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Modulation of stability properties of bovine trypsin after in vitro structural changes with a variety of chemical modifiers

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Keywords: chemical modification/protein structure–function/thermal inactivation/trypsin

Introduction

The motivation for altering protein structures originates from a desire to put them ultimately to use in applications that nature never intended them for, such as in synthetic procedures for making peptides, pharmaceuticals and products such as esters needed for a variety of purposes. Many of these synthetic procedures need to be carried out in organic solvents and many of the applications may be limited to other extreme conditions such as pH, temperature and a possible presence of inhibitors.

In most cases man looks for the microbes to produce proteins tailored to perform extremely specific functions. Given the fact that for an average protein, that is say 200 amino acids long, even if 10 positions are targeted for change one has to confront millions of different proteins only some of which may possess some of the properties we are looking for. In addition, not all useful enzymes are prokaryotic in origin. If we are interested in eukaryotic enzymes then one has to clone them before any engineering can be undertaken. Thus whether it is site-directed mutagenesis (SDM) or directed evolution (DE) the procedures are labour intensive and costly.

The molecular mechanism of protein inactivation may involve a simple one step mechanism of an enzyme (N) leading to a denatured state (D) with k being the rate constant denoting the denaturation process (Lumry and Eying, 1954) or a native protein (N) may go through an intermediary state (I) before being denatured (D). This involves two steps with two equilibrium rate constants k1 and k2 (Ray and Koshland, 1961; Tanford, 1968; Henley and Sadana, 1985).

Since the objective is to create stabilized proteins and cost being understandably always a common denominator, it is prudent not to assume that structural changes may be brought about only by intervention at the DNA level. That chemical modification of proteins is a real alternative to solve these problems has already been demonstrated in the case of the sulphydryl protease papain (Rajalakshmi and Sundaram, 1995), which upon modification showed a ΔT

1/2 at 60°C. Naturally occurring glycoproteins are often more stable than their counterparts lacking the carbohydrate. Consequently it is logical to try to glycosylate proteins using enzymes or by chemical methods. It is also possible to crosslink proteins by reagents with a variety of structures (Schmid, 1979; Marshall, 1978). However a detailed analysis of the basic issues involved in the stabilization mechanism needs to be understood before any rationale can be advanced, based on (i) the type and size of the protein, (ii) the structure and size of the modifying reagent and (iii) other variables such as the chemical reaction involved in the modification procedure and the actual conditions of modification.

This prompted us to stabilize and transform a labile mesophilic bovine trypsin to a form resembling a thermophilic enzyme by chemical modification with various modifiers such as monomeric glutaraldehyde (MGA), polymeric glutaraldehyde (PGA), oxidized sucrose and oxidized sucrose polymers (OSP 70 and OSP 400). Virtually no loss in activity occurred upon modification. Temperature optima of trypsin shifts from 45–76°C and T50 from 54–76°C for the best modified sample made with OSP. The efficiency of the modifiers in stabilization was ranked in the order: OSP 400-T > OSP 70-T > PGA-T > MGA-T > Sucrose-T. Half-life of modified enzymes also followed the same trend. Both stabilization factor and t1/2 decreased with increasing temperatures. The free energy of activation for inactivation Δ(ΔG≠) varies from 12–20 kJ/mol and the activation enthalpy Δ(ΔH≠) of the modified trypsin by 80–120 kJ/mol indicating stabilization. Inactivation of modified trypsin by urea is less noticeable. The character of the two-step inactivation process of trypsin changes with the degree of stabilization in that the duration of phase I one increased noticeably as stabilization increases. Native trypsin fluoresces less intensely showing a red shift under the influence of denaturation. Such a fluorescence change is not so obvious for the modified enzymes indicating conformational stability acquired by modification.

Keywords: chemical modification/protein structure–function/thermal inactivation/trypsin

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Oxidation of sucrose polymer

Sucrose polymer (100 mg) dissolved in 10 ml water was oxidized at pH 4.6 with 500 mg sodium metaperiodate. The oxidation was carried out at 25°C for 2 h in the dark with constant stirring. The excess of unreacted periodate was removed by dialysis against distilled water followed by pH 8.0 borate buffer (0.05 M).

Oxidation of sucrose

Sucrose (2 g) was oxidized with 0.8 g of NaIO₄ in 6 ml water at pH 4.6 for 3 h in the dark. Excess unreacted periodate was removed by passing through an activated neutral alumina column (Whistler and Bemiller, 1972). The oxidized sucrose solution was lyophilized and then used for modification. Polyglutaraldehyde was prepared according to the method of Tor et al. (1989).

