0273-2289/89/2021—0293$02.80

Enhanced Stability of Glucoamylase Through Chemical Crosslinking

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ABSTRACT

A series of bifunctional chemical modification reagents, presenting variations in both the chemistry of the functional groups and in the length of the spacer between the two reactive groups, have been evaluated as agents for enhancing the thermal stability of purified *Aspergillus niger* amyloglucosidase by means of intramolecular crosslinking. Several chemical modifiers (e.g., diimidoesters) were identified that more than double the half-life of this industrially important enzyme during incubation at 65°C in the absence of substrate. The increased stability of the modified enzymes has been correlated with changes in the fluorescence-monitored thermal denaturation curves of the modified enzymes, relative to that of the native enzyme.

Index Entries: Glucoamylase; chemical crosslinking; starch; thermostability; chemical modification.

INTRODUCTION

Amyloglucosidase (EC 3.2.1.3; glucoamylase) from *A. niger*, is an excellent model for industrially important, hydrolytic enzymes. This enzyme, an exo-splitting amylolytic enzyme that attacks amylose, amylopectin, and glycogen, is used widely in the second stage hydrolysis of starch to glucose and fructose syrups. The first stage in the process is the cleavage of the original polymer to oligosaccharides by bacterial α-amylase and is carried out at a temperature of 90°C. The lesser thermostability and lower temperature optimum of glucoamylase make it necessary to cool the reac-

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tor and its contents to 55–60°C before addition of the enzyme to start the second stage of the process, in which the oligosaccharides are completely converted to glucose (*1,2*). Were the glucoamylase more thermostable, it would be possible to initiate the second stage of hydrolysis immediately, without delay for cooling, and to carry out the hydrolysis of the oligosaccharides at a higher rate because of the higher reaction temperature. Use of a more thermostable glucoamylase would therefore increase the speccific activity of the digestion mixture and therefore the throughput of the reactor, and would also simplify the equipment required by removing the need for rapid cooling. Since enzyme (catalyst) production is a major contributor to conversion costs in most processes, enhanced stability will significantly improve overall process economics.

Cost sensitivities in starch processing industries continue to drive the development of alternative methods of high-temperature enzyme utilization, including glucoamylase immobilization (*3–5*), special fluidized bed reactor designs for such catalyst systems (*6*), and the search for more thermostable glucoamylases in nature (*7,8*). The modification of enzymes currently available from proven sources such as *A. niger*, which exhibits high enzyme productivity, holds considerable promise for immediate application, however. In addition, since intramolecular crosslinking of proteins has the potential for enhancing mechanical and chemical stability as well as thermal stability, the modified enzyme could be expected to maintain high activity for longer periods of time in standard stirred reactors and delivery systems. Application of such methodology is expected to be of particular value in production of industrial catalysts intended for continuous or repeated use, i.e., in the production of stabilized enzymes for subsequent covalent or ionic immobilization, or for use as soluble enzymes that are then recovered at the end of the reaction by adsorption on ion-exchangers.

Reticulation of enzymes (the addition of external, intramolecular "braces" by crosslinking the protein with bi- or poly-functional reagents) has been shown to increase the chemical, thermal, and mechanical stability of many enzymes studied (*2,9–14*). A wide variety of protein-crosslinking reagents are now available (*15*). In addition to the glutaraldehyde and carbodiimide/diamine systems used so heavily to connect amino and carboxyl groups, respectively, in the early reticulation studies (*9*), there are now available bifunctional reagents with *N*-hydroxysuccinimide and imidate moieties to react with amino groups (*16,17*), maleimido (*18*), and haloalkyl (*19*) moieties for reaction with sulfhydryls, and glyoxals for reaction with arginine (*20*). The recent addition of the extremely reactive nitrene- and carbene-generating reagents (*21,22*) to the list of bifunctional reagents means that even the relatively inert peptide nitrogens and aliphatic hydrocarbon side-chains of amino acids are subject to chemical crosslinking.

In crosslinking studies, as in any chemical modification study, there is the possibility that unless the active site of the enzyme is protected in

some way, the chemical reagents may react with catalytically essential groups in the active site, and thereby inactivate the enzyme. In experiments in which the object is to produce active modified enzymes, it is, therefore, advisable to protect the active site with substrate or with a competitive inhibitor, either of which will occupy the active site with a high frequency during modification and shield it from the reagent (*23,24*).

In an attempt to reduce interference from nonglucoamylase protein and other components found in the supernatant of fungal growth broth, and the difficulties in interpretation thus produced, the glucoamylase enzyme was purified prior to use in chemical modification studies.

MATERIALS AND METHODS

Materials

Soluble starch (Lintner), gamma-cyclodextrin, maltose, maltohexaose, triethanolamine, *N*-(5-azido-2-nitrobenzoyloxy) succinimide, *N*-(gamma-maleimidobutyryl) succinimide, glutaraldehyde, 2-iminothiolane, dimethylsuberimidate·2HCl, trichloroacetic acid, and bovine serum albumin, were obtained from Sigma Chemicals. Bifunctional dimethylimidoesters (malonimidate, succinimidate, glutarimidate, adipimidate, pimelimidate, azelaimidate, and sebacimidate) were obtained from United States Biochemical. Dextrans (T-10, T-40, and T-70) were from Pharmacia Fine Biochemicals.

