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COMMUNICATIONS

Enhancement of the stability of thrombin by polyols: microcalorimetric studies

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Abstract—Glycerol increased the transition temperature (T_m) of thrombin in a concentration-dependent fashion up to a concentration of 50% glycerol in aqueous buffer solution. Glycerol showed a comparable effect on T_m of trypsin. This effect on T_m of thrombin was not seen in the presence of excess sodium chloride (1·2 M) in aqueous buffer solution. The stabilizing effect of glycerol may be due to increased energy demand to unfold the protein molecule, as reflected by an increase in T_m. This stabilizing effect, as measured by T_m, was seen for other polyols, including sucrose, and was also dependent on the concentration of the stabilizing agent. Microcalorimetry may be used as an effective tool to screen for the protective action of compounds in enzyme stabilization studies before conducting the time-consuming and expensive stability studies of proteins in the presence of additives under different storage conditions.

With the advent of biotechnology, proteins and peptides are expected to become a major source of drugs. However, the stability of such products in aqueous solution is of concern. The subject of chemical and physical instability of proteins has been extensively reviewed by Manning et al (1989). Different systems have been investigated so as to extend the shelf-life of such aqueous products. Glycerol was used by Jarabak et al (1966) and Jarabak (1972) and polyhydric and monohydric alcohols were used by Gerlsma & Stuur (1972) to stabilize proteins. Gekko & Timasheff (1981a, b) found that using glycerol-water as the solvent for proteins was accompanied by an increase in the chemical potential of glycerol. This also could decrease the surface interaction between glycerol and proteins.

Thrombin has been therapeutically used topically to prevent the oozing of blood from tissues during surgery and to control the loss of fluids resulting from burns. Because this enzyme is unstable in aqueous solution, it is currently available as sterile freeze-dried preparations which need to be reconstituted under aseptic conditions before use. It is desirable to have a stable, liquid, ready-to-use, formulation of thrombin for immediate use during emergency surgical procedures rather than the current dry powder.

Several attempts to formulate thrombin as an aqueous solution have been unsuccessful. Acylation of thrombin completely inactivated its proteolytic activity (Landaburu & Seegers 1957, 1959) and thrombin activity decreased rapidly at room temperature (Lundbald 1971) or at 37°C (Miller-Anderson et al 1980).

Seegers (1944) investigated the loss in thrombin activity at room temperature and at 50°C in solution in the absence and presence of glycerol. At room temperature and glycerol concentration of 50% v/v in solution, thrombin clotting activity was maintained for a month; at glycerol concentrations of less than 25% v/v, there was very little stabilization. Similar stabilization of thrombin activity in the presence of glycerol concentrations of greater than 50% v/v in solution was observed at 50°C over 48 h.

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Recently, Silbering et al (1987) showed that thrombin clotting activity could be maintained at room temperature for at least six months in buffered solutions containing 50% glycerol.

This report describes the use of microcalorimetry to compare the influence of different agents on the transition temperature (T_m) of thrombin and trypsin as it relates to enzyme stability. The use of this simple technique to predict the stability of an aqueous protein product could save valuable time and expense for the pharmaceutical industry.

Materials and methods

Ultra-pure thrombin was prepared using column chromatography (unpublished data) and was stored in a solution of 0·025 M sodium phosphate buffer, pH 6·5 containing 0·4 M sodium chloride and 0·02% sodium azide (buffer A).

Total protein concentration of thrombin was determined using the Bio-Rad reagent (Richmond, CA). The transition temperature (T_m) of freshly prepared protein solutions was determined using an MC-2 Scanning Calorimeter (MicroCal, Inc, Northampton, MA) at a scanning rate of 60°C h^{-1} up to 85°C. All solutions were degassed under vacuum before use. The reference cell was filled with a vehicle having a composition similar to that of the test sample, but without protein. Both sample and reference cells were originally cooled to about 10°C using an RC6 cooling system (Brinkman, Westbury, NY). Data acquisition in the heating mode was initiated when the temperature difference between the reference cell and experimental cell was about 1°C as indicated by the instrument's control. The baseline was scan-rate-normalized using two reference points on the low temperature baseline and two points at the high temperature baseline. The excess heat capacity, ΔC_p, temperature curve was then calculated by the computer.

Test agents (other than glycerol) were originally prepared in buffer A and then added to thrombin in solution to give a final enzyme protein concentration of 1–1·5 mg mL^{-1}.

Trypsin from bovine pancreas (Sigma, St Louis, MO) was dissolved in dilute HCl (pH 2·5) before the addition of glycerol.

Results

The microcalorimeter measures the heat flow into a sample while the sample is being heated. This measurement is simultaneously compared with that for the vehicle or solvent for the sample in a differential scanning mode. The instrument measures, therefore, the heat flow or heat capacity, C_p, into the sample only, since the solvent is used as a reference. The heat capacity of a native protein is small at low temperatures, and increases as the temperature increases. At the transition temperature (T_m), when the protein is transformed from the native to the denatured form, an excess heat capacity accompanies this denaturation.

