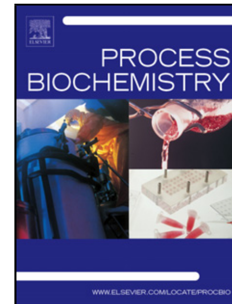
Accepted Manuscript



Title: Purification and characterization of a keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876

Author: Ivana A. Cavello Roque A. Hours Natalia L. Rojas Sebastián F. Cavalitto

PII: S1359-5113(13)00142-6
DOI: http://dx.doi.org/doi:10.1016/j.procbio.2013.03.012
Reference: PRBI 9799

To appear in: *Process Biochemistry*

Received date: 19-12-2012
Revised date: 20-3-2013
Accepted date: 22-3-2013

Please cite this article as: Cavello IA, Hours RA, Rojas NL, Cavalitto SF, Purification and characterization of a keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876, *Process Biochemistry* (2013), http://dx.doi.org/10.1016/j.procbio.2013.03.012

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Highlights

►This is the first report of a serine protease with keratinolytic activity from *P.lilacinus LPS* #876.

► Enzyme stability in broad pH range, and up to 65 °C, suggests its suitability as a detergent additive.

► Oxidant/detergent stability strengthens the enzyme's potential application as laundry additive.

►The production of this enzyme could be an alternative for solid waste management processes, an added valued product for tanneries

Accepted Manuscript

ACCEPTED MANUSCRIPT

1 Purification and characterization of a keratinolytic serine protease from *Purpureocillium*
2 *lilacinum* LPS # 876
3
4
5 **Ivana A. Cavello, Roque A. Hours, Natalia L. Rojas* and Sebastián F. Cavalitto**
6
7 Research and Development Center for Industrial Fermentations (CINDEFI, UNLP; CCT-
8 La Plata, CONICET). Calle 47 y 115. (B1900ASH) La Plata. Argentina
9
10 *Corresponding author
11 E-mail: rojas@biotec.org.ar
12 Tel.: (+54 221) 483-3794. Fax: (+54 221) 483-3794, ext. 103.
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

Accepted Manuscript

ACCEPTED MANUSCRIPT

35 **ABSTRACT**

36 A keratinolytic serine protease secreted by *Purpureocillium lilacinum* (formerly
37 *Paecilomyces lilacinus*) upon culture in a basal medium containing 1% (w/v) hair waste as
38 carbon and nitrogen source was purified and characterized. After purification the
39 keratinase was resolved by SDS- PAGE as a homogeneus protein band of molecular mass
40 37.0 kDa. The extracellular keratinase of *P. lilacinum* was characterized by its appreciable
41 stability over a broad pH range (from 4.0 to 9.0), and up to 65 °C, along with its strong
42 inhibition by phenylmethylsulphonyl fluoride among the protease inhibitors tested (98.2%
43 of inhibition), thus suggesting its nature as a serine protease. The enzyme was active and
44 stable in the presence of organic solvents such as dimethylsulfoxide, methanol, and
45 isopropanol; certain surfactants such as Triton X-100, sodium dodecylsulfate, and Tween
46 85; and bleaching agents such as hydrogen peroxide. These biochemical characteristics
47 suggest the potential use of this enzyme in numerous industrial applications.

48

49

50 Keywords: Enzyme purification, Keratinase, Serine protease, Hair waste, *Purpureocillium*
51 *lilacinum*

52

53

54

55 **1. Introduction**

56 Keratins are insoluble proteins highly cross-linked with disulfide bonds, which
57 linkage in addition to a tightly packed supercoiled polypeptide chain results in high
58 mechanical stability and resistance to proteolytic hydrolysis [1]. Keratin sources such as
59 feather, horn, nails, and hair are abundantly available in nature as wastes. In particular,

Accepted Manuscript

ACCEPTED MANUSCRIPT

60 hair waste—a solid refuse generated by the hair-saving unhairing processes—constitutes a
61 troublesome biodegradable product that is produced in large quantities by tanneries. With
62 feather waste a considerable portion is converted to feather meal and is used as a dietary
63 supplement for animal feed [2]; but with hair-waste, its disposal is the only option at the
64 present time. Therefore, the bioconversion of this kind of residue is an attractive
65 possibility of biotechnological interest since such a utilization might represent an
66 alternative means of waste management that could result in the production of valuable
67 products such as slow-release nitrogen fertilizers, cosmetics, and biodegradable films [3]
68 in addition to being a source of useful enzymes. With respect to this latter possibility, the
69 keratinases produced when keratin-containing wastes are used as substrates could have
70 practical biotechnologic uses: for example, unhairing capabilities of these enzymes would
71 avoid the environmental problems caused by traditional methods such as treatment with
72 sulfide in the leather industry, while the keratinases could also prove useful in the
73 detergent and cosmetics industries [4].

74 Alkaline keratinases from different microorganisms, and with different
75 biochemical properties, have been extensively purified and characterized [4]. The optimal
76 activity of these keratinases lies in the neutral to alkaline pH range, *i. e.*, 7.0–9.0. A few
77 keratinases exhibiting extremely alkalophilic pH optima (*e. g.*, 11.0), however, have been
78 reported [5,6]; but those hydrolases have proven not to be resistant to bleach and
79 detergents.

80 In order to determine the extent of its potential industrial application, the study
81 of the properties of the *Purpureocillium lilacinum* keratinase is fundamental in order to
82 design a biocatalyst suitable to endure industrial conditions, thus making large-scale
83 applications ultimately feasible. *P. lilacinum* LPS # 876 was found to produce
84 keratinase activity when it was grown in liquid cultures with chicken feathers as

Accepted Manuscript

ACCEPTED MANUSCRIPT

85 substrate [7]. In the present investigation, the purification and characterization of a
86 keratinolytic serine protease secreted by this same fungus, but with hair waste as the
87 substrate, is described.
88

89 2. Materials and Methods

90 *2.1. Microorganism and culture conditions*

91 *P. lilacinum* LPS # 876 (formerly *Paecilomyces lilacinus*), isolated from soils in
92 public places in the city of La Plata, Argentina [8], is a non-pathogenic fungal strain,
93 which was deposited at the Spegazzini Institute fungal culture collection (La Plata
94 National University, Argentina). It was maintained in tubes containing potato-dextrose
95 agar under mineral oil at 4 ºC. Cultures were established in 1,000-ml Erlenmeyer flasks in
96 200 ml of hair basal medium containing (per liter) 10 g hair waste, 496 mg NaH_2PO_4,
97 2.486 g K_2HPO_4, 16 mg $FeCl_3.6H_2O$, 13 mg $ZnCl_2$, 10 mg $MgCl_2$, and 0.11 mg $CaCl_2$ (pH
98 7.0) [9]. Hair waste, obtained from a local tannery, was washed extensively with tap
99 water; dried at 60 ºC for 2 days; and used as the source of carbon, nitrogen, and sulfur.
100 The culture flasks were autoclaved at 121 ºC for 15 min for sterilization and then, after
101 cooling, inoculated with 2 x 10^6 conidia per ml. The cultures were incubated in an orbital
102 shaker at 200 rpm and 28 ºC for 117 h. Samples of 5 ml were withdrawn at regular
103 intervals, centrifuged (5,000 ×g, 20 min, 4 °C) and the supernatant was used for pH,
104 protein content and enzyme activities determinations. When purification of the enzyme
105 was achieved, all the contents of each flask withdrawn and centrifuged at 5,000 ×g and 4
106 ºC for 20 min in order to precipitate the fungal biomass. The supernatant was then used for
107 enzyme purification.
108

109 *2.2. Enzyme-activity determination*

Accepted Manuscript

ACCEPTED MANUSCRIPT

110 After each purification step the keratinolytic and proteolytic activities were both
111 measured as described elsewhere [10], with the latter activity determination being used for
112 enzyme characterization.
113
114 *2.3. Protein determination*
115 Proteins were quantified after Bradford [11] with bovine-serum albumin (Sigma)
116 as a standard.
117
118 *2.4. Scaning electron microscopy (SEM)*
119 To characterize the degradation of hair waste by *P. lilacinum*, digested and
120 undigested hair waste samples were freeze-dried and then coated with gold palladium.
121 SEM was accomplished using a Jeol JSM-840 microscope at an accelerating voltage of
122 25kV.
123
124 *2.5. Purification of keratinase*
125 The culture supernatant was filtered through cheesecloth at 4 ºC and concentrated
126 under reduced pressure at 30 ºC. Solid ammonium sulfate was then added to the
127 concentrated extract to up to 85% saturation. The precipitated proteins were dissolved in
128 20 ml of Tris-HCl buffer (20 mM, pH 7.0; TB) and then applied to a Sephadex G-25 gel-
129 filtration column (XK 16/60, General Electric Little Chalfont, UK) equilibrated with TB
130 and eluted isocratically with the same buffer at a flow rate of 1.0 ml min^{-1}. Fractions of 5
131 ml were collected and those exhibiting keratinase activity pooled, concentrated by
132 lyophilization, resuspended in the same buffer up to 4 ml, and loaded onto a DEAE-
133 Sephadex (XK 26/10, General Electric) anion-exchange column. The column was washed
134 with TB, and the bound proteins were then eluted with a linear gradient of NaCl (0.0–1.0

