Stabilization of Invertase by Molecular Engineering

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Extracellular invertase (EC 3.2.1.26) of Saccharomyces cerevisiae *was stabilized against thermal denaturation by intermolecular and intramolecular crosslinking of the surface nucleophilic functional groups with diisocyanate homobifunctional reagents (O═C═N(CH$_2$)$_n$N═C═O) of various lengths (*n = 4, 6, 8*). Crosslinking with 1,4-diisocyanatobutane (*n = 4*) proved most effective in enhancing thermostability. Stability was improved dramatically by crosslinking 0.5 mg/mL of protein with 30 μmol/mL of the reagent. Molecular engineering by crosslinking reduced the first-order thermal denaturation constant at 60°C from 1.567 min^{-1} (for the native enzyme) to 0.437 min^{-1} (for the stabilized enzyme). Similarly, the best crosslinking treatment increased the activation energy for denaturation from 391 kJ mol^{-1} (for the native protein) to 466 kJ mol^{-1} (for the stabilized enzyme). Crosslinking was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 26: 111–117, 2010
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Introduction

Enzymes are widely used in industrial processes (Uhlig, 1998). Novel processes designed for sustainable production of goods and services increasingly rely on enzymes (Gavrilescu and Chisti, 2005). Use of enzymes is limited by their poor thermostability. Enzymes typically denature rapidly at temperatures of >40°C. Thermostable enzymes can improve productivity of processes because the rate of a reaction typically doubles with every 10°C increase in temperature. Consequently, improvement of an enzyme's ability to withstand a relatively high temperature is an important objective. This work demonstrates the thermostabilization of a poorly heat stable invertase (EC 3.2.1.26) by introducing crosslinks within and between enzyme molecules. The impact of crosslinking on enzyme stability and kinetics is reported for various stabilization scenarios used in this study.

Previously, enzymes have been frequently stabilized by crosslinking to solid supports and entrapment within matrices (Betancor and Luckarift, 2008; Bryjak et al., 2008; Dizge et al., 2008; Mateo, et al., 2007; Polizzi et al., 2007; Vallejo-Becerra et al., 2008). The relevant immobilization methods have been reviewed by Sheldon (2007). Immobilization involving a solid support or matrix inevitably introduces diffusion limitations that can substantially slow the rate of a reaction particularly if the substrate or the products of the reaction are large molecules (Chisti, 1999; Goldstein, 1976). Therefore, the present study did not use solid supports. The enzyme was crosslinked such that its stability improved but it remained fully soluble.

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Invertase (β-D-fructofuranosidase, β-fructofuranoside fructohydrolase, saccharase, or sucrase) is commercially used as a biocatalyst in the hydrolysis of α-(1, 2)-glycosidic bond in sucrose. The resulting mixture of glucose and fructose is sweeter than sucrose and does not crystallize as readily as sucrose. Enhanced sweetness and a reduced tendency to crystallize are important advantages in confectionary processing (Uhlig, 1998).

Surface functional groups of enzyme molecules can be covalently crosslinked by using various bifunctional reagents (Wold, 1972). Crosslinking within the same molecule is intramolecular crosslinking. Intermolecular crosslinks are formed when the bifunctional reagent joins two enzyme molecules. Both intramolecular and intermolecular crosslinking can reduce a protein's tendency to unfold at temperatures that would normally denature it (Ó Fágáin, 1995, 2003; Reiner et al., 1977a,b; Torchilin et al., 1978, 1979). Since the crosslinking reagent must tightly bridge two protein functional groups, the distance between the surface groups determines the minimum length of an effective intramolecular crosslinker.

Different bifunctional reagents are required to crosslink different types of surface functional groups. The latter include, for example, the thiol group of cysteine, the amino group of lysine, the N-terminal amine, the C-terminal carboxylate, the carboxylic acid groups of aspartic and glutamic acids, the imidazol group of histidine, and the thioether group of methionine (Wong and Wong, 1992). Bifunctional reagents are classified as homobifunctional or heterobifunctional crosslinkers (Ó Fágáin, 1995). The two reactive groups of a homobifunctional reagent are identical whereas the two reactive groups of a heterobifunctional reagent are different. Bifunctional reagents may be further subdivided into those

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producing noncleavable crosslinks and those producing cleavable crosslinks (Han et al., 1984). The nature of the crosslinked protein depends on the nature of the reactive groups of the reagent, the size of the reagent, the location of the reactive functional groups on the surface of the enzyme, the ratio of the reagent-to-enzyme used during crosslinking, and the concentration of the protein being crosslinked.

