

Rapid report

## Enhanced catalytic and conformational stability of Atlantic cod trypsin upon neoglycosylation

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This article is dedicated to Prof. P. V. Sundaram

### Abstract

The applicability of psychrophilic enzymes is limited because of their lower thermodynamic stability in spite of their higher catalytic rate. In this study, we have shown that the thermodynamic stability of the psychrophilic Atlantic cod trypsin could be enhanced appreciably by covalent chemical modification with oxidized sucrose polymer without affecting its hydrolytic activity. The acquired stability of cod trypsin was found to be on par with the mesophilic porcine trypsin.

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Naturally occurring enzymes are often not optimized to be used in biotechnological applications. A detailed characterization of the structure, function and stability of homologous proteins from different sources will certainly enable the design of non-natural enzymes with pre-determined characteristics. Enzymes that are adapted to cold conditions are of high utility, as their specific activity at low temperatures is superior to their mesophilic or thermophilic counterparts [1–4]. However, decreased packing of the amino acid residues and higher dynamics render them unstable against thermal inactivation [1,5,6]. These characteristics make them unfit for their application in many of the industrial processes. Changes in the physical and/or chemical properties of these biocatalysts could be achieved either by changing the solvent properties by the use of additives, by redefining the primary structure of the enzyme by site-directed mutagenesis or by

covalent chemical modification of specific amino acid residues [7]. The covalent coupling of mono-, oligosaccharides to the enzyme has been found to enhance the kinetic and thermodynamic stability of certain enzymes [8–11]. Chemical modification with polymeric sucrose has been found to effectively increase the stability of enzymes. In order to test whether the conformational flexibility of psychrophilic enzymes could be decreased, polymeric sucrose (OSP) was coupled to lysine residues in trypsin (from Atlantic Cod) by reductive alkylation. Our results, for the first time, indicate that the conformational stability of a psychrophilic enzyme could be greatly enhanced and the acquired properties are comparable to the mesophilic porcine trypsin without compensation in the catalytic activity.

Enzyme concentration was estimated by the method of Lowry et al. [12]. Chemical modification of both porcine and cod trypsins were carried out as described in Venkatesh and Sundaram [10].

The activity of the native and modified trypsins was assayed using  $N\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as the substrate at 25 °C. The Michaelis–Menton kinetic parameters are given in Table 1. The catalytic efficiency of CT is 6.55 times higher than that of PT,

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Table 1

Enzymatic and stability-related parameters deduced for native and chemically modified PT and CT

Parameter	PT		CT	
	Native	OSP PT	Native	OSP CT
Michaelis–Menton parameters (determined within 5% error)				
$K_m$ (mM)	0.90	0.69	0.098	0.14
$k_{cat}$ ( $s^{-1}$ )	0.26	0.28	0.185	0.28
$k_{cat}/K_m$ ( $M^{-1}s^{-1}$ )	288	406	1887	2000
Activation and inactivation (determined within 5% error)				
$E_a$ ( $kJ\ mol^{-1}$ ) <sup>a</sup>	29.50	19.73	11.31	10.90
$t_{1/2}$ (h, at 60 °C)	28	650	0.35	15
$E_{ai}$ ( $kJ\ mol^{-1}$ ) <sup>b</sup>	210.40	301.00	121.00	262.00
Guanidinium chloride denaturation (determined from the two-state fit (Ref. [13]))				
$C_m$ (M)	2.40	2.71	1.74	3.89
$\Delta G_{H_2O}$ ( $kJ\ mol^{-1}$ )	30.24	45.47	24.65	40.40
$m$ ( $kJ\ mol^{-1}\ M^{-1}$ )	12.56	16.81	14.19	10.38

<sup>a</sup> Energy of activation for the activation process.

<sup>b</sup> Energy of activation for the inactivation process.

indicating that the specific activity of the psychrophilic (CT) enzyme is superior to the mesophilic enzyme (PT). Upon modification with OSP, both enzymes exhibit a positive shift in the catalytic efficiency values. In CT, both the dissociation constant of the enzyme-substrate complex ( $K_m$ ) and the hydrolysis rate ( $k_{cat}$ ) showed increase upon modification, whereas a slight decrease in the  $K_m$  value was observed for OSP PT. The increase in the  $k_{cat}$  for both the psychrophilic and mesophilic enzymes, upon modification, is the consequence of a decreased energy of activation for the substrate hydrolysis (Table 1). Overall, the covalent modification of both psychrophilic and mesophilic enzymes with OSP resulted in increased catalytic efficiency.