Accepted Manuscript

ACCEPTED MANUSCRIPT

135 M) in TB over 10 column volumes at a flow rate of 2 ml min^{-1}. Fractions (5 ml) were
136 collected and analyzed for keratinase activity. Those fractions with keratinase activity
137 were pooled and loaded onto a Sp-Sepharose-FF column (Hiload 16/10, General Electric)
138 preequilibrated with TB. The proteins were eluted on a linear gradient of NaCl (0.0–1.0
139 M) in TB over 10 column volumes at a flow rate of 2 ml min^{-1} in the same
140 chromatographic system. The fractions (10 ml) containing keratinase activity were pooled,
141 concentrated by lyophilization, resuspended in TB to up to 2 ml, loaded onto a Superdex-
142 75 column (XK 16/60, General Electric) equilibrated with Tris-HCl buffer (20 mM Tris,
143 0.15 M NaCl; pH 7.0), and eluted isocratically with the same buffer at a flow rate of 0.75
144 ml min^{-1}. The purified enzyme was stored at –20 ºC and used for further biochemical
145 characterization. All chromatographic steps were carried out on an Amersham FPLC-
146 U900 system (General Electric).
147
148 *2.6. Molecular-weight determination*
149 After a prior desalting step, the molecular weight of the protein isolate was
150 estimated by sodium-dodecylsulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) in a
151 12% (w/v) gel calibrated with low-molecular-weight markers (LMW Kit, General
152 Electric) according to Laemmli [12]. The protein bands were stained with Coomassie
153 brilliant blue R-250.
154
155 *2.7. Biochemical characterization of purified keratinase*
156 *2.7.1. Effect of pH on enzyme stability and activity*
157 The pH stability of the purified enzyme was studied over a range of 3.0–13.0,
158 while the pH optimum of the protease activity was determined within the range 6.0–13.0
159 with azocasein as substrate (Azocasein is insoluble at pHs <6.0.). For measurement of the

Accepted Manuscript

ACCEPTED MANUSCRIPT

160 pH stability, the enzyme was incubated at a given pH for 1 h at 37 °C and the residual
161 protease activity determined under standard assay conditions. In both instances, a mixture
162 of buffers (glycine, 2-(*N*-morpholino)ethanesulfonic acid, and Tris-HCl; 20 mM each)
163 adjusted to the required pH was used.
164

165 *2.7.2. Effect of temperature on enzyme stability*

166 The thermostability of the purified enzyme was examined through incubations at
167 different temperatures (40–65 ºC) for 180 min. Aliquots were withdrawn at regular time
168 intervals and the protease activity measured under standard assay conditions. The activity
169 remaining at each time point was expressed as a percent of the value recorded with the
170 unheated crude protease.
171

172 *2.7.3. Effect of inhibitors and metal ions on protease stability*

173 The effect of the following inhibitors of protease activity was investigated:
174 phenylmethylsulphonyl fluoride (PMSF, 1 mM), iodoacetate (10 mM),
175 ethylendiaminetetraacetate (5 mM), 1,10-phenanthroline (1 mM) and Pepstatin A
176 (chlorambucil, 100 μg ml^{-1}). The keratinase was preincubated in the presence of each
177 inhibitor for 1 h at room temperature (20 ºC) and the protease activity remaining during
178 subsequent assay expressed as a percent of the control value with enzyme not exposed to
179 inhibitor.

180 The effect of different metal ions (at a concentration of 1 mM) on protease activity
181 was studied by addition of the cations Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, and Hg^{2+} to the enzyme
182 solution followed by incubation for 1 h at room temperature. The protease activity
183 remaining upon subsequent assay was expressed as a percent of the control value with
184 enzyme not exposed to cations.

Accepted Manuscript

ACCEPTED MANUSCRIPT

185

186 *2.7.4. Effect of surfactants and oxidizing agents on protease stability*

187 The keratinase stability towards selected surfactants (SDS, Triton X-100, Tween

188 20, and Tween 85), and oxidizing agents (H_2O_2, sodium perborate) was tested by

189 incubating the enzyme with each additive for 1 h at room temperature. The remaining

190 protease activity was determined under standard conditions and expressed as percentage of

191 the control value with enzyme not exposed to an oxidizing agent.

192

193 *2.7.5. Determination of kinetic parameters*

194 The kinetic constants K_m and V_{max} of the purified enzyme were calculated by

195 fitting the activity data at increasing substrate concentrations (azocasein; [13]) to a linear

196 regression after Hanes-Hultin transformation [14].

197

198 *2.8. Protein identification*

199 Peptide mass fingerprinting of selected protein spots was carried out by in-gel

200 trypsin (Sequencing-grade, Promega) treatment during an overnight electrophoresis at 37

201 ºC. The trypsinized peptides were extracted from the gels with 60% (v/v) acetonitrile in

202 0.2% (w/v) trifluoroacetic acid, concentrated by vacuum-drying, and desalted on C18

203 reverse-phase microcolumns (OMIX pipette tips, Varian). The peptides from the

204 microcolumn were eluted directly onto the mass-spectrometer sample plates in 3 μl of

205 matrix solution (α-cyano-4-hydroxycinnamic acid in 60% (v/v) aqueous acetonitrile in

206 0.2% (w/v) trifluoroacetic acid. Mass spectra of the digestion mixtures were generated in a

207 4800 MALDI-TOF/TOF instrument (Applied Biosystems) in reflector mode and were

208 externally calibrated by means of a mixture of peptide standards (Applied Biosystems).

209 Collision-induced MS/MS dissociations of selected peptides were performed. Proteins

Accepted Manuscript

ACCEPTED MANUSCRIPT

210 were identified by NCBInr database by searching with peptide m/z values through the
211 MASCOT program and by means of the search parameters monoisotopic mass tolerance,
212 0.08 Da; fragment-mass tolerance, 0.2 Da; and methionine oxidation as possible
213 modifications with one missed tryptic cleavage being allowed.
214
215 *2.9. N-terminal sequencing*
216 The N-terminal–amino-acid sequence of the purified keratinase was determined
217 with a ABI 494 protein sequencer at Tufts University, Boston, MA USA.
218
219 **3. Results and Discussion**
220
221 *3.1. Keratinase production during the growth of* P. lilacinum
222 Figure 1A shows the time course of the production of extracellular keratinase
223 activity in hair basal medium containing 1% (w/v) hair waste. A maximum production of
224 protease and keratinase activities of 2.46 U/ml and 25 U/ml were achieved by between
225 111 and 117 h of incubation, respectively. Both activities increased in parallel, thus
226 maintaining a constant keratinolytic:proteolytic (K:P) activity ratio of 11.32 ± 1.06.
227 Because of this constant production ratio, the proteolytic activity was chosen as an indirect
228 estimation of keratinolytic activity for the purpose of the biochemical characterization of
229 the purified enzyme. During the whole fermentation the pH of the medium increased
230 because of the ammonia produced by the deamination of peptides and aminoacids derived
231 from keratin solubilization. On the other hand, soluble protein concentration showed a
232 similar behavior to that of the proteolytic activity except for the fact that the maximum
233 peak was reached a few days later (Fig. 1A). Since keratin is used as medium component,
234 keratinase production is accompanied by subsequent degradation of keratin substrate

Accepted Manuscript

ACCEPTED MANUSCRIPT

235 leading to an increase in soluble protein concentration. The increment of soluble protein
236 has been reported as a measure for keratin degradation [15]. The decrease of soluble
237 protein may be due to an increment of the proteolysis degree, thus releasing peptides
238 which were not detected by Bradford's reagent.
239 A comparable kinetics of keratinase production has been reported for microorganisms
240 such as *Bacillus subtilis* MTCC (9102) with horn-meal as the substrate [16] and
241 *Microbacterium* sp. strain kr10 grown in feather-meal medium [17]. In cultures of
242 *Aspergillus fumigatus* [18], *A. oryzae* [19], and *Trichophyton vanbreuseghemii* [20], the
243 keratinase production reached a maximum after 21 days of incubation.
244 The extent of degradation of hair waste by *P. lilacinum* during culture was established by
245 SEM. Fig. 1(B) and Fig. 1(C) shows SEM images of uninoculated hair fibres after hair-
246 saving unhairing process using sodium sulfite/lime as unhairing agent and hairs fibres
247 after 5 days of submerged culture, respectively. It could be observed a considerable
248 degradation and disorganization of the fibre due to the fungal attack during the culture and
249 also fungal aggregates with an extracellular matrix, adhered to degraded surfaces.
250
251 *3.2. Enzyme purification*
252 The extracellular keratinase produced by *P. lilacinum* cultivated on hair-waste
253 medium was purified from the concentrated culture extract (72 U/mg protein) by
254 ammonium-sulfate precipitation, gel filtration, and ion-exchange chromatography to
255 obtain a 19.8-fold enrichment and a specific activity of about 1,430 U/mg protein at a
256 yield of 1.3% (Table 1). Although purification resulted in a low total yield, the procedure
257 was chosen in order to obtain a homogeneous fraction of keratinase for the purpose of
258 biochemical characterization. In accordance with that objective, analysis of the enzyme on
259 SDS-polyacrylamide gels revealed a single band of apparent molecular weight 37 kDa