Modification of trypsin

Trypsin was modified with various modifiers that react with the -NH₂ group of lysine in proteins such as monomeric glutaraldehyde (MGA), polyglutaraldehyde (PGA), periodate oxidized sucrose and sucrose polymers (OSP) in 0.05 M pH 8.0 borate buffer containing 20 mM CaCl₂.

The molar ratios of enzyme to modifier and the conditions employed for each of the modifiers during chemical modification are as follows:

(a) 1:250 for modification with MGA the duration of the reaction time being 15 min at room temperature.
(b) 1:120 for modification with PGA the time of reaction being 1 h at room temperature.
(c) 1:7000 for modification with oxidized sucrose conducted for 14 h at room temperature.
(d) 1:0.53 for modification with OSP 70 and 400, the reaction time being 15 min at room temperature.

Enzyme assay with BAPNA and BAEE

The initial reaction rates of amidase and esterase activities were measured with BAPNA (Geiger and Fritz, 1988) and BAEE (Schwert and Takenaka, 1955) as substrates.

Enzyme assay using BAEE as substrate

Native and modified trypsins were assayed against the natural substrate bovine serum albumin (BSA). BSA concentrations in the range 0.0154 to 0.616 mM were prepared in borate buffer (0.05 M) containing 20 mM CaCl₂. 1 ml of BSA was reacted with 20 µg enzyme for 15 min at 37°C, the reaction was stopped by adding 80 µl of 100% TCA, centrifuged at 15 000 r.p.m. for 10 min and absorbance of the supernatant measured at 280 nm. A molecular weight of 64 000 Da for BSA and extinction coefficient value of 6000 for complete digestion of the protein was used in calculating the kinetic parameters. This assay was carried out according to the method of Kunitz (1947).

Temperature optimum

The temperature optimum of native and modified trypsin were measured with BAPNA at different temperatures ranging from 30 to 80°C for 30 min. The optimum temperature and energy of activation (Eₐ) were calculated from Arrhenius plots of log velocity of the reaction against the reciprocal of absolute temperature.

Kinetic parameters

The kinetic parameters of native and modified trypsin were determined by measuring the initial rates of hydrolysis of BAPNA and BAEE as substrates in the range of 0.21–3.3 mM and 0.005 to 0.4 mM respectively.

Thermal stability

The stability of native and modified trypsin in a temperature range from 25 to 80°C was monitored by incubating the enzymes at each temperature for 1 h. The residual activity was measured with BAPNA as substrate after cooling the heated enzymes to 25°C. T₉₀ is defined as the temperature at which 50% of activity is retained after 60 min.

Thermal inactivation

The native and modified trypsin were incubated at each temperature for varying periods of time. At regular intervals samples were removed for measurement of residual activity. The thermal inactivation parameters of native and modified enzymes were determined from an Arrhenius plot of the inactivation rate constant kᵢ.

Stability towards urea

The stability of native and modified trypsin towards different concentrations of urea was determined by incubating the enzyme for 1 h at 37°C. The stability towards 8 M urea in the presence and absence of 5 mM β-mercaptoethanol was also assessed for different time periods at 37°C. Mercaptoethanol was included in urea to hasten the inactivation. The residual activity was determined at room temperature.

Measurement of fluorescence

The fluorescence emission spectra of native and modified trypsins before and after denaturation with either heat (65°C) or 8 M urea were measured with 2.085 nmol enzyme in pH 8.0 borate buffer (0.05 M) using a RF-1501 Shimadzu spectrofluorophotometer. The protein solution was excited at 280 nm and emission scanned between 290–450 nm. Light scattering experiments of native and modified enzymes were monitored at 400 nm.

Results

Modification of trypsin

Bovine pancreatic trypsin was modified with modifiers ranging in molecular weight from 100 to 400 000 Da. Of these, the monomeric glutaraldehyde (MGA) and polyglutaraldehyde (PGA) of molecular weight 100 and 1000 Da, respectively, do not bear any hydroxyl groups whereas sucrose (306 Da) and sucrose polymers (70 and 400 kDa) carry from a few to many -OH groups on their backbone. Sucrose and sucrose polymers are functionalized to possess aldehydic groups by NaIO₄ oxidation. Thus the reactive groups in all the modifiers are similar and react mostly with lysine -NH₂ groups, the reaction finally being terminated and stabilized by NaCNBH₃ reduction. These chemical modifications were optimized by varying the pH, temperature, duration of the modification reaction and the ratios of enzyme to modifiers (data not shown). Molar ratios
of enzyme to polymer of 1:250 for MGA, 1:120 for PGA, 1:7000 for oxidized sucrose, 1:0.53 for both OSP 70 and OSP 400 were found to be ideal when conducted in pH 8.0 borate buffer (0.05 M) containing 20 mM CaCl$_2$ for different lengths of time as described under Materials and methods.