Enzyme Assays

Glucoamylase activity was assayed by adding an aliquot of the protein solution to the substrate solution (consisting of 300 μL of 2% starch and 200 μL water) and incubating for one hour at 55°. Reaction mixtures were then quenched by the addition of 1 *M* trichloroacetic acid. The resulting solutions were assayed for glucose yield using a YSI (Yellow Springs Instruments) glucose analyzer. A procedure identical except for use of cyclodextrin as substrate was used to screen the preparations for contaminating endoglucanase activities.

Enzyme Purification

Glucoamylase activity was isolated from a Boehringer-Mannheim amyloglucosidase preparation (product number 102 857), which is an ammonium sulfate suspension of concentrated fermentation broth from *A. niger*. This product was found to contain less extraneous protein than other commercial *A. niger* supernatant preparations examined. After exhaustive dialysis against the elution buffer (100 m*M* phosphate buffer, pH 6 with 100 m*M* NaCl) the enzyme was loaded on a semi-preparative 2.1×60 cm Toyo Soda G3000 SWG high pressure size exclusion chroma-

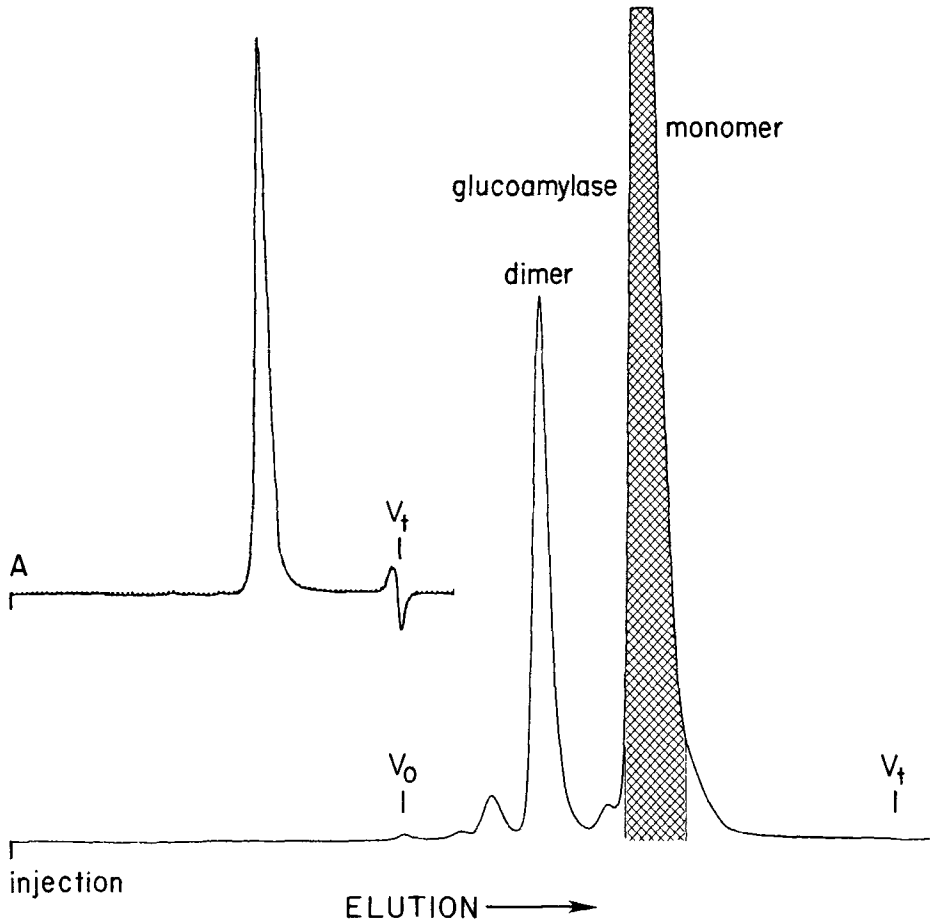

Fig. 1. Chromatographic purification of fungal glucoamylase from Boehringer-Mannheim preparation. Chromatogram shows the elution of activity (cross-hatched area) from a Toyo Soda G3000 SWG semi-preparative column and insert (A) shows the rechromatography of this fraction on a Toyo Soda G3000 SW analytical system.

tography (HPSEC) column. The HPSEC system used consisted of a Beckman model 110B pump, an Altex model 210 injection valve fitted with a 2 mL sample loop, and dual detection using a Beckman model 153 ultraviolet (254 nm) detector and a model 156 refractive index detector. Figure 1 shows a typical chromatogram from these preparative runs, along with the rechromatography (1A) on an analytical HPSEC column (0.75×60 cm) of the "heart cut" from the later-eluting of the two preparative peaks showing activity on starch and gamma-cyclodextrin. Based on its elution volume, and well-known column calibration methods (25), the low molecular weight starch degrading component was assigned a molecular weight of 65,000±9,000. The active fractions from preparative HPSEC were also subjected to polyacrylamide gel electrophoresis in the presence

of sodium dodecylsulfate (SDS-PAGE) following the methods of Laemmli (*26*). *N*-terminal sequence analysis was performed on a Beckman model 890C sequencer using the 0.2 *M* Quadrol program (Beckman No. 121178) following methods described by Fox et al. (*27*) and Himmel et al. (*28*).