Although many different bifunctional reagents can be used for crosslinking (Han et al., 1984; La Rotta Hernandez et al., 2005; Ó Fágáin, 2003; Wong and Wong, 1992), this study focussed on diisocyanates ($O{=}C{=}N(CH_2)_nN{=}C{=}O$) homobifunctional reagents because they are readily soluble in water, and this simplifies reaction protocols. Furthermore, crosslinking with diisocyanates requires mild reaction conditions that reduce the likelihood of damaging the enzyme during crosslinking. Diisocyanates form noncleavable crosslinks that are not likely to be damaged under the conditions of use of the stabilized enzyme. They are readily commercially available. They react with the nucleophilic functional groups on the enzyme molecule. Such groups include the free amino groups ($-NH_2$), hydroxyl groups ($-OH$), thiols ($-SH$) and carboxylic acids ($-COOH$). Free amino groups that may be available for crosslinking are most commonly provided by the amino acid residues of lysine, arginine, asparagine, and glutamine. Free hydroxyl groups occur on amino acid residues of serine, threonine, and tyrosine. Carboxylic acid functional groups are available on amino acid residues of glutamic acid and aspartic acid. Reaction is possible with the N-terminal amino group and the C-terminal carboxylic acid group. Because of its relatively high frequency of occurrence on different amino acids, the free amino group is perhaps one of the most common reactive functional groups that are available on the surface of globular proteins. Crosslinking with diisocyanates is potentially applicable to a wide range of globular proteins because they have numerous reactive surface functional groups available. Despite their broad usefulness, diisocyanates have not been used in stabilizing invertase. Use of bifunctional reagents has been reviewed elsewhere (Govardhan, 1999; Han et al., 1984; Lafuente et al., 1995; Ó Fágáin, 2003; Torchilin, 1979; Torchilin et al., 1979; Wong and Wong, 1992). Chemistry of isocyanates has been reviewed by Ozaki (1972).

Materials and Methods

Extracellular invertase (EC 3.2.1.26; 100–200 units/mg) of the yeast *Saccharomyces cerevisiae*, 1,4-diisocyanatobutane, 1,6-diisocyanatohexane, 1,8-diisocyanatooctane and 1-isocyanatobutane ($O{=}C{=}NCH_2CH_2CH_2CH_3$) were purchased from Sigma Chemical Company (St. Louis, MO). High molecular weight markers for SDS-PAGE were purchased from Amersham Biosciences (Piscataway, NJ).

Crosslinking of invertase

Enzyme was dissolved in 0.1 M citrate buffer, pH 6, at a protein concentration of 0.5 mg/mL. Diisocyanate reagent (1–50 μmol) was added to 1 mL of protein solution, vortex mixed and allowed to stand at room temperature for 15 min (Snyder et al., 1974). Crosslinked enzyme was separated from the residual reagent by a single use PD-10 gel filtration column (GE Healthcare, Buckinghamshire, UK; Sephadex^{TM} G-25 Medium, 13.5 mL column with 8.3 mL of stationary phase). A 2.5 mL sample of the reaction mixture was applied to the column and eluted under gravity with 0.1 M acetate buffer, pH 6. The first 2.5 mL of the eluted fraction was discarded. The next 3.5 mL of eluting material was collected as the protein containing fraction.

Invertase activity assay

Invertase activity was measured at 25°C with 100 g/L sucrose as substrate at pH 4.6 in 0.1 M citrate buffer. The produced reducing sugars were determined by dinitrosalicylic acid method (Boyer, 2000). One unit of invertase was defined as the quantity that liberates 1 μmol of reducing sugars per minute at pH 4.6 and 25°C. Spectrophotometric absorbance was measured at 540 nm (UV-visible spectrophotometer; Hitachi, Model 2000, Hitachi High-Technologies, Tokyo, Japan). A standard curve was prepared using dilutions of an equimolar solution of glucose and fructose. The absorbance (A_{540}) and standard sugar concentration (C, mg/L) correlated (regression coefficient = 0.986) as follows: $A_{540} = 7 \times 10^{-3}\ C$.