Accepted Manuscript

ACCEPTED MANUSCRIPT

260 (Fig. 2). Similar molecular weight values were found for proteinases secreted by *P.*
261 *lilacinum* strains (33.5 kDa, [21] and 33 kDa, [22]), *Bacillus licheniformis* (33 kDa, [23]),
262 and *Trichophyton vanbreuseghemii* (37 kDa, [20]).
263
264 *3.3. Biochemical characterization of purified keratinase*
265 Enzymes with keratinolytic activity have constituted a focus of interest in various
266 studies because of their wide spectrum of potential industrial applications—*e. g.*, in the
267 catalysis required in the production of fertilizers or animal feed, as additives in detergent
268 formulation, and as dehairing agents in tanneries [4,24 - 26]. Keratinases can also be used
269 in skin-care cosmetics and for feather-waste degradation in the poultry industry [27]. As a
270 rule, naturally available enzymes are not optimally suitable for such industrial
271 applications, and this incompatibility often stems from the lack of stability of those
272 proteins under the conditions of the particular process needed. Although sometimes an
273 adaptation of industrial processes to mild and environmentally benign conditions can be
274 suitable, the use of extreme conditions is often unavoidable. For example, proteolytic
275 enzymes incorporated into detergent formulations should exhibit certain special
276 characteristics: activity and stablity at alkaline pHs and/or at relatively high temperatures
277 (40–50 ºC or more) and compatiblity with other detergent components such as surfactants,
278 perfumes, bleaches, and oxidizing and sequestering agents [28]. In general, the majority of
279 commercially available enzymes are not stable in presence of bleaching or oxidizing
280 agents. Regardless of the conditions of the process in question, the stability of the
281 biocatalyst is often a relevant economic consideration.
282 The pH stability of the *P. lilacinum* enzyme was tested at values between 3.0 and
283 13.0. The enzyme was fully stable over a wide pH range (from 4.0 to 9.0). Outside this
284 range, the keratinase catalysis was only moderately stable, retaining 50% of the native

Accepted Manuscript

ACCEPTED MANUSCRIPT

285 activity at pH 3 and 40% at pH 12 (Fig. 3A). Keratinases have occasionally exhibited this
286 degree of stability—*e. g.*, the keratinases from *Kocuria rosea* within the range of pH 10.0–
287 11.0 [29]; from *Norcardiopsis* sp. TOA-1, at pH 12.0 [5]; and from *Bacillus* sp. AH-101,
288 between pH 11.0 and 12.0 [6]. The notably wide pH range throughout which the *P.*
289 *lilacinum* keratinase is both active and stable may enhance its biotechnological
290 applications, especially in the leather and detergent industries.

291 Fig. 3B shows the effect of pH on enzyme activity. The *P. lilacinum* keratinase
292 was found to be active at pHs ranging from 6.0 to 13.0 with a constant maximum activity
293 between pHs 7.0 and 12.0.

294 The thermal stability of the keratinase was evaluated by incubating the purified
295 enzyme at different temperatures (between 40–65 °C) for 180 min (Fig. 4). The enzyme
296 was stable below 50 °C and also retained more than 40% of the initial activity after 3 h of
297 incubation at that temperature. The half-life of the enzyme was estimated at 137 min at 50
298 °C and 68 min at 55 °C. In comparison, the keratinase from *Bacillus* sp. P7 had a half-life
299 of 53 min at 50 °C and less than 10 min at 55 °C [30]; while the keratinase from *K. rosea*
300 remained fully active after 1 h of incubation at 10–60 °C, with 40% of the initial activity
301 remaining after 1 h at 90 °C [29]. We therefore conclude that the keratinase from *P.*
302 *lilacinum* exhibits moderate thermotolerance and thermostability, which features might be
303 conducive to the efficient use of the enzyme in processes involving protein hydrolysis
304 [31]. Moreover, a recent trend in the detergent industry has resulted in the requirement of
305 alkaline proteases that remain active at washing temperatures (between 20 and 30 ºC),
306 with that prerequisite aimed at maintaining fabric quality along with low energy demands
307 [32]. As indicated by additional assays, the *P. lilacinum* keratinase proved to be
308 completely active over this temperature range, thus pointing to the enzyme's usefulness
309 within that specific industrial context (data not shown).

Accepted Manuscript

ACCEPTED MANUSCRIPT

310 Most of the keratinases that have been reported belong to the serine or
311 metalloprotease classes [33]. In the present study, the enzymatic activity was strongly
312 inhibited by PMSF, a serine-protease inhibitor; whereas other inhibitors assayed affected
313 the enzyme activity only slightly (Table 2). Accordingly, from the inhibition
314 characteristics observed for the *P. lilacinum* keratinase—a 98.2% inhibition with PMSF
315 and a 92.1% inhibition in the presence of Hg^{2+}—this keratinase is highly likely to be a
316 thiol-dependent serine protease [22].

317 After purification of the enzyme from the fungus Ca^{2+} was found to slightly
318 decrease keratinase catalysis (Table 2). Since the enzyme´s stability therefore does not
319 depend on the presence of Ca^{2+}, the likelihood of the keratinase's usefulness in the
320 detergent industry is increased, mainly because in that process—it commonly employing
321 alkaline proteases—chelating agents are included to avoid the problem of hardness in the
322 water. In the presence of such chelating agents, Ca^{2+} could be easily removed, thus greatly
323 affecting the activity of a Ca^{2+}-dependent hydrolase. For this reason, enzymes without any
324 metal-ion requirement for stability offer considerable potential for use in the manufacture
325 of detergents.

326 The organic solvents dimethylsulfoxide, isopropanol, methanol, and ethanol, in the
327 concentrations tested, had no effect on keratinase activity (Table 2), as had been reported
328 to be true for the keratinases from *K. rosea* [29], from *Bacillus* sp. P7, and from
329 *Nocardiopsis* sp. TOA-1 [5]. The purified keratinase also proved highly stable in the
330 presence of nonionic surfactants, retaining 100% of its initial activity in the presence of
331 1% (v/v) Triton X-100, 1% (v/v) Tween 20, and 1% (v/v) Tween 85 after 1 h of
332 incubation at room temperature. SDS at 0.5% (w/v), a strong anionic surfactant, produced
333 only a minor inhibition of enzyme activity, with the keratinase retaining approximately
334 70% of the initial levels after 1 h of incubation at room temperature. This percent retention

Accepted Manuscript

ACCEPTED MANUSCRIPT

335 was greater than the figure of 45.8% that had been reported for a keratinase from
336 *Chryseobacterium* L99 sp. nov. after a 1-h incubation with only 0.2% (w/v) SDS [34].
337 In the inactivation process of proteins by oxidizing agents, methionine residues
338 have been identified as primary targets. All subtilisins (serine proteases) contain a Met
339 residue next to the Ser of the catalytic site so that they are strategically positioned for the
340 enzyme to undergo oxidative inactivation in the presence of oxidizing agents such as
341 hydrogen peroxide. Thus, many of the available alkaline proteases have been found to
342 exhibit a low activity and stability towards the oxidants that are common ingredients in
343 modern bleach-based detergents. To overcome these shortcomings, several attempts have
344 been made to enhance enzyme stability through protein engineering [35]. In addition, the
345 search for enzymes with a high stability against surfactants and oxidants for industrial
346 applications has gained an equally high priority. Accordingly, when the *P. lilacinum*
347 keratinase was incubated in the presence of 1% (v/v) H_2O_2 or 1% (w/v) sodium perborate
348 for 1 h at room temperature, no inactivation occurred. This substantial stability toward
349 oxidizing agents was similar to the properties of the proteases from *B. licheniformis* NH1,
350 with those hydrolases retaining 85% and 80% of the initial activity after 1 h of incubation
351 at 40 ºC with 0.5% (v/v) H_2O_2 and 0.2% (w/v) sodium perborate, respectively [36].
352 Moreover, the *P. lilacinum* keratinase proved to be more stable than the *B. licheniformis*
353 RP1 proteases, where those retained only 48% of their activities after a 1-h incubation at
354 40 ºC in the presence of this same concentration of sodium perborate [28].
355
356
357
358 *3.4. Kinetic parameters*

