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Physical-chemical characterization and stability study of α-trypsin at pH 3.0 by differential scanning calorimetry

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Abstract

α-Trypsin is a serine-protease with a polypeptide chain of 223 amino acid residues and six disulfide bridges. It is a globular protein with predominance of antiparallel ß-sheet secondary structure and it has two domains with similar structures. In the present work, a stability study of α-trypsin in the acid pH range was performed and some physical-chemical denaturation parameters were measured by using differential scanning calorimetry (DSC). The α-trypsin has a shelf-life ($t_{95\%}$) of about 10 months at pH 3.0 and 4 °C and its hydrolysis into the ψ-trypsin isoform is negligible during 6 months. The observed ratio $\Delta H_{cal}/\Delta H_{vH}$ is close to unity, which suggests the occurrence of a two-state transition. At pH 3.0, α-trypsin unfolded with $T_m = 325.9$ K and $\Delta H = 99.10$ kcal mol^{-1}, and the change in heat capacity between the native and unfolded forms of the protein was estimated to be 1.96 ± 0.18 kcal mol^{-1} K^{-1}. The stability of α-trypsin calculated at 298 K was $\Delta G_U = 6.10$ kcal mol^{-1} at pH 3.0. These values are in the range expected for a small globular protein. These results show that the thermodynamic parameters of unfolding of β-trypsin do not change substantially after its conversion to α-trypsin.

Keywords: Shelf-life; Thermodynamics; Trypsin isoforms; Calorimetry; α-Trypsin

1. Introduction

In many cases, the transition of small globular protein from native to denatured state is highly cooperative and such transition may be described as "two-state", where no intermediate states are significantly populated [1,2]. From accurate measurements of the folding/unfolding equilibrium, a transition model can be proposed and both the associated thermodynamic parameters as the native structure stability can be estimated [3]. Successful thermodynamic analyses are dependent upon the protein characteristics such as size, tertiary structure, number of domains and media conditions, such as organic solvents and the presence of chaotropic or kosmotropic agents [4]. Bovine α-trypsin is a powerful proteolytic enzyme [5] that fulfills requirements for a thermodynamic investigation. It is an isoform of β-trypsin that is derived from pancreatic trypsinogen, both molecules belonging to the serine-protease family, which are typical globular proteins (223 amino acid residues and about 24 kDa). Some members of the family have been well characterized in the solid state [6]. β-Trypsin, the most studied of the trypsin isoform derived from N-terminal cleavage of trypsinogen, is a single polypeptide chain. After hydrolysis of the peptide bond between Lys-131 and Ser-132 it gives rise to the α-trypsin isoform, which has two polypeptide chains linked by a disulfide bond [5]. Concerning the tertiary structure α-trypsin is similar to β-trypsin, it is composed of two closely related domains, stabilized by six disulfide bonds, with antiparallel β-pleated sheets in each domain [7]. A second isoform of β-trypsin, which is present in smaller amount (in commercial preparations) is originated from hydrolysis of α-trypsin at the peptide bond between Lys-176 and Asn-177 producing ψ-trypsin [5] that has three polypeptide chains linked by disulfide bond. Both β- and α-trypsin show

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significant amidasic activity (β-trypsin is about 50% more active than α-trypsin under the conditions of hydrolytic assay of N_α-benzoyl-DL-arginine-4-nitroanilide [8]). However the ψ-trypsin isoform presents some differences such as a higher affinity for positively charged ligands like N_α-benzoyl-L-arginine ethyl ester and benzamidine, and its poor amidasic activity [5]. The isoform α-trypsin can now be purified in large quantities [8] and it undergoes reversible thermally-induced or denaturant-induced transitions in acidic media. The aim of our work was to elucidate if there is thermodynamic significant differences between α- and β-trypsin isoforms at pH 3.0 [9] using direct measurements by differential scanning calorimetry (DSC). Previous calorimetric investigation of thermal denaturation of β-trypsin [9] has suggested a two-state transition between the native and denatured forms.

