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# Heat of protonation of endoglucanase V catalytic domain (EGV-core) from *Humicola insolens*

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**Abstract**

Endoglucanase V catalytic core is a single chain protein of 213 amino acids. The titration of the ionizable groups of the macromolecule has been performed by an isothermal calorimetric technique and the $pK_i^0$ and $\Delta H_i^0$ related to the groups have been estimated and no apparent abnormality was observed in the titration behavior. These values are in agreement with the expected theoretical ones. © 1998 Elsevier Science B.V.

*Keywords:* Cellulase; Heat of protonation; Protein calorimetry; Protein titration

*Abbreviations:* EGV, Endoglucanase V; EGV-core, Endoglucanase V catalytic domain; ITC, isothermal titration calorimetry

## 1. Introduction

Ever since the pioneering work on calorimetric study of potentiometric titration of myoglobin [1], many other proteins have been studied by microcalorimetry [2–9] and experimental values compared with theoretical calculated ones. This procedure allows the confirmation of the number of ionizable groups exposed to the solvent compared with the amino-acid composition, the ionization p*K*s and the heats of ionization of the groups present in the macromolecule. Furthermore in the case of a structural modification, such as an unfolding transition characterized by a large enthalpic contribution, a large heat effect would be observed during the pH variation.

With the currently available instrumentation, high precision measurements are possible in a short time with the use of a small amount of macromolecules in diluted solution.

The Endoglucanase V (EGV) produced by the soft-rot fungus *Humicola insolens* is a cellulase (Mr=43 000).

It consists of a catalytic core connected by a flexible linker to a cellulose binding domain, similar to other fungal cellulases [10,11]. The catalytic EGV-core is a protein in a single chain of 213 amino acids (Mr=22 865) and its 3D structure has been determined by X-ray analysis at 1.6 Å resolution [12,13]: it is an overall spheroid with approximate dimensions 42×42×22 Å.

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## 2. Materials and methods

### 2.1. Protein preparation

The EGV cellulase has been cloned from *Humicola insolens* [14]. Initially, the catalytic active core was obtained by proteolytic cleavage of the linker; later, by a stop codon introduced after amino-acid residue 213. The amino-acid composition can be seen in the Table 1. The catalytic core has been crystallized and the 3D structure solved [12,13].

The gene of EGV-core was transformed into *Aspergillus oryzae* using a plasmid with the gene inserted between the gene coding for fungal amylase promoter and the gene coding for the AMG terminator from *Aspergillus niger* [15].

The cellulase was separated from the *Aspergillus* proteins by cation exchange chromatography on the extracellular proteins as follows. The fermentation broth was adjusted to pH 3.5 and filtered to remove the precipitating proteins. Then, the proteins were subjected to ultra-filtration (concentrated and washed with water) on a DOW GR81PP membrane with a cut-off at 6 kDa until the conductivity of the eluate was below 1000 mS/cm. The sample was finally applied to a S-Sepharose column equilibrated with a 20 mM citrate buffer pH 3.5. The enzyme will bind to the S-Sepharose at this low pH and it is eluted as a single peak using a NaCl gradient from 0 to 500 mM. The eluted pure enzyme was concentrated on an Amicon cell with a DOW GR81PP membrane and lyophilized. The cellulase was stable over a large range of pH (3–9) and lyophilized without any loss of biological activity.

Table 1
Amino-acid composition of EGV-core from *Humicola insolens* [25]

| AA | No. | AA | No. |
|---|---|---|---|
| Pro [P] | 15 | Leu [L] | 7 |
| Gly [G] | 26 | Tyr [Y] | 5 |
| Ala [A] | 20 | Phe [F] | 15 |
| Val [V] | 11 | Lys [K] | 8 |
| Cys [C] | 14 | His [H] | 1 |
| Met [M] | 1 | Trp [W] | 6 |
| Asp [D] | 16 | Glu [E] | 5 |
| Asn [N] | 12 | Gln [Q] | 9 |
| Thr [T] | 10 | Arg [R] | 11 |
| Ser [S] | 15 | Ile [I] | 6 |

The lyophilized powder was dissolved in a de-aerated 50 mM NaCl solution without any buffer and then gel filtrated in a BioGel P10 column (350 mm h; 0.8 mm $\phi$). The pH of the final solution was corrected at a value of 8.35±0.03 (Orion, Boston, MA, USA) with small amounts of NaOH solution.