Chemical Modification

Different samples of the monomeric form of glucoamylase, purified by column chromatography, were subjected to the following chemical modifications

1. Crosslinking agents with different chemical functional groups (some homo- and some hetero-bifunctional) were examined for effectiveness. These groups included an aldehyde (glutaraldehyde), imidate (dimethylsuberimidate dihydrochloride), carboxyl (*N*-(gamma-maleimidobutyryl) succinimide), azide (*N*-(5-azido-2-nitrobenzoyloxy) succinimide), and imine and sulfuryl (2-iminothiolane).
2. Crosslinking agents with the same chemical functional groups but different carbon chain lengths were used in a study of the effect of spacer length. These modifiers were dimethyldiimidates with chain lengths ranging from that of malonic acid (1 carbon between the functional sites) to that of sebacic acid (8 carbons between the functional sites).
3. Dextrans of different sizes (T-10, T-40, and T-70) were attached to glucoamylase following activation by cyanogen bromide.

Other experiments tested the effect of the presence of two active-site protectors, maltose and maltohexaose, on the activity and stability of the modified enzyme.

The products of all chemical modification schemes were checked for possible intermolecular crosslinking by HPSEC using the analytical system described in *Enzyme Purification*.

An aliquot of the purified enzyme to be used in chemical modification studies was assayed for protein content using the BCA (bicinchoninic acid) protein assay (Pierce). A value of 0.38 mg/mL was found. Stock solutions of bifunctional reagents were prepared in water and consisted of 1% wt/wt glutaraldehyde and 3–5 m*M* solutions of all other reagents. Exceptions were *N*-(5-azido-2-nitrobenzoyloxy)succinimide and *N*-(gamma-maleimidobutyryl)succinimide, solutions of which contained 5% ethanol to ensure solubility of the samples in water. The general procedure for preparation of the reaction cocktail entailed the addition of 1.00 mL of enzyme stock, 50 μL of crosslinking reagent, and enough 50 m*M* pH 8 Tris buffer to bring the final volume to 5.00 mL. When required, the active-site protectors maltose and maltohexaose were dissolved in water and added to the reaction cocktail to produce a final concentration of 1 m*M*. The mix-

Table 1
Thermostability of Modified and Native Glucoamylase

Chemical modifiers	Activity,[a] mg/dL glucose	Activity temperature optimum, °C	Apparent conformational transition,[b] °C
glucoamylase (monomer)	145	48	60
glucoamylase (dimer)	114	48	59
N-(5-azido-2-nitrobenzoyloxy) succinimide	121	47	64
glutaraldehyde	113	48	59
N-(gamma-maleimidobutyryl) succinimide	123	48	60
2-iminothiolane	118	49	67
dimethylmalonimidate	188	49	62
dimethylsuccinimidate	186	48	63
dimethylglutarimidate	111	48	61
dimethyladipimidate	100	46	61
dimethylpimelimidate	191	49	65
dimethylsuberimidate	197	49	63
dimethylazelaimidate	132	46	63
dimethylsebacimidate	97	46	62
dextran T-10	121	42	63
dextran T-40	114	42	62
dextran T-70	132	42	61

[a]Determined under standard assay conditions. Data obtained from duplicate experiment to that shown in Figs. 2 and 3.

[b]From studies of intrinsic protein fluorescence as a function of temperature (*see* Materials and Methods). Midpoint of change in wavelength of maximum emission.

tures were stirred at room temperature for 24 h on a rotator at 50 rpm. The resulting solutions were dialyzed against 50 m*M* acetate buffer, pH 5 with 100 m*M* NaCl, for 3 d and then concentrated about tenfold in an Amicon stirred cell concentrator fitted with a PM-10 membrane. Dextran-linked enzyme was prepared following the procedure of Marshall et al. (*29*), except that in this work acetate buffer was used.

The solutions of modified enzymes were diluted to an absorbance (280 nm) of 0.004 before use in assay procedures. The thermal stabilities of the chemically modified enzymes were evaluated both with and without substrate at various time intervals and temperatures (37–70°C). During preincubation activity studies, the enzymes were exposed to each temperature without substrate; aliquots (100 μL) were removed at various time intervals, and assayed as described above under *Enzyme Assays*. The values listed in Table 1 were obtained by normalizing the results from each modified enzyme with that of a native enzyme control. In this study, each crosslinking scheme was carried out at least twice and all enzyme assays were performed in duplicate to ensure repeatability.

Structural Stability Analysis

Thermal-scanning fluorescence spectroscopy was carried out using a SPEX Fluorolog Spectrofluorometer fitted with a water-jacketed cell housing for sample temperature control. Actual sample temperature was monitored by inserting an Omega microthermocouple (stainless steel sheathed) into the out-of-beam volume in the cell. Following the method of Elwell and Schellman (*30*), each sample was excited at 280 nm at a series of temperatures ranging from 30 to 90°C, while the emission spectrum was scanned from 310 to 450 nm. Samples were allowed to equilibrate for 15 min between temperature increments (typically, 2°C). For the native enzyme (both monomer and dimer) and for each of the chemically modified enzyme preparations, the wavelength of maximum emission intensity was plotted as a function of temperature. The temperature of maximum change in the wavelength of maximum emission as a function of temperature was found by numerical differentiation of the primary data plots of maximum emission wavelength vs temperature.

RESULTS AND DISCUSSION

Glucoamylase, obtained from Boehringer-Mannheim and purified to homogeneity by high pressure size-exclusion chromatography, was found to exist in both monomeric and dimeric forms. The ratio of monomer to dimer was found to be 3:1. HPSEC (Fig. 1) and SDS-PAGE (not shown) demonstrated the homogeneity of the resulting enzyme preparations. The amino acid sequence for *A. niger* glucoamylases is known and this purified enzyme was found to have the same *N*-terminal sequence (first 30 residues) as that reported by Svensson et al. (*31*).