2. Materials and methods

2.1. Materials

Tris (hydroxymethyl) aminomethane, benzamidine-hydrochloride, glycine, citric acid, sodium citrate and bovine trypsin type III (T8253) LOT 125HO676-EC 3.4.21.4 and N_α-benzoyl-DL-arginine-4-nitroanilide (BA*p*NA) were purchased from Sigma (St. Louis, MO, USA). Calcium chloride was purchased from Merck (Darmstadt, Germany). Sodium chloride was from Carlo Erba (Rodano, MI, Italy). Acetic acid and hydrochloride acid were purchased from Synth (Diadema, SP, Brazil). Formic acid was from Reagen (Rio de Janeiro, Brazil). Matrix cation exchanger SE-SEPHADEX-C50 (capacity: 2.3 mequiv./g and particle size: 40.0–120.0 μm) was purchased from Pharmacia Fine Chemicals INC (Uppsala, Sweden). Type I water was purified by Barnstead Nanopure Diamond™ Ultrapure water system (Barnstead International, Dubuque, IA, USA). All chemicals used were of reagent grade.

2.2. Protein purification and characterization

All steps of the purification of trypsin isoforms were conducted according to the method of Santos et al. [8]. After protein isolation, only the fractions with purity close to 100% of α-trypsin were used. Purity of the isoform was detected by mass spectrometry (ESI–MS, Micromass Q-ToF).

2.3. Shelf-life determination

Shelf-life determination was accomplished by storing several aliquots (1.0 mg) of freeze-dried α-trypsin at 4 °C with 50% of relative humidity and the enzymatic activities were measured in intervals of 15 days during about 6 months. Enzymatic activity [10,11] of α-trypsin was quantified by measuring its ability to cleave the amide bond in a small molecular weight synthetic substrate, N_α-benzoyl-DL-arginine-4-nitroanilide [12]. The results were plotted and analyzed on Sigma Plot 8.0 software by using the pharmacology tool. The data are expressed as the mean with $n = 3$.

2.4. Determination of transformation percentage of α-trypsin into ψ-trypsin as a function of storage time

To certify that the thermodynamic data collected is related to a unique isoform, it becomes necessary to monitor the transformation of lyophilized α-trypsin into the ψ-trypsin isoform (its proteolytic product) as a function of storage time at 4 °C and 50% of the relative humidity. For this proposal, once for month (during 12 months) aliquots of α-trypsin were re-chromatographed at a similar system, but at a smaller scale than that previously described [8]. Resultant products were analyzed by UV absorption (280 nm) using the molar extinction coefficient value of 40,000 M^{-1} cm^{-1} and identified by mass spectrometry (Q-ToF, Micromass). Areas of individual peaks were calculated (areas below 1% were considered as being 1%) and plotted at Origin Scientific™ 5.0 software. The data are expressed as the mean ± S.D. ($n = 3$).

2.5. Differential scanning calorimetry

DSC measurements were performed by using a Microcal (Northampton, MA, USA) model VP-DSC scanning microcalorimeter. Protein was dissolved in several buffer types containing stabilizer or denaturing agents, and the protein concentration was verified spectrophotometrically by using the trypsin molar extinction coefficient ($\varepsilon = 40{,}000\ M^{-1}\ cm^{-1}$ at 280 nm) [13]. The samples were degassed at low pressure for 30 min, placed into the microcalorimeter cell and scanned relative to the reference buffer over the temperature range from 283 to 353 K at a scan rate in the range from 1 to 90 K h^{-1} and at a constant pressure of 20 psi. For each solution composition studied three independent measurements were carried out, providing the mean and standard deviation. Buffer versus buffer baseline scans were determined and subtracted from transition scans prior to normalization and analysis of protein denaturation [14]. Finally, the values of the excess heat capacity were obtained after subtraction of the baseline [15]. Raw data from the DSC runs were curve fitted using the Microcal Origin™ DSC (version 4.1) software.

2.6. The effect of scan rate on T_m and ΔH_{cal} thermodynamics parameters

Both α-trypsin sample and a reference (cell that contain only 50.0 mmol L^{-1} glycine buffer, pH 3.0, with 20.0 mmol L^{-1} $CaCl_2$) were scanned from 283 to 353 K at seven different scan rates (0.5, 3, 5, 15, 30, 60 and 90 K h^{-1}) in triplicate. The calorimetric experiments were performed under an extra pressure of 20 psi. The comparison among the T_m and ΔH_{cal} values obtained at different scan rates defines if the process is thermodynamically or kinetically driven.

