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BIOTECHNOLOGY AND INDUSTRY - RESEARCH PAPER

Biochemical features and biotechnological potential of a proteolytic extract from a psychrophilic Antarctic bacterium

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Abstract
Proteases are hydrolases that act on peptide bonds, releasing amino acids and/or oligopeptides, and are involved in essential functions in all organisms. They represent an important segment of the global enzyme market, with applications in the food, leather, detergent, and pharmaceutical industries. Depending on their industrial use, proteases should exhibit high activity under extreme conditions, such as low temperatures, *e.g.* cold-active protease may have potential uses in the detergent industry. Cold-active enzymes show high catalytic constants (k_{cat}) at low temperatures and thermolability, allowing their inactivation at moderate temperatures. This work aimed to characterize an extracellular proteolytic extract produced by an Antarctic isolate identified as *Flavobacterium* sp. strain AU13, and to evaluate its biotechnological potential as a detergent additive. By mass spectrometry analysis, we identified a major 50 kDa protease, with high identity with an epralysin from *Pseudomonas fluorescens* Pf0-1, an alkaline extracellular metalloprotease belonging to the serralysin subfamily. The AU13 proteolytic extract showed metalloprotease activity and, maximal activity over a wide pH range (pH 5 to 8); it also showed maximal activity at 40 °C, suggesting that this extracellular protease is a cold-active enzyme. The AU13 proteolytic extract demonstrated stable and compatible activity with surfactants and oxidants, making it a promising additive for commercial laundry detergents. Its ability to function effectively in cold-water washing conditions offers a significant advantage over conventional enzymes, potentially improving energy efficiency in industrial processes. The biochemical properties and performance of the AU13 proteolytic extract in the presence of laundry detergents, suggest that AU13 produces an extracellular protease with a biotechnological potential.

Keywords Antarctic bacterium · Cold-active enzymes · *Flavobacterium* sp. · Metalloprotease

Introduction

Proteases (also known as peptidases or proteolytic enzymes) are hydrolases that catalyze the cleavage of peptide bonds. They are involved in essential cellular and physiological processes in all organisms, including the digestion of diet proteins, protein turnover, post-translational protein processing, and cellular regulation [1]. Based on the functional group responsible for the catalysis, these enzymes can be classified as aspartic, cysteine, glutamic, metallo, serine, and threonine proteases [2]. For instance, an initial classification of the protease mechanism is achieved based on the enzyme susceptibility to group-specific protease inhibitors. A limited set of four inhibitors is used for the identification of the catalytic mechanisms, *e.g.* iodoacetamide (for cysteine-proteases), phenylmethylsulfonyl fluoride (PMSF, for serine-proteases), ethylenediaminetetraacetic acid (EDTA, for metalloproteases) and pepstatin (for aspartic-proteases) [3]. Another inhibitor, 1,10-phenanthroline, is preferred as a metal chelator for zinc metalloproteases because, unlike EDTA, it has a much lower affinity for calcium. This selectivity makes it more suitable for distinguishing between zinc-specific metalloproteases and calcium-dependent proteases, as it can effectively inhibit zinc-dependent enzymes even in the presence of 10 mM Ca^{2+}. However, this classification does not consider their structural diversity and the complexity of

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proteases. A more accurate and efficient classification system was proposed based on their structural and sequence features, reflecting their evolutionary relationship (MEROPS system) [4].

This group of enzymes has a wide range of substrates and conditions of activity that make them suitable for many biotechnological uses. For instance, they belong to one of the largest segments in the global enzyme market, with applications in the food, leather, detergent, and pharmaceutical industries [5]. They are obtained from animals, plants, and microorganisms. However, microbial proteases have a dominant role in industrial applications because most microbes are easily cultivated, and produce high amounts of enzymes in a limited cultivation area [6, 7]. In general, extracellular proteases are preferred to intracellular ones due to their simpler downstream processing, lowering production costs [5, 8].

Proteases have long been used in many industries. They broke into the detergent market in 1960 with the presentation of Alcalase® from Novozymes [9]. Among other characteristics, these enzymes must remove persistent proteinaceous stains caused by blood, grass, and food, avoiding aggressive cleaning methods, such as higher water temperatures or stronger chemicals, which can damage fabrics [10]. Therefore, these proteases should be active in surfactants, anti-redeposition agents, bleaching agents, and perfumes. Modern detergents include enzymes such as proteases, lipases, and glycosidases; multi-enzymatic systems improve washing performance and reduce environmental footprint [11]. In addition, the activity of these enzymes must be compatible with the components in the detergent and stable under the pH and temperature conditions expected during conventional washing.

