J. Biochem. **142,** 293–300 (2007)
doi:10.1093/jb/mvm132

Proton Inventories Constitute Reliable Tools in Investigating Enzymatic Mechanisms: Application on a Novel Thermo-stable Extracellular Protease from a Halo-Alkalophilic *Bacillus* sp.

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Received May 9, 2007; accepted June 13, 2007

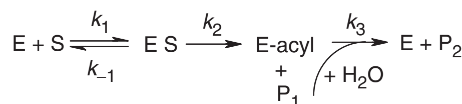
A novel protease designated protease-A-17N-1, was purified from the halo-alkalophilic *Bacillus* sp. *17N-1*, and found active in media containing dithiothreitol and EDTAK$_2$. This enzyme maintained significant activity from pH 6.00 to 9.00, showed optimum k_{cat}/K_m value at pH 7.50 and 33°C. It was observed that only specific inhibitors of cysteine proteinases inhibited its activity. The pH-(k_{cat}/K_m) profile of protease-A-17N-1 was described by three pK_as in the acid limb, and one in the alkaline limb. Both are more likely due t3o the protonic dissociation of an acidic residue, and the development and subsequent deprotonation of an ion-pair, respectively, in its catalytic site, characteristic for cysteine proteinases. Moreover, both the obtained estimates of rate constant k_1 and the ratio k_2/k_{-1} at 25°C, from the temperature-(k_{cat}/K_m) profile of protease-A-17N-1, were found similar to those estimated from the proton inventories of the same parameter, verifying the reliability of the latter methodology. Besides, the bowed-downward proton inventories of k_{cat}/K_m, as well as the large inverse SIE observed for this parameter, in combination with its dependence versus temperature, were showed unambiguously that $k_{cat}/K_m = k_1$. Such results suggest that the novel enzyme is more likely to be a cysteine proteinase functioning *via* a general acid-base mechanism.

Key words: cysteine proteinases, enzyme kinetics, enzyme mechanisms, halo-alkalophiles, proton inventories.

Abbreviations: DMSO, dimethyl sulphoxide; E-64, 1-[[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl] amino]-4-guanidinobutane; ONp, p-nitrophenyl; PI, proton inventory and/or inventories; PCMB, 4-hydroxy-sodium-mercury-benzoate; pNA, p-nitroanilide; SIE, solvent isotope effect; Suc, succinyl.

Halo-alkalophiles are extremophilic microorganisms requiring high salt concentrations and high pH-values for growth (*1*). Their development is also influenced by other environmental factors (*2*). Conversely, high salt is toxic for most microorganisms and has been used traditionally in storage of natural products, as well as in industrial processing. However, deteriorations were observed in products maintained in high salt due to their degradation by microbial extracellular proteases. Such enzymes secreted by extremophilic microorganisms have found many applications (*2*, *3*).

Proteases catalyse the hydrolysis of proteins into peptides and amino acids, which are crucial with respect to their applications in physiological, commercial and industrial fields, and are classified on the basis of their mode of catalysis (*4*). The kinetics and mechanisms of proteolysis have been studied to some extent to improve the applications of these enzymes, as controlled proteolysis is essential, among others, for the consistent quality of commercial products (*5*). Generally, proteases hydrolyse amide or ester bonds according to the known three-step mechanism of Scheme 1, where the steady-state parameters, k_{cat}/K_m, k_{cat} and K_m, are related to the rate constants k_1, k_{-1}, k_2 and k_3, as it is shown in Eq. 1–3, where $K_S = (k_{-1} + k_2)/k_1$ (*6*).



Scheme 1. **Minimal kinetic mechanism for proteinase catalysis.**

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{1}$$

$$K_m = \frac{k_3 K_S}{k_2 + k_3} = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)} \tag{2}$$

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_S} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{3}$$

Proton inventories (PI) have been widely used as probes for the recognition of the mechanism of proteinase action, and comprise kinetic studies of solvent isotope effects (SIE) in a series of mixtures of H_2O and D_2O (*7*, *8*). In this methodology, the kinetic parameters are expressed as $k_n(n)$ functions of deuterium atom fraction

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n present in the isotopic solvent according to equation $k_n = k_0[\Pi_{i=1}^{\mu}(1 - n + n\phi_i^T)]/[\Pi_{j=1}^{\nu}(1 - n + n\phi_j^G)]$, as it has been described previously in details (*6*, *9*). The shape of the $k_n(n)$ functions, the magnitude of SIE and the number of the transferred protons are diagnostic of the reaction mechanism (*6*, *9*, *10*).

Herein, we deal with the validity of PI as a reliable and useful tool in investigating enzymatic mechanisms. In this regard, we describe the purification, and the kinetic characterization of a novel enzyme, named protease-A-17N-1, from the halo-alkalophilic *Bacillus* sp. *17N-1* originated from Lake Elmenteita at East Africa (*11*). By taking into account also previous studies (*6*, *9*, *12*), the results obtained from the present studies confirm that properly treated PI may achieve excellent estimates of all suitable kinetic parameters. Furthermore, by using a novel combination with other methods, has led to an unambiguously accepted mechanism.

MATERIALS AND METHODS

Reagents—Amino acids, D_2O (99.9%, pD 7.4), molecular mass standards and other chemicals were purchased from Sigma, and BioRad. Peptide substrates Suc-FL-pNA, and Suc-FL-ONp were synthesized, and purified as described previously (*6*).