2.7. Determination of heat capacity change

For analyses in acidic media the α-trypsin samples were resuspended in ß-alanine buffers at pH 2.00, 2.25, 2.50, 2.75,

3.00 and 3.25 (50.0 mmol L^{-1} ß-alanine and 20.0 mmol L^{-1} $CaCl_2$), or acetate buffer at pH 3.25, 4.00 and 4.20 (50.0 mmol L^{-1} sodium acetate and 20.0 mmol L^{-1} $CaCl_2$). Other runs were performed with addition of denaturant or stabilizing agents and were carried out by the method described above. Guanidinium hydrochloride was used as a denaturing agent at 0.25, 0.50 and 0.75 mol L^{-1} in buffer (50.0 mmol L^{-1} ß-alanine, pH 3.0, with 20.0 mmol L^{-1} $CaCl_2$) [16], and sorbitol was used as a stabilizing agent at 0.5, 1.0, 1.5 and 2.0 mol L^{-1} in buffer (50.0 mmol L^{-1} of ß-alanine, pH 3.0, with 20.0 mmol L^{-1} $CaCl_2$). Protein concentration was determined spectrophotometrically, by using a molar extinction coefficient of 40,000 M^{-1} cm^{-1} at 280.0 nm [13] to be approximately 1.0 mg mL^{-1}. The runs were carried out from 283 to 353 K temperature range at a scan rate of 60 K h^{-1} and at constant pressure of 20 psi.

2.8. Determination of thermodynamics parameters $\Delta H_{(T_m)}$, CI (cooperative index), $\Delta S_{(T_m)}$, $\Delta S_{(298\,K)}$, $\Delta H_{(298\,K)}$, $\Delta G_{U(T)} \times T$, $\Delta G_{U(298\,K)}$, T_{max} (temperature of maximum stability) and T_m' (cold melting temperature) at pH 3.0

Ten microcalorimetric runs of α-trypsin at 1.0 mg mL^{-1} in 50.0 mmol L^{-1} ß-alanine buffer, pH 3.0, containing 20.0 mmol L^{-1} $CaCl_2$ were conducted to determine the average and standard deviation of T_m and ΔH_{T_m} values in the specific condition (pH 3.0) with high accuracy.

3. Results

3.1. Shelf-life

The enzymatic activity data shown as open circles (Fig. 1) has a linear regression line superimposed (*R*-value of about 0.98) by the computational algorithm from the tool Pharmacology of Sigma Plot™ 8.0. The lower 95% confidence line is also plotted in solid triangles [17]. The accepted definition of the shelf-life

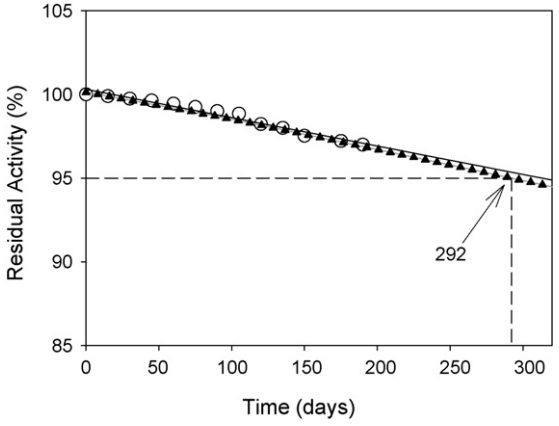

Fig. 1. Shelf-life estimation of enzymatic activity of lyophilized α-trypsin stored at 4 °C. The enzymatic activity data are shown as open circles (◯) and has a linear regression line superimposed shown as solid line (—). The lower 95% confidence line is also plotted as triangle up line (▲,) and $t_{95\%}$ line is shown as dash line (- - -). The intercept of lines (shelf-life time) is indicate by an arrow.