The objective of this investigation was to determine the effect of certain agents, especially glycerol, on the stability of proteins in solution by measuring the T_m.

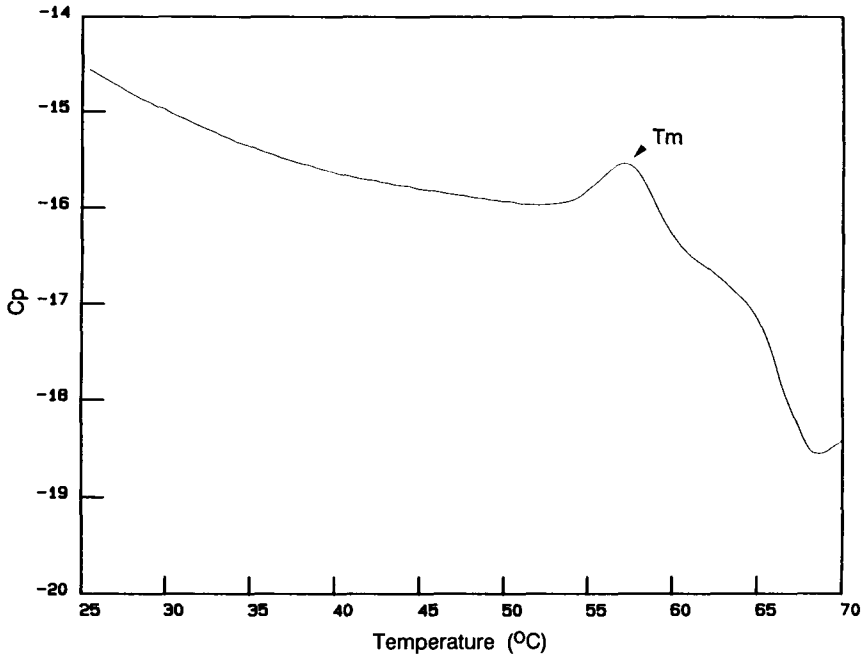

FIG. 1. Thrombin (1·5 mg mL^{-1}), in 0·025 M phosphate buffer containing 0·4 M sodium chloride, pH 6·5, was heated in the microcalorimeter, and the heat capacity (C_p) was recorded as described in the text.

It is important to note that as the thrombin solution was heated to 85°C, as described under Materials and methods, it lost its clotting activity irreversibly, and the solution became turbid due to the irreversible denaturation of thrombin.

A typical microcalorimetric scan of thrombin in aqueous solution at pH 6·5 is shown in Fig. 1. The T_m is 56·5°C. This value for T_m was independent of the thrombin protein concentration in solution (1–5 mg mL^{-1}). Table 1 shows the effect of pH of the thrombin solution on T_m. At pH 6·5, T_m was 57·4°C; and on increasing the pH to 7·9 and 9·9, T_m decreased to 54·0 and 47·6°C, respectively.

The effect of glycerol concentration in aqueous buffered thrombin solutions was investigated. In the presence of glycerol, T_m increased in a concentration-dependent manner with a correlation coefficient r = 0·995 (Fig. 2).

In a similar study, trypsin was dissolved in different glycerol concentrations, and T_m was determined. The data indicate that the T_m for trypsin was also increased by increasing glycerol concentration (Table 2).

Table 1. Effect of pH on the transition temperature (T_m) of thrombin in solution*.

pH	T_m
6·5	57·4
7·9	54·0
9·9	47·6

* Thrombin was diluted to a concentration of 1 mg mL^{-1}, and the pH was adjusted using 0·1 M NaOH. Thrombin was loaded in the sample cell of the microcalorimeter and scanned for T_m measurement, as described in the text.