The concentration of the protein solution was determined by spectrophotometry using a Cary 219 instrument (Varian, Palo Alto, CA, USA) at 280 nm with $E^{1\%}_{1\,\mathrm{cm}} = 1.846$ assuming a molecular mass of 22 865 [16]. The absorbance measured was corrected for light scattering according to the method of Leach et al. [17].

### 2.2. Experimental calorimetry (ITC)

Microcalorimetric experiments were performed at 25°C with an isothermal titration microcalorimeter (ITC) (Microcal, Northampton, MA, USA) [18] equipped with a microsyringe of 250 µl.

The protein concentration was $4–8\times10^{-5}$ mol $l^{-1}$ and the effective volume of the calorimetric cell is 1.3622 ml. A $5\times10^{-3}$ mol $l^{-1}$ HCl solution, also containing $50\times10^{-3}$ mol $l^{-1}$ NaCl, was placed in the calorimetric syringe and 28 injections of 4–16 µl were made. The stirring device was at 500 rpm. Observed heat effects were corrected for the heats of dilution of the solutions, the latter being always very small.

The calculation of the enthalpy of ionization from the areas of the peaks after drawing the baseline (Fig. 1) was performed by the Origin software program (Microcal, Northampton, MA, USA).

## 3. Results

### 3.1. Theoretical approximation

The heat of protonation of a protein can be calculated assuming that all the ionizable amino acids (Table 1) present in the macromolecule are all exposed in the solution and they contribute with their protonation enthalpy change according to the extent of protonation at different pH values.

$$\Delta H^0_{\mathrm{ion}} = \sum n_i \Delta H^0_i \qquad (1)$$

where $n_i$ is the number of groups of type $i$ ionized out of a total of $n^0_i$, and $\Delta H^0_i$ the value of the heat of

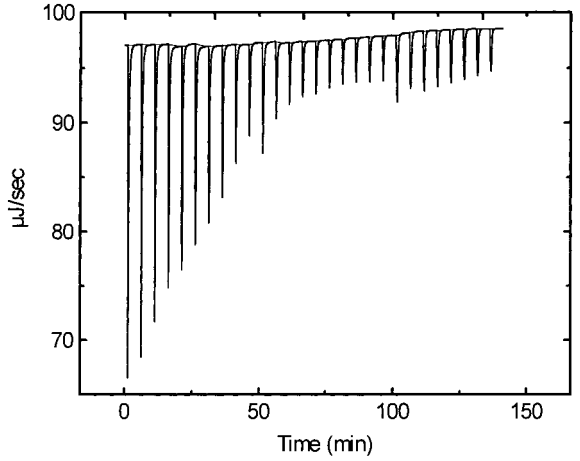


Fig. 1. Raw data obtained for 28 injections, 10 of 4 µl, 10 of 8 µl and 8 of 16 µl, of 5 mM HCl in the presence of 50 mM of NaCl into a sample cell containing EGV-core solution at a concentration of $6.94\times10^{5}$ M in the same concentration NaCl solution. The starting pH of the solution was 8.35.

ionization observed in model compounds [19,20] or standard behaving proteins [1–9].

$n_i$ as a function of pH can be estimated by the Lindestrøm–Lang approximation theory [21,22]:

$$\mathrm{pH} - \log\left[\frac{n_i}{n_i^0 - n_i}\right] = \mathrm{p}K_i^0 - \left(\frac{1}{2.3\,RT}\right)wZ \quad (2)$$

where $\mathrm{p}K_i^0$ is the intrinsic p$K$ of this group of side chain, $R$ the gas constant, $T$ the temperature, $w$ a constant depending on the solution's ionic strength and on the size and the shape of the protein molecule and $Z$ the charge of the molecule at a given pH.