Modifications of about 75–85% of $\varepsilon$-lysine amino groups resulted in the production of modified enzymes with no appreciable alteration in the catalytic activity of the protein. SDS–PAGE and gel filtration analysis revealed that modification of trypsin with MGA and PGA resulted in the introduction of intramolecular cross-links. This conclusion is based on the evidence that native and modified trypsin migrate to the same extent ($R_i = 0.96$) on SDS–PAGE and by their similar elution profile on Sephadex G-100 as also by the existence of similar light scattering intensity for native and modified enzymes. On the other hand, sucrose polymer modified trypsin failed to migrate through the gel and remained at the origin. This indicates that these high molecular weight adducts are too large to migrate through the gel. In gel filtration through Sephadex G 200 both OSP 400 and OSP 70 trypsin elute separately as a single peak after the other quite ahead of native trypsin. There was no activity or peak where unmodified enzyme should elute. These sucrose polymer-modified enzymes also displayed light scattering at 400 nm similar to the native enzyme implying the existence of intramolecular cross-links.

Table I shows $T_{50}$, $T_{opt}$ and the classical Arrhenius activation energy ($E_a$) of thermal activation of native and modified enzymes. The native trypsin values for these parameters were found to be 54, 45 and 21.17 kJ/mol respectively. On the other hand modified enzymes exhibit a remarkable upward shift in $T_{opt}$ values for BAPNA as substrate. In contrast no significant differences were found with BAEE as the substrate.

A $K_m$ value of 0.24 mM for native trypsin towards BSA is not altered much upon modification (data not shown) although activity was reduced considerably, that is by about 50%. However $T_{opt}$ increases by 2–7°C over native trypsin towards BSA.

**Thermal inactivation**

Thermal inactivation experiments showed a progressive loss in activity with time of both the native and modified trypsin when exposed to different temperatures. Figure 1A–C shows the biphase nature of thermal denaturation (Table II and III), $k_1$ and $k_2$ representing the first-order inactivation rate constants of the two phases. However the modified enzymes possessed lower inactivation rate constants indicating an enhancement in their stability.

Progressive loss in activity with time of native and modified enzymes occurred when exposed to different temperatures during thermal inactivation experiments. The inactivation of most proteins follows first-order kinetics. In contrast, a biphase mode of inactivation was observed in the case of trypsin indicating the complexity of the phenomenon as described in the Introduction.

The inactivation rate constants $k_1$ and $k_2$ were determined from the slope of log % residual activity plotted against time (Figure 1A–C). The values of $k_1$ and $k_2$ increased logarithmically with temperature for both native and modified enzymes. However, the modified enzymes possessed lower inactivation rate constants than native enzyme which implies an enhancement in their stability.

The values of $k_1$ were greater than $k_2$ for both native and modified enzymes because phase I is a sensitive phase of inactivation than phase II. At temperatures between 40 and 60°C the second phase of inactivation could not be observed with some of the modified enzymes. This is because the onset of the second phase is considerably delayed due to stabilization although in extreme cases, such as when OSP 70 or OSP 400 is the modifier, even in 48 h, a limit set by us in our experiments for brevity, the second phase does not appear at all thus pointing to the increased stability. In the present study, the final end process of thermal inactivation of native and modified enzymes was found to be irreversible. Besides, a closer look at Figure 1A–C shows that their is no evidence to suggest that the denaturation pattern changes after modification, for example in Figure 1A and B, containing the data for 40 and 50°C, the activities for OSP 400 trypsin does not change and shows a straight line for 50 h. However at 70°C (Figure 1C) at around 100 min a change of slope indicates the start of the second phase.

### Table I. Temperature optima, energy of activation ($E_a$), $T_{50}$, $U_{50}$ and $U_{1/2}$ of native and modified trypsin

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>$T_{opt}$ (°C)</th>
<th>$E_a$ (kJ/mol)</th>
<th>$T_{50}$ (°C)</th>
<th>$U_{50}$ (M)</th>
<th>$U_{1/2}$ (min)</th>
<th>$U_{1/2}$ (min) with 5 mM β-ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>45±2.0</td>
<td>21.17±0.252</td>
<td>54±1.0</td>
<td>5.2</td>
<td>25</td>
<td>9.0</td>
</tr>
<tr>
<td>MGA</td>
<td>60±2.0</td>
<td>21.34±0.126</td>
<td>63±2.5</td>
<td>NM</td>
<td>NM</td>
<td>30</td>
</tr>
<tr>
<td>PGA</td>
<td>68±2.4</td>
<td>17.80±0.210</td>
<td>68±2.0</td>
<td>NM</td>
<td>NM</td>
<td>75</td>
</tr>
<tr>
<td>Sucrose</td>
<td>55±3.0</td>
<td>21.34±0.294</td>
<td>64±3.0</td>
<td>6.7</td>
<td>42</td>
<td>12</td>
</tr>
<tr>
<td>OSP 70</td>
<td>74±2.5</td>
<td>13.35±0.300</td>
<td>74±3.0</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>OSP 400</td>
<td>76±2.1</td>
<td>10.75±0.340</td>
<td>76±2.0</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
</tbody>
</table>