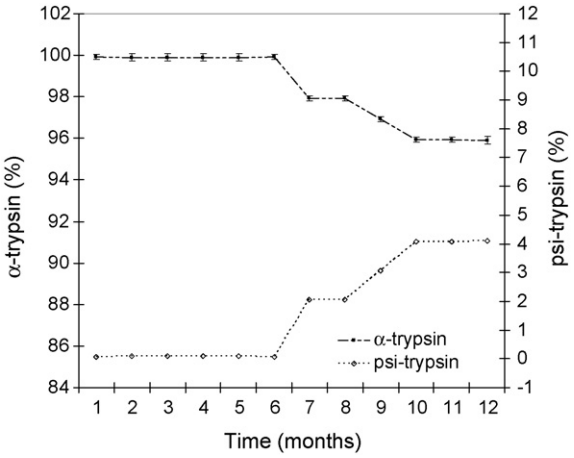

Fig. 2. Evaluation of percentage of transformation of lyophilized α-trypsin into ψ-trypsin as a function of storage time at 4 °C.

time by the Food and Drug Administration (www.fda.org) is the *x*-axis coordinate for the intersection of the lower 95% confidence line with line of 90% or in the specific work 95% enzyme or drug activity [18]. This exactly determined intersection is shown on the graph by the dotted drop lines and the estimated $t_{95\%}$ value is 292 ± 15 days or about 10 months.

3.2. Time course of transformation of α-trypsin lyophilized at 4 °C into ψ-trypsin

Results for α-trypsin transformation into ψ-trypsin showed three main intervals of transformation of isoforms as a function of time (Fig. 2). The first one was from month 1 to 6, the second one from month 7 to 8 and the last one from 10 to 12 months. Within the first mentioned interval the ψ-trypsin concentration does not exceed 1% of the total protein amount, despite small oscillations. Overall, the α-trypsin percentage decreased by 4% over 1 year.

3.3. The effect of scan rate on DSC thermograms

The α-trypsin (1.0 mg mL^{-1} in 50 mmol L^{-1} of β-alanine buffer, pH 3.0, with 20 mmol L^{-1} $CaCl_2$) thermal denaturation was monitored from 283 to 353 K temperature range, on seven different scanning rates 0.5, 3, 5, 15, 30, 60 and 90 K h^{-1}. The seven analyses furnished similar thermograms (data not shown). The corresponding calorimetric enthalpies–ΔH_{cal} and the melting temperature–T_m values obtained from these thermograms are presented in Table 1. No significant scan rate effect is observed.

3.4. Thermal denaturation

The repeatability of the scans was excellent and the data for triplicate scans performed for each sample fell within a differential power band of 2 μcal min^{-1} (±0.15 μW) at all temperatures. The standard deviation for these experiments showed an average of the only 0.35 μcal min^{-1} (0.025 μW). The reproducibility

Table 1
Evaluation of T_m and ΔH_{cal} values dependence of scan rate for α-trypsin at 50 mmol L^{-1} β-alanine buffer pH 3.0, containing 20 mmol L^{-1} $CaCl_2$

Scan rate (K h^{-1})	T_m (K)	ΔH_{cal} (kcal mol^{-1})
0.5	325.5 ± 0.2	100.3 ± 0.6
3	325.4 ± 0.2	101.2 ± 0.3
5	325.7 ± 0.1	98.4 ± 0.7
15	325.6 ± 0.2	99.3 ± 0.4
30	325.5 ± 0.1	96.5 ± 0.4
60	325.9 ± 0.1	97.5 ± 0.5
90	325.6 ± 0.1	98.2 ± 0.2

The data are expressed as the mean ± S.D. ($n = 3$).

of the baseline at repeated filling of the cell is not worse than 1.25 μcal K^{-1} (at scan rate of 60 K h^{-1}) and the precision of temperature recording was ± 0.1 K. The thermogram for α-trypsin aqueous solutions showed a single symmetric endotermic transition peak, typical of a "two state" process. (Fig. 3) that is typical for protein denaturation [19]. This figure is resultant from subtraction of the baseline and then of the heat capacity difference between the denatured and the native states followed by normalization of protein concentration and fitting of the resultant curve.

3.5. Determination of heat capacity change

Only the experimental points that showed $\Delta H_{cal}/\Delta H_{vH}$ ratio close to unity were used for ΔC_p determination. The ΔH_{cal} and the respective T_m are shown in Fig. 4. In accordance to the Kirchhoff's relation [15] the slope of the plot provides the ΔC_p value (1.96 ± 0.18 kcal mol^{-1} K^{-1}).

