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THE INVOLVEMENT OF SALT LINKS IN THE STABILIZATION OF BAKER'S YEAST INVERTASE

EVIDENCE FROM IMMOBILIZATION AND CHEMICAL MODIFICATION STUDIES

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Summary

Baker's yeast invertase was immobilized onto microcrystalline cellulose DEAE- and CM-Sephadex and insolubilized concanavalin A, with 46, 41, 70 and 73% recovery of activity, respectively.

The concanavalin A and microcrystalline cellulose invertase complexes exhibited spreading of their pH optimum curves, possessed similar K_m and V and were more thermally stable in the absence and presence of 0.12 M sucrose, than the soluble enzyme.

DEAE- and CM-Sephadex · invertase complexes were extremely unstable towards heat, compared to the soluble enzyme, losing all activity within 1 min of heating to 65°C. However, normal thermal stability of these complexes was observed in the presence of 20 mM succinic acid and 20 mM ethylenediamine, respectively.

The treatment of purified invertase with citraconic anhydride, which converts the amino group function to carboxyl groups, resulted in the loss of enzyme activity. The enzyme, with 50% activity after such treatments, was less thermally stable than the untreated enzyme. Similar results were obtained using ethylene diamine and a water-soluble carbodiimide that converts carboxyl group function to amino group function. Treatment of invertase with methylacetimidate (which modifies amino groups of proteins but retains the positive charge) resulted in no loss in activity and very little effect on thermal stability.

It is suggested that salt linkages may be important in maintaining the conformational stability of invertase. Modification of either amino or carboxyl groups chemically or by immobilization on to charged supports, would result in the

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loss of conformational stability, unless these stabilizing salt linkages were preserved.

Introduction

Purified baker's yeast invertase is a phosphomannan protein [1] containing 50% (w/w) carbohydrate [2] which catalyses the hydrolysis of sucrose to an equimolar solution of glucose and fructose. The mannan moiety is not essential for catalytic activity [3] and can be removed from the protein with little or no effect on its thermal stability [3,4].

Little is known about the mechanism whereby, during its biosynthesis, this enzyme protein achieves conformational stability, but it is presumably due to amino acid interactions (e.g. hydrogen bonding, hydrophobic and ionic interactions) in the protein tertiary (and, perhaps, quaternary) structure [5]. Any methods for immobilizing invertase should not interfere with such stabilizing interactions. Yeast invertase bound to DEAE-cellulose exhibits lower thermal stability than the soluble enzyme [6—9], suggesting that the stabilizing interactions have been disturbed by such immobilization.

We have immobilized baker's yeast invertase on to concanavalin A insolubilized on agarose. Concanavalin A binds α-D-mannans [10] and has been shown to bind invertase [11]. For comparison, invertase was immobilized, via its hydroxyl groups, on to microcrystalline cellulose and DEAE- and CM-Sephadexes. It was found that the agarose-concanavalin A and microcrystalline cellulose complexes with invertase, but not ionically-bound invertase, exhibited greater thermal stability than the soluble enzyme. Soluble invertase treated with reagents that modify amino and carboxyl groups of proteins exhibited lower thermal stability compared to the native soluble enzyme.

These results are consistent with our hypothesis that salt-links may be important in maintaining the conformational stability of invertase [12].

Materials and Methods

Enzyme source and assay

Commercial baker's yeast invertase concentrate was a gift from Honeywill and Stein Co. Ltd., Wallington, Surrey and contained 4000 units/ml. The enzyme was also isolated and purified from baker's yeast [3]. The purified enzyme had a specific activity of 2537 units/mg protein at 25°C, which was similar to previously published results [13]; we found a mannan content of 62% (w/w).

Invertase activity was assayed as previously described [14]. The agarose-concanavalin A and microcrystalline cellulose complexes with invertase were assayed at pH 4.7; the pH optima of these bound enzymes were similar to that of the soluble enzyme. The DEAE- and CM-Sephadex · invertase complexes were assayed at pH 4.0 and pH 5.6, which were their respective pH optima. The reason for the change in the pH optimum of an enzyme immobilized on to a charged carrier has been described [15].