The electrostatic factor $w$ can be approximately calculated according to the Debye–Hückle theory for a protein of Stokes' radius $R_s$=1.693 nm and an ion exclusion radius $a$=$R_s$+0.2 nm [19] in a solvent with a dielectric constant $D$ (78.36 at 25°C) by the equation:

$$w = \frac{e^2}{2DR_skT}\left(1 - \frac{kR_s}{1+ka}\right) \quad (3)$$

where $e$ is the electron elementary charge and $k$ the Debye–Hückle parameter:

$$k = \left(\frac{8\pi Ne^2}{1000DkT}\right)^{1/2} I^{1/2} = 7.357\times10^{8}\ \mathrm{m}^{-1} \quad (4)$$

where $N$ is the Avogadro's number, $k$ the Boltzmann's constant and $I$ the ionic strength (50 mol/m$^3$).

From Eqs. (3) and (4) the calculated $w$ is 0.101.

The best $\mathrm{p}K_i^0$, $w$ and $\Delta H^0$ values were estimated by fitting the calorimetric data to Eqs. (1) and (2). $Z$ as a function of pH can be drawn from Eq. (2).

### 3.2. Experimental isothermal calorimetry

Fig. 1 shows the raw calorimetric data obtained by injecting a HCl solution in a EGV-core solution from a starting pH of 8.35±0.03.

The heat developed as a function of the amount of HCl added is shown in the Fig. 2. From the theoretical curve the amount of protons, [$H^+$], which reacted with the ionizable groups and the amount of free protons in solution which modify the pH have been calculated. As a first approximation, the heats of protonation of the single groups reported for other proteins [1–9] have been utilized to draw a theoretical curve and then the differences from the experimental ones have been minimised by an iterative method. The heat of hydroxyl groups protonating between pH 8.3 and 7.0 with a $\Delta H\approx$43.2 kJ/mol has been considered in the theoretical calculations. The pH of the solution during the calorimetric titration can be evaluated assuming that the sum of the protons bound to the protein and the change in free ones correspond to the amount added in each injection. The calculated line of the heat evolved is reported in Fig. 2(b) and Table 2 indicates the values of p$K_i$, the number of ionizable groups in the molecule and the $\Delta H$s of protonation calculated.

Table 2
p$K_a$ and $\Delta H^0$ values for amino-acid residues in EGV-core calculated by best-fitting approximation. I=50 mM NaCl, $T$=25.0°C

| | $n$ | p$K_a$ | $\Delta H^0$/(kJ mol$^{-1}$) |
|---|---|---|---|
| α-COOH | 1 | 3.13±0.01 | 5.0±0.4 |
| R-COOH | 21 | 3.87±0.01 | 4.68±0.04 |
| Imidazole | 1 | 6.90±0.02 | 28.42±0.04 |
| α-Amino | 1 | 7.90±0.01 | 39.7±0.4 |
| Phenolic | 5 | (10.10±0.01) [a] | (25.5±0.4) [a] |
| ε-Amino | 8 | (10.50±0.05) [a] | (25.6±0.4) [a] |
| Guanidyl | 11 | (12.6±0.1) [a] | (58±4) [a] |
| $w$ | 0.097±0.002 | | |
| p$I_c$ | 5.14±0.05 | | |

[a] See text.223

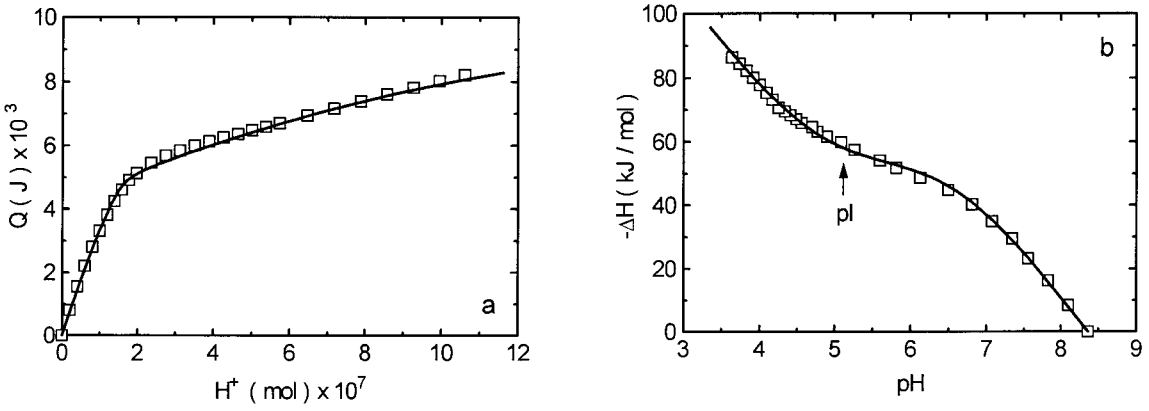


Fig. 2. (a) Heat developed during the thermal titration of EGV-core in the function of the amount of HCl added. (b) Enthalpy titration curve for EGV-core in the pH range from 8.35 to 4.0. (□) Experimental data points, (———) calculated best-fitting curve.