$T_{opt}$ optimum temperature for activity, $E_a$ is the energy of activation of thermal activation, the classical Arrhenius activation energy, assays are performed by adding enzyme solution to substrate preheated to the desired temperature (see Figure 1A in Rajalakshmi and Sundaram, 1995), $U_{50}$ is the temperature where 50% of the initial activity is retained. $U_{1/2}$ is the temperature required to inactivate 50% of the initial activity at 8 M urea concentration in the presence and absence of 5 mM β-mercaptoethanol (β-ME). Values are given as ±SD of triplicates or average of duplicates. NM, not measurable.
**Fig. 1.** Log % residual activity versus time of (■) native trypsin, (x-x) MGA-trypsin, (□-□) PGA-trypsin, (●-●) sucrose trypsin, (+++) OSP 70-trypsin and (○-○) OSP 400-trypsin at (A) 40°C, (B) 50°C and (C) 70°C.

$E_a$, the energy of activation of inactivation, values are given in Table II and III for both phases of denaturation (see table footnotes). Not to be confused with $E_a$, $E_a$ is a true indication of the effect of thermal inactivation of the enzymic protein before it is allowed to react with the substrate. Obtained from plots (not shown) similar to Figure 3 for chymotrypsin (Sundaram and Venkatesh, 1998). $E_a$ values increase upon modification depending upon the degree of stabilization, OSP 400-T being the most stable. These values obtained from an average of triplicates had a correlation coefficient ranging between 0.978 and 0.99. Larger values of $E_a$ (Table II and III) indicate that more energy is required to inactivate the catalyst.

$E_a$ values resemble $\Delta H^\#$, values as can be expected from the equation $\Delta H^\# = E_a - RT$.

The activation free energy, enthalpy and entropy of inactivation at each temperature were determined using the equations as described in the earlier work on papain (Rajalakshmi and Sundaram, 1995).

The values of $\Delta G^\#$, $\Delta H^\#$ and $\Delta S^\#$ for the first and second phase of native and modified enzymes are summarized in Table II and III. Modified enzymes showed higher $\Delta G^\#$, $\Delta H^\#$ and $\Delta S^\#$ values than the native enzyme to varying extents as follows: OSP 400-T > OSP 70-T > PGA-T > MGA-T > SUC-T > N-T. These values change with temperature.

These elevated levels of Gibbs free energy ($\Delta G^\#$), enthalpy ($\Delta H^\#$) and entropy ($\Delta S^\#$) of modified enzymes imply that more energy and heat are required for the inactivation to occur. Upon stabilization $\Delta S^\#$, the activation entropy of inactivation, also goes up similar to that observed for thermophilic enzymes.

**Half-life of native and modified trypsins**

$t_{1/2}$ for native trypsin ranged between $29.4 \pm 1.3$ h at $40^\circ$C to $0.035 \pm 0.003$ h at $80^\circ$C. This increased to $6635 \pm 309$ h at $40^\circ$C and $1.6 \pm 0.081$ h at $80^\circ$C for the best preparation modified with OSP 400. These $t_{1/2}$ values decreased with increasing temperature for both phases of inactivation of the native and modified enzymes (Tables II and III).

**Stability towards urea with and without $\beta$-mercaptoethanol**

Modified trypsin resisted loss of activity through unfolding after exposure to 8 M urea in the presence or absence of 5 mM $\beta$-mercaptoethanol, whereas native enzyme lost its activity in 20 and 120 min, respectively, under these two conditions. Table I contains $U_{1/2}$ values of activities measured under these two conditions. Relative stability towards urea was as follows: OSP 400-T > OSP 70-T > PGA-T > MGA-T > Sucrose-T > native trypsin. In the presence of $\beta$-mercaptoethanol the stability of native, MGA-T, PGA-T and sucrose-T are considerably reduced whereas OSP 70-T and OSP 400-T were still very stable.

**Fluorescence spectrum**

The fluorescence emission spectra of native and modified enzymes before and after denaturation are shown in Figure 2A–E. Upon excitation at 280 nm both native and modified enzymes exhibited essentially identical emission maxima at 340 nm. This is characteristic of tryptophan residues partially shielded from the surrounding medium.