Strain, Culture and Treatment of Cell-Free Supernatants—The strain halo-alkalophilic *Bacillus* sp. *17N-1* used in this work was a gracious gift from Professor W.D. Grant (University of Leicester, U.K.) and Dr B.E Jones. (Genencor International, Leiden, The Netherlands). The strain was characterized by 16S-RNA gene sequence analysis (Grant, W.D. and Jones, B.E., personal communication). The cells of the strain were preserved in stocks containing 50% v/v glycerol/culture medium, and stored at -80°C. The organism was grown in 3 l aqueous culture media, containing (w/v) yeast extract 5%, peptone 5%, K_2HPO_4 0.1%, $MgSO_4$ 0.02%, glucose 1%, Na_3CO_3 1% and 0.7 M (4%) NaCl, adjusted to a starting pH-value of 10.00. Culture flasks were inoculated with 5×10^6 cells and were gently agitated at 30°C, up to an $O.D._{600\,nm}$ of 0.5. Cell-free culture medium was collected by centrifugation at 8,000 rpm (Sorvall - GS-3) for 10 min, at 2°C, and stored at -80°C. Approximately 1 l of cold acetone was added in drops to 3 l of cell-free supernatant at 0°C. The mixture was centrifuged at 8,000 rpm (Sorvall - GS-3) for 10 min, at 2°C. The precipitate was lyophilized to remove acetone, and it was stored at -80°C until the following treatment.

Isolation and Purification of Extracellular Protease-A-17N-1—Gel-filtration chromatography

About 60 g of dry Sephadex G-25 was swollen in 500 ml of H_2O, in a boiling water-bath for 5 h. The swollen material was chilled at room temperature, loaded onto a glass column of 2.5 cm diameter and 50 cm height (Pharmacia) and equilibrated by 1.5 l of phosphate buffer 0.05 M, pH 7.00. The dry acetone powder was dissolved in 50 ml of the equilibration buffer, and applied to the equilibrated Sephadex G-25 column. Fractions of 10 ml were collected, and assayed for protein content and enzyme catalytic activity (*6*). Protease active fractions were combined (80 ml) and used to load the ion-exchange column (*13–16*).

Ion-exchange chromatography

Approximately 2 g of DEAE Sephadex A-50 were suspended successively for 30 min in sufficient quantities of: (i) HCl 0.5 M, (ii) H_2O, (iii) NaOH 0.5 M and (iv) H_2O. The resulting swollen material was loaded onto a glass column of 1.0 cm diameter and 25 cm height (Pharmacia), and equilibrated using 200 ml of buffer Tris–HCl 20 mM, pH 8.50, containing 1 mM $EDTAK_2$ and 1 mM 2-mercaptoethanol. A mixture of 80 ml of the combined fractions from Sephadex G-25, and 20 ml of five-time concentrated equilibration buffer were applied to the equilibrated anion exchanger at room temperature. An excess of 20 ml of the resulting equilibration buffer was eluted the column. The enzymatically active fractions were obtained by a NaCl gradient of the equilibration buffer.

Electrophoresis of Protease-A-17N-1—SDS–PAGE

An SDS–PAGE was performed as previously (*17*) using a Bio-Rad Mini-Protean II gel apparatus. Aliquots of the enzyme preparations, as well as molecular mass standards were combined with sample buffer containing 100 mM Tris–HCl, 16% (v/v) glycerol, 3.2% (w/v) SDS, 8% (v/v) 2-mercaptoethanol and 0.01% (w/v) Bromophenol blue at pH 6.8. The samples were heated at 98°C for 10 min, incubated at 25°C and used to load 12% (w/v) 0.75-mm thick gels. The gels were electrophoresed at constant current, initially at 10 mA (for 10 min) and followed by 35 mA for 5 h. After electrophoresis, gels were stained with a solution containing 0.05% Coomassie brilliant blue G 250 in 50% of methanol, and 10% acetic acid (*18*). Distaining was performed by shaking the gels for 6 h in 7.5% acetic acid/methanol, and then for 1 h in H_2O. The molecular mass of the purified protease was determined by comparing its mobility with the following protein Mr-standards: Lysozyme (14.3 kDa), soya trypsin inhibitor (20.1 kDa), carboxyl-anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa) and phosphorylase B (97 kDa) (*19*).

Native-PAGE

Omitting out SDS as well as the reducing DTT from the standard Laemmli SDS–PAGE protocol (*20*), an electrophoresis under native conditions was also performed for protease-A-17N-1, by using specific Mr-standards (*21*). Gels were run at constant current, initially at 5 mA, for 30 min, followed by 15 mA for 8 h, to avoid heat denaturation of the enzyme.

2D-Electrophoresis (sample preparation)

An aliquot of 300 µl of the purified protease-A-17N-1 preparation were transferred, lyophilized and re-dissolved in 20 µl of deionized H_2O. To this sample, 2 µl of loading buffer containing 10% w/v SDS, and 2.3% w/v DTT were added, and the mixture was heated at 95°C for 10 min, chilled at room temperature and centrifuged for 5 min at 10,000 rpm (Sorvall-GS-3). Prior to sample application, 10 µl of a solution of 8 M urea, 4% CHAPS, 40 mM Tris, 65 mM DTT and a trace of bromophenol blue, were added in the sample.