The effect of other polyols on T_m of thrombin in solution at

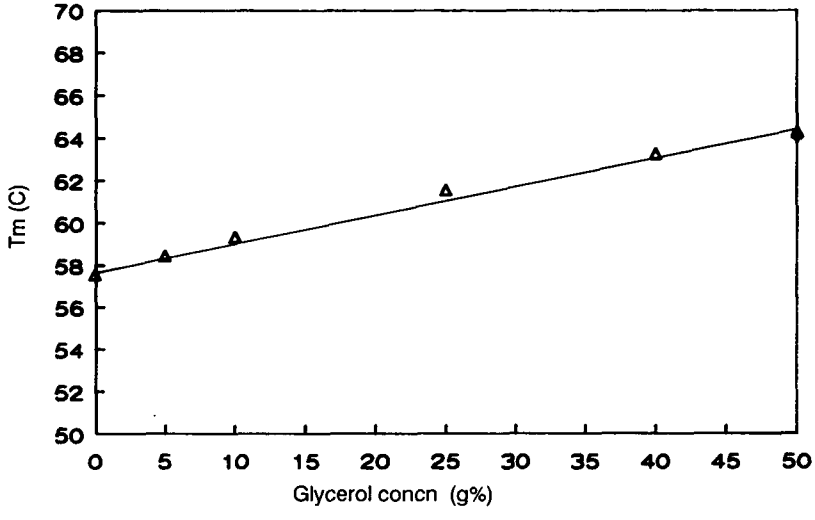

FIG. 2. Transition temperature T_m change of thrombin in solution at pH 6·5 as a function of glycerol concentrations.

Table 2. Effect of glycerol on the transition temperature (T_m) of trypsin in solution*.

Glycerol concentration (%)	T_m (°C)	ΔT_m (°C)
0	52·2	—
25	55·8	3·6
50	60·2	8·0

* Trypsin was prepared in HCl, pH 2·5, 1·5 mg mL^{-1}, loaded in the sample cell and scanned for T_m measurement as described in the text.

Table 3. Effect of polyhydric compounds on the transition temperature change (ΔT_m) of thrombin in solution*.

Additive	ΔT_m (°C)
48% Sucrose	4·8
20% Dextrose	4·7
20% Sorbitol	5·0
20% Dextrose + 20% sorbitol	8·8
50% Glycerol	9·8
25% Glycerol	3·35
25% Glycerol + 25% sorbitol	7·20
25% Glycerol + 12·5% sorbitol	5·40
1·2 M Sodium chloride**	1·7
20% Dextran (mol. wt 18 000)**	1·9
16% Dextran (mol. wt 51 000)**	0·7
12% Mannitol	2·8

* Thrombin was diluted to contain 1–1·5 mg mL^{-1}, loaded in the sample cell of the microcalorimeter and scanned for T_m measurement as described in the text. All polyols tested were dissolved in 0·025 M sodium phosphate buffer, pH 6·5 containing 0·4 M sodium chloride and 0·02% sodium azide, with the exception of glycerol. **These compounds were included for comparison with the polyols.

pH 6·5 was evaluated and is shown in Table 3. Sucrose and sorbitol are examples of sugars that contain no reducing groups, while dextrose is an example of a reducing sugar. All these sugars increased the T_m of thrombin; some combinations of these compounds showed a transition temperature change (ΔT_m) comparable with that observed using 50% glycerol, e.g. 20% dextrose + 20% sorbitol and 25% glycerol + 25% sorbitol. Dextran in solution showed only a minor effect (Table 3).

Discussion

Activity of enzymes could be influenced by many factors including temperature. However, at a critical temperature, the enzyme protein separates out of solution and loses its enzymatic activity. This situation is described as the transition point of heat denaturation. The heat required to cause a chemical or physical change is one of the basic thermodynamic parameters of the protein molecule. Our results show that heat denaturation of thrombin was accompanied by change in heat capacity, Δc_p (Fig. 1), in a manner similar to that observed by Gekko & Timasheff (1981b) for other proteins.

According to studies conducted by Fenton et al (1977), thermal denaturation occurs rapidly at temperatures above 40°C with loss of thrombin clotting activity but without changes in the electrophoretic behaviour in SDS polyacrylamide gels. This may suggest the occurrence of little change in the primary structure of the thrombin molecule when it is heated under these conditions.

Furthermore, it has been observed that thrombin in aqueous solution is unstable at room temperature, but, in the presence of glycerol, the clotting activity could be stabilized (Seegers 1944; Silbering et al 1987). In addition, the present data indicate that this stabilizing action of glycerol was dependent on glycerol concentration as measured by an increase in T_m. Sorbitol also has a stabilizing action on thrombin. These results compare well with data obtained by Back et al (1979) for ovalbumin in phosphate buffer pH 7·0.

Although the exact mechanism of stabilization of thrombin is unknown, it may be related to the folding and unfolding of the protein molecules. Tsong & Baldwin (1978) have shown that glycerol accelerated the refolding of ribonuclease. Although the folded state does not necessarily represent the native state, this compact state could favour stabilization of the molecule. The present data show that T_m was increased by increasing glycerol concentration in a concentration-dependent manner. This phenomenon may suggest that T_m change may be related to the degree of compactness of the protein molecule.

