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Glass-immobilized glycated-trypsin: A novel modified trypsin that is remarkably thermostable

Van Thong Pham [a], Illimar Altosaar [a,*], Michael Norman Duhig [b], Harvey Kaplan [b]

[a] Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Canada
[b] Department of Chemistry, University of Ottawa, Canada

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ABSTRACT

Porcine trypsin was glycated with glucose and covalently immobilized through its carboxyl groups onto aminated glass beads to produce porcine immobilized glycated-trypsin (IGT). On incubation at 60 °C and pH 8, IGT retained its full activity for 8 h and 50% of its activity after 24 h. In comparison, under the same conditions porcine native trypsin lost 80% of its activity in 2 h and was completely inactivated in less than 4 h. The rate of autolysis of porcine glycated-trypsin at 37 °C was 40% that of native trypsin and with IGT there was no significant autolysis, even at elevated temperatures as high as 60 °C. Glycation significantly increased the stability of trypsin and immobilization also significantly increased the stability of trypsin. The remarkable thermostability of IGT is attributed to a synergistic effect when these two modifications are combined. Tryptic fragmentation of denatured proteins with IGT can be performed at 60 °C for shorter digestion times and with smaller amounts of enzyme than normally employed to achieve complete digestion with soluble forms of trypsin. Prior denaturation of proteins for tryptic digestion is not required with IGT as in situ denaturation and digestion can be achieved simultaneously at 60 °C with an enzyme:protein mass ratio as low as 1:1000.

1. Introduction

Trypsin is the most widely used protease for the fragmentation of proteins [1–3], especially in proteomic applications involving the use of mass spectrometry (MS) to analyze and characterize peptides. This is due to the predictability of tryptic proteolytic fragmentation patterns based on its specificity for cleavage at lysine and arginine residues. Trypsin readily autolyzes [4,5] and the autolytic peptides generated can complicate the analysis of MS spectra [3]. This problem has been addressed by reducing the number of potential autolytic sites in trypsin using reductive methylation [6] to dimethylate the ε-amino groups of the lysine side-chains, but even this modified trypsin can still undergo some autolysis [5,7]. Another problem is that native proteins are not readily digested by trypsin, so they must be denatured to achieve efficient fragmentation [8]. The need to denature proteins, usually by the addition of chemicals, incurs additional steps and further complicates analytical procedures. It would be advantageous if tryptic fragmentation of proteins could be achieved without contamination from autolytic peptide products from trypsin itself or from the use of chemical denaturants.

There have been several successful strategies for increasing the stability of trypsin by chemical modification [5,9–15]. Among the modifiers that confer the greatest stability are methoxypolyethylene glycol (MPEG) [12], cyclodextrins [14], and glucose [15]. A common feature of these modifiers is that they enhance the stability of trypsin in two ways: (1) Covalent adducts are formed on lysine side-chains and thereby reduce inactivation caused by autolysis; (2) they add hydrophilic moieties to the protein that enhance its structural stability. Another successful stabilization strategy has been the immobilization of native trypsin on solid supports, which reduces autolysis [16–18] due to the fact that the immobilized enzyme molecules have greatly restricted contact with each other. It is our hypothesis that combining the stabilizing effects of chemical modification and immobilization will have a substantial synergistic effect. This will yield a form of trypsin that will have a sufficiently high thermostability to perform tryptic digestion of native proteins in situ without the use of denaturants, yet will be autolysis-free. To test this hypothesis we have covalently immobilized porcine glycated-trypsin [15] on aminated glass beads to form porcine immobilized glycated-trypsin (IGT).

2. Experimental

2.1. Materials

Bovine pancreatic trypsin and porcine pancreatic trypsin were purchased from Sigma–Aldrich Chemical Company (St.

* Corresponding author. Tel.: +1 613 562 5459; fax: +1 613 562 5452.
E-mail address: altosaar@uottawa.ca (I. Altosaar).

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Louis, Missouri). N-benzoyl-L-arginine ethyl ester (BAEE) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma–Aldrich Chemical. Standard 0.100N NaOH was from Canada VWR (Montreal PQ, Canada). D-(+)-glucose was purchased from the BDH Chemical Company. Whatman 3MM paper was purchased from Sigma–Aldrich Chemical Company. Aminated glass beads (3 μm) were purchased from Varian Inc. (Mississauga, ON, Canada). All other chemicals, reagents and solvents were high purity preparations obtained from commercial sources.