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Immobilized pH-gradients and 2D-Electrophoresis

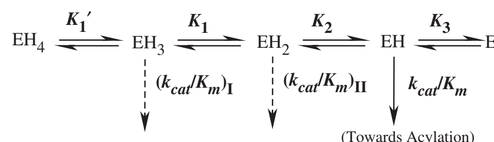
Gel preparations as those described above (SDS–PAGE) were placed in 17-cm long appropriate pH gradient strips (from pH 4.00 up to 7.00), following the manufacturer's protocol (Bio-Rad) with minor modifications. The sample, containing 400 µg of protein was prepared and loaded as described above (SDS–PAGE), and the isoelectric focusing was performed for a total of 14.6 KVh, at 20°C (PROTEAN IEF Cell apparatus: 200 V for 2 h, 500 V for 2 h and 800 V for 16 h). After isoelectric focusing, strips were equilibrated for 15 min in 0.5 M Tris–HCl buffer, pH 8.80, containing 10% (w/v) SDS and 0.05% (w/v) bromophenol blue. Strips were transferred to 12% poly-acryl amide gels as in SDS–PAGE, and ran in a PROTEAN II xi cell tank at 120 V for 11 h. Gels were stained by silver nitrate, and distained as previously (*22*).

Solutions—Phosphate buffers for the PI studies allowing different values of deuterium atom fraction n in the solvent were prepared gravimetrically as referred previously (*6*). Catalytic site titrations and temperature-activity profiles were carried out in aqueous 0.1 M phosphate buffers, pH 7.20, containing 1 mM $EDTAK_2$ and 2 mM DTT. The pH-activity profiles were evaluated by kinetic measurements in buffers of 0.1 M ionic strength at different pH-values (acetate: pH 5.00, phosphate: pH 5.25–8.00 and borate: pH 8.25–10.00) containing 1 mM $EDTAK_2$ and 2 mM DTT. Reaction mixtures contained 5% ($^v/_v$) DMSO.

Kinetic Measurements—All measurements were performed spectrophotometrically by initial velocities at 410 nm and/or 347.5 nm, for the pNA and/or ONp substrates, respectively, and by following a typical kinetic run (*6*, *9*, *12*), at 25°C. The active concentration of enzyme was varied from 180 nM up to 480 nM and the reaction was initiated by addition of 10–40 µl of appropriate substrate solution in DMSO. In addition, the substrate concentrations were varied from about 20 µM to 6,300 µM and the release of the leaving group was recorded. Self-hydrolysis of Suc-FL-ONp was determined by substituting the enzyme solution by buffer solution, per n value, and per substrate concentration (*10*). Eleven different values of n ranging from 0 to 0.99 were utilized for each substrate and eight substrate concentrations were used per n value, to measure the parameters $(k_{cat})_n$ and $(K_m)_n$. Every single kinetic measurement was repeated eight times. Catalytic site titrations were performed by using the irreversible inhibitors of cysteine proteinases PCMB and E-64 (*23*–*25*) and the Suc-FL-pNA substrate, at pH 7.50, and 25°C.

Dependence of k_{cat}/K_m *of Protease-A-17N-1 versus pH, and/or Temperature*—Measurements were performed by the method of initial velocities using the substrate Suc-FL-pNA, in buffers of 0.1 M ionic strength, at different pH-values and then at different temperatures but at pH 7.50, as it is described in the Solutions section (*12*, *26*).

Proton Inventories—All measurements were performed by means of substrates Suc-FL-pNA and Suc-FL-ONp in mixtures of eleven different values of deuterium atom fraction $n = [D_2O]/[H_2O]$ ranging from 0 to 0.99 per substrate, as previously (*6*, *9*, *12*). The parameters



Scheme 2. **Five hydrogenic forms of protease-A-17N-1, where only one is active.**

k_{cat}, K_m and k_{cat}/K_m were determined from initial velocity measurements, in buffer phosphate 0.1 M containing 1 mM $EDTAK_2$ and 2 mM DDT, at pL 7.50 and at 25°C (*6*, *7*, *9*, *12*). For each n value, the experimental data were best fitted by the Michaelis–Menten equation $v = k_{cat}[E]_0[S]/([S]+K_m)$.

Analysis of Data—The experimental data of the dependence of k_{cat}/K_m versus pH, of protease-A-17N-1, were analysed by means of Scheme 2 and Eq. 4, comprising five hydrogenic forms, and one operative reactive state. Equations comprising less hydrogenic forms, and/or more operating reactive states as those corresponding to $(k_{cat}/K_m)_I$ and $(k_{cat}/K_m)_{II}$ were also considered (*12*).

$$(k_{cat}/K_m)_{obs} = \frac{(k_{cat}/K_m)^{lim}}{\left(\begin{array}{l} 1+10^{pK_1'+pK_1+pK_2-3pH}+10^{pK_1+pK_2-2pH} \\ \quad +10^{pK_2-pH}+10^{pH-pK_3} \end{array}\right)} \tag{4}$$

The experimental data of the dependence of k_{cat}/K_m, of protease-A-17N-1 versus temperature, were best fitted by Eq. 5. In Eq. 5, $k_{1,0}$ and a_0, E_1, T, R and T_0, represent the values of rate constant k_1 and the ratio k_2/k_{-1} *i.e.* the stickiness of substrate (Scheme 1) at 298.15° K (25°C), the activation energy corresponding to the rate constant k_1, the absolute temperature in °K, the gas constant (8.314 $Jmol^{-1}K^{-1}$) and the temperature 298.15° K (25°C), respectively (*12*, *26*). Moreover, $E_a = E_{-1} - E_2$, where E_{-1} and E_2 hold similar meanings as E_1.

$$\frac{k_{cat}}{K_m} = \frac{k_{1,0}\, a_0\, e^{\left[\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]}}{1 + a_0\, e^{\left[\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]}}\, e^{\left[\frac{-E_1}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]} \tag{5}$$