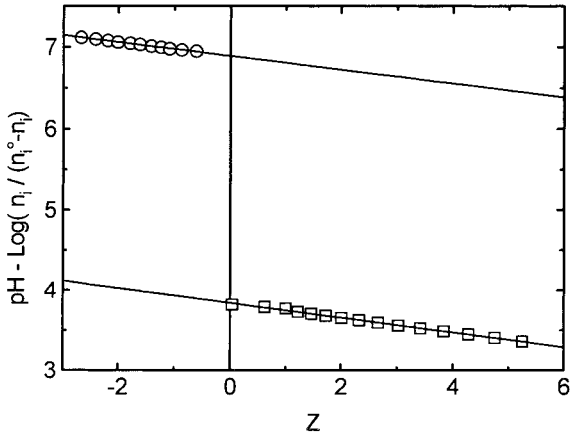


Fig. 3. Binding data of the experiment plotted according to the Eq. (2) for carboxyl (□) and histidine groups (○).

The calculated isoelectric point is $pI_c$=5.14±0.05 and corresponds to the calculated pH where the electrical charge of the protein is nearly zero.

The experimental results can be plotted (Fig. 3) according to the Eq. (2) for each ionizable group: the intercept indicates the experimental value of $pK_i^0$ and from the slope $w$ can be calculated. The $pK^0$ of carboxyl groups is relatively low, $pK^0$=3.84± 0.01; $w$ =0.096±0.002, and their protonation does not overlap with the histidine group, $pK^0$=6.89±0.01; $w$=0.097±0.002. This fact avoids the controversial considerations on calorimetric protein titration reported by Bjurulf [5].

## 4. Discussion

The results reported in the Table 2 confirm that all the ionizable groups expected from the amino-acid composition are exposed to the solvent and are titrated by the acid solution: this is in agreement with the structure determined by X-ray measurements [12,13]. Furthermore, the heat per mole of protein measured over the entire pH range does not imply any drastic transition of the structure of the molecule usually associated with larger heat values as was reported for oxidised cytochrome C, chymotrypsinogen A [6] and α-chymotrypsin [9]. The parameter $w$ is also connected with the shape and the dimension of the macromolecule [21] and the possible uncertainty calculated from the fitting ($w$=0.097±0.002) does not implicate large structural modifications. The cellulase retains >50% relative catalytic activity over the pH range of 6 to 10 [16].

The calculated $\Delta H$s are very similar to those reported in similar titration curves for other proteins [1–9] confirming that the protonation of the ionizable groups is not thermodynamically abnormal [23].

It should be noted that the apparent p$K$ values obtained for guanidyl- and ε-amino-groups cannot be determined with the same precision as those of the other groups because of the relatively low starting pH of the experiments (8.35) and their consequent low weight in the minimization procedure. It was not possible to increase the starting point without compromising the stability of the macromolecule.

α-Carboxyl groups are usually protonated in proteins with a enthalpy change ranging from 1 to 6 kJ/mol and the apparent p*K*s are not easily distinguished unless the side chains are in markedly different environment [5,23]. The low value compared with other proteins can be justified because of the sequential and spatial proximity of many of the carboxyl groups. These are found between 3–5 Å as seen from the molecular structure [12,13] using Insight II (Biosym Technologies, San Diego, USA).

The precision of the parameters for histidine reflects to the large value of enthalpy change of protonation compared with the value of carboxyl groups. Preliminary NMR studies on this protein have reported that the p$K_a$ value for imidazolyl group titration was 6.98±0.03 [24] in agreement with the value reported in the Table 1.

## 5. Conclusions

The calorimetric titration carried out on this protein confirms that the structural conformation of the macromolecule is stable over a large range of pH and that all the ionizable groups predicted from the amino-acid composition are present and exposed to the solvent.

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