Enzyme immobilization

A suspension of concanavalin A insolubilized on to agarose was purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The coupling of invertase to concanavalin A-agarose was performed as follows. 1 ml concanavalin A suspension was added to the purified invertase solution (3 mg enzyme (≡891 units), in 5 ml 10 mM sodium phosphate buffer, pH 7.2). The mixture was shaken for 30 min at room temperature. The resulting invertase · concanavalin A-agarose complex was centrifuged and the supernatant fraction assayed for invertase activity. The complex was then washed with buffer until no activity was detectable in the washings and finally resuspended in 6 ml of the same buffer. Purified invertase was used for coupling to concanavalin A-agarose since the more crude commercial invertase was likely to contain "non-invertase" mannan, which might preferentially bind to the carrier.

Commercial invertase (0.4 ml = 1600 units) was used for covalent immobilization on to microcrystalline cellulose (Sigma cell type 20) using the metal-link titanium chloride method of Emery et al. [16].

Immobilization of commercial invertase on the DEAE-Sephadex A-50 and CM-Sephadex C-50 (from Pharmacia Ltd., London) was performed as follows. The anion and cation exchanger (0.5 g dry weight of each) was equilibrated in 100 ml 10 mM sodium phosphate buffer (pH 7.0) and 10 mM sodium acetate buffer (pH 3.6), respectively, for 3 days at room temperature. Commercial invertase (0.1 ml = 400 units) was added to 10 ml of each equilibrated Sephadex (≡50 mg dry weight) and mixed for 30 min at room temperature. The Sephadex · invertase complexes were centrifuged and washed with their equilibrating buffers until no enzyme activity could be detected in the washings. The complexes were then resuspended in their equilibrating buffer to give 10 ml.

Chemical modification studies

Protein ϵ-amino groups react with citraconic anhydride at alkaline pH and are converted to anionic residues [17]. For the modification of invertase amino groups, purified invertase (5 mg = $18.5 \cdot 10^{-3}$ μmol) was diluted to 5 ml with 100 mM sodium phosphate buffer (pH 8.0) and allowed to react at room temperature with a range of citraconic anhydride (B.D.H., Poole, Dorset; 55.75—557.5 μmol). The pH of the solution was maintained at 8.0 by the addition of 1 M NaOH. After each addition of citraconic anhydride, the effect upon enzyme activity was determined using a suitably diluted aliquot of the reaction mixture.

Methylacetimidate reacts specifically with free ϵ-amino groups of proteins the resulting acetamidination preserving the positive charges near these sites [18]. Purified invertase (1 mg = $3.7 \cdot 10^{-3}$ μmol) was dissolved in 0.9 ml 200 mM-sodium carbonate buffer (pH 10.0) and $7.0 \cdot 10^{-2}$ μmol methylacetimidate (Pierce Chemicals, Rockford, Illinois, U.S.A.) was added. The reaction was allowed to proceed for 1 h at room temperature, after which a 0.1-ml aliquot was diluted to 1 ml with 100 mM sodium acetate buffer (pH 4.7) and 0.1 ml of this diluted enzyme was assayed for activity. The remainder was dialysed overnight against buffer (control experiments in buffer pH 10.0, without methylacetimidate).

The water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide modifies carboxyl groups of proteins [19] and in the presence of ethylenediamine the carboxyl groups are converted to cationic (possitively charged) residues. Purified invertase (1 mg = $3.7 \cdot 10^{-3}$ μmol) was dissolved in 1 ml 0.5 M ethylenediamine (pH 4.7) and the reaction initiated by the addition of carbodiimide to 0.1 M. During the reaction at 25°C, the pH was maintained at 4.7 with 1 M HCl. It should be noted that the reaction was done in water (not sodium acetate buffer) and the pH adjusted as described. At prescribed intervals of time, 0.1-ml aliquot of the mixture was removed and quenched in 0.1 M sodium acetate buffer (1.9 ml) and the remaining activity determined.

Thermal stability studies

These studies were carried out at 65°C in the absence and presence of 0.12 M sucrose [14]. Heating was performed in 0.1 M sodium acetate buffer (pH 4.7) except for the DEAE- and CM-Sephadex · invertase complexes which were heated in the same buffer at pH 4.0 and 5.6, respectively.