2.2. Preparation of in vacuo glycated enzymes

Trypsin was glycated with glucose as previously described [19,20]. In the case of the trypsin preparations, these samples were treated with TPCK to inactivate any pseudotrypsin or chymotrypsin present in the stock preparations [21]. All enzyme samples were dialyzed (3.5 kDa cutoff) before use to remove any traces of lower molecular weight impurities. Enzyme (50 mg) was dissolved in 10 mL distilled water (dH_2O) and glucose (10 mg) was added. The pH was adjusted to 7.0 with 0.100N NaOH and 2.0 mL aliquots (~10 mg protein) were lyophilized in Pyrex borosilicate tubes (size 16 mm × 100 mm). The glass tubes were narrowed and sealed under a vacuum of 50 mTorr, and subsequently placed in an oven at 85 °C for 40 h. After thermal incubation, the protein samples were reconstituted with 20 mL of 0.01% (v/v) acetic acid, placed in a dialysis membrane (6–8 kDa cutoff) and dialyzed against a 3000 mL volume of distilled water acidified with 0.100 mL glacial acetic acid (2 exchanges) and subsequently with only dH_2O (3 exchanges) over a period of 24 h before final re-lyophilization for storage at 4 °C.

2.3. Non-denaturing acidic polyacrylamide gel electrophoresis

Glycated-trypsin was analyzed by acidic non-denaturing polyacrylamide gel electrophoresis (PAGE) with the Bio-Rad Mini-Protean II electrophoresis system. The electrophoresis was performed with the acid/urea/Triton (AUT) gels described by Bonner et al. [22] but modified by omitting urea and Triton X-100. Protein (5–10 μg) was loaded onto an acidic 15% acrylamide gel (1.5-mm thickness) with 100 mM glycine containing 1% (v/v) glacial acetic acid as running buffer. After electrophoresis at constant current of 5 mA for 180–225 volts-hour (VH), the gel was stained with Coomassie Brilliant Blue R-250.

2.4. Quantification of tryptic activity

Tryptic activity was quantified by determining the rate of hydrolysis of BAEE at pH 7.5 and 25 °C. The activity was monitored by a Radiometer Copenhagen type PHM26 pH meter fitted with a Beckman Futura™ refillable combination electrode coupled to a Titration 11/Ole Dich autotitrator pH-stat assembly. A 5.00-mL aliquot of substrate solution (1.00 mM BAEE; 100 mM KCl; and 1.00 mM $CaCl_2$) was transferred to the reaction vessel with a constant stream of nitrogen over the solution surface and subsequently titrated to a pH of 7.5 using 0.020N NaOH added via a micro-syringe. A 100 μL sample containing 5.00 μg of the enzyme was added to the reaction vessel, and the volume of base (0.020N NaOH) added per unit time to maintain a constant pH of 7.5 (endpoint) by the autotitrator was used to quantify the rate of hydrolysis.

2.5. Quantification of tryptic activity after incubation at 45 °C

Trypsin (0.1 mg/mL) in 5.00 mL volume (20 mM Tris, pH 8; 2 mM $CaCl_2$) was incubated at 45 °C. Aliquots of 100 μL were removed at timed intervals and diluted to a concentration of 0.050 mg/mL by the addition of 100 μL of 0.05% (v/v) acetic acid and placed on ice. The enzyme's tryptic activity was measured at 25 °C as described above and the rates were reported as percentage of the initial rates.

2.6. Use of pH-stat to measure rates of tryptic autolysis at pH 8.5

A sample (3.00 mg) of either native, reductively methylated or glycated porcine trypsin prepared in a 3.0 mL solution (100 mM KCl, 1 mM $CaCl_2$) was placed in a reaction vessel of a pH-stat (see above). The rate of autolysis was monitored at 37 °C by the amount of 0.010N NaOH required to maintain a constant pH of 8.5.