Accepted Manuscript

ACCEPTED MANUSCRIPT

359 The kinetic parameters K_m and V_{max}, measured with azocasein as substrate, were
360 0.72 mg/ml and 3.6 U/min, respectively (Fig. 5). This K_m value resulted to be similar to
361 that reported by Silveira et al. [37] for *Chryseobacterium* sp. strain kr6 (Km, 0.75 mg/ml)
362 and lower from those reported by Ghosh et al. [38] from *Bacillus cereus* DCUW (Km,
363 0.161 mg/ml) and by Daroit et al. from *Bacillus* sp. P45 (Km, 2.85 mg/ml)[39].
364
365 *3.5. Protein identification and N-terminal–sequence analysis*
366
367 After trypsin hydrolysis and MALDI-TOF/TOF analysis, a search in the NCBI nr
368 database identified peptide similarities (52% homology, including both N- and C-terminal
369 peptides) to a previously reported *P. lilacinus* serine protease (Swiss-Prot Accesion No.
370 Q01471; NCBI Accesion 3F7O_A), indicating the similarity of the keratinolytic protease
371 purified in this work to that earlier described *P. lilacinus* enzyme [21]. Table 3 shows the
372 amino acid sequence coverage of *P. lilacinum* serine protease obtained from micro
373 sequencing and MALDI/TOF MS data. Additional results concerning MALDI-TOF/TOF
374 results can be obtained from
375 http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20110406/FttpInSTt.dat&
376 hit=1.
377 The N-terminal–amino-acid sequence of the enzyme was A-Y-T-Q-Q-P-G-A-I,
378 thus showing complete identity to the N-terminal–amino-acid sequence of the serine
379 protease from still another *P. lilacinus* strain (CBS 243.75; [21]), but did not match the
380 N-terminal sequence of a thiol-dependent serine protease (G-A-T-T-Q-G-A-T-G/I-
381 Xxx-G) isolated from a fourth *P. lilacinus* strain (VKM F-3891;[22]).
382
383 **4. Conclusions**

Accepted Manuscript

ACCEPTED MANUSCRIPT

384 A keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876 would
385 appear to be a protease with significant industrial possibilities as a result of its catalytic
386 stability over a broad pH and temperature range in addition to its tolerance to bleaching
387 and chelating agents. The enzymatic properties of the enzyme suggest its potential use in
388 detergent formulations and the leather industry (*i. e*., for the processes of dehairing and
389 bating). These characteristics of the fungal keratinase and its prospective application in
390 other commercial contexts—such as in the cosmetic and pharmaceutical industries—are
391 indeed promising.
392 The production of the keratinase from *P. lilacinum* is a simple process and
393 amenable to a scaling-up since the enzyme is excreted into the extracellular medium when
394 the microorganism is cultured with hair waste as a sole nitrogen, energy, and carbon
395 source. Finally, the production of the enzyme with such attractive biochemical
396 characteristics from a cheap substrate constitutes an economically attractive process for
397 industrial applications because of its low production cost.
398

399 **Acknowledgements**

400 This research work was supported by CONICET. RAH and SFC are members of the
401 Research Career of CONICET; NLR and IAC hold a fellowship of CONICET. The
402 authors wish to thank Dr. Donald F. Haggerty, a retired career investigator and native
403 English speaker, for editing the final version of the manuscript.

404

405

406

407

408 References

409

Accepted Manuscript

ACCEPTED MANUSCRIPT

410 [1] Böckle B, Galunsky B, Müller R. Characterization of a keratinolytic serine
411 proteinase from *Streptomyces pactum* DSM 40530. Appl Environ Microbiol 1995;
412 61:3705-3710.

413 [2] Odetallah NH, Wang JJ, Garlich JD, Shih JCH. Keratinase in starter diets
414 improves growth of broiler chicks. Poultry Science 2003; 82:664-670.

415 [3] Schrooyen PMM, Dijkstra PJ, Oberthür RC, Bantjes A, Feijen J. Partially
416 carboxymethylated feather keratins. 2. Thermal and mechanical properties of films.
417 Journal of Agricultural and Food Chemistry 2001; 49:221-230.

418 [4] Gupta R, Ramnani P. Microbial keratinases and their prospective applications: an
419 overview . Appl Microbiol Biotechnol 2006; 70:21-33.

420 [5] Mitsuiki S, Ichikawa M, Oka T, Sakai M, Moriyama Y, Sameshima Y, Goto M,
421 Furukawa K. Molecular characterization of a keratinolytic enzyme from an
422 alkaliphilic *Nocardiopsis sp*. TOA-1. Enzyme Microb Technol 2004; 34:482-489.

423 [6] Takami H, Akiba T, Horikoshi K. Production of extremely thermostable alkaline
424 protease from *Bacillus* sp. no. AH-101. Appl Microbiol Biotechnol 1989; 30:120-
425 124.

426 [7] Cavello I, Cavalitto S, Hours R. Biodegradation of a Keratin Waste and the
427 Concomitant Production of Detergent Stable Serine Proteases from *Paecilomyces*
428 *lilacinus*. Appl Biochem Biotechnol 2012; 167:945-958.

429 [8] Gortari C, Cazau MC, Hours R. Hongos nematófagos de huevos de *Toxocara*
430 *canis* en un paseo público de La Plata, Argentina. Revista Iberoamericana de
431 Micología 2008; 24:24-28.

432 [9] Galarza B, Goya L, Cantera C, Garro ML, Reinoso HM, Lopez LMI. Fungal
433 biotransformation of bovine hair part I: Isolation of fungus with keratinolytic
434 activity. J Soc Leather Technol Chem 2004; 88:93-98.

435 [10] Cavello IA, Hours R, Cavalitto SF. Bioprocessing of "Hair Waste" by
436 *Paecilomyces lilacinus* as a source of a bleach-stable, alkaline, and thermostable
437 keratinase with potential application as a laundry detergent additive:
438 characterization and wash performance analysis. Biotechnology Research
439 International 2012; 2012:1-12.

440 [11] Bradford MM. A rapid and sensitive method for the quantitation of microgram
441 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem
442 1976; 72:248-254.

443 [12] Laemmli UK. Cleavage of structural proteins during assembly of head of
444 *bacteriophage* T4. Nature 1970; 227:680-685.

445 [13] Ageitos M, Vallejo JA, Sestelo ABF, Poza M, Villa TG. Purification and
446 characterization of a milk-clotting protease from *Bacillus licheniformis* strain
447 USC13. J Appl Microbiol 2007; 103:2205-2213.

Accepted Manuscript

ACCEPTED MANUSCRIPT

448 [14] Hultin E. Statistical calculations of the accuracy of the Michaelis constant from
449 viscosimetric determinations of polymetaphosphatase and dextranase activity. Acta
450 Chemica Scandinava 1967; 21:1575-1580.

451 [15] Bálint B, Bagi Z, Tóth A, Rákhely G, Perei K, Kovács K. Utilization of keratin-
452 containing biowaste to produce biohydrogen. Appl Microbiol Biotechnol 2005;
453 69:404-410.

454 [16] Balaji S, Kumar MS, Karthikeyan R, Kumar R, Kirubanandan S, Sridhar R, Seghal
455 PK. Purification and characterization of an extracellular keratinase from hornmeal-
456 degrading *Bacillus subtilis* MTCC (9102). World J Microb Biotech 2008; 24:2741-
457 2745.

458 [17] Thys RCS, Brandelli A. Purification and properties of a keratinolytic
459 metalloprotease from *Microbacterium* sp. J Appl Microbiol 2006; 101:1259-1268.

460 [18] Do JH, Anderson MJ, Denning DW, Bornberg-Bauer E. Inference of Aspergillus
461 fumigatus pathway by computational genome analysis: tricarboxylic acid cycle
462 (TCA) and glyoxilate shunt. J Microb Biotech 2004; 14:74-80.

463 [19] Jousson O, Lechenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni
464 M, Monod M. Multiplication of an ancestral gene encoding secreted fungalysin
465 preceded species differentation in the dermatophytes *Trichophyton* and
466 *Microsporum*. Microbiology 2004; 150:301-310.

467 [20] Moallaei H, Zaini F, Larcher G, Beucher B, Bouchara JP. Partial purification and
468 characterization of a 37 KDa extracellular proteinase from *Trichophyton*
469 *vanbreuseghemii*. Mycopathologia 2006; 161:369-375.

470 [21] Bonants PJ, Fitters PF, Thijs H, den Belder E, Waalwijk C, Henfling JW. A basic
471 serine protease from *Paecilomyces lilacinus* with biological activity against
472 *Meloidogyne hapla* eggs. Microbiology 1995; 141:775-784.