The enzyme was found to be stable between pH 2.5 and 7.5 with a pH optimum of 4.5 (date not shown) and retained its full activity at temperatures up to 50°C. The temperature optimum was found to be between 45 and 50 °C, similar to that reported for the enzyme from other sources (*32*).

The chemically modified enzymes were initially analyzed for activity during a 1-h incubation with 2% starch at various temperatures. This procedure proved to show too little sensitivity to differences in enzyme stability, owing to the strong protection provided by substrate at elevated temperatures. With a 1-h assay time, the temperature optima for all enzyme samples, both modified and native, were between 55 and 60°C.

Various lengths of preincubation period (ranging from 15 min to 3 h) were evaluated for use in studies of the stability of the enzyme at elevated temperatures in absence of substrate. Similar trends were observed for native and modified enzymes at all preincubation times examined; the data for one hour preincubations is used to illustrate the trends. Based on the YSI activity data, enzyme modified with diimidate crosslinkers with chain lengths of 1 carbon (malon-), 2 carbons (succin-), 5 carbons (pimel-),

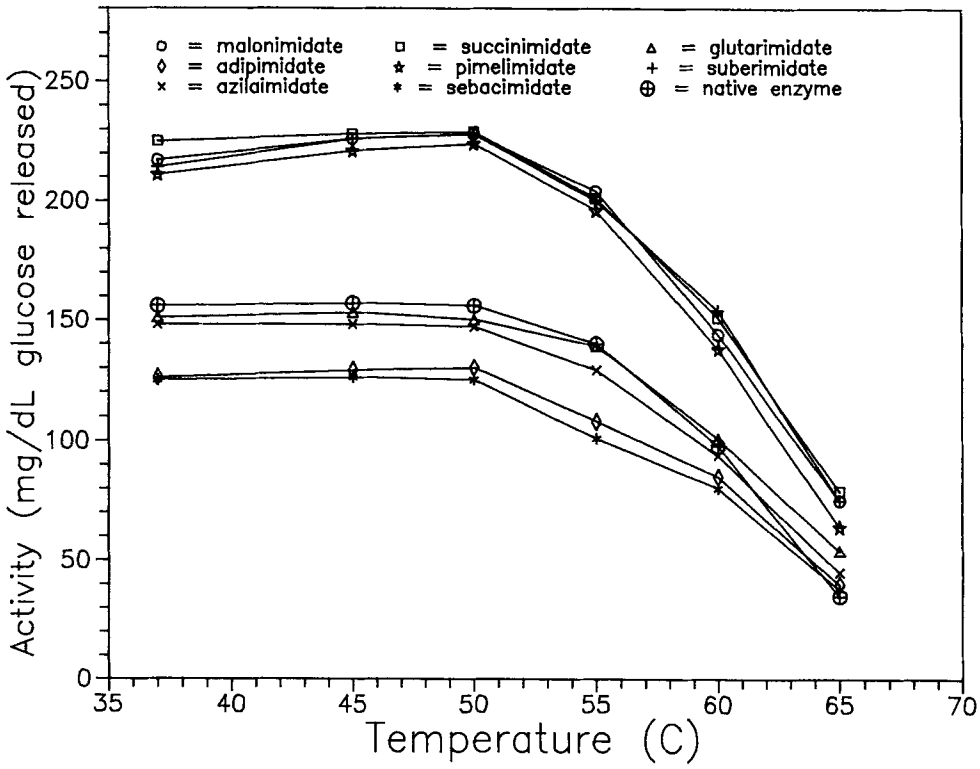

Fig. 2. Thermal-activity profiles for native and modified glucoamylase enzymes after preincubation for 1 h at the indicated temperatures with subsequent assay under standard conditions (*see* text). The crosslinking reagents tested were dimethylesters that ranged in carbon spacer length from one carbon (malonimidate) to eight carbons (sebacimidate).

and 6 carbons (suber-) retained considerably greater glucoamylase activity after high-temperature preincubation than did the native enzyme (i.e., 60% greater at 65°C for dimethylsuccinimidate-modified enzyme), whereas enzyme modified with diimidates of other chain lengths released much less glucose from starch (Figs. 2 and 3). Figure 3 shows modified enzyme activities normalized against surviving native enzyme activity at the indicated temperatures so that differences may be amplified. Also, the activity temperature optima and activities at optimal temperature for native and modified enzymes on starch are summarized in Table 1.

Preincubation-type activity studies on enzyme subjected to modifiers of different chemistry did not show conclusive evidence that one modifier was more successful than the other chemical types in increasing the temperature optima (Fig. 4). In fact, treatment with most modifiers produced a negative effect (lower activity values) at temperatures below the optimum. At higher preincubation temperatures (more than 10°C above optimum), however, all but one of the crosslinked enzymes showed greater