2.7. Immobilization of trypsin and glycated-trypsin

Aminated glass beads (1 g) were washed with a 1% (w/v) NH_4HCO_3 solution and then washed twice with a volume of ddH_2O and resuspended in ddH_2O to form a slurry. Enzyme (20.0 mg) was dissolved in ddH_2O and the slurry of the glass beads was added to the enzyme solution to a final volume of 4.0 mL. The pH was adjusted to 7.00 and the enzyme/glass beads mixture was evenly distributed among four Pyrex® borosilicate glass test tubes, flash frozen, and lyophilized. The sample tubes were purged with nitrogen and subsequently narrowed and sealed under vacuum (50 mTorr) using an oxygen enriched flame. The sealed tubes were placed in an oven at 85 °C for 24 h. The sealed sample tubes were opened and 5 mL of a 0.5% acetic acid solution was added. All of the samples were transferred to a 15-mL microfuge tube and centrifuged to collect the pellets.

To remove enzyme that was not covalently attached but adsorbed to the glass beads, the pellets were washed twice with a volume of 0.5% acetic acid and twice with phosphate buffered saline (PBS). A 3-mL volume of ddH_2O was added to the washed pellet and vortexed. The suspension was brought up to a volume of 10.0 mL with ddH_2O, transferred to a 20-mL screw-capped vial, and incubated at 60 °C for 3 h with continuous stirring. The beads were recovered by centrifugation and the pellet was washed twice with PBS, twice with 0.5% acetic acid and once with ddH_2O. The pellet was resuspended in 3 mL of ddH_2O and lyophilized for storage.

2.8. Determination of the thermostability of trypsin samples

A suspension of immobilized native trypsin or IGT (1.00 mg/mL) was prepared by the addition of a pH 8.00 buffer containing 0.100 M KCl, 1.00 mM $CaCl_2$ and 0.200 mM Tris. The sample was placed in a shaker bath set to a constant temperature of 60 °C. It was necessary to allow the sample to equilibrate at 60 °C, therefore the first sample aliquot (t = 0 h) was removed after a 5 min incubation period. Aliquots (5.00 mL) were taken from the bulk sample after timed periods of incubation (t = 0 h, 0.5 h, 1 h, 2 h, 4 h and 24 h) at 60 °C, and the tryptic activity was quantified at 25 °C on a pH-stat as previously described. The method used to obtain the thermostability data for the free native porcine trypsin was essentially identical to the method stated above for the immobilized sample. The native enzyme was incubated in the 0.100 M KCl, 1.00 mM $CaCl_2$ and 0.200 mM Tris pH 8.0 buffer at a concentration of 0.200 μg/mL, and 5.00 mL sample aliquots were taken after timed intervals of incubation in the 60 °C shaker bath.

2.9. In situ digestion of native lysozyme at 60 °C

Hen egg white lysozyme (2.0 mg, 1.0 mg/mL, 50 mM NH_4HCO_3) was incubated with porcine native trypsin (2 μg), porcine glycated-trypsin (2 μg) or IGT (0.8 mg), where the mass ratio was 1000:1 (lysozyme to trypsin). It was determined that 1 mg of the 3 μm-IGT glass beads had the solution equivalent of 2.5 μg of soluble native porcine trypsin. Therefore, 0.8 mg of IGT had the equivalent activity of 2.00 μg of soluble porcine trypsin. The various trypsin

samples (2 μg) in a volume of 1.800 mL (50 mM NH_4HCO_3) were incubated at 60 °C for 5 min before the addition of 0.200 mL solution of lysozyme (10 mg/mL). After 24 h incubation at 60 °C, an aliquot (100 μL) of each digestion was transferred to a 1.5-mL microfuge tube containing 200 μL pH 2.1 buffer (8% acetic acid and 2% formic acid) and lyophilized on a Savant SpeedVac™ apparatus. The samples were reconstituted with 50.0 μL of pH 2.1 buffer. An aliquot (40.0 μL) of each sample (80.0 μg of protein) was applied to a 3.3 cm wide strip on a 20 cm × 20 cm piece of Whatman chromatography paper and subjected to high voltage paper electrophoresis (HVPE) at 20 V/cm for 2 h (800 VH). The paper was stained by dipping in a cadmium-ninhydrin solution to visualize the peptides generated from lysozyme by tryptic digestion. HVPE was performed by using the Hunter Thin Layer Electrophoresis equipment (HTLE 7002; CBS Scientific Ins., Del Mar, CA).