Parameters $k_{cat}(n)$ and $K_m(n)$ of this study were estimated from initial velocities of hydrolysis of the substrates by non-linear curve fitting of Michaelis–Menten equation to the experimental data (*27*). The PI were estimated by fitting the corresponding experimental data with simplified forms of equation $k_n = k_0[\prod_{i=1}^{\mu}(1-n+n\phi_i^T)]/[\prod_{j=1}^{\nu}(1-n+n\phi_j^G)]$ as previously (*6*, *9*, *12*). As reported before and in case of the ester substrate Suc-FL-ONp, the relation $k_{3(Suc-FL-ONp)} = k_{3(Suc-FL-pNA)}$ is valid (*6*–*8*); accordingly, $(K_S)_n$ values for the Suc-FL-ONp were estimated by using the relations $k_2 = k_{cat}k_3/(k_3 - k_{cat})$, and $K_m = k_3K_S/(k_2+k_3)$ and Eq. 6 (*6*, *12*).

$$\frac{(k_2)_n}{(k_{cat}/k_m)_n} = \frac{1-n+n\phi^{G,k_1}}{1-n+nC_1} \times \left[\frac{k_2}{(k_{cat}/k_m)} + K_R\left(\frac{1-n+nC_2}{1-n+n\phi^{T,k_1}} - 1\right)\right] \tag{6}$$

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Alternative fitting procedures of all series of the above experimental data were performed also by non-parametric curve fitting methods, and in most cases we reached the same results through approaching global minima, as it has been reported earlier (*6*).

RESULTS AND DISCUSSION

Purification of Protease-A-17N-1 up to Homogeneity—The elution profile from Sephadex G-25 column is shown in Fig. 1. Fractions from 150 ml up to 220 ml were found to be active in proteolysis, were collected, combined (80 ml) and loaded on the ion-exchange column. Then, 60 fractions of 5 ml were collected and one major proteolytic peak was eluted by 50 mM ≤ [NaCl] ≤ 500 mM gradient of the equilibrium buffer (Fig. 2). Fractions from 190 ml up to 205 ml (Component-A) were combined, and designated as protease-A-17N-1. Minor proteolytic peaks (Components B and C) were collected from fractions 250 ml up to 265 ml, and from 330 ml up to 345 ml, respectively, and were combined separately. These two proteolytic peaks were disregarded since they contained very low activities. The protease isolated by this protocol was purified 91.9 times with a yield of 21% (Table 1). SDS and native-PAGE showed a single protein band at about 16 kDa, with a pI-value of 7.60 as estimated by 2D-electrophoresis (Figs 3 and 4, respectively).

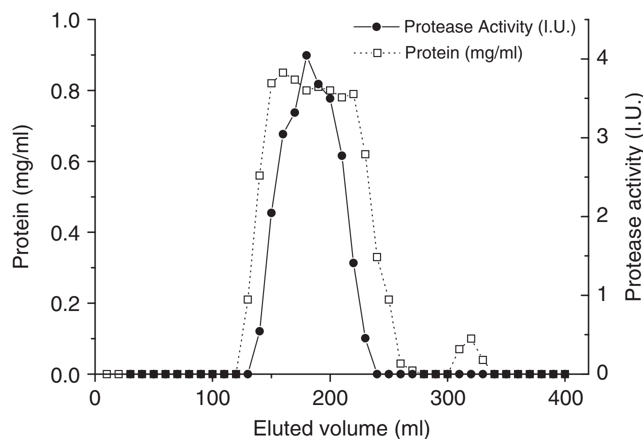

Fig. 1. **Elution profile from the Sephadex G-25 column.** Fractions from 150 ml up to 220 ml were collected, combined and loaded on the ion-exchange column.

Effect of Substrates and Irreversible Inhibitors—Protease-A-17N-1 was found to be active *versus* the synthetic substrates Suc-FR-pNA, Cbz-FR-pNA, Suc-FL-pNA and Suc-FL-ONp at assay conditions relatively specific for cysteine proteinases; no activity was detected in buffers lacking DDT. Instead, its activity increased ~30% in buffers containing $EDTAK_2$. Furthermore, protease-A-17N-1 was found to be almost inactive versus the synthetic substrates Suc-AAPF-pNA, MeOSuc-AAPV-pNA and Suc-AAA-pNA, which are more specific for serine proteinases, regardless of the presence of DDT and/or $EDTAK_2$. Specific irreversible inhibitors of serine proteinases acting by blocking the active Ser residue, such as 3,4-dichloro-isocoumarine (*28*), and phenyl-methyl-sulphonyl-fluoride (*29*) showed no effect on protease-A-17N-1. However, irreversible inhibitors of cysteine proteinases, specific to block the active Cys residue, such as PCMB (*30*), and E-64 (*24*, *25*, *31–33*), both were found to irreversibly inhibit protease-A-17N-1. Moreover, catalytic site titrations of protease-A-17N-1 were performed, and were found equal by using both irreversible inhibitors PCMB and E-64.

pH-(k_{cat}/K_m) Profile—Using the substrate Suc-FL-pNA, the bell-shaped pH-(k_{cat}/K_m) profile of protease-A-17N-1 was obtained, exhibiting a maximum at