Enzyme stability measurement

Native and modified invertase in 0.1 M citrate buffer, pH 6, was incubated at various temperatures (25–60°C) for up to 2 h. Samples were taken at specified time intervals, cooled instantly in ice and assayed for activity.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 40 g/L acrylamide stacking gel and 65 g/L acrylamide running gel. SDS concentration in both gels was 1 g/L (Laemmli, 1970). Gels were completely polymerized in the usual way (Laemmli, 1970). Samples were dissociated by heating at 95°C for 4 min. The operating current was 15 mA. The gel had developed fully by 45 min. Gel was stained for 30 min in Coomassie solution (1 g/L Coomassie brilliant blue R-250 dissolved in 40% (vol/vol) methanol and 10% (vol/vol) acetic acid). Excess background color was removed by washing the gel in a solution of 40% methanol and 10% acetic acid for 1–3 h. A mini vertical electrophoresis unit (Hoffer Scientific, San Francisco, CA) was used for preparing gels that were 8 cm wide and 7.3 cm high.

Kinetics and Thermodynamics of Enzyme Denaturation

An enzyme's stability can be quantified in terms of its thermal denaturation rate constant, k_d and the activation energy of denaturation, E_d.

For otherwise fixed conditions, the rate v of an enzymatic reaction generally depends on the concentration of active enzyme (Shuler and Kargi, 2002; Verábel et al., 1997), which are as follows:

$$v = k[E] \tag{1}$$

where, k is a temperature-dependent rate constant and $[E]$ is the molar concentration of the active enzyme. At any fixed temperature, the concentration of active enzyme declines with time because of thermal denaturation. Thermal denaturation rate is typically first-order in the concentration of the active enzyme (Shuler and Kargi, 2002; Verábel et al., 1997); thus,

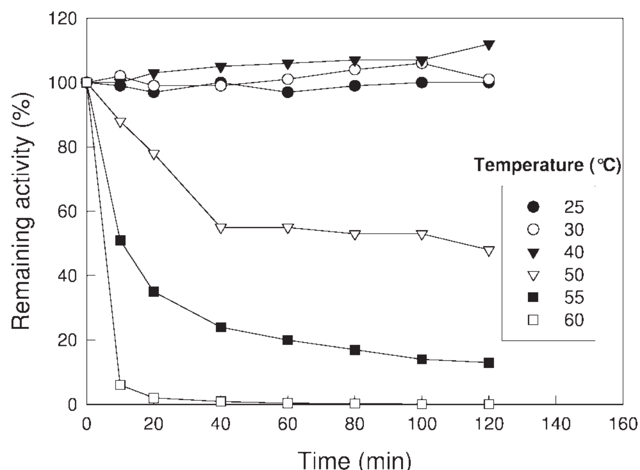

Figure 1. Thermal denaturation of native invertase.

Percent of initial activity remaining after incubation at various temperatures in 0.1 M citrate buffer, pH 6.

$$-\frac{d[E]}{dt} = k_d[E] \qquad (2)$$

where k_d is the denaturation rate constant and t is time.

At a constant temperature, Eq. 2 can be integrated between the limits $t = 0$, $[E] = [E_o]$ and $t = t$, $[E] = [E]$, or:

$$-\int_{[E_o]}^{[E]} \frac{d[E]}{[E]} = k_d \int_0^t dt \qquad (3)$$

where $[E]$ and $[E_o]$ are the active enzyme concentrations at times t and zero, respectively. Concentrations $[E]$ and $[E_o]$ in Eq. 3 can be replaced with the corresponding activities without any impact on the outcome. Solution of Eq. 3 is as follows:

$$\ln\frac{[E]}{[E_o]} = -k_d t, \qquad (4)$$

or

$$[E] = [E_o]e^{-k_d t}. \qquad (5)$$

Substitution of Eq. 5 in Eq. 1 leads to the following:

$$v = k[E_o]e^{-k_d t}, \qquad (6)$$

where both k and k_d depend on temperature. From Eq. 6 the rate of reaction at $t = 0$, i.e. the initial activity v_i at a given temperature, is

$$v_i = k[E_o] \qquad (7)$$

From Eqs. 6 and 7, the fraction of initial activity (i.e. v_f) remaining at any time t, is:

$$v_f = \frac{v}{v_i} = e^{-k_d t}, \qquad (8)$$

Therefore,

$$\ln v_f = -k_d t. \qquad (9)$$

Thus, a plot of ln v_f vs. t should be a straight line of slope $-k_d$. This allows calculation of the denaturation rate constant.

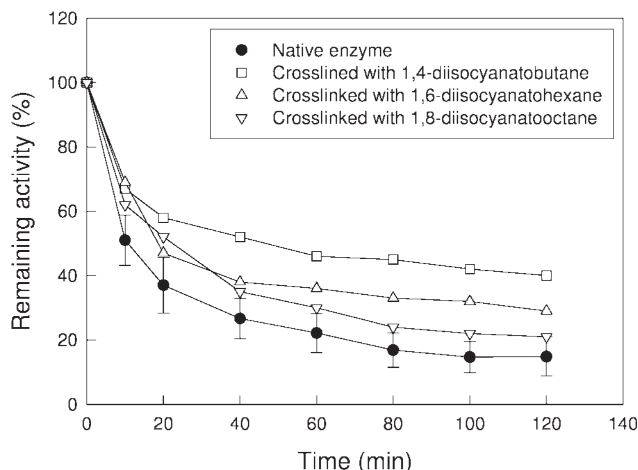

Figure 2. Thermal denaturation of native invertase (control) and the enzyme modified by crosslinking with diisocyanate crosslinkers of various chain lengths.

Incubation was at 55°C in 0.1 M citrate buffer, pH 6. In all cases, 0.5 mg/mL of protein was reacted with 1 μmol/mL of the crosslinker.

As for the rate constant of any chemical reaction, the rate constant for enzyme denaturation depends on absolute temperature T, which is as follows (Copeland, 2000):

$$k_d = Ae^{-E_d/RT} \qquad (10)$$

where A is the Arrhenius parameter, E_d is the deactivation energy (J mol^{-1}), and R is the gas constant (= 8.314 J mol^{-1} K^{-1}). Activation energy for thermal denaturation of enzymes is typically in the range of 165–550 kJ mol^{-1}. Arrhenius parameter is constant at a constant temperature.

Equation 10 can be linearized as follows:

$$\ln k_d = \ln A - \frac{E_d}{RT}. \qquad (11)$$

Denaturation energy for the enzyme can be determined by calculating k_d at various constant temperatures, plotting ln k_d vs. $1/T$, and reading the slope of the resulting straight line as $-E_d/R$.

Results and Discussion

The model native invertase was fully stable at an incubation temperature of up to 40°C in 0.1 M citrate buffer at pH 6 (Figure 1). Under these conditions, 100% of the initial activity remained after 120 min of incubation. The native enzyme was rapidly denatured at incubation temperatures of ≥50°C (Figure 1). As expected, the rate of thermal denaturation increased with increasing temperature. At an incubation temperature of 60°C, nearly 95% of the original activity had been lost within 10 min (Figure 1).

Effect of crosslinking on enzyme denaturation at 55°C is shown in Figure 2. Compared to the native enzyme (i.e. the control), crosslinking with all the diisocyanate reagents improved enzyme stability. For example, the enzyme crosslinked with 1,4-diisocyanatobutane retained nearly 50% of its original activity after 80 min of incubation compared with less than 20% of the original activity retained by the noncrosslinked control (Figure 2). Enzyme denaturation profiles were highly reproducible, as shown for the representative case of the native enzyme in Figure 2. The error bars