Denaturation of native and modified trypsins either by heat treatment at $65^\circ$C or with 8 M urea shows that native trypsin unfolds leading to a distinct red shift in the wavelength maximum, accompanied by a decrease in the fluorescence intensity. On the contrary, the modified enzymes exhibited lesser red shift and lesser decrease in their fluorescence intensity indicating that covalently modified enzymes resisted denaturant-induced unfolding.

Thus the fluorescence spectra show that trypsin adducts made with OSP 70 and OSP 400, the large carbohydrate polymers, are far more conformationally stable.

**Discussion**

Most of the proteases are marginally stable, the prominent cause of their reversible inactivation being their autoproteolytic digestion. This leads to a complex denaturation pattern that consists of a reversible unfolding step followed by an irreversible step (Eijssink et al., 1990). It has been shown that native enzymes
Table II. Half-life and kinetic activation parameters for the first phase ($k_1$) in the thermal inactivation of native and modified trypsin

<table>
<thead>
<tr>
<th>Type of trypsin</th>
<th>$t_{1/2}$ (h)</th>
<th>$E_a$ (kJ/mol)</th>
<th>$\Delta G_i$ (kJ/mol)</th>
<th>$\Delta H_i$ (kJ/mol)</th>
<th>$\Delta S_i$ (J/mol K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>29.4 ± 1.3</td>
<td>173 ± 6.1</td>
<td>107.5 ± 0.5</td>
<td>172.8 ± 6.3</td>
<td>217.4 ± 6.3</td>
</tr>
<tr>
<td>to</td>
<td>0.035 ± 0.003</td>
<td>102.6 ± 0.4</td>
<td>172.7 ± 1.6</td>
<td>198.5 ± 5</td>
<td></td>
</tr>
<tr>
<td>MGA-Trypsin$^a$</td>
<td>717 ± 55</td>
<td>256 ± 4.1</td>
<td>119.5 ± 0.7</td>
<td>255.8 ± 3.8</td>
<td>426 ± 5.5</td>
</tr>
<tr>
<td>to</td>
<td>0.43 ± 0.024</td>
<td>109.7 ± 1.25</td>
<td>255 ± 1.4</td>
<td>411 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>PGA-Trypsin$^a$</td>
<td>1153 ± 75</td>
<td>257 ± 1.6</td>
<td>121.1 ± 0.17</td>
<td>256.8 ± 1.7</td>
<td>420.5 ± 3.3</td>
</tr>
<tr>
<td>to</td>
<td>0.48 ± 0.02</td>
<td>110 ± 0.4</td>
<td>256.5 ± 1.5</td>
<td>415 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Sucrose- Trypsin$^a$</td>
<td>1310 ± 15.6</td>
<td>255.4 ± 2.6</td>
<td>121.6 ± 1.05</td>
<td>255 ± 2.5</td>
<td>408.5 ± 5</td>
</tr>
<tr>
<td>to</td>
<td>0.41 ± 0.005</td>
<td>110.9 ± 1.26</td>
<td>254.8 ± 0.15</td>
<td>404 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>OSP 70- Trypsin$^a$</td>
<td>1814 ± 111</td>
<td>263 ± 5.8</td>
<td>122 ± 0.5</td>
<td>262 ± 5.4</td>
<td>435.1 ± 6.8</td>
</tr>
<tr>
<td>to</td>
<td>1.32 ± 0.05</td>
<td>113 ± 0.17</td>
<td>262.4 ± 2.1</td>
<td>418 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>OSP 400- Trypsin$^a$</td>
<td>6635 ± 3.9</td>
<td>291 ± 1.35</td>
<td>126 ± 0.15</td>
<td>290.5 ± 0.7</td>
<td>560 ± 2</td>
</tr>
<tr>
<td>to</td>
<td>1.6 ± 0.082</td>
<td>113.2 ± 0.4</td>
<td>290.3 ± 0.7</td>
<td>532 ± 2.9</td>
<td></td>
</tr>
</tbody>
</table>

Experimental data collected at 40–80°C. The values are given as ± SD from triplicates.

$^a$Not measurable at 40°C.

Otherwise the data presented are from the two extremes, that is 40 and 80°C, 50 and 80°C.

$E_a$ is the activation energy of inactivation. It is obtained by plotting log $k_i$, the first order inactivation constant, against reciprocal of temperature as per the Arrhenius equation. $k_i$ is obtained from the slope of log % residual activity plotted against time in hours. The enzymes in assay buffer were incubated at various temperatures. At regular intervals, aliquots were removed to measure the residual activity expressed relative to that of the unheated control.

