis by an imidazolium cation, leading to an electron-deficient intermediate which is stabilised by an active centre carboxylate anion. We suggest that the external and internal yeast invertases have identical mechanisms of reaction.

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