In recent years, cold-water washing gained increasing popularity because it reduces energy costs [12]. Since 1998, some cold-active proteases, such as Kannase® and Polarzyme® (from Novozymes) and Purafect Prime® and Properase® (from Genecor), were developed and are now widely used in many commercial detergent formulations [13]. However, the search for novel enzymes with properties such as low-temperature activity and high stability/activity in a surfactant environment is currently an active area of investigation [14, 15]. Cold-active enzymes (also known as psychrophilic enzymes) have high catalytic efficiency at low temperatures and thermolability that allow their inactivation at moderate temperatures [16]. These properties enable cold-active enzymes to perform industrial processes at room temperature, offering economic benefits through energy savings [17]

Where to find cold-active enzymes? In cold habitats, such as polar regions, on top of mountains, or Frigidaire, among others. Natural cold habitats constitute most of the Earth´s area and present a diversity of psychrophilic/psychrotolerant organisms that can thrive at low temperatures [18]. Among different biochemical adaptations to low temperatures [19], organisms synthesize cold-active enzymes. Previously, we searched for cold-active enzyme-producing bacteria in the Antarctic environment [20–22] and, characterized some of these enzyme's biochemical and biotechnological potential. Regarding proteases, we reported the recombinant production, purification, and biochemical characterization of an extracellular metal-dependent serine-protease produced by a psychrotolerant Antarctic isolate identified as *Pseudomonas* sp. AU10 [23]. However, a second bacterium emerged as a candidate for producing a highly cold-active and detergent-tolerant protease. This bacterium showed an increased proteolytic activity in the presence of surfactants, as compared with the protease produced by *Pseudomonas* sp. AU10.

Thus, the current work aimed to characterize the extracellular proteolytic extract produced by a psychrophilic Antarctic bacterium, identified as *Flavobacterium* sp. strain AU13 by Martínez-Rosales & Castro-Sowinski [21]. We evaluated a few biochemical properties and the biotechnological potential of the extracellular proteolytic extract in the detergent industry. We show information that suggests AU13 produces a protease that could be included in the portfolio of enzymes for a new laundry formulation release.

Material and methods

Proteolytic microorganism

The bacterial isolate AU13 was obtained from a water sample collected from a temporal gully located near the Base Científica General Artigas (Fildes Peninsula, King George Island, South Shetland Islands), Antarctica (62°11′4" S, 58°51′7" W). This is an extracellular-protease-producing strain previously identified as a member of the genus *Flavobacterium* by amplification and sequencing of the 16S rDNA encoding gene [21].

Production of the proteolytic extract

The microorganism was grown in 5 mL Luria Bertani (LB; 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) medium until the exponential phase and used as pre-inoculum. To produce the proteolytic extract, the pre-inoculum was transferred to 0.2 L of 20-fold diluted LB medium supplemented with 5% (w/v) of skim milk, and cells were grown at 16 °C and 200 rpm, and culture was observed daily until the clotting of milk casein was evident. Then, we separated the cells and the proteolytic extract by centrifugation (35 min at 6,654 g and 10 ºC), and the supernatant was filtered through a 0.45 µm membrane. The resulting cell-free supernatant was lyophilized in 10 mL fractions and stored at

−20 °C until used. For subsequent studies, each fraction was dissolved in 2 mL of 0.2 M sodium phosphate buffer, at pH 6.0, and referred to as AU13 proteolytic extract (AU13PE).

Protease assay

The proteolytic activity was determined by the modified method of Andrews and Asenjo [24]. Briefly, we incubated 340 µL of 1% (w/v) azocasein (prepared in water) with 340 µL of activity buffer (0.2 M sodium phosphate buffer, at pH 7.5) at 30 ºC for 5 min; then, we added 340 µL of an appropriate dilution of AU13PE, and after 10 min at 30 °C we stopped the reaction by adding 340 µL of 10% (w/v) trichloroacetic acid. After 20 min at 30 °C, the mixture was centrifuged (13,200 g and 25 ºC for 30 min), and the absorbance of the supernatant was recorded at 337 nm. One proteolytic activity unit (U) was defined as the amount of proteolytic extract required to cause a change of 1 unit of absorbance (337 nm) per minute, under the experimental conditions.