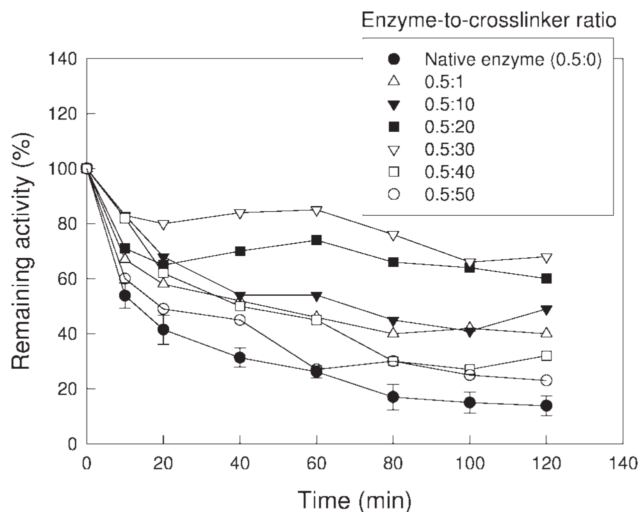

Figure 3. Thermal denaturation of native invertase (control) and the enzyme (0.5 mg/mL protein) crosslinked with 1,4-diisocyanatobutane (1–50 μmol/mL) at various ratios of the enzyme to crosslinker.

Incubation was at 55°C in 0.1 M citrate buffer, pH 6.

denote the standard deviation of the measurements of eight separate denaturation experiments (Figure 2).

Crosslinking with 1,4-diisocyanatobutane had the greatest stabilizing effect (Figure 2). Because of its short length, compared to the other crosslinkers, 1,4-diisocyanatobutane could only crosslink surface functional groups that were relatively close together on a given molecule of the enzyme. Furthermore, during any intermolecular crosslinking, this short crosslinker held the crosslinked enzyme molecules tightly together. In contrast, a longer crosslinker could crosslink all those surface groups that could be crosslinked by a shorter crosslinker and possible additional groups that were farther apart. Therefore, for a long crosslinker, once one end of the molecule had attached to the enzyme, the other end had a statistically larger choice of functional groups to attach to, compared to a shorter crosslinker. However, for a longer crosslinker, many of the possible binding scenarios produce loose or floppy crosslinks as opposed to a taut crosslink. Only a relatively taut crosslink is likely to be effective in substantially restricting thermally induced unfolding of a protein molecule. Thus, if the crosslinkable surface groups are assumed to be uniformly distributed on the surface of a globular protein, a crosslinker that can just physically link the two closest functional groups has a greater probability of producing effective crosslinks, i.e. crosslinks that lead to stabilization, compared with a crosslinker that can link the functional groups that are farther apart than the average distance between two adjacent crosslinkable groups. This explains the greater stabilizing efficacy of 1,4-diisocyanatobutane compared to the two longer crosslinkers.

In view of its stabilizing efficacy, further experiments used only 1,4-diisocyanatobutane for stabilization. For a fixed concentration of protein (i.e. 0.5 mg/mL), the effect of different concentrations (0–50 μmol/mL) of 1,4-diisocyanatobutane during crosslinking was tested on the stability of the crosslinked enzyme. The results are shown in Figure 3. The native control enzyme was more rapidly denatured compared to enzyme samples that had been crosslinked using any of the tested concentrations of the crosslinker (Figure 3). A crosslinker concentration of 30 μmol/mL in a solution containing 0.5 mg/mL protein had the greatest stabilizing effect on the crosslinked enzyme. Enzyme that had been crosslinked at this combination of concentrations retained more than 70% of its initial activity after 120 min of incubation at 55°C (Figure 3). In contrast, under the same conditions, the native enzyme retained less than 20% of its initial activity.

For 1,4-diisocyanatobutane, increasing the concentration from 1 μmol/mL to 30 μmol/mL at a fixed concentration (0.5 mg/mL) of protein in the reaction mixture progressively increased the degree of stabilization achieved (Figure 3). Increasing the crosslinker concentration to >30 μmol/mL actually reduced the thermal stability of the crosslinked enzyme compared to the crosslinking scenario that used the crosslinker at a concentration of 30 μmol/mL (Figure 3). This behavior is easily explained: so long as the crosslinking process does not interfere with the active site of the enzyme and its ability to interact with the substrate, an increasing number of taut crosslinks is likely to progressively improve thermostability. An increasing number of effective links are likely to be produced with an increasing molar ratio of crosslinker-to-protein, so long as the protein has surface groups available to react.