473 [22] Kotlova EK, Ivanova MN, Yusupova MP, Voyushina TL, Ivanushkina NE,
474 Chestukhina GG. Thiol-Dependent Serine Proteinase from *Paecilomyces lilacinus*:
475 Purification and Catalytic Properties. Biochemistry (Moscow) 2007; 72:117-123.

476 [23] Lin X, Lee C, Casale E, Shih JCH. Purification and characterization of a keratinase
477 from a feather degrading *Bacillus licheniformis* strain. Appl Environ Microbiol
478 1992; 58:3271-3275.

479 [24] Anbu P, Gopinath SC, Hilda A, Lakshmipriya T, Annadurai G. Optimization of
480 extracellular keratinase production by poultry farm isolate *Scopulariopsis*
481 *brevicaulis*. Bioresour Technol 2007; 98:1298-1303.

482 [25] Godfrey G. Protease in waste treatment. In: Godfrey G,West S editors. Industrial
483 Enzymology. London: Macmillan Press Ltd; 1996. p. 315-316.

484 [26] Macedo AJ, Beys da Silva WO, Gava R, Driemeier D, Pêgas Henriques JA,
485 Termignoni C. Novel keratinase from Bacillus subtilis S14 exhibiting remarkable
486 dehairing capabilities. Appl Environ Microbiol 2005; 71:594-596.

Accepted Manuscript

ACCEPTED MANUSCRIPT

487 [27] Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S. A review: potentials
488 for biotechnological applications of keratin-degrading microorganisms and their
489 enzymes for nutritional improvement of feathers and other keratins as livestock
490 feed resources. Bioresour Technol 1998; 66:1-11.

491 [28] Sellami-Kamoun A, Haddar A, El-Hadj Ali N, Ghorbel-Frikha B, Kanoun S,
492 Moncef N. Stability of thermostable alkaline protease from *Bacillus licheniformis*
493 RP 1 in commercial solid laundry detergent formulations. Microbiologial Research
494 2008; 163:299-306.

495 [29] Bernal C, Cairó J, Coello N. Purification and characterization of a novel
496 exocellular keratinase from *Kocuria rosea*. Enzyme Microb Technol 2006; 38:49-
497 54.

498 [30] Correa AP, Daroit DJ, Brandelli A. Characterization of a keratinase produced by
499 *Bacillus sp*. P7 isolated from an Amazonian environment. Int Biodeter Biodegr
500 2010; 64:1-6.

501 [31] Rao MB, Tanksale A.M., Ghatge MS, Deshpande VV. Molecular and
502 biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998;
503 62:597-635.

504 [32] Venugopal M, Saramma AV. Characterization of alkaline protease from *Vibrio*
505 *fluvialis* strain VM 10 isolated from a mangrove sediment sample and its
506 application as a laundry detergent additive. Process Biochem 2006; 41:1239-1243.

507 [33] Brandelli A. Bacterial Keratinases: useful enzymes for bioprocessing
508 agroindustrial wastes and beyond. Food Bioprocess Technol 2008; 1:105-116.

509 [34] Lv LX, Sim MH, Li YD, Min J, Feng WH, Guan WJ, Li YQ. Production,
510 characterization and application of a keratinase from *Chryseobacterium* L99 sp.
511 nov. Process Biochem 2010; 45:1236-1244.

512 [35] Estell DA, Graycar TP, Wells JA. Engineering an enzyme by site-directed
513 mutagenesis to be resistant to chemical oxidation. J Biol Chem 1985; 260:6518-
514 6521.

515 [36] Hmidet N, El-Hadj Ali N, Haddar A, Kanoun S, Sellami-Kamoun A, Nasri M.
516 Alkaline proteases and thermostable α-amylase co-produced by *Bacillus*
517 *licheniformis* NH1: Characterization and potential application as detergent
518 additive. Biochem Eng J 2009; 47:71-79.

519 [37] Silveira ST, Jaeger MK, Brandelli A. Kinetic data and substrate specificity of a
520 keratinase from *Chryseobacterium* sp. strain kr6. Chemical Technology and
521 Biotechnology 2009; 84:361-366.

522 [38] Ghosh A, Chakrabarti K, Chattopadhyay D. Degradation of raw feather by a novel
523 high molecular weight extracellular protease from newly isolated *Bacillus cereus*
524 DCUW. J Ind Microbiol Biotechnol 2008; 35:825-834.

Accepted Manuscript

ACCEPTED MANUSCRIPT

525 [39] Daroit DJ, Corrêa AP, Segalin J, Brandelli A. Characterization of a keratinolytic
526 protease produced by the feather-degrading Amazonian bacterium *Bacillus* sp.
527 P45. Biocatalysis and Biotransformation 2010; 28:370-379.
528
529
530
531

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 1

Steps involved in the purification of *P. lilacinus* keratinase

Purification step	Volume (ml)	Total protein (mg)	Total activity (U_k)	Specific activity (U_k/mg)	Yield (%)
Concentrated extract	290	503.8	36274.9	72.0	100
Precipitation	20	302.5	30982.8	102.4	85
G-25	120	37.2	3950.7	106	11
DEAE Sepharose	26.5	4.14	2759.8	625.8	7.6
Sp Sepharose FF	26.5	1.40	954.3	640	2.6
Superdex 75	13.25	0.32	458.5	1432.7	1.3

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 2

Effect of protease inhibitors, metal ions, detergents and solvents on protease activity

Chemical	Concentration	Residual activity (%)
None		100
Inhibitors		
PMSF	1 mM	1.8 ± 0.5
Iodoacetate	10 mM	79.4 ± 0.6
EDTA	5 mM	93.0 ± 1.8
1,10-Phenantroline	1 mM	88.3 ± 1.0
Pepstatin A	100 µg/ml	88.7 ± 2.3
Metal ions		
Mg^{2+}	1 mM	72.7 ± 0.6
Zn^{2+}	1 mM	74.9 ± 1.5
Ca^{2+}	1 mM	81.7 ± 1.8
Hg^{2+}	1 mM	7.9 ± 0.4
K^{+}	1 mM	83.5 ± 1.0
Detergents		
Triton X-100	1 % (v/v)	100 ± 0.4
Tween 20	1 % (v/v)	98.3 ± 1.9
Tween 85	1 % (v/v)	101.5 ± 2.6
SDS	0.5 % (v/v)	69.5 ± 2.5
Bleaching agents		
H_2O_2	1 % (w/v)	99.4 ± 5.5
Sodium perborate	1 % (w/v)	99.7 ± 2.4
Solvents		
DMSO	1 % (v/v)	99.0 ± 0.6
Ethanol	1 % (v/v)	100 ± 5.8
Methanol	1 % (v/v)	100 ± 2.9
Isopropanol	1 % (v/v)	88.7 ± 5.5