3. Results and discussion

3.1. Preparation and homogeneity of glycated-trypsin

Glycation of trypsin was performed by the in vacuo glycation procedure developed by Kaplan and co-workers [15,19,20]. In this procedure protein and glucose are simply lyophilized together at neutral pH, and incubated under vacuum. Glucose becomes covalently attached to lysine residues by a ketoamine linkage. In vacuo glycation has the advantage that it is more economical and much simpler to perform than solution-based chemical modifications. It also minimizes inactivation due to denaturation and autolysis often encountered using activated chemical reagents in solution to chemically modify proteolytic enzymes such as trypsin. In vacuo glycation with glucose has been shown to increase the structural stability of several proteins [15].

Porcine and bovine glycated-trypsins had significantly decreased electrophoretic mobilities compared to the native unmodified forms in acid native PAGE (Fig. 1). This showed that the glycation had significantly increased the size of the protein resulting in a decrease in electrophoretic mobility due to the effect of molecular sieving. As the size of glucose is small relative to the protein, this indicated that extensive glycation had taken place. The presence of distinct bands with no intermediate glycated forms of trypsin indicated that the vast majority of the 14 lysine residues in bovine trypsin and 11 in porcine trypsin [4] had been glycated. This was confirmed by the deconvoluted mass spectrum of the bovine glycated-trypsin in Fig. 2 which showed that species with 9–14 glucosyl adducts were present, with the majority containing more than 11 glucosyl adducts. Each of the major peaks differed by 162 mass units corresponding to the expected value of a glucosyl ketoamine adduct.

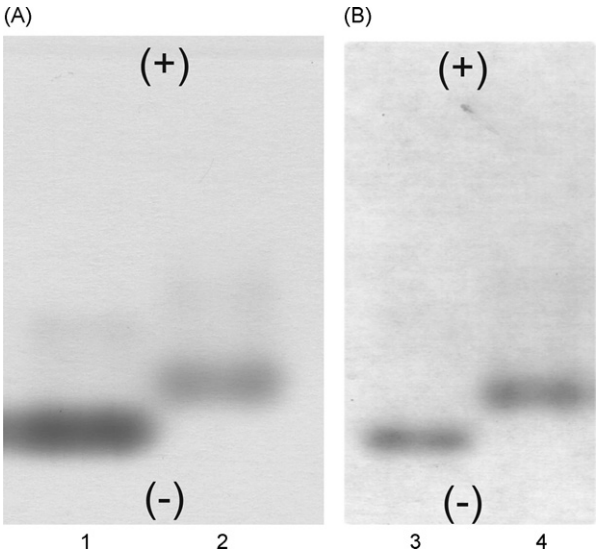
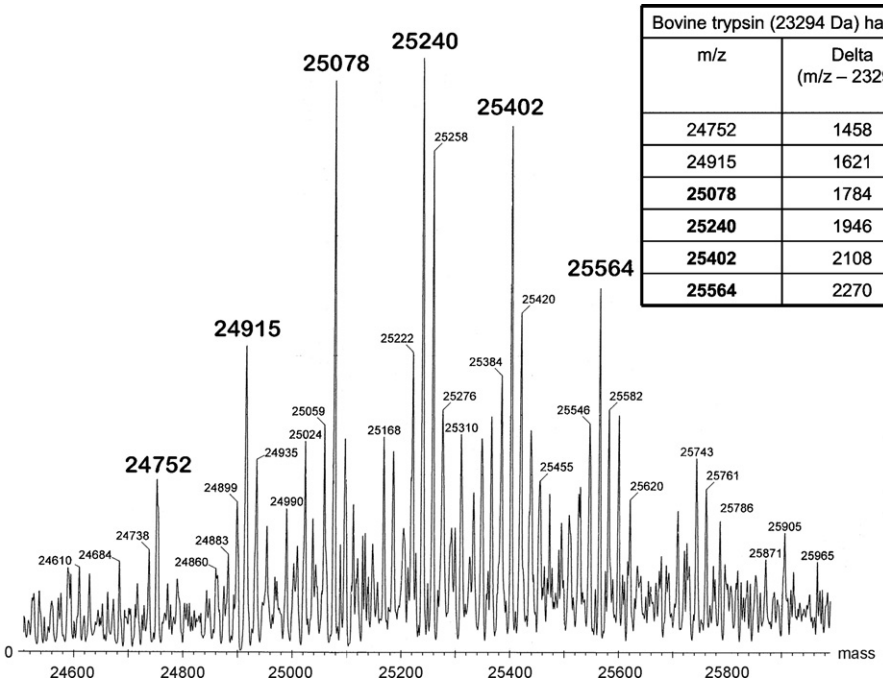


Fig. 1. Non-denaturing PAGE of porcine (panel A) and bovine (panel B) trypsins. Lane 1: porcine native trypsin (5 μg); Lane 2: porcine glycated-trypsin (5 μg); Lane 3: bovine native trypsin (5 μg); Lane 4: bovine glycated-trypsin (10 μg). Gels were stained with Coomassie Blue R-250.