Results and Discussion

Invertase was bound by all supports tested (Table I). Invertase-concanavalin A-agarose exhibited high retention of specific activity (73%) which might be expected, since this method of immobilization does not involve binding of the protein moiety (and hence essential catalytic groups) to the support. The invertase · microcrystalline cellulose complex exhibited 46% retention of specific activity, which is considerably higher than the value of 3.6% reported previously [20]. 50—70% of invertase activity was lost when the enzyme was ionically bound to anion exchanges such as DEAE- and DEAA-cellulose [6,9,21]. Our results are in agreement with these findings for the immobilization of invertase on to DEAE-Sephadex. We found however that invertase bound to CM-Sephadex had high retention of specific activity (70%). No loss in the original enzyme activity of tomato invertase was found when it was bound to CM-cellulose [22]. A summary of some of the properties of the bound invertases compared to the soluble enzyme is given in Table II.

The concanavalin A-agarose and microcrystalline cellulose to invertase complexes exhibited spreading of their pH optimum and stability curves and their K_m and V values were similar to the native enzyme, suggesting freedom from

TABLE I

PREPARATION OF IMMOBILIZED INVERTASE

Type of support	Invertase added (units)	Invertase in washings (units)	Bound invertase (A) maximum (units)	Activity of complex (B) (units)	(B)/(A)
Concanavalin-A-Agarose	891	85	806	588	73
Microcrystalline cellulose	1600	1000	600	275	46
DEAE-Sephadex A-50	400	0	400	164	41
CM-Sephadex C-50	400	100	300	210	70

TABLE II

KINETIC PROPERTIES OF THE IMMOBILIZED INVERTASE PREPARATIONS

Type of support	pH optimum	pH maximum stability	K_m (mM)	V (μmol · min^{-1})
None *	3.5—6.0	3.5—6.0	28.0	20.5
Concanavalin-A-Agarose	2.5—7.0	2.5—6.0	28.5	19.6
Microcrystalline cellulose	2.5—7.0	2.5—6.0	28.5	33.0
DEAE-Sephadex A-50	3.5—5.6	3.5—5.6	n.d. **	n.d. **
CM-Sephadex C-50	4.7—7.0	4.7—7.0	100.0	25.0

* The purified and commercial invertases possessed similar properties.
** n.d. = not determined.

diffusional restrictions of substrate into, and products out of, the bound enzyme.

We found that both the concanavalin A-agarose and microcrystalline cellulose-invertase complexes exhibited improved thermal stability at 65°C in the absence and presence of 0.12 M sucrose compared to the native soluble enzymes (Fig. 1 and 2). It should be noted that both the soluble and immobilized invertases exhibited greater thermal stability in the presence of substrate, than in its absence. In 0.12 M sucrose, the progress of sucrose hydrolysis

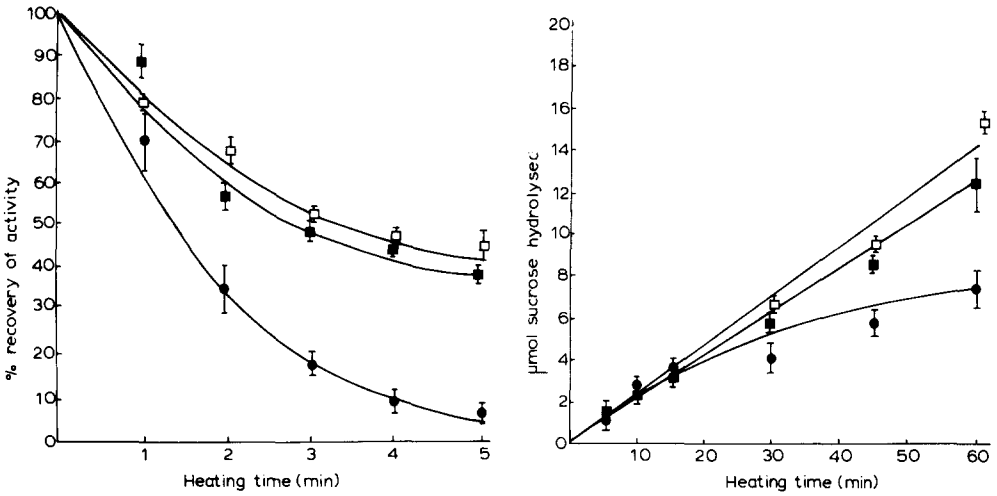

Fig. 1. Thermal stability (65°C) of soluble (●———●) and microcrystalline cellulose-invertase (□———□) and agarose-concanavalin A-invertase (■———■) in the absence of substrate. [Suitably diluted aliquots (0.1 ml) of the soluble and immobilized enzymes were heated in 2.0 ml of sodium acetate buffer (pH 4.7) for the times indicated and the residual activity determined [14].] Values are mean ± S.D.