Protein quantification

Protein quantification was performed by the Bradford method [25], using a standard curve of bovine serum albumin (BSA) in the concentration range of 0.1 to 1.0 mg/mL.

Denaturing gel electrophoresis (SDS-PAGE)

The protein profile of AU13PE was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [26], using 12% and 5% polyacrylamide as resolving and stacking gels, respectively. Samples were boiled for 5 min in sample buffer containing 5% (v/v) 2-mercaptoethanol, and separated at 20 mA for 60 min. We visualized the protein bands by staining with Coomassie Blue R-250.

Protein identification by mass spectrometry

The major protein band was cut for further identification by mass spectrometry (performed by the Service of the Unidad de Bioquímica y Proteómica Analíticas, Institut Pasteur de Montevideo). In-gel tryptic digestion and MALDI-TOF MS/MS analysis, using a 4800 MALDI TOF/TOF equipment (Applied Biosystems), was done as described by Piñeyro et al. [27]. The protein was identified by NCBI protein database searching with peptide m/z values using the MASCOT Software.

Gelatin zymogram technique

Samples of AU13PE (without boiling, non-reducing conditions, and diluted in sodium dodecyl sulfate (SDS) sample buffer) were loaded onto 11% polyacrylamide gel co-polymerized with 0.1% (w/v) gelatin (JT Baker 2124–01) containing SDS under nonreducing conditions [28]. Electrophoresis was carried out in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) for 60 min at 20 mA and 4 °C. After electrophoresis, the gel was cut into two strips, which were then washed and developed either without additives or with 10 mM 1,10-phenanthroline. Washing was carried out by incubating the gel strips in 0.2 M phosphate buffer (pH 6.0) containing 2.5% (v/v) Triton X-100 for 1 h at room temperature (either without additives or with 1,10-phenanthroline). The strips were then developed in 0.2 M sodium phosphate buffer, pH 6.0 (either without additives or with 1,10-phenanthroline) for 45 min at 37 °C. Finally, the gels strips were stained with 0.025% (w/v) Coomassie Blue R-250 prepared in 10% (v/v) acetic acid and proteolytic bands were visualized as clear bands on a blue background. BSA (A2153, Sigma) and thermolysin from *Geobacillus stearothermophilus Rokko* (T7902, Sigma) were used as negative and positive controls, respectively.

The effects of temperature on proteolytic activity and thermostability

The effect of temperature on activity was assayed as outlined previously (Protease assay) at different temperatures, from 15 to 60 °C (with 5 °C intervals), at pH 6.0 using azocasein as substrate.

The thermostability was studied by incubating AU13PE for 0, 15, 30, 60, 90, 120, 180, 240, and 300 min at 25 or 40 °C, followed by a fast cooling in an ice bath, until residual activity was determined at 30 ºC, as described above.

The effect of pH on the proteolytic activity and stability

The effect of pH on the activity of the AU13PE was studied over a pH range of 4.5 to 10, at 30 ºC, and using azocasein as substrate. We used three-component (acetic acid/MES/Tris buffer) constant ionic strength buffers (I = 0.1M), as described by Ellis and Morrison [29]. The buffer contains 0.05 M acetic acid, 0.05 M MES, 0.1 M Tris with variable amounts of HCl or NaOH to adjust the pH in the range of 4.5 to 10.0. We also used the ACES/Tris/ethanolamine buffer (0.1 M ACES, 0.051 M Tris, 0.051 M ethanolamine) with variable amounts of HCl or NaOH to adjust the pH in the range of 9.0 to 10.0. The pH stability was studied by incubating AU13PE for 0, 60, and 180 min at pH 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 (at 25 or 40 °C). After incubation, the residual activity was determined at pH 6.0 and 30 ºC as outlined previously.

The effect of inhibitors on proteolytic activity

The AU13PE (0.15 U) was independently pre-incubated with the following protease inhibitors: iodoacetamide (100 μM), PMSF (1 mM), EDTA (10 mM), 1,10-phenanthroline (10 mM) and pepstatin (1 mM), for 15 min at 30 ºC. After incubation, we determined the proteolytic activity as described above. Finally, we determined the residual activity. The activity without inhibitor represented 100% activity.