Bovine trypsin (23294 Da) has 14 lysine residues		
m/z	Delta (m/z – 23294)	# glycated amino group (Delta+162.05)
24752	1458	9
24915	1621	10
25078	1784	11
25240	1946	12
25402	2108	13
25564	2270	14

Fig. 2. Deconvoluted electrospray mass spectrum of bovine glycated-trypsin. Each ketoamine adduct has a mass of 162.05 amu.

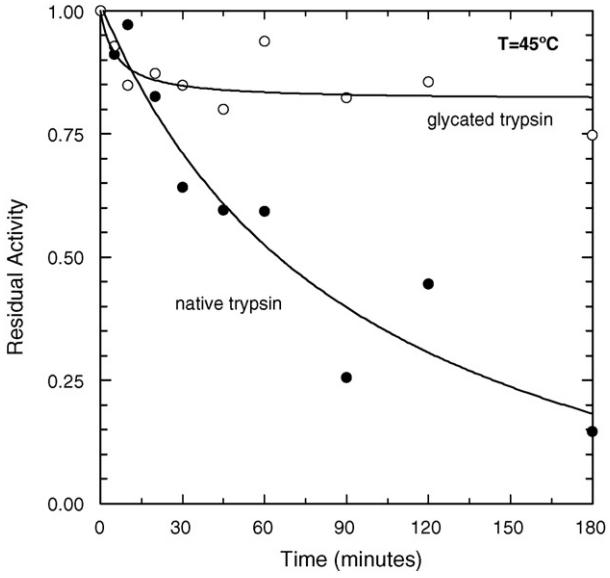


Fig. 3. Effect of glycation on the activity of bovine trypsin. The rate of BAEE hydrolysis by native trypsin (●) and glycated-trypsin (○) was measured after incubation of the enzyme sample for times at 45 °C. The activity is shown as relative to the initial activity of the samples.

3.2. Autolysis of trypsin and modified trypsins

At 45 °C and pH 8, the native bovine trypsin rapidly lost activity (Fig. 3). This has been attributed to autolytic cleavage of peptide bonds at lysine and arginine [5]. In sharp contrast to this, the glycated-trypsin maintained its activity over a 3 h period and glycation of the lysine residues, which blocks potential autolytic cleavage sites, is consistent with a decrease in autolysis. This was confirmed by the direct quantification of the rates of autolysis of native, reductively methylated, and glycated forms of porcine trypsin, using a pH-stat in which the release of protons on cleavage of peptide bonds at 37 °C and pH 8.5 was monitored (Fig. 4). Native

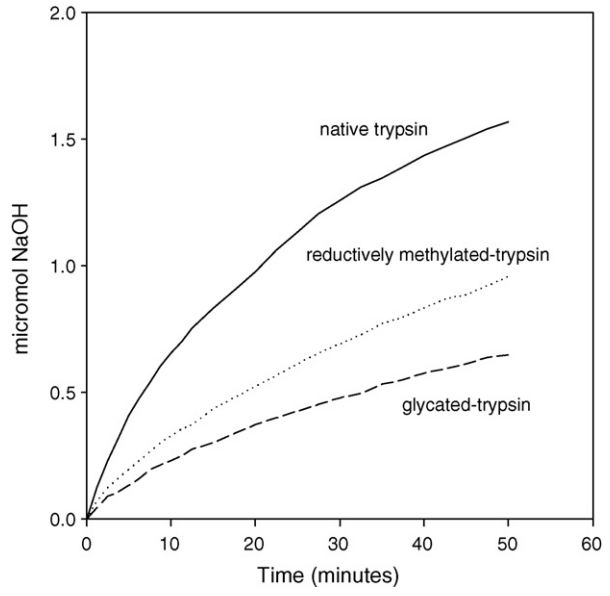


Fig. 4. Autolysis of native porcine trypsin (—), reductively methylated porcine trypsin (···), and porcine glycated-trypsin (– – –) at 37 °C. Enzyme samples (3.0 mg) were incubated at a concentration of 1 mg/mL at 37 °C and the extent of autolysis was measured on a pH-stat at pH 8.5 by addition of a volume of 0.01N NaOH to compensate for a pH drop resulting from the release of acid by cleavage of peptide bonds.