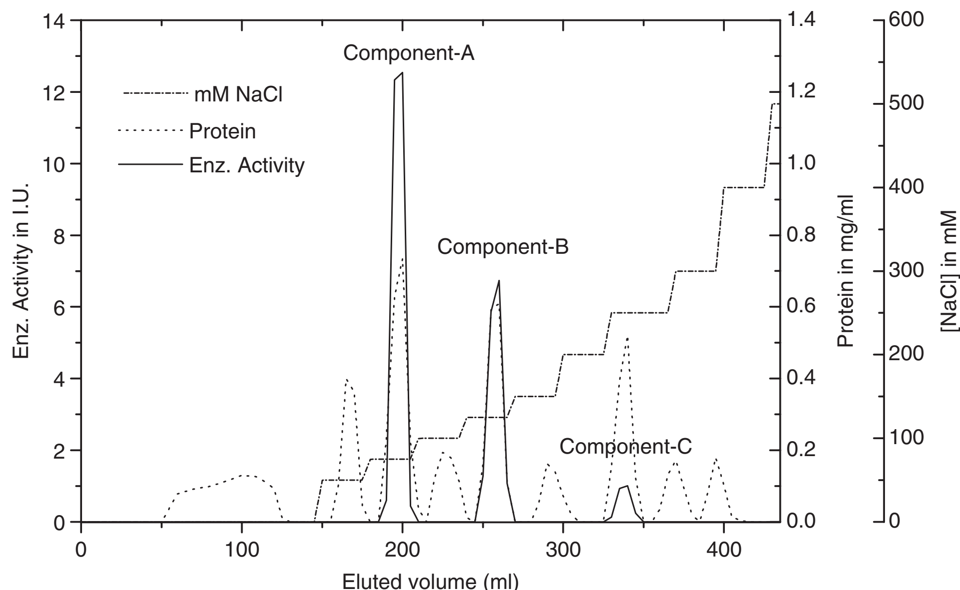

Fig. 2. **Elution profile from the DEAE Sephadex A-50 column.** One major proteolytic peak (Components A) was eluted by a gradient of 50 mM ≤ [NaCl] ≤ 500 mM, of the equilibrium buffer, and the fractions from 190 ml up to 205 ml were collected, combined and designated protease-A-17N-1.

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Table 1. **Purification protocol of the novel protease-A-17N-1 from cell-free extracts of cultures of the halo-alkalophilic *Bacillus* sp. *17N-1*.**

Purification step	Volume (ml)	Protein (mg)	Enzyme activity (I.U.)[a]	Specific activity (I.U./mg)	Purification (times)	Yield (%)
Cell-free extract	3,000	6,000	120	0.020	–	100
Acetone powder re-dissolved in equilibration buffer	50	5,700	110	0.019	1.0	95
Eluate from Sephadex G-25	80	62.5	26	0.416	20.8	22
Eluate from DEAE Sephadex A-50 (Component-A)	10	13.6	25	1.838	91.9	21

[a]I.U. were estimated by using the substrate Suc-FL-pNA.

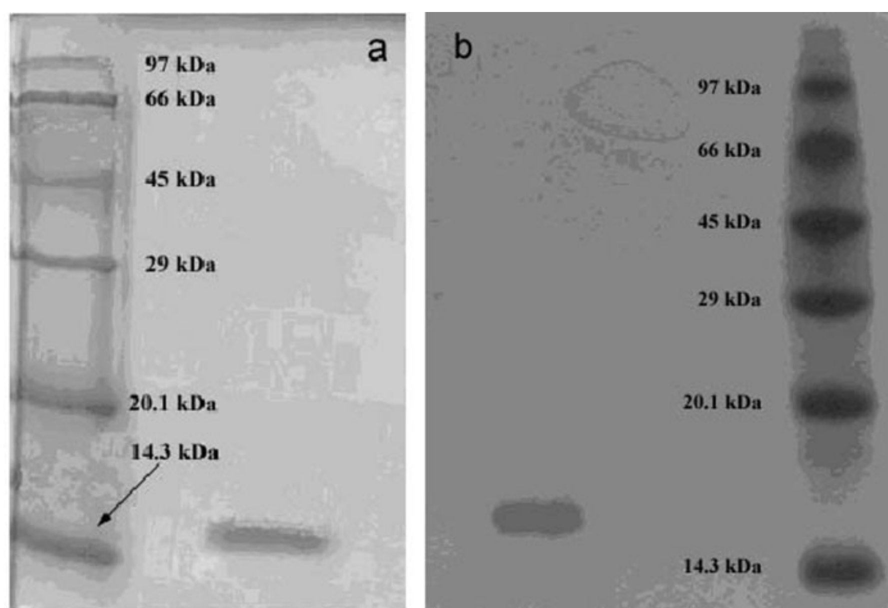

Fig. 3. **SDS–PAGE (a), and Native-PAGE (b), of protease-A-17N-1, stained by Coomassie brilliant blue G 250, showing one single band of ~16 kDa.**

pH = 7.50 (Fig. 5). The corresponding experimental data were best fitted by Eq. 1, whose parameter values were estimated as: $(k_{cat}/K_m)^{lim} = 56.64 \pm 0.35E{-}02\,M^{-1}\,s^{-1}$, $pK_1^{/} = 2.78 \pm 0.90$, $pK_1 = 4.57 \pm 0.40E{-}02$, $pK_2 = 6.25 \pm 0.02E{-}02$ and $pK_3 = 8.69 \pm 9.97E{-}05$. Attempts to fit these data by equations comprising less hydrogenic forms, and/or more operating reactive states were failed (*12*).

The results taken with the observed pI-value of 7.60 are somewhat surprising for a protease isolated from a halo-alkalophilic microbe that grows in culture media of starting pH-value 10.00. This paradox may be explained by the fact that the culture medium of the halo-alkalophilic *Bacillus* sp. *17N-1* reaches a pH-value of 7.50 at the end of its stationary phase (Fig. 6). This may be due the secretion of CH_3COOH, identified by GLC-IF (data not shown).