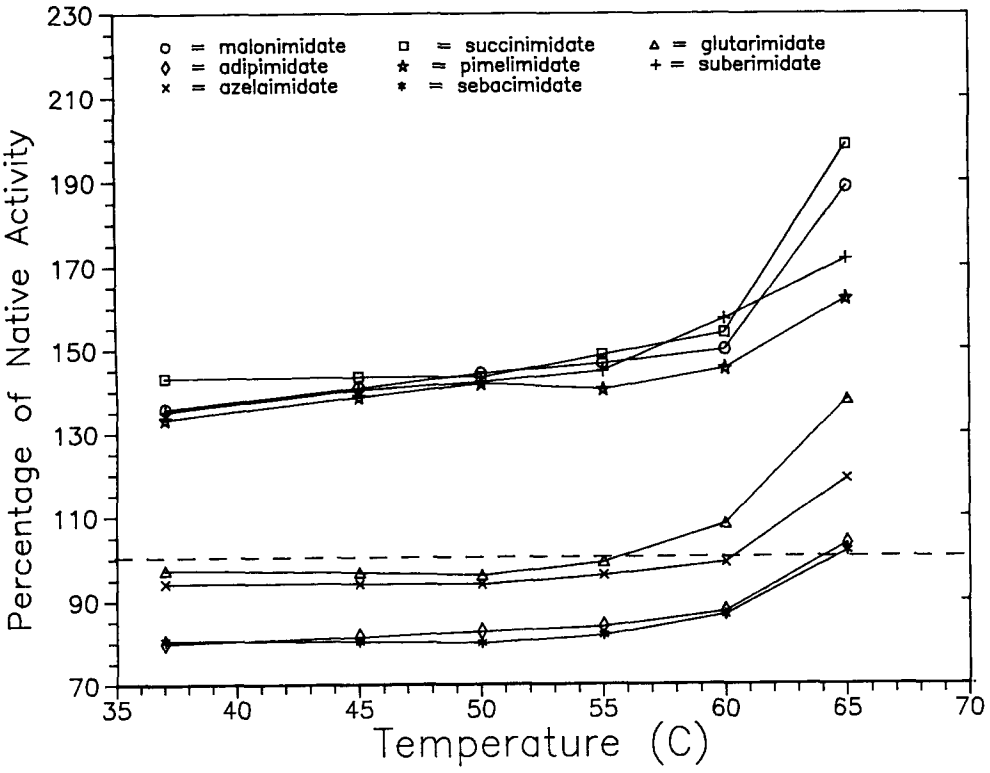

Fig. 3. Profiles of remaining activity vs perincubation temperature for modified glucoamylase enzymes. Activity data in Fig. 2 have been normalized (values plotted = 100 × [modified/native]) to that of the native enzyme to emphasize detail in activity differences compared to the native enzyme. The horizontal dashed line at the normalized value of native enzyme activity (= 100%) is provided as an aid in interpretation.

activities than the native enzyme. Enzymes modified with glutaraldehyde and 2-iminothiolane demonstrated higher activities than the native enzyme near (±10°C) the optimal temperature.

The dextran-conjugated enzymes showed lesser activities than native enzyme at temperatures below the optimum and somewhat greater activities at temperatures above the optimum (data not shown because of small differences). The differences in the molecular weight of dextrans attached to the enzyme did not seem to have an effect on activity or temperature optimum.

Maltose and maltohexaose were used as active-site conformational templates during the harsh crosslinking process, in order to allow the native enzyme to maintain the most favorable structure for binding to the substrate (starch). The use of maltose as an active-site protector during the crosslinking process increased activities for some modified enzymes (relative to those modified in absence of maltose) at higher temperatures

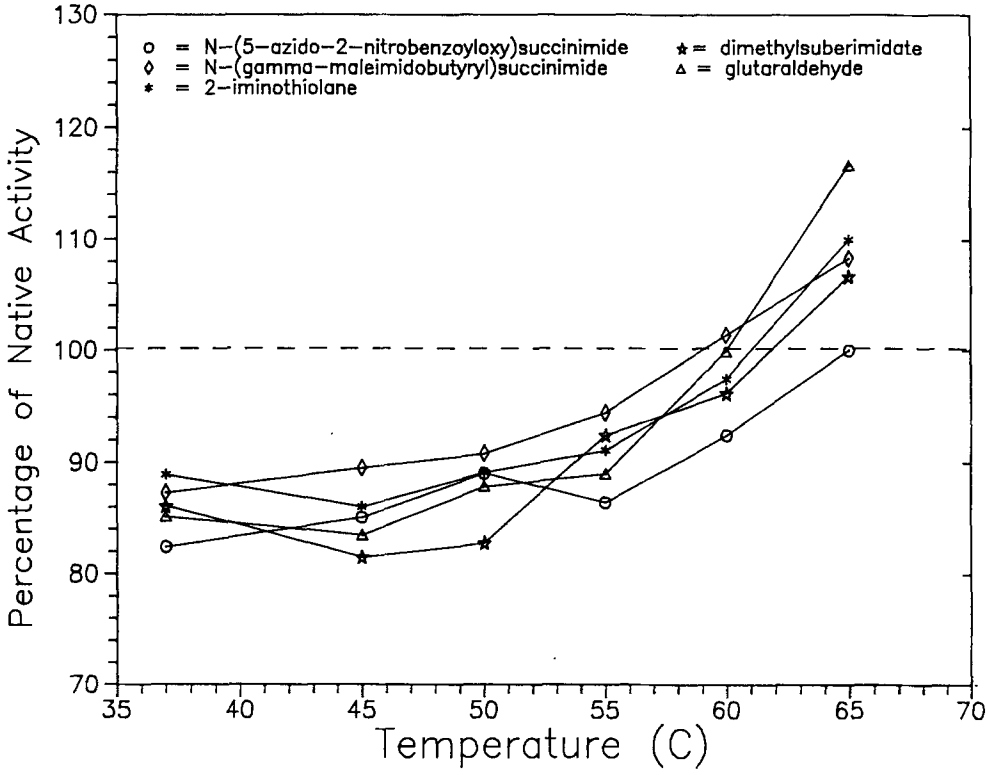

Fig. 4. Profiles of remaining activity vs preincubation temperature for glucoamylase enzymes modified with crosslinkers having chemically different functional groups. Experimental procedures as in Fig. 2. Data has been normalized as in Fig. 3.