Table 1
Retention of activity[a] by porcine IGT on repeated use.

Number of trials (washing)	Percent original activity
	100%
1	94%
2	93%
3	95%
4	95%

[a] The 3-μm IGT beads have a solution equivalent of 2.5 μg of native trypsin per mg of glass beads. After each trial, the glass-immobilized trypsin beads (15 mg) were recovered by centrifugation and washed with 10 mL 10 mM Tris pH 8 and followed by 2 × 10 mL of dH_2O. The activity was quantified by the rate of hydrolysis of BAEE.

trypsin released protons more rapidly than the glycated-trypsin and hence had a higher rate of autolysis. The direct measurement of the protons released on incubation of reductively methylated-trypsin confirmed that it did indeed have a reduced rate of autolysis, but the rate was greater than that of the glycated-trypsin. The initial relative rates of autolysis over the first 2 min were the following: native trypsin (1.0); reductively methylated-trypsin (0.60); glycated-trypsin (0.40).

3.3. Immobilization of glycated-trypsin

Porcine glycated-trypsin was lyophilized with 3-μm amino functionalized glass beads and subjected to the in vacuo cross-linking procedure [23] to achieve covalent immobilization. After multiple washings, the beads did not lose esterolytic activity toward BAEE confirming the effectiveness of the immobilization (Table 1). The amount of immobilized glycated-trypsin was estimated based on the activity of trypsin in solution. It was found that IGT had a solution equivalent of 2.5 μg trypsin per mg of glass bead.

The glycated-trypsin was immobilized by the formation of amide linkages between its carboxyl groups and amino groups on the glass beads [23]. Most immobilization procedures use free amino groups on the protein to couple to activated carboxyl groups on the solid support [24] because this does not require any prior chemical modification of the protein. However, in glycated-trypsin the amino groups are blocked and conventional immobilization procedures would require chemical activation of the carboxyl groups [25,26] on the protein but, like conventional chemical modifications in solution, this often leads to some inactivation of the enzyme. In vacuo cross-linking provides a facile method for immobilizing proteins in which there are no free amino groups. Another advantageous feature is that no activating chemicals are required to effect the immobilization, thus eliminating or minimizing inactivation of the enzyme.

3.4. Thermostability of IGT

In the first part of this investigation, bovine rather than porcine trypsin was used (Figs. 1–3) because bovine trypsin is less stable than porcine trypsin. It was felt that this would provide a much more rigorous test of the ability of glycation to reduce autolysis and increase stability of the trypsin. Having successfully demonstrated this, porcine trypsin was used in the second part as it is the most commonly used source of trypsin in proteomic applications.

Immobilization on glass beads improved the thermostability of native porcine trypsin (Fig. 5). After 2 h at 60 °C, it retained 100% of its activity whereas the free native trypsin in solution lost 80% of its activity. In comparison, IGT retained 100% of its activity for almost 8 h of incubation at 60 °C (Fig. 5). Even at 70 °C, it retained 90% of its activity after 30 min of incubation (data not shown). The proteolytic specificity and efficiency of glycated-trypsin and IGT at 60 °C were compared with native trypsin at 37 °C by determining the electrophoretic peptide profiles obtained on digestion of CM-