The bell-shaped pH-(k_{cat}/K_m) profile of protease-A-17N-1, for the substrate Suc-FL-pNA, reflects both its ionization and the catalytic states, which maintain an active conformation. It seems more likely, that protease-A-17N-1 exists in five protonic forms (Scheme 2) as three ionizable groups affect the acid limb of the bell-shaped curve, and one its alkaline limb, though, only one (EH) is active (Fig. 5). Alternatively, the fitting of the experimental data by an equation comprising four hydrogenic forms, showed a less steep line (Fig. 5 - dashed line) (*12*). Nevertheless, from the best fitting of the experimental data of pH-(k_{cat}/K_m) profile of protease-A-17N-1 by Eq. 4, it is possible that a $pK_1^{/} = 2.78$ reveals the occurrence of the protonic dissociation of an acidic residue whose anion influences the most likely ion pair of protease-A-17N-1 for catalytic competence. Additionally, it is reasonable to suggest that this ion pair is formed by protonic dissociation across a $pK_2 = 6.25$, while a $pK_3 = 8.69$, indicates its subsequent deprotonation. However, a $pK_1 = 4.57$ could be due to the development of a hydrogen bond, which stabilizes the ion pair, and it is formed between the anion (whose $pK_1' = 2.78$) with the charged imidazole of an active His in the catalytic site (*12*, *34*).

Temperature-(k_{cat}/K_m) *Profile*—The temperature-(k_{cat}/K_m) profile of protease-A-17N-1 was obtained for the substrate Suc-FL-pNA (Fig. 7). Subsequently, from the best fit of the experimental data by Eq. 5 were estimated the value of $k_{1,0} = 50.36 \pm 0.65\,M^{-1}\,s^{-1}$, which corresponds to the rate constant k_1 at 25°C (*31*), the value of $a_0 = k_2/k_{-1} = 24.14 \pm 4.28$, as well as the values of activation energies $E_a = 157.47 \pm 6.12\,kJ\,mol^{-1}$, and

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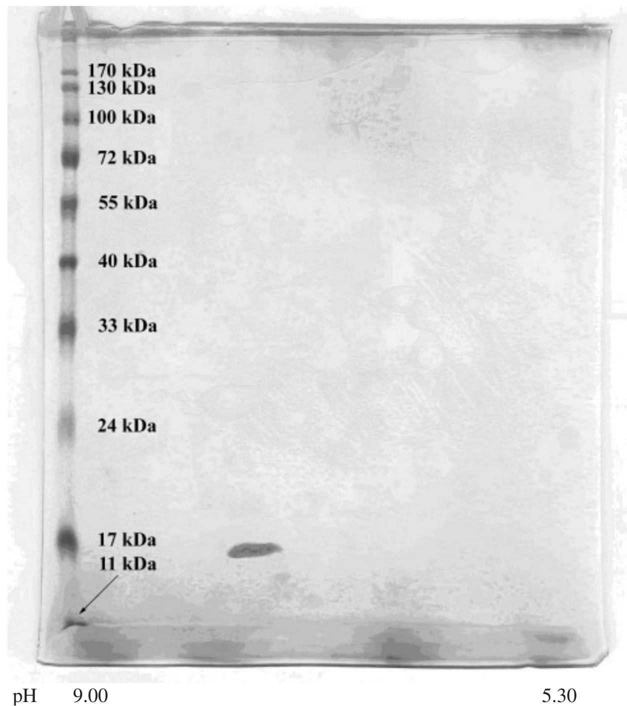

Fig. 4. **Two-dimensional electrophoresis of protease-A-17N-1 under denaturation conditions, stained by $AgNO_3$, showing a pI-value at 7.60 (scale from pH 5.30 up to 9.00).**

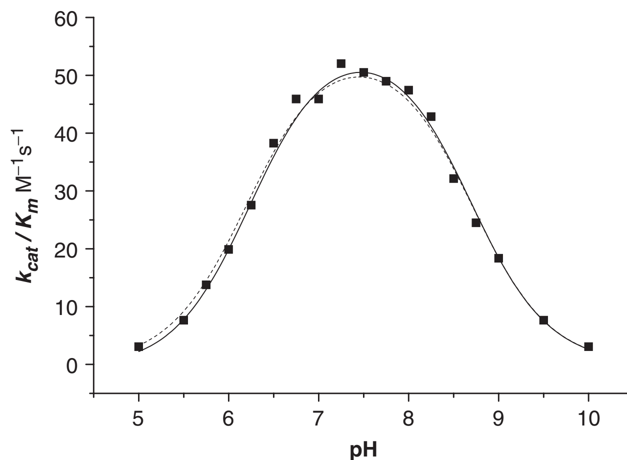

Fig. 5. **The dependence of k_{cat}/K_m versus pH in the hydrolysis of substrate Suc-FL-pNA by protease-A-17N-1.** Solid line represents the best fit of the experimental data by Eq. 4, (all the estimated parameter values are given in the text). Dashed line corresponds to the fit of the same data by equation $(k_{cat}/K_m)_{obs} = 56.58/(1 + 10^{4.00+6.20-2pH} + 10^{6.20\text{-}pH} + 10^{pH\text{-}8.63})$.

$E_1 = 29.81 \pm 0.71\,kJ\,mol^{-1}$. Besides, protease-A-17N-1 retained full activity at 25°C and 33°C after 1-h incubation at 5°C, as well as at 45°C. Such a property is uncommon in plant proteases, and was interpreted in terms of change in the individual rate constants that compose k_{cat}/K_m (*6*, *12*, *26*, *35*).