The stabilization of thrombin and trypsin by polyols may be related to the interactions between the enzyme, water and polyol. Jarabak et al (1966) and Ruwart & Suelter (1971) have proposed that the stabilization of enzymes by glycerol results from the formation of water-glycerol structure around the protein molecule. Gekko & Timasheff (1981a, b) explained the stabilization of enzymes in glycerol-water mixtures by the exclusion of the stabilizing solvent, glycerol, from the domain of the protein and the preferential hydration of the protein molecule. An increase in the chemical potential of glycerol in the presence of protein results in a thermodynamically unfavourable interaction which would tend to minimize the surface of contact between protein and glycerol, thereby stabilizing the globular structure of protein. The studies of Combes & Monsan (1984) and Combes et al (1988) on the stabilization of invertase in polyol-water solutions indicate that these additives interact more strongly with water molecules than with enzyme molecules, thereby organizing the water molecules into aggregates. This organization of water limits the unfolding of the enzymes thrombin and trypsin. The resulting immobilization stabilizes the enzyme against thermal degradation (Klibanov 1983) and increases the resistance to denaturation of the protein with a concomitant increase in the T_m of the enzyme in glycerol-water solutions.

Studies conducted by Lundbald (1971) indicate that thrombin loses 50% of its proteinase or clotting activity and not the esterase activity in a pH 7·5 solution at 23°C in 26 h. The clotting activity is considerably more stable at 4°C. Since thrombin is a highly specific proteolytic enzyme, the nature of the binding site is exceedingly complex. Thus, it is possible to have distortion of the enzyme molecule which causes a loss in clotting activity without affecting the catalytic property due to the less specific esterase activity.

Studies on enzyme inactivation by Ahern & Klibanov (1985) and Yoshioka et al (1991) indicate that enzyme inactivation follows first-order kinetics, and the rate constants conform to the Arrhenius relationship. Hence, studies based on the T_m allow for stability predictions at ambient temperature. In addition, the similarity between the stabilizing effect of glycerol on thrombin and on trypsin suggests that the determination of the T_m provides a powerful screening method for predicting the protective effect of additives on proteins before conducting expensive and time consuming routine stability studies of protein solutions.

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Failure of tetrodotoxin to inhibit the prostaglandin-induced secretory response of rat small intestine in-vitro

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Abstract—Tetrodotoxin (TTX, 10 μM) did not inhibit prostaglandin E_2 (PGE_2)-stimulated increases in electrical activity in intact or stripped sheets of rat small intestine, although it reduced basal electrical activity in the intact preparation. Basal and PGE_2-stimulated cAMP production by enterocytes isolated from the small intestine was unaffected by TTX. Thus its reported ability to inhibit prostaglandin-induced fluid secretion in-vivo does not appear to represent a direct interaction of the neurotoxin with the mechanism of prostaglandin action at the enterocyte.

There is evidence that the secretory response of the intestinal mucosa involves not only a direct action of secretagogues on the transporting cells, but also the participation of the enteric nervous system (ENS, Lundgren et al 1989). Prostaglandins are potent stimulants of intestinal secretion in-vivo (Matuchansky & Coutrot 1978; Hardcastle et al 1981) and the observation that in such preparations the neurotoxin, tetrodotoxin (TTX), abolishes the secretory response to prostaglandin E_1 (PGE_1) (Coupar 1986) suggests that the prostaglandin does not directly affect the enterocyte but acts via a neural pathway. The fact that prostaglandins can stimulate secretion in in-vitro preparations of intestine does not exclude this possibility as such preparations retain a large proportion of their intramural nerve networks. It is, however, less easy to reconcile with reports that prostaglandins can stimulate transporting cells in the absence of neural networks; they can enhance cAMP production in isolated enterocytes (Hardcastle et al 1981) and increase the short-circuit current in a colonic cell line (Dharmsathaphorn et al 1984). To account for the abolition of prostaglandin-induced intestinal secretion by TTX in-vivo, despite the direct action of prostanoids on the transporting cells, it has been proposed that the toxin might release an endogenous inhibitor or that it might exert a direct antisecretory action on the transporting cells (Coupar 1986). This latter possibility was examined by testing the ability of TTX to influence the secretory actions of PGE_2 in in-vitro preparations of rat small intestine.

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Materials and methods

Experiments were carried out on male albino rats (Sheffield strain, 230–250 g) allowed free access to food and water.

Measurement of transintestinal electrical activity. Transintestinal electrical activity was measured in-vitro in paired sheets of mid-intestine, removed from anaesthetized rats (60 mg kg^{-1} sodium pentobarbitone, i.p.). In some experiments the muscle layers were removed (stripped sheets), while in others they were not (intact sheets). The sheets were clamped between two Perspex chambers (exposed tissue area = 1·925 cm^2) and incubated at 37°C in Krebs bicarbonate saline (5 mL in each chamber) gassed with 95%O_2–5%CO_2. The potential difference (PD) was measured with salt bridge electrodes, connected via calomel half-cells to a differential input electrometer. Current was applied through Ag/AgCl electrodes in contact with mucosal and serosal solutions via wide-bore salt bridges. When determining the short-circuit current (SCC) a correction for fluid resistance was