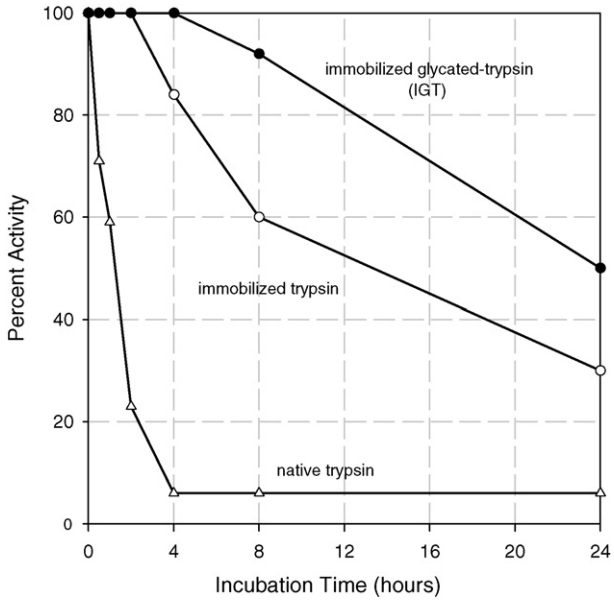


Fig. 5. Stability at 60 °C of native porcine tryspin (△), immobilized porcine trypsin (○), and immobilized porcine glycated-trypsin (●). The rate of BAEE hydrolysis was measured at a temperature of 25 °C after continuous incubation for various periods of time at 60 °C. The activity is shown as % values relative to the initial activity of the samples.

RNase A (Fig. 6). After 1 h incubation at 60 °C, the glycated-trypsin and IGT (enzyme:protein mass ratio of 1:200) generated the same peptide profile as the 24 h incubation with native trypsin at 37 °C (enzyme:protein mass ratio of 1:50).

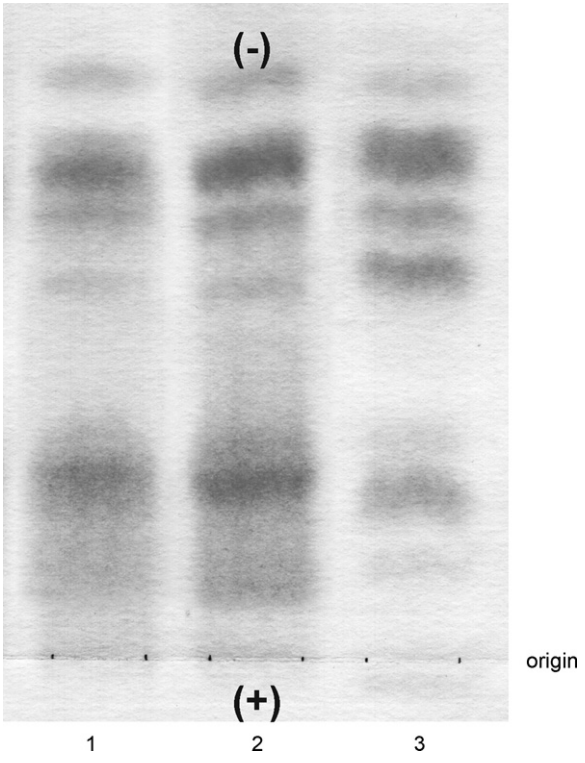


Fig. 6. Electrophoretogram of enzymatic digests of CM-RNase. HVPE was performed at pH 2.1 at 20 V/cm for 2 h and stained with cadmium-ninhydrin. Lane 1: porcine glycated-trypsin (enzyme:CM-RNase 1:200, 1 h, 60 °C); Lane 2: porcine IGT (enzyme:CM-RNase 1:200, 1 h, 60 °C); Lane 3: porcine trypsin (enzyme:CM-RNase 1:50, 24 h, 37 °C).

3.5. Fragmentation of proteins with IGT

Tryptic fragmentations are usually performed on denatured proteins at 37 °C for 8–24 h at enzyme:protein mass ratios ranging from 1:20 to 1:200 [8]. The thermostability of IGT makes it possible to perform tryptic fragmentation at elevated temperatures. This is advantageous because the increased rate of peptide bond hydrolysis due to elevated temperature permits shorter incubation times or less enzyme. In the results reported for digestion of denatured RNase A (Fig. 6), an enzyme:protein mass ratio of 1:200 was used, however, from the native lysozyme digestion result (see Section 3.6), it is clear that a much lower enzyme:protein mass ratio could have been used, at least as low as 1:1000.