To favor intramolecular crosslinking over intermolecular crosslinking, the concentration of protein during crosslinking should be such that the average distance between protein molecules is longer than the length of the crosslinker molecule. Some intermolecular crosslinking is acceptable, but it must not be extensive. Extensive intermolecular crosslinking produces large, insoluble multienzyme complexes that are severely diffusion limited especially if the substrate is also a large molecule (Chisti, 1999; Goldstein, 1976). Such large complexes invariably have many active sites that are completely inaccessible to the substrate.

If, for an otherwise effective crosslinker, the molar ratio of crosslinker-to-protein during the crosslinking process exceeds a certain value, a situation arises in which each reactive group on the bifunctional reagent is competing for a relatively few nucleophilic groups on the surface of the protein. If this occurs, only one end of the crosslinker will bind to the protein and the other end will remain free. Therefore, relatively few crosslinks will be produced. Consequently, for an otherwise effective crosslinker, exceeding the optimal molar ratio of crosslinker-to-protein will actually reduce the protein stability, as observed in Figure 3. An exact value for the optimal molar ratio is difficult to estimate because of the uncertainty in the molar mass of the enzyme.

In principle, reaction of the functional groups of an enzyme with certain monofunctional reagents can stabilize the enzyme without producing any crosslinks (Torchilin, 1979). This effect is caused by changes in surface charge or hydrophobicity of the protein. To clearly demonstrate that the observed stabilization was because of the crosslinking, the enzyme was reacted with 1-isocyanatobutane, the monofunctional analog of the bifunctional reagent 1,4-diisocyanatobutane. The concentration of the monofunctional reagent used during stabilization was 60 μmol/mL, or twice the molar concentration of the bifunctional reagent. This ensured that the monofunctional reagent provided exactly the same number of potential reactive groups as were provided by the bifunctional reagent. The concentration of protein in the reaction mixture was 0.5 mg/mL for both cases. The stability data are shown in Figure 4. Clearly, the enzyme treated with the monofunctional reagent is barely more stable than the

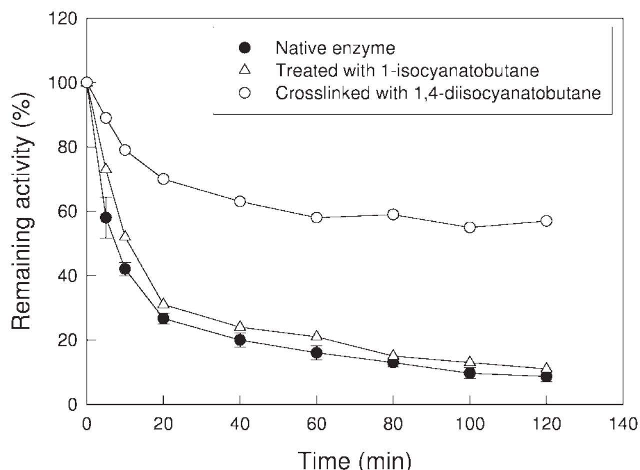

Figure 4. Thermal denaturation of native invertase (control), crosslinked invertase and invertase treated with the monofunctional reagent.

Incubation was at 55°C in 0.1 M citrate buffer, pH 6. Protein (0.5 mg/mL) was treated with 60 μmol/mL of the monofunctional reagent or 30 μmol/mL of the bifunctional reagent.

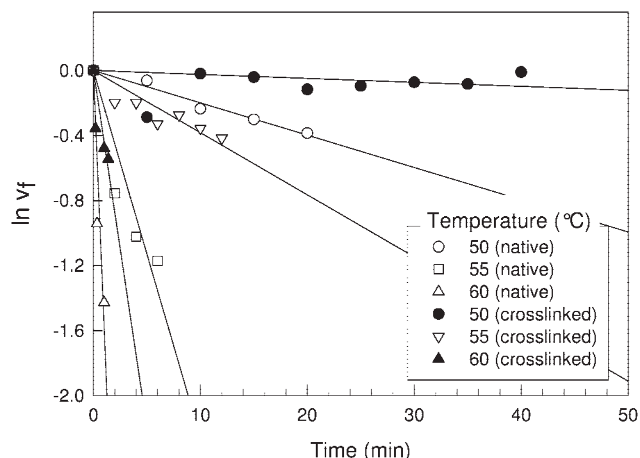

Figure 5. Estimation of denaturation rate constant (k_d) for native and crosslinked invertase at various temperatures in 0.1 M citrate buffer, pH 6.