Table III. Half-life and kinetic activation parameters for the second phase ($k_2$) in the thermal inactivation of native and modified trypsin

<table>
<thead>
<tr>
<th>Type of trypsin</th>
<th>$t_{21/2}$ (h)</th>
<th>$E_a$ (kJ/mol)</th>
<th>$\Delta G_i$ (kJ/mol)</th>
<th>$\Delta H_i$ (kJ/mol)</th>
<th>$\Delta S_i$ (J/mol K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>58 ± 0.9</td>
<td>146 ± 3</td>
<td>110 ± 0.7</td>
<td>145.8 ± 2.9</td>
<td>116 ± 2.1</td>
</tr>
<tr>
<td>to</td>
<td>0.22 ± 0.04</td>
<td>108 ± 0.6</td>
<td>145 ± 1.1</td>
<td>105 ± 1</td>
<td></td>
</tr>
<tr>
<td>MGA-Trypsin$^a$</td>
<td>276 ± 18.7</td>
<td>198 ± 2</td>
<td>120 ± 1.25</td>
<td>197.8 ± 1.7</td>
<td>247 ± 1.5</td>
</tr>
<tr>
<td>to</td>
<td>3.45 ± 0.35</td>
<td>112 ± 0.6</td>
<td>197.5 ± 2.09</td>
<td>242 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>PGA-Trypsin$^b$</td>
<td>168 ± 2</td>
<td>216 ± 3.5</td>
<td>121 ± 1.3</td>
<td>218 ± 3.3</td>
<td>294 ± 4.2</td>
</tr>
<tr>
<td>to</td>
<td>2.4 ± 0.14</td>
<td>115 ± 0.85</td>
<td>215 ± 1.9</td>
<td>294 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Sucrose-Trypsin$^b$</td>
<td>38.7 ± 2.5</td>
<td>167.6 ± 3.3</td>
<td>116 ± 0.7</td>
<td>167 ± 3</td>
<td>161 ± 1.4</td>
</tr>
<tr>
<td>to</td>
<td>1.72 ± 0.02</td>
<td>114 ± 0.3</td>
<td>165 ± 1</td>
<td>152 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>OSP 70-Trypsin$^b$</td>
<td>524.2 ± 36.1</td>
<td>293 ± 1.5</td>
<td>124 ± 0.5</td>
<td>292 ± 1.3</td>
<td>507 ± 4</td>
</tr>
<tr>
<td>to</td>
<td>2.3 ± 0.12</td>
<td>115 ± 0.6</td>
<td>291.6 ± 1.7</td>
<td>503 ± 3</td>
<td></td>
</tr>
<tr>
<td>OSP 400-Trypsin$^c$</td>
<td>254 ± 8</td>
<td>307 ± 3.5</td>
<td>124 ± 1.5</td>
<td>306 ± 3.2</td>
<td>540 ± 6.7</td>
</tr>
<tr>
<td>to</td>
<td>2.07 ± 0.2</td>
<td>115 ± 1.3</td>
<td>305 ± 8.4</td>
<td>440 ± 9.5</td>
<td></td>
</tr>
</tbody>
</table>

Experimental data collected at 40–80°C. The values are given as ± SD from triplicates.

$^a$Not measurable at 40°C.

$^b$Not measurable at 40 and 50°C.

$^c$Not measurable at 40, 50 and 60°C.

Otherwise the data presented are from the two extremes, that is, 40 and 80°C, 60 and 80°C, and 65 and 80°C.

$E_a$ is the activation energy of inactivation. It is obtained by plotting log $k_i$, the first order inactivation constant, against reciprocal of temperature as per the Arrhenius equation. $k_i$ is obtained from the slope of log % residual activity plotted against time in hours. The enzymes in assay buffer were incubated at various temperatures. At regular intervals, aliquots were removed to measure the residual activity expressed relative to that of the unheated control.

in their folded state are less prone to proteolysis than their corresponding partially or fully unfolded form (Pace and Barret, 1984). In addition autoproteolysis has been shown to occur under slightly denaturing conditions (Fassina et al., 1986).

Based on the above facts, it is felt that stabilized proteases can be produced by curtailing the reversible unfolding step. Several methods of increasing the stability of proteases by protein engineering, such as deletion and replacement of labile residues or loops (Ejssink et al., 1992), introduction of S–S bonds (Van den Burg et al., 1993) and cavity filling (Ejssink
et al., 1992) have been attempted in spite of their limited success. Stabilization of proteolytic enzymes has been explained based on thermodynamic principles and hydrophobic interactions (Frigerio et al., 1996).