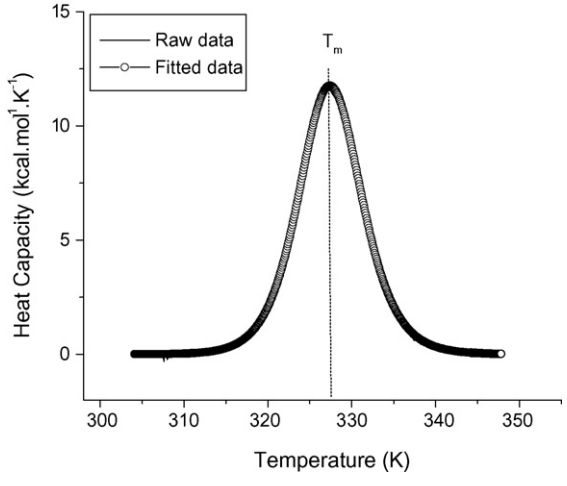

Fig. 3. Differential scanning calorimetry curve of α-trypsin. The protein was solubilized at 1.0 mg mL^{-1} in 50 mmol L^{-1} β-alanine buffer pH 3.0, containing 20 mmol L^{-1} $CaCl_2$ at scan rate of 60 K h^{-1}. The solid line (—) shows the processed data after subtraction of the baseline and normalization of protein concentration. Open circle line (-◯-) indicates the fitting of the data to the mathematical model of a two-state transition. T_m is the temperature midpoint of transition for the enthalpy change that occurs when the protein goes from native to denatured form.

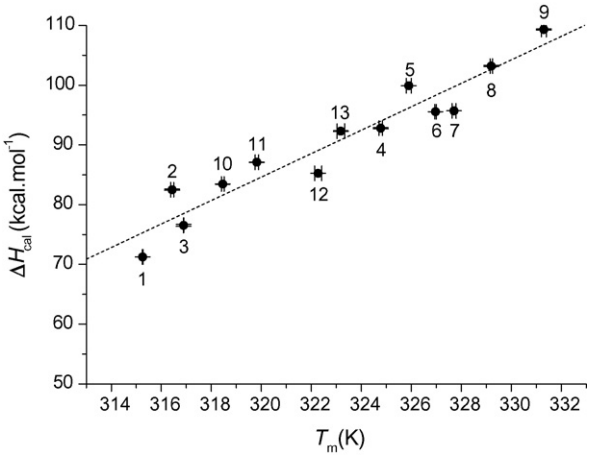

Fig. 4. Plot of $\Delta H_{cal} \times T_m$ for α-trypsin. Experimental conditions: 50 mmol L^{-1} of a specific buffer (sodium acetate or β-alanine), 20 mmol L^{-1} $CaCl_2$ plus: 1 (0.50 mol L^{-1} guanidine hydrochloride - Gdn–HCl, pH 3.0); 2 (0.40 mol L^{-1} Gdn–HCl, pH 3.0); 3 (pH 2.2); 4 (pH 2.8); 5 (pH 3.0); 6 (0.50 mol L^{-1} Sorbitol, pH 3.0); 7 (pH 3.0 without $CaCl_2$); 8 (pH 3.2); 9 (1.50 mol L^{-1} Sorbitol, pH 3.0); 10 (pH 2.5); 11 (pH 2.6); 12 (pH 2.7) and 13 (pH 2.8). The data are expressed as the mean ± S.D. ($n = 3$).

3.6. Determination of thermodynamics parameters: T_m, ΔH_{T_m}, CI (cooperative index), ΔS_{T_m}, $\Delta S_{298\,K}$, $\Delta H_{298\,K}$, $\Delta G_{U(T)} \times T$, $\Delta G_{U(298\,K)}$, T_{max} (temperature of maximum stability) and T_m' (temperature of cold denaturation) at pH 3.0

The average results for ten independent calorimetric runs of α-trypsin solution (1.0 mg mL^{-1} in 50.0 mmol L^{-1} of β-alanine buffer, pH 3.0, with 20.0 mmol L^{-1} $CaCl_2$) yielded the parameters shown in Table 2. The reproducibility of independent experiments (triplicate) for a given sample under the same conditions was of ± 0.1 K for T_m, and about ±3% for ΔH_{cal} values.