Proton Inventories (PI) of k_{cat}/K_m, k_2, k_3 and K_S—Equation $k_n = k_0\,(1-n+n\phi^T)/(1-n+n\phi^G)$, a particular case of the general equation $k_n = k_0[\Pi_{i=1}^{\mu}(1-n+n\phi_i^T)]/[\Pi_{j=1}^{\nu}(1-n+n\phi_j^G)]$, best fitted the experimental data of k_{cat}/K_m, k_2 and k_3 parameter for the substrates Suc-FL-pNA, and Suc-FL-ONp. The PI showed bowed-downward shapes and high inverse SIE with a mean value ${}^D(k_{cat}/K_m) = 0.33$, while the PI for k_2, and k_3 (and/or k_{cat}) were found linear with large normal SIE, and values ${}^D(k_2) = 2.20$ and ${}^D(k_3) = 2.10$, respectively. The experimental data for K_S were calculated through relation $K_m = k_3K_S/(k_2 + k_3)$, and were best fitted by equation $K_n = K_0(1-n+n\phi^P)/(1-n+n\phi^G)$ showing linear PI and large normal SIE with a value ${}^D(K_S) = 6.40$ (*22*). The linear character of PI of k_2, k_3 and K_S was validated as described previously (*6*, *12*) and deviations of individual experimental points due to small errors were taken into account by robust weighting requirements. Such results were obtained by alternative treatments of the experimental data. The estimates of $k_2 = 6.91E\text{-}2 \pm 0.11E\text{-}2\,s^{-1}$,

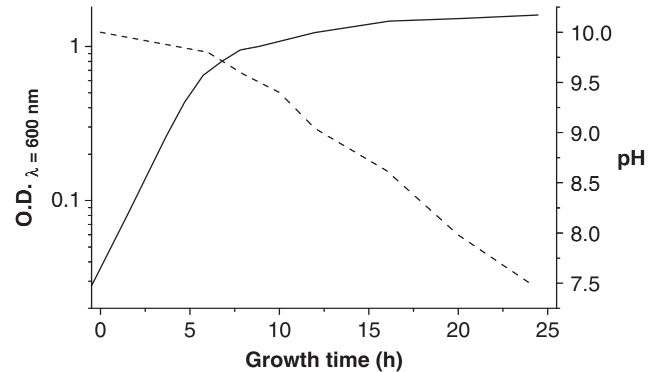

Fig. 6. **The halo-alkalophilic *Bacillus* sp. *17N-1* properties.** Solid line represents the growth of cells in culture media at starting pH-value 10.00 up to $O.D._{600\,nm} = 0.5$. Dashed line shows the variation of the pH-value of the culture medium during growth. Data points are not shown for clarity.

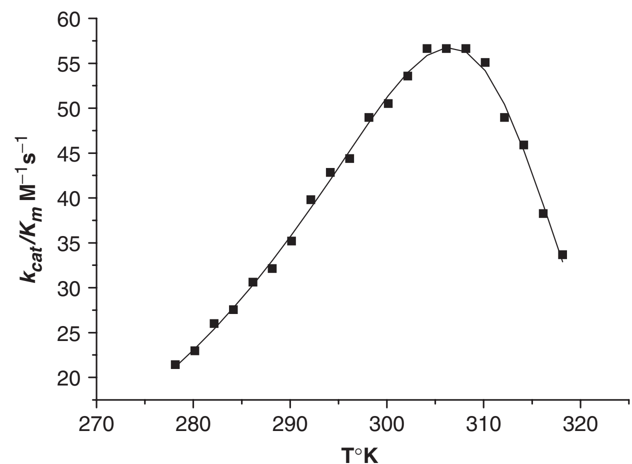

Fig. 7. **The dependence of k_{cat}/K_m versus absolute temperature in the hydrolysis of substrate Suc-FL-pNA by protease-A-17N-1.** The line represents the best fit of the experimental data by Eq. 5 (estimated parameter values are given in the text).

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$k_3 = 0.38 \pm 0.01\,\text{s}^{-1}$ and $K_S = 13.76\text{E-}4 \pm 0.42\text{E-}4\,\text{M}^{-1}$ were obtained at 25°C. Examples of these results are shown in Table 2, and in Figs 8 and 9. Together as by Eq. 6, were obtained estimates of the rate constants $k_1 = 52.78 \pm 2.12\,\text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 3.52\text{E-}3\,\text{s}^{-1}$ (standard error was not calculated) for the substrate Suc-FL-pNA, and thus $k_2 \gg k_{-1}$ ($k_2/k_{-1} = 19.6$). Moreover, the fitting of Eq. 6 showed fractionation factors whose values were found as $\phi^{T,k} = 0.46 \pm 10\text{E-}2$ and $\phi^{G,k_1} = 0.15 \pm 5\text{E-}2$ very close to the corresponding data for the PI of k_{cat}/K_m (Fig. 8a).

By applying the PI method and Eq. 6 at 25°C, we re-estimated the values of k_1, k_2/k_{-1} and k_2; the results were found almost equal to those estimated from the best fit of the experimental data of temperature-(k_{cat}/K_m) profile by Eq. 5. The latter experiments are also strongly supported by the estimated activation energies values $E_a = E_{-1} - E_2 = 157.47\,\text{kJ mol}^{-1}$, and $E_1 = 29.81\,\text{kJ mol}^{-1}$.