Protein (0.5 mg/mL) was crosslinked with 1,4-diisocyanatobutane (30 μmol/mL).

Table 1. Denaturation Rate Constants at Various Temperatures

	k_d (min^{-1})	
Temperature (°C)	Native invertase	Crosslinked invertase*
50	1.99×10^{-2}	2.4×10^{-3}
55	22.54×10^{-2}	3.82×10^{-2}
60	156.72×10^{-2}	43.73×10^{-2}

* Protein (0.5 mg/mL) was crosslinked with 1,4-diisocyanatobutane (30 μmol/mL).

native enzyme (Figure 3). In contrast, the enzyme crosslinked with the bifunctional reagent has a dramatically improved stability (Figure 3).

In concurrence with Eq. 9, semilog plots of v_f vs. time were linear for both the native and the crosslinked enzyme (Figure 5). Slopes of these plots provided the values of the first-order denaturation rate constants (Table 1). For both the native and the crosslinked enzyme, the k_d values increased with increasing temperature; however, the crosslinked

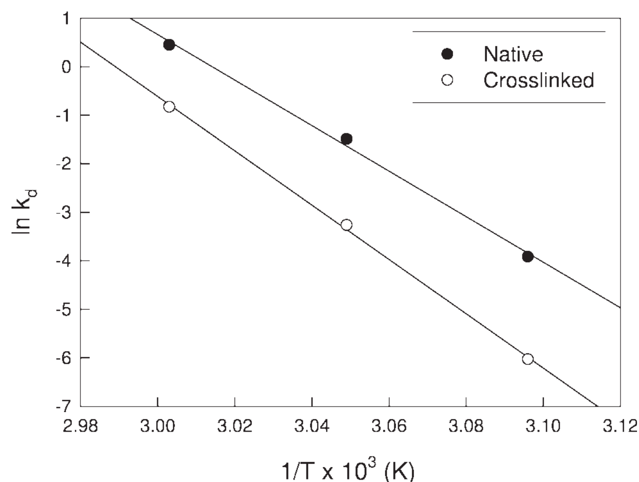

Figure 6. Estimation of activation energy of denaturation (E_d) for native and crosslinked invertase in 0.1 M citrate buffer, pH 6.

Protein (0.5 mg/mL) was crosslinked with 1,4-diisocyanatobutane (30 μmol/mL).

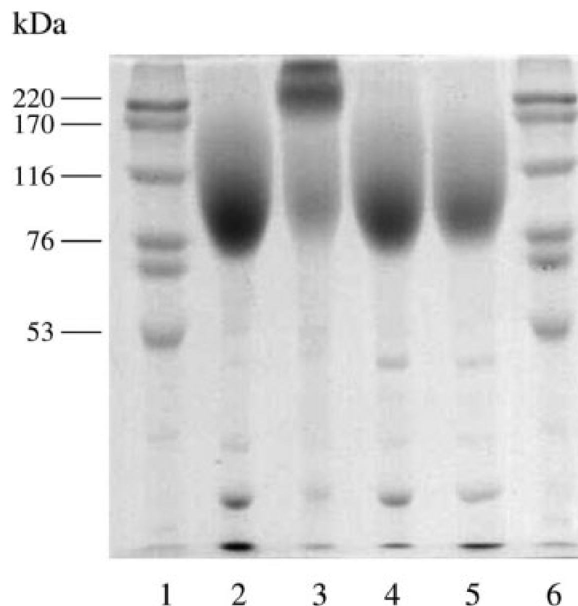

Figure 7. SDS-PAGE pattern of crosslinked and native invertase.