The ΔS_{T_m}, ΔH_T and ΔS_T were calculated accordingly to standard thermodynamic equations [15] and the parameters are shown in Table 2. The calculation of the parameter of the cooperative index was done accordingly to Relkin [20].

Once ΔH_{T_m}, T_m and ΔC_p are known, the curve of protein stability, ΔG_U as a function of (T), at the temperature range investigated can be calculated according to the following equation [21]:

$$\Delta G_{(T)} = \Delta H_{(T_m)} \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m}\right)\right] \quad (1)$$

From 200 to 353 K the comparative $\Delta G_U \times T$ plots for α- and β-trypsin [9] are shown in Fig. 5.

The thermodynamic parameters obtained from Fig. 5 and the comparison with those obtained for β-trypsin [9] are presented in Table 3.

Table 2
Comparative thermodynamic parameters between α and β-trypsins obtained at pH 3.0

	T_m[a] (K)	$\Delta H_{(T_m)}$[b] (kcal mol^{-1})	$\Delta H_{(298\,K)}$[c] (kcal mol^{-1})	$\Delta S_{(T_m)}$[d] (kcal mol^{-1} K^{-1})	$\Delta S_{(298\,K)}$[e] (kcal mol^{-1} K^{-1})	CI[f] (K)
α-trypsin	325.9 ± 0.1	99.10 ± 0.83	44.41	0.30 ± 0.01	0.12	10.0 ± 0.2
β-trypsin [9]	327.0 ± 0.1	101.8 ± 0.58	29.30	0.31 ± 0.01	0.08	8.0 ± 0.1

[a] Melting point at denaturation transition when 50% molecules remain folded.
[b] Calorimetric enthalpy of transition.
[c] Total enthalpy of the system at 298 K.
[d] Denaturation entropy at T_m.
[e] Total entropy of system at 298 K.
[f] Cooperative index.

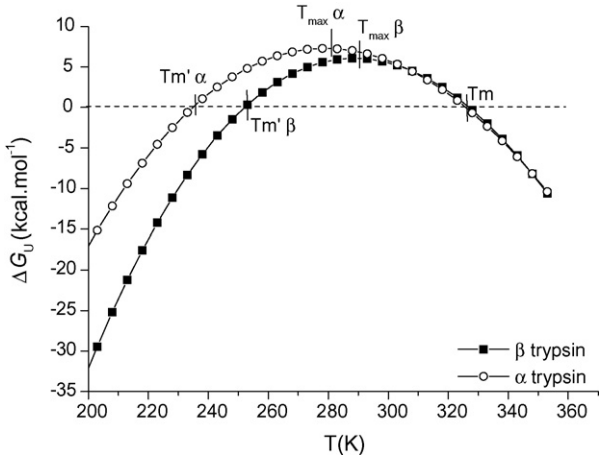

Fig. 5. Comparative stability curves for α and β-trypsin: ΔG_U as a function of temperature (K), at pH 3.0, where T_m is the midpoint denaturation temperature, T_m is the theoretical midpoint cold denaturation temperature and T_{max} is the temperature of maximum stability for the protein under these experimental conditions.

4. Discussion

The shelf-life results (Fig. 1) and the percentage of transformation of β-trypsin into ψ-trypsin were important data for decision about the quality control for the selection of samples to be studied by DSC. Such parameters are necessary to certify that the samples assayed (molecules population) comprise only the α-trypsin isoform with highly preserved enzymatic activity (≥95%). Hence, all the experiments were performed with α-trypsin stored up to 5 months based on the results of Fig. 2. Thus all experiments accomplished had at least 99% of the α-trypsin isoform or minus than 1% of the isoform ψ-trypsin as a contaminant.