(Fig. 5), which may indicate that a greater proportion of enzyme molecules were thermally stabilized in a favorable conformation. The results of maltohexaose addition (Fig. 6) also showed that the resulting enzyme activities were generally greater than those of the enzymes modified without inhibitor (*compare* Figs 5 and 6 with Fig. 4).

Figure 7 illustrates the decay of activity during preincubation at 65°C for enzyme preparations modified with a series of homobifunctional crosslinking agents having the same chemical-functional groups (imidoester) but different carbon chain lengths. The half-lives of these modified enzymes are shown in Table 2. From these data it can be seen that modification with diimidate crosslinkers with spacers 2 or 3 carbons in length produces a very active enzyme that has more than twice the half-life of the native enzyme. Except for the enzyme preparations modified with the two longest crosslinkers, the 7- and 8-carbon azelaimidate and sebacimidate, all of the modified enzymes retain significant activity after an incubation period as long as 5 h, at which time the native enzyme has been

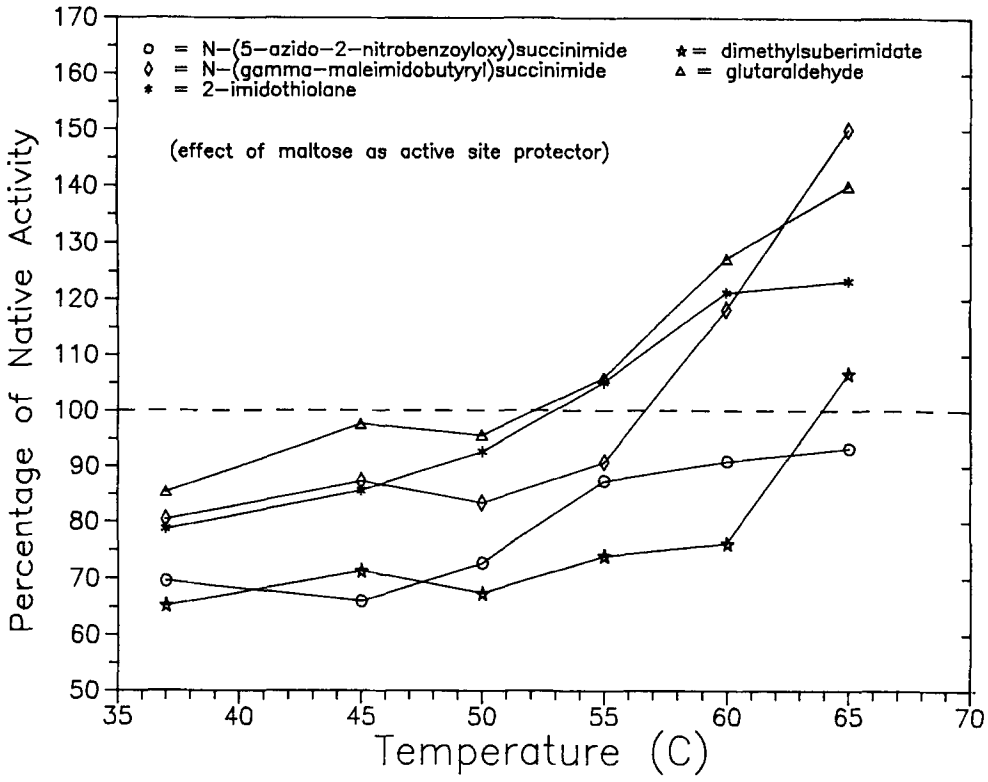

Fig. 5. Profiles of remaining activity vs preincubation temperature for glucoamylase enzymes modified in the presence of 1 m*M* maltose. Experimental procedures and data normalization as in Fig. 3. Compare results to those shown in Fig. 4.

almost completely inactivated. The observation that the activities of two of the preparations (glutarimidate- and adipimidate-treated) are virtually unchanged between 3 and 5 h (Fig. 7), suggests that a small proportion of the enzyme molecules may have been converted to enzyme species of extremely long half-life. Adjustment of crosslinking conditions to maximize production of these extremely stable species would, therefore, appear to be a worthwhile goal for further research.

Fluorescence-detected thermal denaturation studies were also carried out, and data (Table 1) showed results similar to the activity study. Here thermal-scanning fluorescence spectroscopy was used to follow the tryptophan emission of the native and modified enzymes. This technique is useful in determining the changing environment of tryptophan residues as fluorescence emission wavelength shifts during temperature-induced protein denaturation (*33*). The results from peak maximum shift studies for the diimidate-modified preparations showed a 4–6 °C higher conformational stability than the native enzyme for the case of the more activity-stable