Table 2. **Proton inventories of k_{cat}/K_m, k_2, k_3 and K_S, where Equations $k_n = k_0(1 - n + n\phi^T)/(1 - n + n\phi^G)$ and $K_n = K_0(1 - n + n\phi^P)/(1 - n + n\phi^G)$, respectively, best fitted the experimental data, using Suc-FL-pNA as substrate.**

Parameter	SIE	ϕ^T	ϕ^G
k_{cat}/K_m	**1/3.00**	$0.46 \pm 1E{-2}$	$0.15 \pm 4E{-3}$
k_2	**2.20**	$0.44 \pm 2E{-3}$	$0.96 \pm 3E{-2}$
k_3	**2.10**	$0.48 \pm 2E{-2}$	$1.05 \pm 4E{-2}$
K_S	**6.40**	$0.16 \pm 5E{-3}$	$1.06 \pm 3E{-2}$

Therefore, it has been verified by two independent methods that the K_S (of protease-A-17N-1) is equal to k_2/k_1, *i.e.* a dynamic equilibrium constant. This by extrapolation implies that the relation $k_{cat}/K_m = k_1$ is valid (*6*, *12*, *26*, *35*, *36*). These relations, along with the large inverse SIE associated with bowed-downward PI observed for the reaction governed by the k_{cat}/K_m, and the relatively low estimates of this parameter, they may be explained on a basis of full commitment to catalysis due to sticky substrates (*8*, *12*, *26*). Additionally, the linear PI along with large normal SIE, which were observed for K_S, k_2 and k_3, suggest that k_2 affects K_S, and depends strongly on the basicity of the nitrogen atom of the scissile amide bond (*8*, *12*).

In summary, we propose that this novel protease-A-17N-1 is a cysteine proteinase of low molecular mass, about 16 kDa, which performs catalysis *via* a (Cys)-S^-/(His)-Im^+H ion-pair, by a mechanism similar to that described previously (*6*, *12*). Moreover, the validity of PI as a reliable and useful tool in investigating enzymatic mechanisms has a good match by the novel combination with other methods. These results confirmed that properly treated PI may achieve perfect estimates of suitable kinetic parameters, leading to an unambiguously accepted reaction mechanism.

This research was funded by the program "Heraklitos", of the Operational Program for Education and Initial Vocational Training of the Hellenic Ministry of Education under the 3rd Community Support

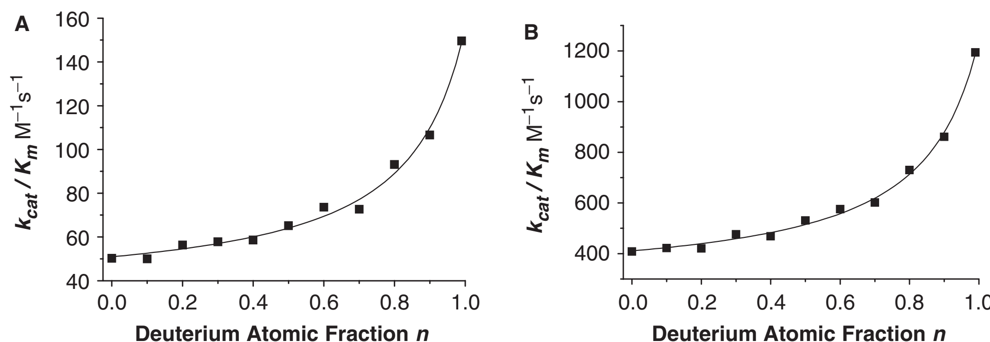

Fig. 8. **The best fits of the experimental data for the parameter k_{cat}/K_m.** The solid lines were drawn by equation $k_n = (50.44\,\text{M}^{-1}\text{s}^{-1})(1-n+n[0.46])/(1-n+n[0.15])$ (curve A), and by equation $k_n = (407.60\,\text{M}^{-1}\text{s}^{-1})(1-n+n[0.45])/(1-n+n[0.15])$ (curve B), corresponding to substrates Suc-FL-pNA, and Suc-FL-ONp, respectively. In all cases, SEs did not exceed 5%.

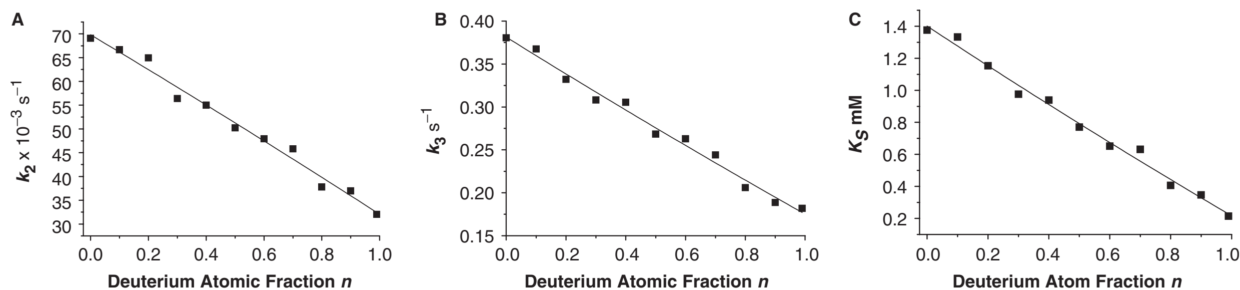

Fig. 9. **The best fits of the experimental data for the k_2 (curve A), k_3 (curve B) and K_S (curve C) parameters for the substrate Suc-FL-pNA.** Solid lines were drawn by equations $k_n = 0.07\,\text{s}^{-1}(1-n+n[0.44])/(1-n+n[0.96])$ (curve A), and $k_n = 0.38(1-n+n[0.48])/(1-n+n[1.05])$ (curve B) and $K_n = 407.60(1-n+n[0.45])/(1-n+n[0.15])$, respectively. In all cases, SEs did not exceed 5%.

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Framework and the European Social Fund. The authors also wish to thank Dr James R. Kinghorn (University of S. Andrews, Scotland, UK) for critical reading of this manuscript.

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