The inactivation kinetics of both PT and CT exhibited a biphasic behavior. Table 1 gives the kinetic parameters associated with enzyme inactivation for both native and modified trypsins at 60 °C for the phase I of the inactivation process. The time required for the enzyme to lose 50% of its

initial activity ( $t_{1/2}$ ) increased from 28 h to 650 h for PT and from 21 s to 15 h for CT, upon modification, implying that OSP PT and OSP CT exhibited stabilization factors (defined as the ratio of the  $t_{1/2}$  values of the modified enzyme to the native enzyme) of 23.2 and 43, respectively. Such increase in the  $t_{1/2}$  values is due to the increased energy of activation for the inactivation process ( $E_{ai}$ ) upon modification (Table 1). Similar changes in the kinetic parameters were also observed in the phase II of enzyme inactivation (data not shown).

In order to assess the global conformational stability of trypsins used in this study, both native and modified enzymes were treated with different concentrations of guanidinium chloride, and the fluorescence spectra ( $\lambda_{ex}=280$  nm) were recorded. Upon increasing the concentration of guanidinium chloride, there was a gradual decrease in the tryptophan fluorescence intensity accompanied by the red shift of the wavelength of maximum fluorescence (from 335 nm to 355 nm for PT and from 345 nm to 356 nm for CT). Fig. 1 shows the fraction of unfolded enzyme molecules as a function of denaturant concentration. The non-linear regression fit [13] of the data indicated that the enzymes, before and after modification, followed a two-state model of protein unfolding. The difference in the free energy of unfolding increased for both PT and CT after modification. An increase of about 15  $kJ\ mol^{-1}$  in the  $\Delta G_{H_2O}$  is observed for both these enzymes (Table 1). The co-operativity associated with the denaturation process seems to have increased in OSP PT, whereas in OSP CT, the corresponding value has decreased. This is reflected in the differences in the  $C_m$  values for PT and CT. In PT, the  $C_m$  has increased from 2.40 M to 2.71 M, whereas CT exhibited an increase from 1.74 M to 3.89 M. It is interesting to observe that the  $C_m$  for OSP CT is higher than that of OSP PT, indicating that the stability of CT has not only increased upon modification but also comparable to PT.

In conclusion, the results obtained in this study indicate, for the first time, that the conformational stability of the psychrophilic Atlantic Cod trypsin could be enhanced by

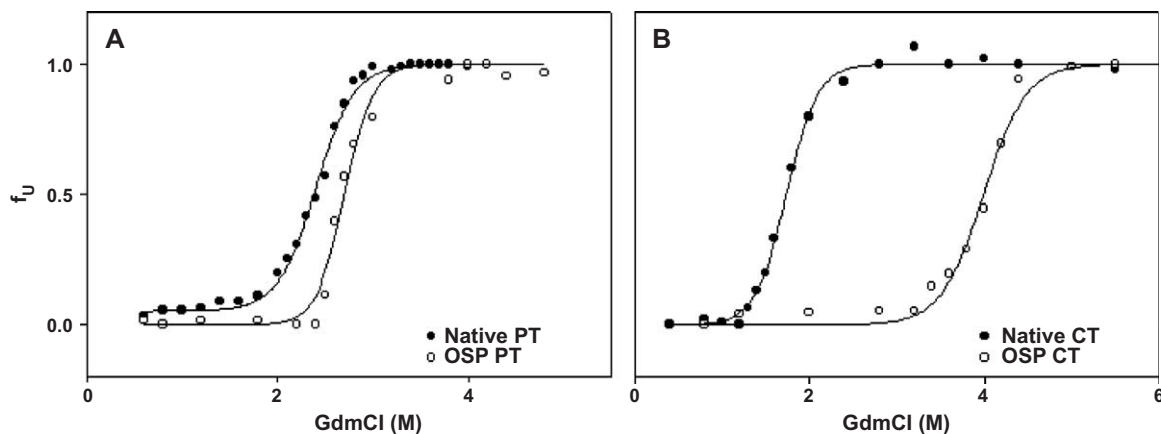


Fig. 1. Guanidinium chloride induced denaturation of PT (A) and CT (B) observed for the native and OSP modified forms. The data points were fit by two state models of protein unfolding to determine the stability related parameters (given in Table 1).

chemical coupling of the lysine amino acid residues with sucrose polymer without decreasing its hydrolytic activity. Such drastic increase in the thermodynamic stability was never observed with either mesophilic or thermophilic enzymes [9–12,14]. This could be due to the fact that psychrophilic enzymes generally exhibit improper packing of the interior amino acid residues that results in increased overall flexibility. Therefore, the covalent modification, described in this study, could have decreased the overall conformational mobility of the enzyme by increasing the interaction(s) of the core residues. Such induced optimization may not be possible in mesophilic enzymes because of their high intrinsic stability. Additionally, the newly formed multi-point covalent linkages and the additional intermolecular hydrogen bonds between the enzyme and the sucrose polymer would aid in the stabilization of the enzyme [11]. In our opinion, the enhancement of the thermodynamic stability of psychrophilic enzymes, as presented here, will greatly increase the usefulness of these enzymes in biotechnological applications.

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