Fig. 2. Thermal stability (65°C) progress curves of soluble and immobilized invertase in the presence of 0.12 M sucrose. Approximately 0.1 unit of soluble or immobilized invertase was added to 3.4 ml 0.12 M sucrose in 0.1 M sodium acetate buffer (pH 4.7) for initiating the reaction. At the indicated times the invert sugar formed was measured [14]. Key as Fig. 1. Values are mean ± S.D.

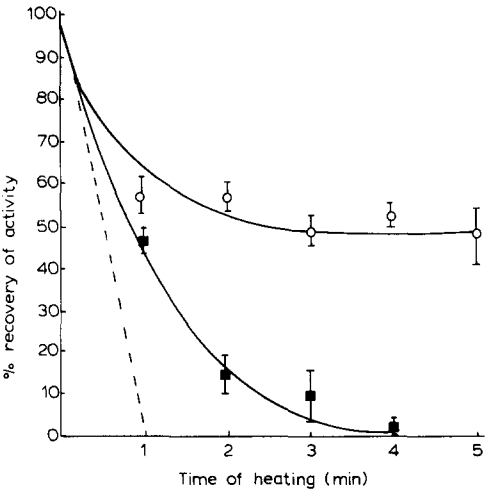

Fig. 3. Thermal stability at 65°C of DEAE-Sephadex- (■———■) and CM-Sephadex- (○———○) invertase complexes in the presence of 20 mM succinic acid and 20 mM ethylenediamine respectively. [Dashed line represent the thermal stability of both complexes in the absence of ethylenediamine or succinic acid showing total loss of enzyme activity within 1 min of heating.] Values are mean ± S.D.

(Fig. 2) for each immobilized enzyme was linear for at least 60 min at 65°C (i.e. no loss of activity). In contrast, the progress of sucrose hydrolysis by soluble invertase departed from linearity after 15 min at 65°C. There was only 30% recovery of activity for both soluble enzymes after 60 min at 65°C.

In contrast, the DEAE- and CM-Sephadex invertase complexes exhibited lower thermal stability than the soluble enzyme at 65°C, losing all activity within 1 min when heated in the absence of substrate. This is in agreement with others who have found that ionically-bound invertase in thermally less stable than the soluble enzyme [6—8]. Significant stabilization of the DEAE- and CM-Sephadex invertase complexes was achieved if they were heated in 20 mM succinic acid and 20 mM ethylenediamine, respectively (Fig. 3). This suggests a beneficial effect in the restoration of lost ionic charge of the appropriate type. No elution of the enzyme from the support or alteration of the pH of the complex suspension was observed in these treatments.

Since the binding of invertase to these ionic supports involves amino and carboxyl groups of the enzyme, it is postulated that salt-linkages may be important in maintaining the conformational stability of invertase. Thermal stabilization of DEAE- and CM-Sephadex invertase complexes was achieved by succinic acid and ethylenediamine, respectively, presumably due to the formation of new stabilizing salt-linkages to replace those which were broken upon immobilization (Fig. 4).

The treatment of purified invertase with citraconic anhydride, or carbodiimide in the presence of ethylenediamine, resulted in eventual inactivation of the enzyme. No inactivation of invertase was observed when it was treated with methylacetimidate. The thermal stability of the citraconylated and carbodiimide-modified invertase with 50% recovery of native activity was lower than that of the native enzyme. Invertase treated with methylacetimidate had similar (or slightly lower) thermal stability compared to the native enzyme (Table III).