* Data are shown as residual activity (%) ± SD

Accepted Manuscript

ACCEPTED MANUSCRIPT

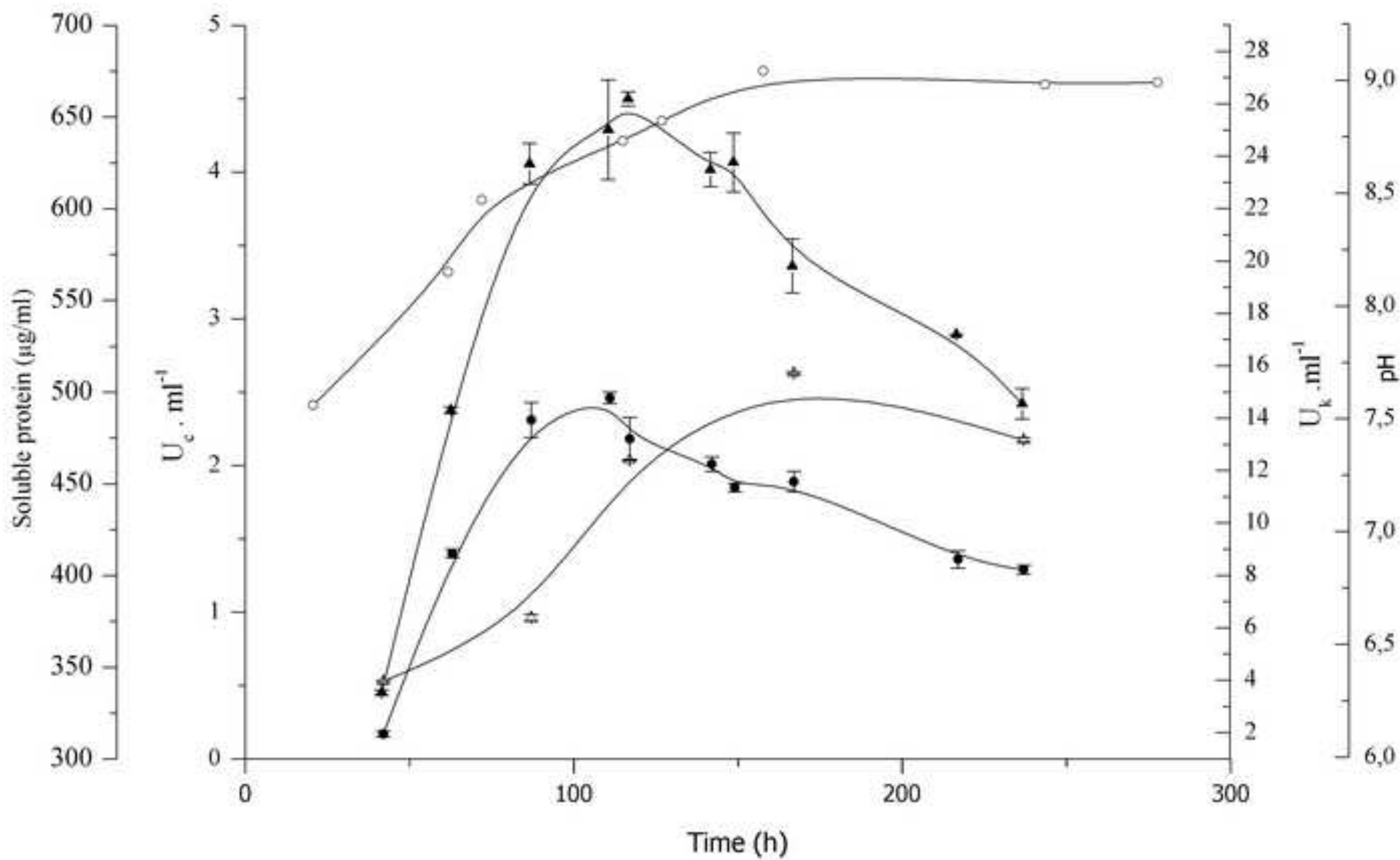
Table 3

Amino acid sequence coverage of *P. lilacinum* serine protease obtained from MALDI/TOF MS data.

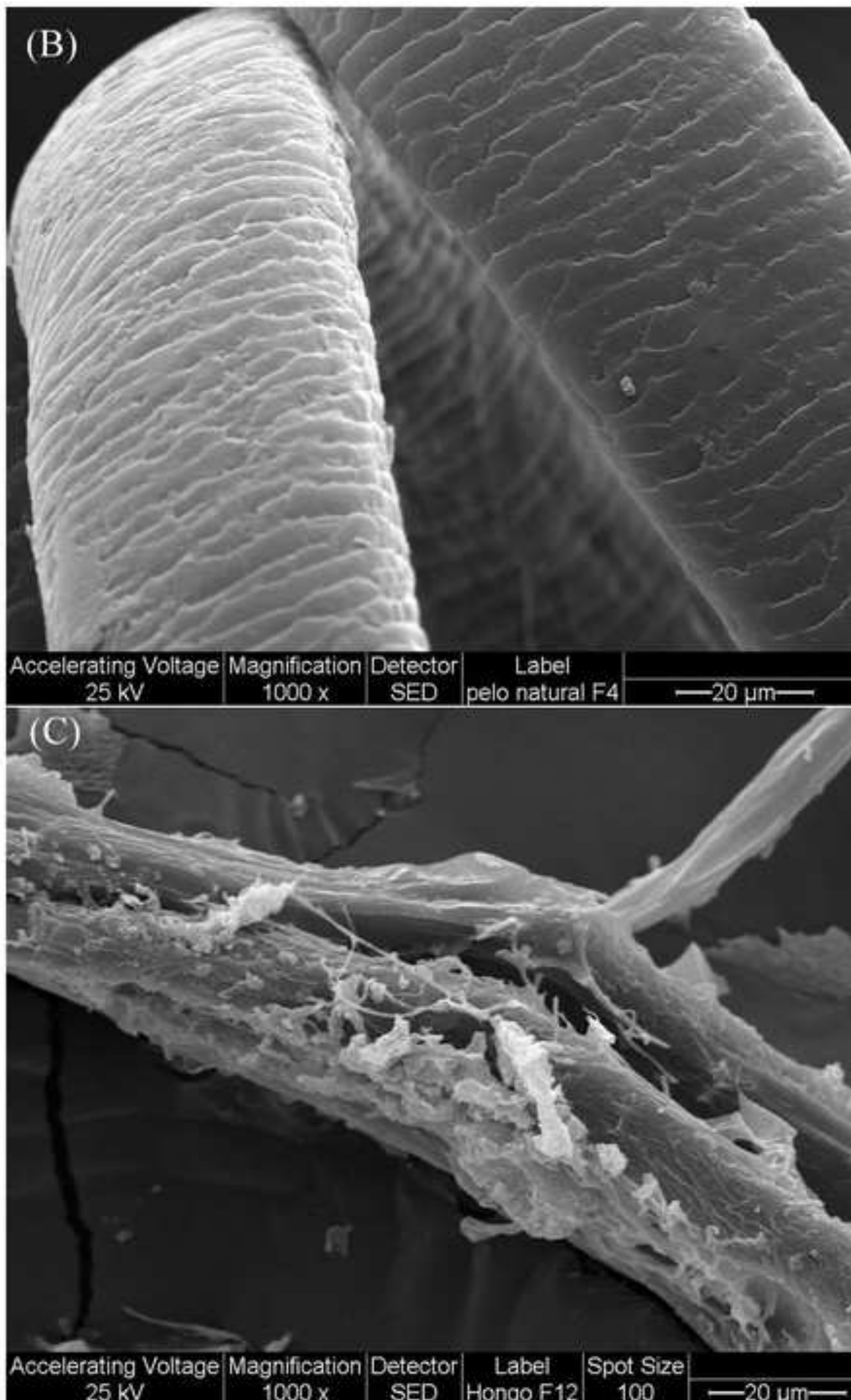
Peptide position	Matched masses	Matched Cys_PAM	Peptide sequence
1-14	1501.7534		AYTQQPGAPWGLGR
21-54	3543.5622	Cys36:3614.5857	GSTTYEYDTSGGSGTCAYVIDTGVEASHPEFEGR
98-122	2534.1885		VLDNSGSGSYSGIISGMDFAVQDSK
98-124	2777.2483		VLDNSGSGSYSGIISGMDFAVQDSKSR
141-153	1303.6455		AQSVNDGAAAMIR
154-192	3740.7996	Cys181:3811.8401	AGVFLAVAAGNDNANAANYSPASEPTVCTVGATTSSDAR
263-284	2192.0811		NVLTGIPSGTVNYLAFNGNPSG

Accepted Manuscript

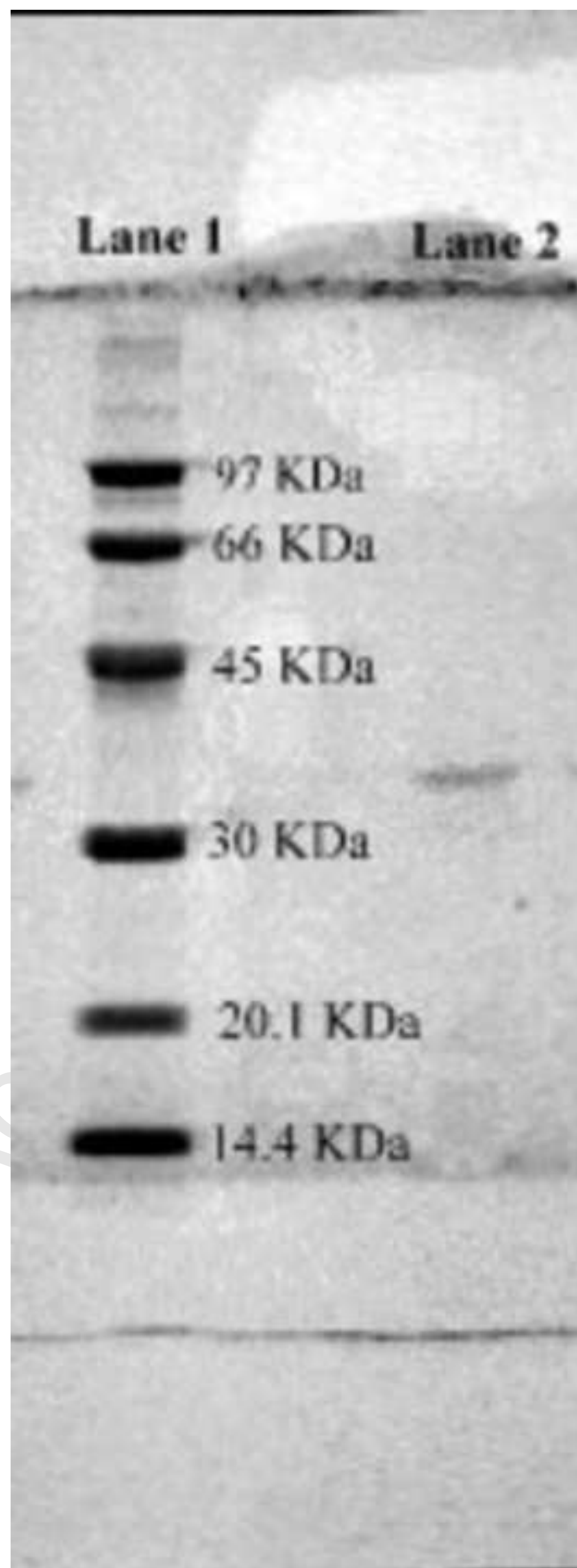
Figure(s)