Lanes 1 and 6: molecular weight markers; lanes 2 and 5: native enzyme partially purified by gel filtration and as purchased, respectively; lane 3: invertase crosslinked by the bifunctional reagent 1,4-diisocyanatobutane; lane 4: invertase modified by the monofunctional reagent 1-isocyanatobutane. Protein (0.5 mg/mL) was reacted with either 1,4-diisocyanatobutane (30 μmol/mL) or 1-isocyanatobutane (60 μmol/mL).

enzyme always denatured more slowly than the native control.

Plots of ln k_d vs. 1/T were linear (Figure 6) as expected from Eq. 11. From the slopes of these plots, the activation energy for deactivation was calculated to be 391 kJ mol^{-1} for the native enzyme and 466 kJ mol^{-1} for the crosslinked enzyme. These values were within the expected range for enzymes.

Extracellular invertase of *Saccharomyces cervisiae* is a dimeric protein of about 120 kDa (Trimble and Maley, 1977), but its apparent molecular weight can be much higher because of the substantial amount of carbohydrate that is

associated with the protein (Trimble and Maley, 1977). In SDS-PAGE analysis, the dimers of approximately equal molecular weight are delinked and move together with the attached carbohydrates as a smudge around the molecular weight of 76 kDa (Figure 7, lanes 2 and 5). This diffuse migration of yeast invertase on SDS-PAGE is well known (e.g. Trimble and Maley, 1977). Treatment of the enzyme with the monofunctional 1-isocyanatobutane does not form crosslinks and barely alters the molecular weight of the enzyme. Consequently, the SDS-PAGE pattern does not change (Figure 7, lane 4) in comparison with that of the native enzyme (Figure 7, lanes 2 and 5). In contrast, the SDS-PAGE pattern for the crosslinked enzyme (Figure 7, lane 3) is totally different from that of the native enzyme: clear protein bands are observed around a molecular weight of 220 kDa. These bands are produced by the crosslinked dimers and the associated carbohydrates moving and some intermolecular crosslinking. Some intermolecular crosslinking is inevitable if the crosslinking reaction involves a relatively concentrated solution of the enzyme. Although intermolecular crosslinking can stabilize proteins by reducing their tendency to unfold, it should be avoided because it can produce insoluble multimeric enzyme complexes that are subject to severe mass transfer limitations. Furthermore, extensive intermolecular crosslinking inevitably blocks access of the substrate to many of the active sites that exist in the crosslinked multimeric enzyme complex.

Concluding Remarks

Compared to controls, a poorly thermostable invertase could be stabilized by crosslinking of the surface functional groups with 1,4-diisocyanatobutane, 1,6-diisocyanatohexane, and 1,8-diisocyanatooctane. 1,4-Diisocyanatobutane proved to be the most effective stabilizing crosslinker. Efficacy of thermostabilization depended on the ratio of the protein and stabilizer concentrations during the crosslinking reaction. Optimal stabilization required mixing of a protein solution containing 0.5 mg/mL protein with 30 μmol/mL of 1,4-diisocyanatobutane. Stabilization was achieved within 15 min at room temperature. Stabilized enzyme retained more than 70% of its initial activity after 120 min of incubation at 55°C whereas over the same period, the native enzyme lost more than 80% of its initial activity. Crosslinking with 1,4-diisocyanatobutane provided a mild, rapid, and highly effective method of stabilizing enzymes against thermal denaturation. The stabilization method demonstrated here is potentially applicable to any enzyme so long as any crosslinks formed do not interfere with the binding of the substrate to the active site and any essential functional groups at the active site do not react with the crosslinker.

Notation

A = Arrhenius parameter
DNS = dinitrosalicylic acid
E_d = activation energy of thermal denaturation
$[E]$ = molar concentration of the active enzyme at time t
$[E_o]$ = initial molar concentration of the active enzyme
k = rate constant of enzyme catalyzed reaction
k_d = thermal denaturation rate constant
R = gas constant (8.314 J mol^{-1} K^{-1})
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
T = absolute temperature
t = time
v = rate of an enzymatic reaction
v_f = fraction of initial enzyme activity remaining at time t
v_i = initial activity of enzyme at a given temperature

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