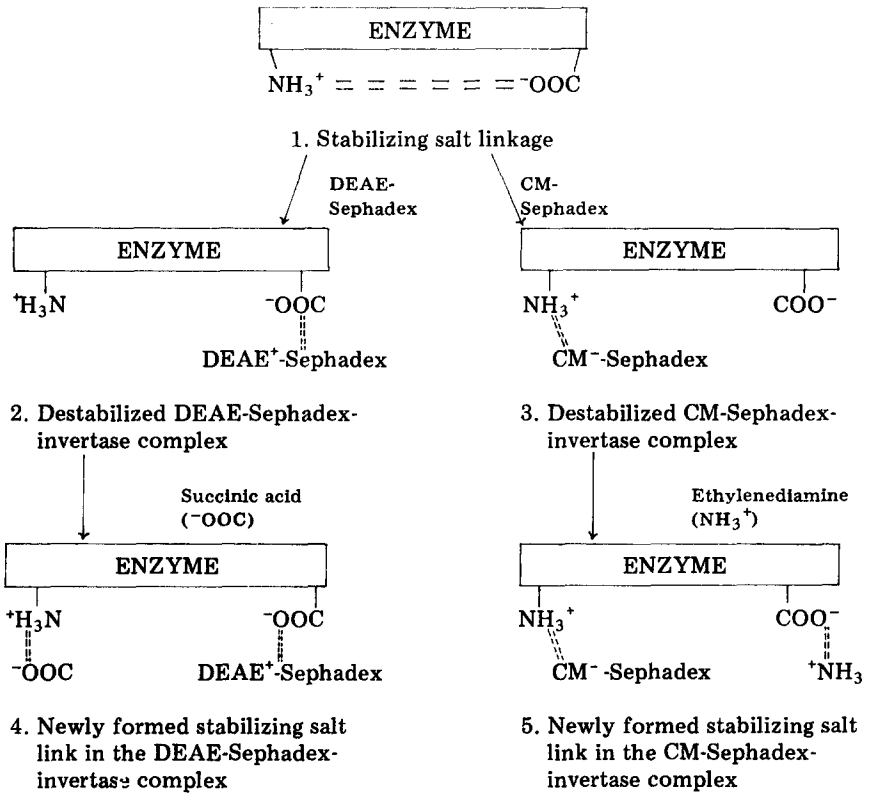

Fig. 4. A hypothetical approach to the thermal stabilization of invertase immobilized on DEAE-Sephadex and CM-Sephadex.

On possible explanation is that invertase is stabilized by salt-linkages on the surface of the enzyme molecule and that these ionic interactions are destroyed during some of these treatments. The modification of invertase amino groups with methylacetimidate has very little effect on thermal stability, presumably because the positive charge is preserved close to the site of modification and therefore could still participate in a stabilizing ionic interaction. The other modifications increase either the net negative or net positive charges (perhaps on the enzyme surface) and may cause a partial unfolding of the enzyme

TABLE III

Thermal stability of chemically modified invertases at 65° C

Modifying reagent	Recovery of Enzyme activity (%)	Pseudo-first order rate inactivation constant (k, min^{-1})
None	100	0.10
Citraconic anhydride	50	0.70
Carbodiimide	50	0.38
Carbodiimide	30	0.65
Methylacetimidate	100	0.14

leading to a disturbance of other stabilizing interactions (e.g. hydrogen bonding and hydrophobic interactions). This destabilization towards heat denaturation of yeast invertase when its amino or carboxyl groups are modified, chemically or by immobilization, is not a general effect for all enzymes. Lactate dehydrogenase and aminoacylase were stabilized against thermal denaturation when they were immobilized on to DEAE-cellulose [23,24] as were tomato invertase and phosphomonoesterase immobilized on to CM-cellulose [22,25]. Recently, lactate dehydrogenase was stabilized against heat denaturation upon acetamidination [26].

We have previously shown that *Candida utilis* invertase was stabilized against heat denaturation by its treatment with the bifunctional reagent, dimethylsuberimidate, which cross-links two ϵ-amino groups in the enzyme molecule and preserved the charge close to the site of modification [27]. Baker's yeast invertase, however, was destabilized against heat denaturation when treated similarly [4]. This suggests that there are subtle differences in the amino acid interactions stabilizing these two invertases. We have also found that baker's yeast invertase is destabilized against heat denaturation when it is heated in the presence of 100 mM $CaCl_2$, whereas *C. utilis* invertase is completely stabilized against heat denaturation when treated similarly (Woodward, J. and Wiseman, A., unpublished data). This suggests that Ca^{2+} binds carboxylate ions (in the baker's yeast invertase molecule) which are involved in stabilizing salt linkages, thereby lowering the conformational stability of the enzyme.

These results suggest that invertase immobilized on to concanavalin A-agarose and microcrystalline cellulose could have interesting industrial applications for the continuous production of invert sugar. We have previously reported that both complexes are stable to continuous hydrolysis of 0.12 M sucrose for at least 15 h at room temperature in a fixed bed reactor [28]. Both immobilized preparations are conformationally stable, because the amino acid-stabilizing interactions (salt linkages) are preserved.

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