The effect of surfactants and oxidizing agents on proteolytic activity

The activity of AU13PE was assayed in the presence of different surfactants such as Tween 20%, Tween 80%, and Triton X 100 at final concentrations of 7 and 20% (v/v). The effect of an oxidant on the activity was determined using hydrogen peroxide (H_2O_2), at final concentrations of 3, 10, 20, and 50% (v/v). The results were expressed as a percent, relative to the proteolytic activity without a detergent or oxidant (100% of activity).

Laundry detergent compatibility and stability of the AU13PE

The activity of AU13PE was determined in the presence of different commercial laundry detergents (Ariel®, Nevex Matic®, Nevex®, Vivere®, Persil®, Skip White®, and Skip Black®). Detergents were diluted with tap water to a final concentration of 1% (w/v or v/v), frozen for 24 h, and treated at 100 °C for 15 min to inactivate any enzymes they might contain. The enzyme-inactivated detergent solutions (at a 0.5% final concentration) were mixed with AU13PE (0.04 U), and used for compatibility and stability assay. We determined the protease activity at pH and temperature of maximal activity, expressing results as percent, compared with the proteolytic activity of control experiments without adding the detergent (100% activity). The stability was determined after 1 h incubation at 25 °C (a regular time and temperature of home washing).

Statistical analysis

All experiments were performed using two biological independent replicates and at least three technical replicates. Results were expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey's test. Means were different at a significance level of 0.05.

Results

Production and identification of the extracellular protease produced by *Flavobacterium* sp.

Flavobacterium sp. AU13 grown in a liquid medium supplemented with milk resulted in the coagulation of milk proteins after 8 days of incubation at 16 °C, evidencing the production of proteins with proteolytic activity. The activity of the cell-free supernatant was 0.020 ± 0.003 U/mL, and the total protein concentration was 1.5 ± 0.1 mg/mL. Upon freeze-dried of 10 mL AU13PE, we suspended the powder in 2 mL 0.2 M phosphate buffer at pH 6.0 (5 × concentrated) and, determined the activity, detecting a nearly 20-fold increase in proteolytic activity (0.37 ± 0.07 U/mL) and an almost five-fold increase in total protein concentration (6.7 ± 0.1 mg/mL).

We analyzed the profile of extracellular proteins produced by AU13 performing an SDS-PAGE analysis of AU13PE. The results show that AU13 mainly produces a 48 kDa extracellular protein (Fig. 1, lane 1). The 48 kDa protein was analyzed by peptide mass fingerprinting, showing the best match with an epralysin (extracellular alkaline metalloprotease from *Pseudomonas fluorescens* Pf0-1) (Fig. 2). According to the results, four peptides matched the epralysin sequence, but only one of them had a statistically significant match (score > 46).

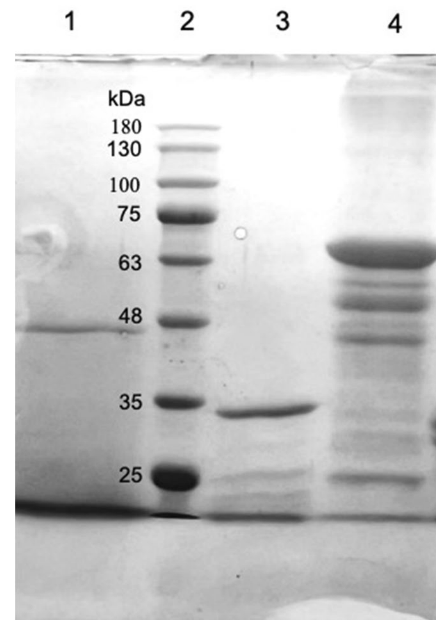

Fig. 1 Denaturing electrophoresis (SDS-PAGE). Lane 1: AU13PE (10 μg protein); lane 2: molecular weight marker; lane 3: Thermolysin (10 μg protein); lane 4 bovine serum albumin (10 μg protein)