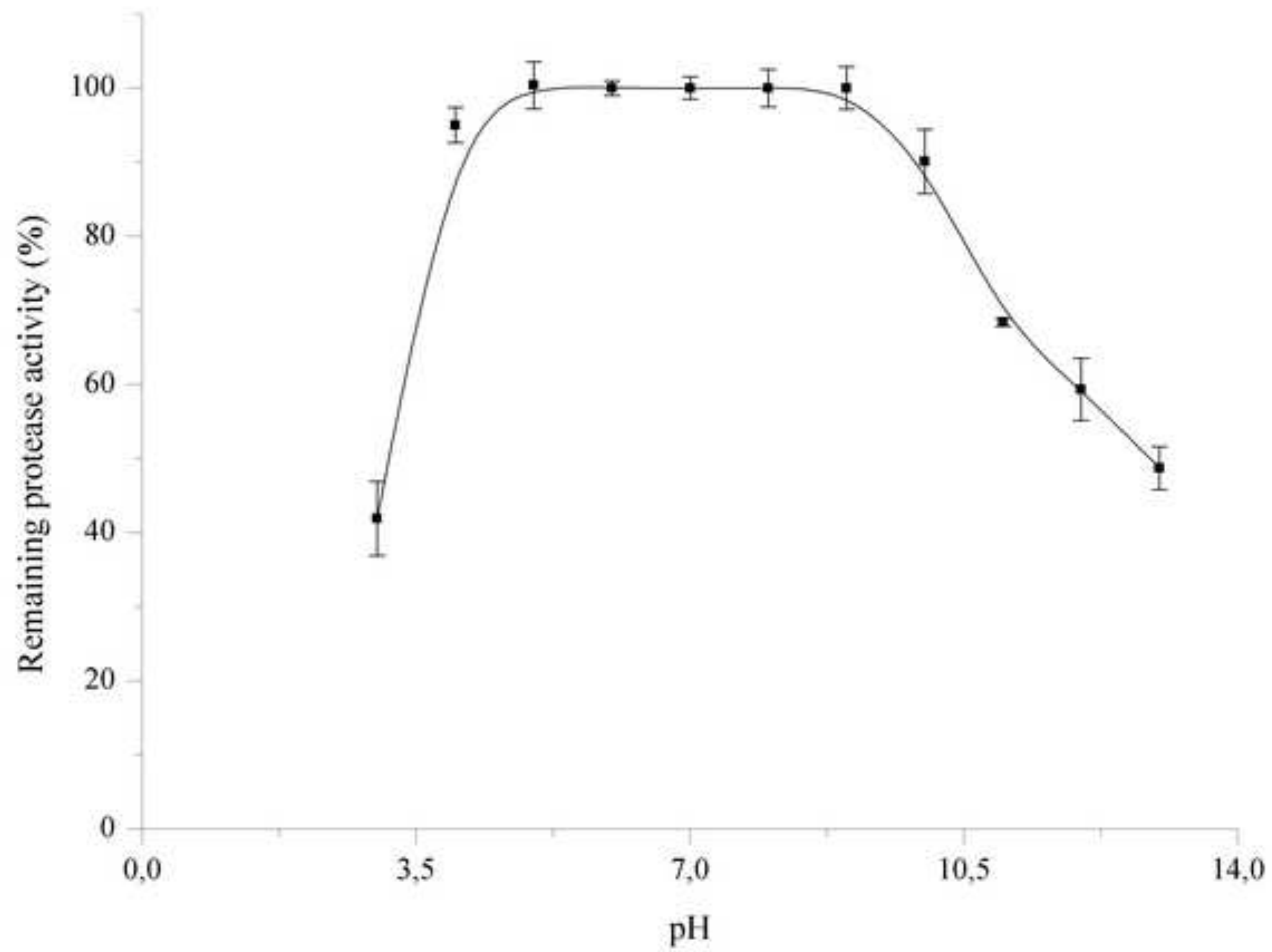
ACCEPTED MANUSCRIPT



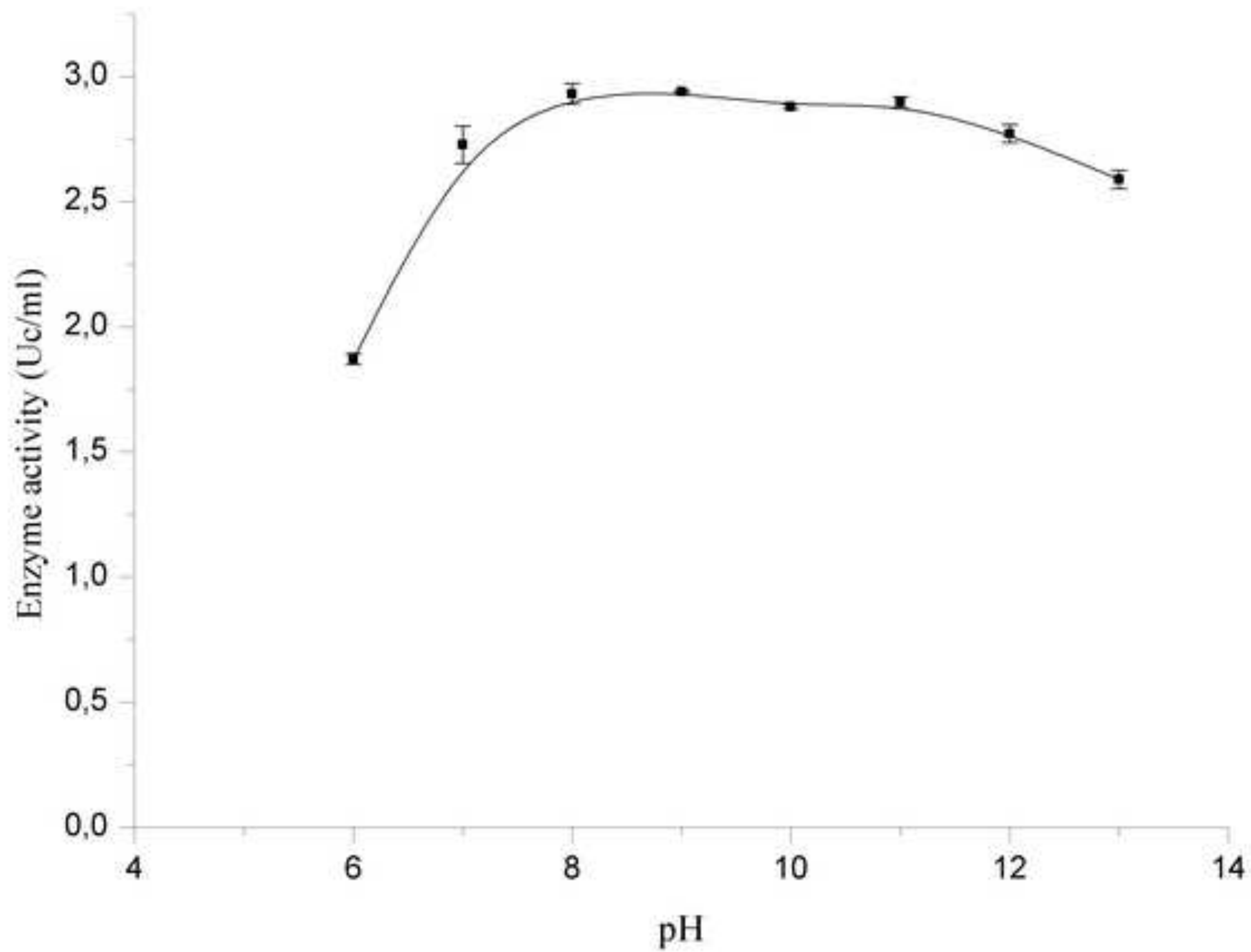
ACCEPTED MANUSCRIPT



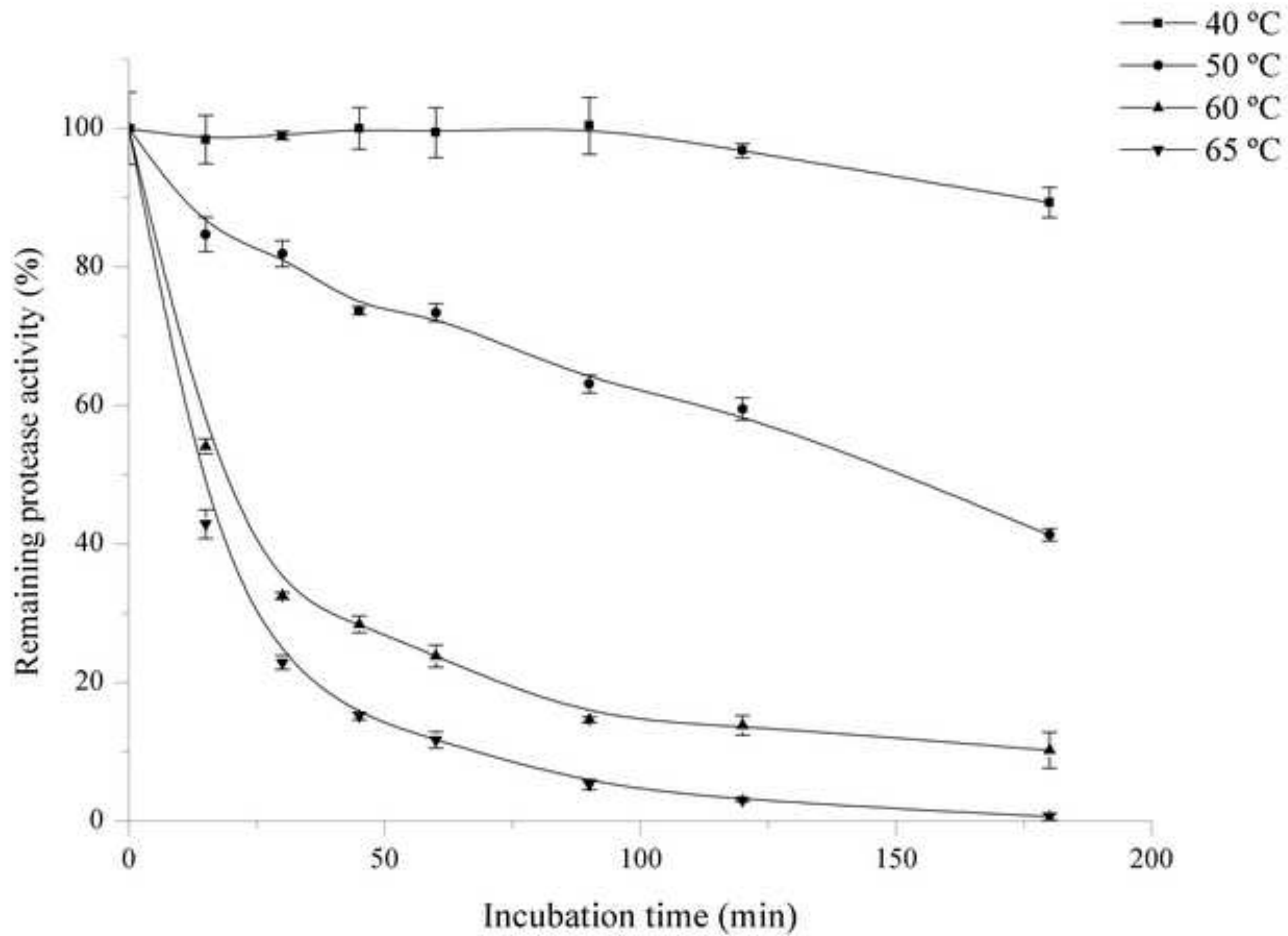
Figure(s)



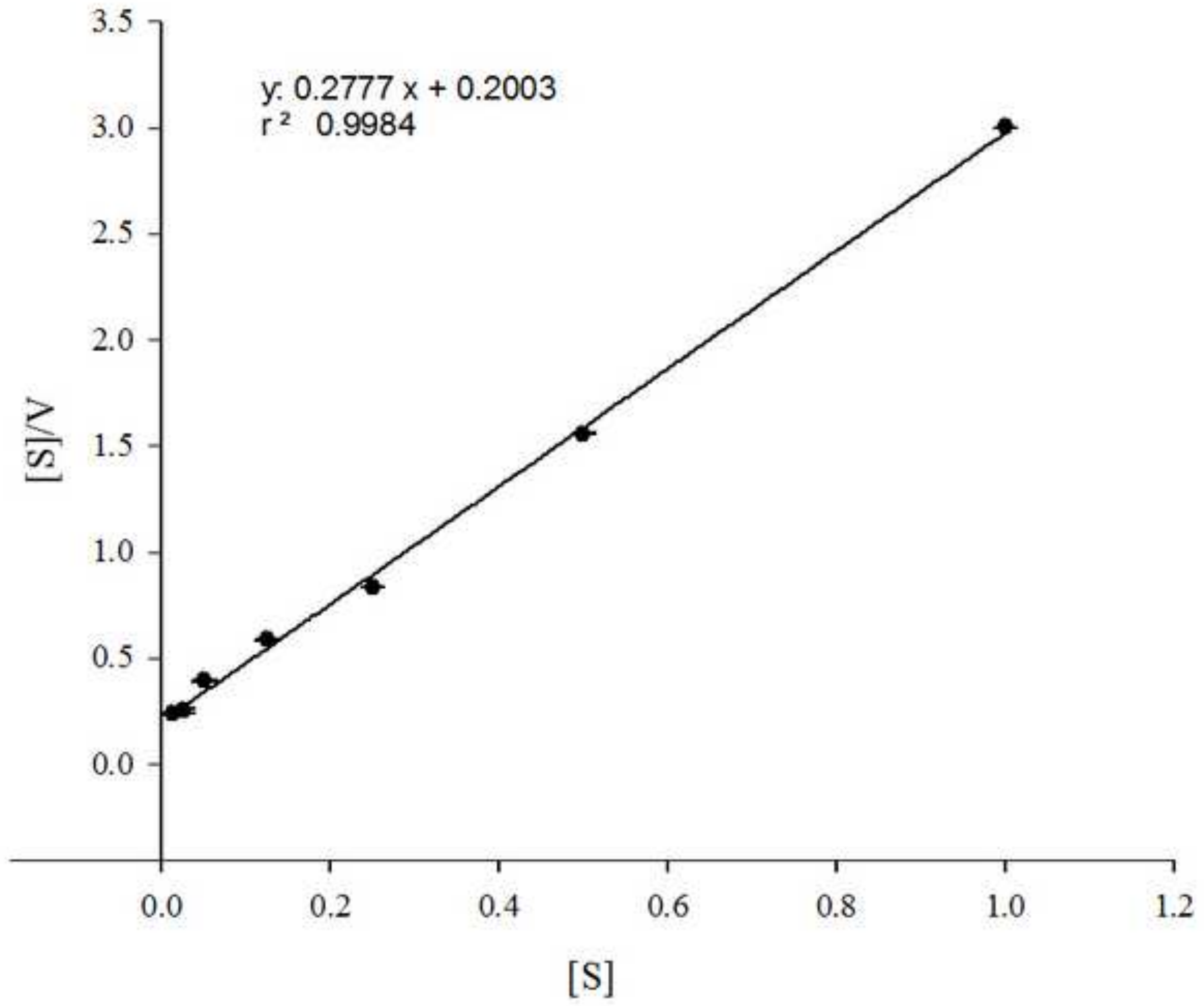
Figure(s)



Figure(s)



Figure(s)



ACCEPTED MANUSCRIPT

Fig. 1 (A) Time course of keratinolytic (▲), proteolytic (●), soluble protein (△) production and pH (○) of *P. lilacinus* in a basal hair medium. Error bars (±S.D.) are shown when larger than the symbol. (B) Scanning electron micrographs of hair waste degradation by *P .lilacinum*. Uninoculated hair fibres after hair-saving unhairing process using sodium sulfite/lime as unhairing agent; (C) degradation of hair fibres by the fungus after 5 days; it can be seen the colonization of *P. lilacinum* on hair surface.

Fig. 2 SDS-PAGE of *P. lilacinus* keratinase. Lane 1: purified keratinase. Lane 2: low molecular weight markers (KDa) Phosphorylase b (97), Albumin (66), Ovalbumin (45), Carbonic anhydrase (30), Trypsin inhibitor (20.1), α-Lactalbumin (14.4).

Fig. 3 Effect of pH on enzyme stability (A) and activity (B). The enzyme activity was measured at 37 ºC for 30 min using azocasein as substrate. Results represent the means of three experiments, and bars indicate ± standard deviation.

Fig. 4 Effect of temperature on enzyme stability. Keratinase was incubated at 40, 50, 60 or 65 ºC up to 180 min, withdrawing samples at different times. Remaining protease activity was measured under standard assay conditions. Results represent the means of three experiments, and bars indicate ± standard deviation.

Fig. 5 Hanes Hultin transformation plot of the purified enzyme using azocasein as susbtrate. *Error bars* correspond to standard deviations from triplicate replicas.

Accepted Manuscript