However, marginally stable proteolytic enzymes can be transformed to possess thermophilic properties by selective chemical modification. These chemical modifications introduce multipoint linkages involving covalent bonds which reinforce the folded protein structure by preventing unfolding (Rajalakshmi and Sundaram, 1995). It appears worthwhile to investigate chemical modification of proteases with simple cross-linkers or with several activated polysaccharides.
the two groups of modifiers differing in their physicochemical properties. These cross-links function as intramolecular bridges and increase the stability and hydration of the proteins (Venkatesh et al., 1996).

**Consequences of chemical modification**

Modification of trypsin amino groups with various modifiers resulted in a 2–5% increase in enzyme activity. However, in the case of MGA-modified trypsin, a 15% reduction in activity was observed. This loss in activity on cross-linking can probably be curtailed or restored by carrying out the modification in the presence of low concentrations of denaturants as in the case of glutaraldehyde cross-linked lactate dehydrogenase (Tsou, 1993).

In the present study, the kinetic parameters, such as \( k_m \) and \( k_{cat} \) of the modified trypsins were not altered significantly. This indicates that the conformation of the enzyme is not changed by covalent modification. On the contrary, the modified enzymes exhibited lesser activity towards macromolecular proteinaceous substrate. This is primarily attributed to the presence of surface attached polymers or cross-linkers which impede normal access of the substrate to the active site.

**Theoretical and practical assessment of cross-links**

Distance mapping studies with bovine trypsin (Nagarajan et al., 1996) show that it has 14 lysines of which four are within a distance of 7–10 Å and the rest above 10 Å apart. This information is valuable in assessing how many intramolecular cross-links are possible with a cross-linker of known length.

Since monomeric glutaraldehyde can adopt two extreme conformations, that is the extended or the bent form with lengths ranging between 5 and 8 Å, a maximum of two cross-links are possible (Nagarajan et al., 1996). Similarly oxidized sucrose can possess a pair of -CHO groups in the hexose and pentose rings. The two extreme -CHO groups are about 7.0 ± 0.2 Å apart. Thus in this case also two intramolecular cross-links in each trypsin molecule are possible.

On the other hand, PGA with a molecular weight of 1000 Da (Tor et al., 1989), which is about 10 times as large as MGA, has a complex and indefinite structure depending on the polymerisation conditions (Wong, 1991). Since a molecule of PGA is around 60 Å long and bears more than two -CHO groups, it is potentially capable of cross-linking several pairs of -NH₂ groups in the protein, but exactly how many is difficult to assess accurately.

The -CHO groups in PGA can occur near a carbon–carbon double bond or in a single bond region (Chaplin and Bucke, 1990). If the protein is cross-linked intramolecularly between two aldehyde groups near a double bond, then it is possible that the portion of the polypeptide sandwiched in between becomes rigid and restricted in motion.

**Light scattering as a method to distinguish between inter- and intramolecular cross-links**

Similar light scattering intensity of native and modified enzymes illustrate the existence of intramolecular cross-links. However during the optimization studies of trypsin modification with MGA, aggregation of trypsin molecules was encountered above 8.34 nmol trypsin. This aggregated trypsin displayed increased light scattering which implies the existence of intermolecular cross-links. In contrast cross-linking in the presence of 1 M urea resulted in the suppression of intermolecular cross-links as evidenced by reduced light scattering. This suppression is attributed to the ability of urea to disrupt the non-specific interactions (Maeda et al., 1996). Thus, light scattering experiments may give an indication about the nature of cross-links.

**Effect of modification on the optimal temperature and activation energy of trypsin**

Increased optimal temperature and \( k_{cat} \) values and decreased \( E_a \) values of certain kinds of modified trypsin clearly indicates that the cross-linked trypsin forms a more stable transition state complex with the substrate than the native enzyme. This higher stability of the transition state complex probably indicates that rigidification of the conformational structure of the enzyme has taken place upon modification and consequently lower energy is required to catalyse the reaction.