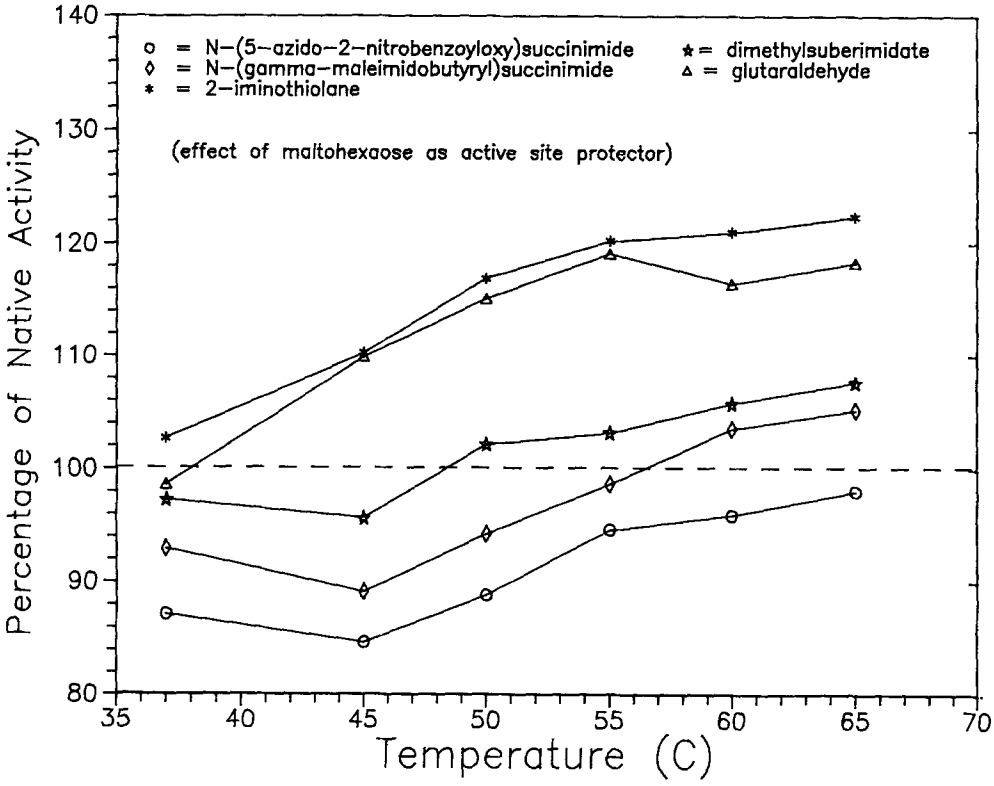

Fig. 6. Profiles of remaining activity vs preincubation temperature for glucoamylase enzymes modified in the presence of 1 m*M* maltohexaose. Experimental procedures and data normalization as in Fig. 3. Compare results to those shown in Figs. 4 and 5.

species (Fig. 8). Generally, all modified enzymes showed conformational thermal stabilities somewhat greater than the native enzyme. The *N*-(5-azido-2-nitrobenzoyloxy) succinimide increased the midpoint of the conformational transition about 5°C and the 2-iminothiolane about 8°C (Table 1). The dextran-crosslinked enzymes showed about the same increase in thermal stability as did the more successful diimidate-modified enzymes (i.e., 2-3°C increase in transition temperature).

Indeed, the high-temperature half-lives exhibited by the best of the modified *Aspergillus* enzymes are near that observed for the native and highly thermostable glucoamylase secreted by *Clostridium thermohydrosulfuricum* (7), although the temperature optima found for the modified enzymes were much lower than that for the bacterial enzyme (7). The thermal stabilities (half-lives) of the modified enzymes produced in our study also compare well with the most stable glucoamylase-dextran conjugates reported in a recent study by Lenders and Crichton (34). In contrast to this study, however, our results indicated that reticulation of the

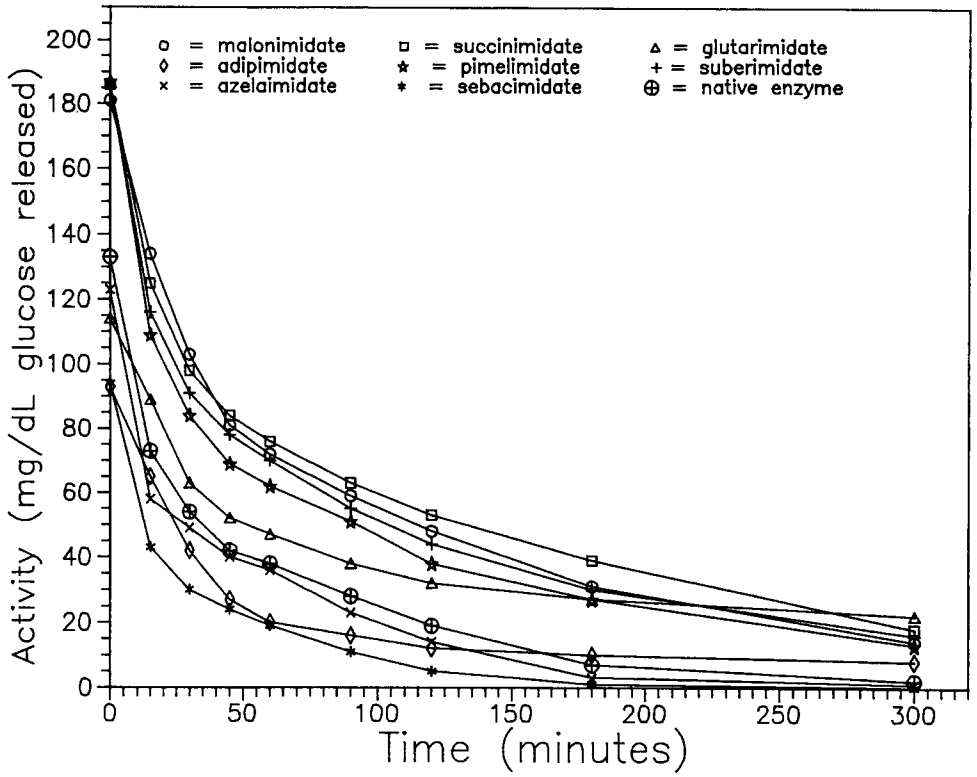

Fig. 7. Activity decay curves of native and dimethylimidoester-modified glucoamylase enzymes preincubated for indicated periods at 65°C.

native enzyme with the correct crosslinker was alone required, and not the introduction of ancillary amino groups with subsequent conjugation to dialdehyde dextrans.