The extraordinary thermostability of IGT makes it attractive for tryptic fragmentation of proteins particularly for proteomic applications where contamination is often a problem. Organic polymers are the solid supports most often used for protein immobilization probably because of the ease of immobilization and flexibility compared to glass [24]. However, for proteomic applications, glass is superior as a support because of the decreased potential for the presence of organic contaminants. For protein fragmentation with IGT, contamination from autolytic products is for all practical purposes eliminated because the retention of full activity for long periods of time at elevated temperatures demonstrated that it does not undergo significant autolytic cleavage. Furthermore, at an enzyme:protein mass ratio as low as 1:1000 (see Section 3.6), even if some autolysis were to occur, only negligible amounts of autolytic products can be produced. Another advantageous feature is that IGT can be easily separated from the digest by centrifugation or filtration and reused (Table 1).

For the purposes of this study, it was convenient to perform digests by adding IGT glass beads to a solution of the protein to be digested as the glass beads are easily removed and HPVE on the solution could be performed directly. Another digestion procedure could be to place the IGT beads in a column where protein fragmentation could be achieved in the column as a solution of protein passes through and the eluate containing the peptides collected. In fact, the aminated glass beads are produced for column applications, and such a column procedure with IGT beads could be useful for MS applications where column eluates are injected directly for analysis.

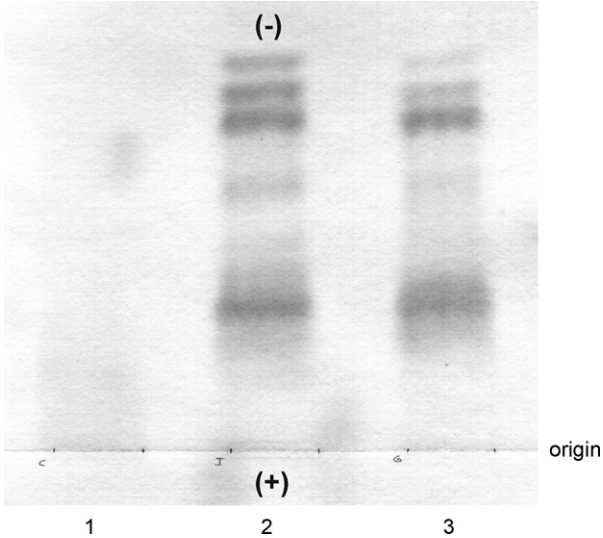


Fig. 7. Electrophoretogram of tryptic digests of native lysozyme. HVPE was performed at pH 2.1 at 20 V/cm for 2 h and stained with cadmium-ninhydrin. Digestions used an enzyme:lysozyme ratio of 1:1000 (w/w) for 24 h at 60 °C in 50 mM ammonium bicarbonate. Lane 1: porcine native trypsin. Lane 2: porcine IGT. Lane 3: porcine glycated-trypsin.

3.6. Fragmentation of native lysozyme with IGT

The ability of the IGT to digest native proteins without prior denaturation was tested by incubation with native lysozyme at 60 °C for 24 h using enzyme:protein mass ratio of 1:1000. Extensive digestion of the native lysozyme did indeed occur as evidenced by the number of peptide bands generated (Fig. 7, lane 2). Native trypsin (lane 1) in comparison generated no detectable peptide bands. Free glycated-trypsin (lane 3) also generated peptide bands, but the digestion was not as extensive as with IGT. Lysozyme has a melting point of 68 °C and is much more thermostable than most proteins [27]. Nevertheless, the results show that it is sufficiently unfolded at 60 °C for IGT to digest it and it is likely that this will be true for most proteins. There will be some variation depending on the protein being digested, but it is possible to optimize tryptic fragmentation with IGT by varying conditions such as the temperature, possibly as high as 70 °C, the enzyme to protein mass ratios, and incubation time.

4. Conclusion

We have been successful in producing a novel form of trypsin that is thermostable at 60 °C. This thermostability is achieved by: (1) a reduction in autolysis due to glycation of the amino groups of lysine side-chains with glucose; (2) an increase in structural stability of the trypsin molecule conferred by the attachment of glucosyl residues; (3) immobilization of the glycated-trypsin on a glass solid support, which further reduces inactivation due to autolysis. The synergistic combination of these three factors gives rise to the remarkably thermostable IGT from native trypsin. Indeed, IGT is more thermostable than a trypsin-like protease from a thermophilic bacterium [28]. To our knowledge, the porcine IGT described here is the most thermostable form of trypsin that has been characterized to date.

Acknowledgements

This paper is dedicated to the memory of Michael Duhig who contributed to the success of this research with his ingenuity and ability to plan and execute critical experimentation.

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