Higher Gibbs activation free energy (\( \Delta G^\# \)), activation enthalpy (\( \Delta H^\# \)) and activation entropy (\( \Delta S^\# \)) of inactivation of modified enzymes also imply that they are rigid and therefore require higher energy and heat for their inactivation. Similar results on stability and rigidity of papain modified with oxidized sucrose polymer (Rajalakshmi and Sundaram, 1995) showed an upward shift of 11°C in \( T_{50} \). Half-life of native papain, which was 48 h and 0.28 h at temperatures of 60 and 90°C, improved to 940 and 0.6 h, respectively, for modified papain. Similarly \( \Delta (\Delta G^\#) \), which is the difference in activation free energy for inactivation between the modified and native papain ranged between 8.3 kJ/mol at 60°C and 2.3 kJ/mol at 90°C, whereas for trypsin the \( \Delta (\Delta G^\#) \) values at 60 and 80°C are 16.3 and 10.6 kJ/mol, respectively. \( \Delta C_m \) in trypsin (Sundaram and Venkatesh, 1997, accompanying paper) modified with dextran, CMC, OSP 70 and OSP 400 showed an upward shift in \( T_{50} \) from 50°C for the native enzyme to 55, 58, 61 and 64°C, respectively, for the four differently modified enzymes. \( t_{1/2} \) values for chymotrypsin in the temperature range 50–60°C shifted from 5.5 h to 0.033 h for the native and 363 h to 1.30 h for the best preparation modified with OSP 400. In addition, it showed a change in \( \Delta (\Delta G^\#) \) by 12 kJ/mol. Thus, in comparison, the increase in \( T_{50} \) from 54°C for native to 76°C for trypsin modified with OSP 400 shows that it is better stabilized than papain and chymotrypsin studied so far in our laboratory. Our results of enhanced stability and rigidity of chemically modified trypsin lead us to categorise it as a ‘thermophilic enzyme’, based on the recommendations proposed by Voordouw et al. (1976).

The molecular events behind the stabilization achieved by covalently modified trypsin with various modifiers may be explained as follows.

(i) The \( \textit{in vitro} \) introduction of several intramolecular cross-links increases the rigidity by reinforcing the enzyme structure. This reinforcement restrains the unfolding of the protein molecule (Wong and Wong, 1992).

(ii) Reduction of the surface non-polar groups on enzymes by shielding or masking with large polymers (Mozhaev et al., 1990).

(iii) Formation of a hydration layer around the enzyme (Perutz, 1978).

(iv) Elimination of lysine as a specific site for attack by trypsin itself (autocatalytic) due to chemical modification.

(v) The modifiers impart a ‘cage’ like effect on the enzyme by bracing the molecule together, that is, the active site has enough mobility whereas the immediate surrounding area is sequestered to become rigid.

(vi) Potential areas which may serve as nuclei of unfolding are eliminated by cross-linking.
(vii) A general micro-environmental change in the immediate vicinity of the modified enzyme including a change in the water structure.

Tolerance towards chaotrophic agents
In addition to the resistance to thermal inactivation and resistance to exogenously added proteases, chemically modified trypsin also displayed noticeable resistance to denaturants such as urea in the presence or absence of β-mercaptoethanol. This indicates that stabilization strategies employed to confer thermal stability also imparts tolerance to chemical denaturants. This correlation indicates that inactivation by different denaturants probably proceeds through the same pathway (Mozhaev, 1993).

The acquisition of stability towards urea is mainly ascribed to intramolecular cross-links on the surface of the modified enzymes. It is presumed that these cross-links on the modified enzymes maintain the tertiary structure intact similar to the S–S bonds. The cumulative effect of the same modifier chain forming several covalent bonds at different sites on the protein should also contribute towards this newly acquired stability of the enzyme. When low concentrations of denaturants like urea or GdmCl were added to inherently less active but rigid thermophilic enzymes, 2–3-fold increase in activity has been reported. Similar activation was noticed in the case of papain covalently modified with OSP-400 (Rajalakshmi and Sundaram, 1995). This probably is indicative of an improvement in flexibility of the active site made possible by urea. However a similar behaviour was not noticed in the case of chemically modified trypsin. This supports our conclusion that we accomplished stabilization by rigidification without a concomitant loss in activity or flexibility. The above observation is unusual and should prove beneficial in practical industrial applications.

Reasons for differences in stability with various modifiers
The acquisition of higher stability by trypsin modified with sucrose polymers when compared with other cross-linkers could be due to the better wraparound capabilities of the larger modifiers such as OSP. The smaller cross-linkers like MGA and PGA introduce merely intramolecular cross-links. This leads to a marginal increase in the stability over the native enzyme. On the contrary, modification with oxidized sucrose leads to a marginal increase in the stability over the native enzyme. On the contrary, modification with oxidized sucrose polymers not only introduce multipoint cross-links, but also profoundly increase the H-bond network around the enzyme primarily due to the existence of numerous hydroxyl groups of the carbohydrate molecules, which are known to interact with the hydrophilic groups on proteins, leading to substantial hydrophilization.

Sucrose is less effective as a stabilizer than OSP. OSP is superior because it is able to form several linkages with the protein and thus wraps around the enzyme better, whereas one molecule of disaccharide is not capable of forming multiple linkages. Preliminary studies of differential scanning microcalorimetry on native and modified trypsin showed a shift in $T_m$ from 54 to 76°C for the OSP 400 modified trypsin (Venkatesh et al., 1996).

Thus the data presented in this paper demonstrates that the inherent rigidity and stability found in the thermophiles can be manifested in a mesophilic labile enzyme by covalent modification without sacrificing its catalytic activity.

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