The success of the 1,2,5, and 6 carbon linked diimidates in producing a modified glucoamylase considerably more thermostable than found in nature is intriguing. Cautious speculation on the matter would indicate that the involvement of surface amino groups spaced optimally for these modifiers and/or the ability of the long chain modifiers (dimethylsebacimidate, for example) to interfere with the active site, could lead to the observed dependence on the length of the crosslinker.

In conclusion, this study showed that in the case of glucoamylase the enhancement of both initial activity and stability (and thus of long-term activity) relative to native enzyme at elevated temperature (specifically, temperatures above the optimum of the native enzyme) can be achieved by chemical crosslinking. The enhancement of stability at high temperature is in this case independent of any effect on the classical temperature optimum value, which is a complex product of both the catalytic and denaturation rates, and which was essentially unchanged by the chemical

Table 2
Activity Decay Results at 65°C for Glucoamylase Modified with Different Chemical Bifunctional Reagents

Modifiers of different chemistry	$T_{0.5}$ (minutes)	$T_{0.1}$
{glucoamylase[a] (monomer)	17	175}
N-(5-azido-2-nitrobenzoyloxy) succinimide	21	170
glutaraldehyde	22	210
N-(gamma-maleimidobutyryl) succinimide	19	175
2-iminothiolane	22	230
Diimidates of varying chain length		
{glucoamylase[a] (monomer)	16	145}
dimethylmalonimidate [1 carbon]	36	230
dimethylsuccinimidate [2 carbon]	35	290
dimethylglutarimidate [3 carbon]	40	>300
dimethyladipimidate [4 carbon]	25	180
dimethylpimelimidate [5 carbon]	23	250
dimethylsuberimidate [6 carbon]	26	270
dimethylazelaimidate [7 carbon]	15	125
dimethylsebacimidate [8 carbon]	13	95

[a]Data for each modifier series was obtained from a different lot of purified enzyme.

modifications studied in this work. This initial study indicates that selective chemical crosslinking holds potential for increasing the stability of many labile enzymes.

ACKNOWLEDGMENT

The authors wish to thank Jay Fox at the University of Virginia Medical School for *N*-terminal sequence data and Karel Grohmann for valuable discussions during the course of this work. This work was funded by the Directors Development Fund of the Solar Energy Research Institute. The Solar Energy Research Institute is funded by and operated for the US Department of Energy.

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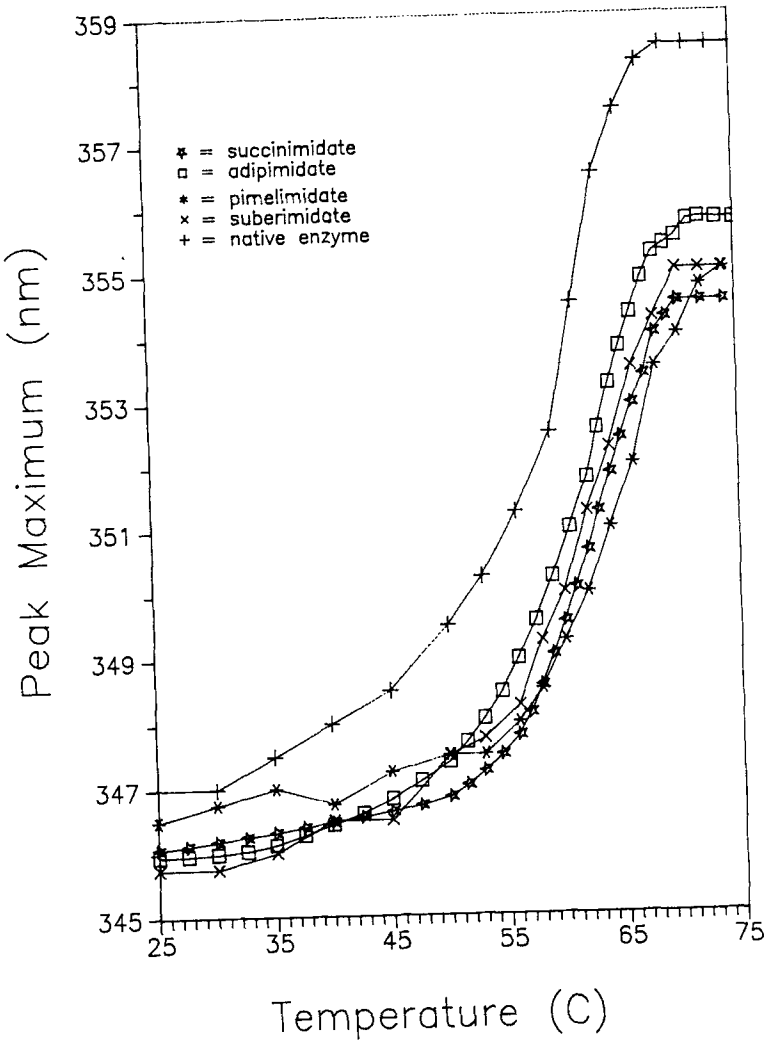

Fig. 8. Fluorescence-detected thermal denaturation of modified and native glucoamylase enzymes.

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