General belief about the interpretation of differential scanning calorimetry results for protein states is that the denaturation calorimetric parameters are independent of the heating rate. Hence, they can be considered as a process under thermodynamic equilibrium [19,22]. When equilibrium is approached on a longer time scale than the scan rate, kinetics effects must be taken into account in interpreting the results of an experiment. Nevertheless, such condition validity was checked thoroughly only in a few cases [2,23,24]. This test is, however, of prime importance to adopt a correct interpretation method for the calorimetric results. The process can be considered under thermodynamic equilibrium or under kinetic control depending on the heating rate effects on the transition parameters (T_m and ΔH_{cal}). The values collected in Table 1 show that the whole denaturation process is not scan-rate-dependent, because the T_m and ΔH_{cal} variations are very small when the scan rate is changed and the values found are small and they do not exceed the experimental errors. A noticeable effect of scan rate is on transition cooperativity, because smaller scan rates broaden the thermal transition of the protein. However the T_m did not change significantly for the α-trypsin transition. These results indicate that denaturation of α-trypsin, taken as a whole, occurs as a thermodynamically controlled process [2,25].

The analysis of experimental DSC thermograms allowed the evaluation of two types of enthalpy change. The calorimetric enthalpy (ΔH_{cal}) represents the area under the denaturation curve, minus the contribution of the variation of heat capacity (ΔC_p) between the native and denatured forms of the protein. This parameter is independent of the chosen model and the units are kcal mol^{-1} of protein (monomer), but can also be expressed as kcal mol^{-1} of dimer, if necessary. Other important parameter is the van't Hoff enthalpy (ΔH_{vH}), which reflects the shape of the denaturation curve and describes the equilibrium constant (K_D) as a function of the temperature. Such parameter is dependent of the transition model (e.g. presence of intermediate states) and is expressed as kcal mol^{-1} of cooperative units, responsible for the

Table 3
Thermodynamic parameters for α and β-trypsin at pH 3.0 obtained from the stability curves

	$\Delta G_{D(298\,K)}$[a] (kcal mol^{-1})	$\Delta G_{D(max)}$[b] (kcal mol^{-1})	T_{max}[c] (K)	T_m'[d] (K)
α-trypsin	6.1 ± 0.5	7.3 ± 0.6	281	236
β-trypsin [9]	5.7 ± 0.2	6.5 ± 0.3	290	249

[a] Change in Gibbs energy between folded and unfolded 298 K at pH 3.0.
[b] Maximum Change in Gibbs energy between folded and unfolded at pH 3.0.
[c] Maximum stability temperature at pH 3.0.
[d] Cold denaturation temperature at pH 3.0.

observed conformational transitions [9]. If the protein denatures via a "two-state" mechanism, calorimetric to van't Hoff enthalpy ratio should be close to unity [19]. Scanning microcalorimetry tests for α-trypsin, provided an $\Delta H_{cal}/\Delta H_{vH}$ ratio near to unity, indicating that the thermal denaturation is well represented by the "two-state" model and that the unfolding of this globular protein is a highly cooperative transition, proceeding without noticeable intermediates under these experimental conditions.

A second heating of the sample solution, which should show a similar transition peak to the one observed in the first heating curve, generally checks the reversibility of the denaturation process. The α-trypsin thermal denaturation reversibility was estimated to be about 95% or higher. Thus the α-trypsin denaturation is considerably reversible (under non-reducing conditions), probably due to the presence of six disulfides bonds that guides its renaturation.

The heat capacity of α-trypsin denaturation (1.96 ± 0.18 kcal mol^{-1} K^{-1}) was smaller than the value observed for the β-trypsin isoform, which was 2.50 ± 0.07 kcal mol^{-1} K^{-1} [9]. The heat capacity effect is a very important parameter because it is mainly determined by the change in protein hydration. Since the hydration effects are proportional to the exposed surface areas of nonpolar groups, the heat capacity change yields information on the extent of polarity of isoforms [26]. Taking this into account, it can be suggested that the α-trypsin exposes less non-polar area under denaturation as compared to β-trypsin, which in turn suggests that α-trypsin is a less compact structure than β-trypsin (the number of contacts between nonpolar groups in native α-trypsin after its conversion was probably decreased).

At pH 3.0, α-trypsin denaturation presented a T_m value of 325.9 ± 0.1 K and a ΔH_{cal} value of 99.10 ± 0.83 kcal mol^{-1}. T_m was slightly smaller than that for β-trypsin isoform whose value is 327 ± 0.1 K [9], but ΔH_{cal} for both isoforms matches with data generally observed for small globular proteins (about 100 kcal mol^{-1}). Thus, compared to α-trypsin, the β-trypsin isoform is slightly more stable at pH 3.0, probably because it has a more compact hydrophobic core than α-trypsin. The cooperative index of α-trypsin (CI = 10.0 ± 0.2 K) is larger than that for β-trypsin (CI = 8.0 ± 0.1 K). Thus α-trypsin is less cooperative during thermal transition than its isoform at pH 3.0, reinforcing the interpretation given above.

Thermodynamic parameter such as $\Delta S_{298\,K}$ and $\Delta H_{298\,K}$ showed significant differences between the two isoforms (Table 2), because individual heat capacities differ significantly between them at pH 3.0. ΔH_{T_m} and ΔS_{T_m} (parameters obtained from denaturation transition curve) do not present noteworthy differences for these isoforms, showing that during thermal transition both isoforms have similar behaviors at pH 3.0. As α- and β-trypsin are supposedly similar in tertiary structure and in other physical-chemical parameters, ΔH_{cal} and ΔS_{T_m} values do not show significant differences (Table 2).

It is easy to observe the high melting temperature (T_m) and the maxima stability temperature (T_{max}) for globular proteins from the stability curves, but special conditions and efforts are required to predict the low melting temperature (T_m'), because it is often found below the water freezing point [27,28]. This parameter can be calculated by an extrapolation using plot of ΔG_U as a function of T (Fig. 5). T_m' is then obtained when $\Delta G_U = 0$ and the temperature is below 273 K. This is an important parameter because if cold denaturation occurs, the spontaneous refolding of freeze-dried protein upon thawing (e.g. upon reconstitution with buffers) could not be completely reversible. Such events can turn a protein into an irreversible degradation pathway [29]. Our results (Fig. 5 and Table 2) show that α-trypsin have a T_m' with value 236 K, which is smaller than that found for β-trypsin (249 K) [9]. These differences are influenced by the ΔC_p values that are statistically different for the two isoforms.

The ΔG_U (T) plot supplies the main thermodynamic parameter ΔG_U at the calculated temperature range. The ΔG_U value at 298 K is the most important parameter of stability of a molecule (Fig. 5 and Table 3), because it provides the quantitative stability at ambient temperature. For α-trypsin the value found was 6.1 ± 0.5 kcal mol^{-1} that is statistically equivalent [30] to that found for the β-trypsin isoform (5.7 ± 0.2 kcal mol^{-1} Bittar et al. [9]) under the same experimental conditions (pH 3.0). From standard thermodynamic equations [15] one can also see that ΔC_p plays a key role in proteins stability, since both the enthalpy and entropy changes for denaturation depend on ΔC_p. Thus, after β-trypsin is hydrolyzed to α-trypsin, a decrease of ΔC_p and an increase in the width of stability curve take place when the protein denatures (Fig. 5). The $\Delta G \times T$ plot also supplies the $\Delta G_{D(max)}$ and T_{max} values, which for α-trypsin are not significantly different [30] from the values found for β-trypsin isoform (Table 3). This small difference found for both isoforms could be due to the small tertiary structural change that occurs when the single-chain β-trypsin is converted to α-trypsin with two polypeptide chains. On the other hand, the lower amidasic activity of α-trypsin, when compared to β-trypsin, could be due to the looser hydrophobic core of the former one. The authors expect to find more significant thermodynamic changes for the ψ-trypsin isoform [5] that does not present amidasic activity but has still an esterase.

5. Conclusions

For α-trypsin the best fittings of DSC experimental results are found with the two-state transition model. The DSC studies for the thermal denaturation process of α-trypsin and its comparison with the β-trypsin isoform [9] show small differences between their unfolding characteristics. In the case of α-trypsin the denaturation proceeds with smaller transition temperatures and with lower ΔC_p. Also, the native-to-unfolded transition is less cooperative than that observed for β-trypsin. On the other hand, their enthalpies of denaturation are similar. The heat capacity versus temperature profiles show some features (no scan-rate-dependence) that are characteristic of a thermodynamically controlled processes. The comparative data of ΔG_U and ΔG_{max} for the two isoforms showed that the values are not significantly different. These results imply that most thermodynamic parameters of unfolding do not change substantially upon the conversion of β-trypsin to α-trypsin, despite a possible loosening of the hydrophobic core.

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