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Protein hits   : gi|77458903  epralysin [Pseudomonas fluorescens Pf0-1]
                 gi|296138753 integrase [Tsukamurella paurometabola DSM 20162]
                 gi|294934066 hypothetical protein Pmar_PMAR018009 [Perkinsus marinus ATCC 50983]
                 gi|159898801 hypothetical protein Haur_2280 [Herpetosiphon aurantiacus DSM 785]
                 gi|21220960  transport ATPase [Streptomyces coelicolor A3(2)]
                 gi|338782050 arginyl-tRNA synthetase [Achromobacter xylosoxidans AXX-A]
                 gi|118588062 Transcriptional regulator [Stappia aggregata IAM 12614]
                 gi|307596174 hypothetical protein Vdis_2070 [Vulcanisaeta distributa DSM 14429]
                 gi|260428060 sensory box sensor histidine kianse/response regulator [Citreicella sp. SE45]
                 gi|254381629 luxR family two-component response regulator [Streptomyces sp. Mg1]
                 gi|312111862 2-oxoglutarate dehydrogenase, E1 subunit [Geobacillus sp. Y4.1MC1]
                 gi|159899479 PASTA sensor-containing serine/threonine protein kinase [Herpetosiphon aurantiacus DSM 785]
                 gi|91779858  hypothetical protein Bxe_B0227 [Burkholderia xenovorans LB400]
                 gi|94310895  gamma-glutamyltransferase 1 [Cupriavidus metallidurans CH34]
                 gi|71003998  hypothetical protein UM00518.1 [Ustilago maydis 521]
                 gi|77553311  zinc finger family protein, putative, expressed [Oryza sativa Japonica Group]
                 gi|66807347  LSM domain-containing protein [Dictyostelium discoideum AX4]
                 gi|239608434 conserved hypothetical protein [Ajellomyces dermatitidis ER-3]
                 gi|154243787 TetR family transcriptional regulator [Xanthobacter autotrophicus Py2]
                 gi|195112971 GI22194 [Drosophila mojavensis]
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gi|77458903      Mass: 50139     Score: 167     Expect: 3.3e-10  Matches: 4
epralysin [Pseudomonas fluorescens Pf0-1]
 Observed    Mr(expt)   Mr(calc)    Delta Start   End Miss Ions  Peptide
1460.6465  1459.6392  1459.6328   0.0064   276 - 289   0   40   R.TGDTTYGFNSNAGR.D
2021.9817  2020.9744  2020.9775  -0.0031   247 - 266   0   ---  K.GGVEAYSSGPLMDDIAAIQK.L
2037.9744  2036.9671  2036.9725  -0.0053   247 - 266   0   ---  K.GGVEAYSSGPLMDDIAAIQK.L + Oxidation (M)
2373.2468  2372.2395  2372.2448  -0.0053    43 -  65   0  112   R.GGNLTVNGKPSFSVDQAATQLLR.D
No match to: 903.5632, 908.9877, 984.2137, 990.4791, 991.4994, 1044.0934, 1050.1030, 1054.4990, 1060.0671, 1066.0778, 1070.4861, 1082.0500, 1088.0599, 1098.0242, 1193.6490,
1222.6241, 1251.7159, 1267.7124, 1277.1042, 1293.0754, 1299.7119, 1309.0490, 1320.6674, 1337.6842, 1367.6837, 1384.7245, 1437.8020, 1439.7623, 1482.6285, 1532.8153, 1635.7802,
1708.8912, 1900.8713, 1961.8958, 2059.9653, 2068.9514, 2090.0486, 2112.0400, 2128.0122, 2139.9880, 2208.0996, 2211.1023, 2215.9863, 2224.0857, 2231.9519, 2247.0801, 2314.2627,
2324.1057, 2374.2363, 2409.1855, 2424.1777, 2539.3276, 2562.3149, 2600.1799, 2675.2036, 2723.3606
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Fig. 2 Protein identification by peptide fingerprint mapping (MALDI-TOF–MS/MS). The summary of the list obtained by MALDI-TOF analysis with the MASCOT search engine is shown. The first 20 hits of the analysis and a list of the peptides matching the first protein are shown. The Mascot gave us a score greater than 50 as significant ($p < 0.05$), and the protein with the high score of 155 is a 50 kDa epralysin from Pseudomonas fluorescens Pf0-1

We evaluated if AU13 produces one or more extracellular proteases performing a specific protease zymogram, as described in Materials and Methods. The results shown in Fig. 3a reveal the presence of a single band with activity (lane 1). We used thermolysin (lane 3) and BSA (lane 5) as positive and negative controls, respectively. As expected, thermolysin hydrolyzed gelatin, while BSA did not show any gelatinolytic activity. Furthermore, the proteolytic band from AU13PE was confirmed as a metalloprotease, as its gelatinolytic activity was completely inhibited by 10 mM

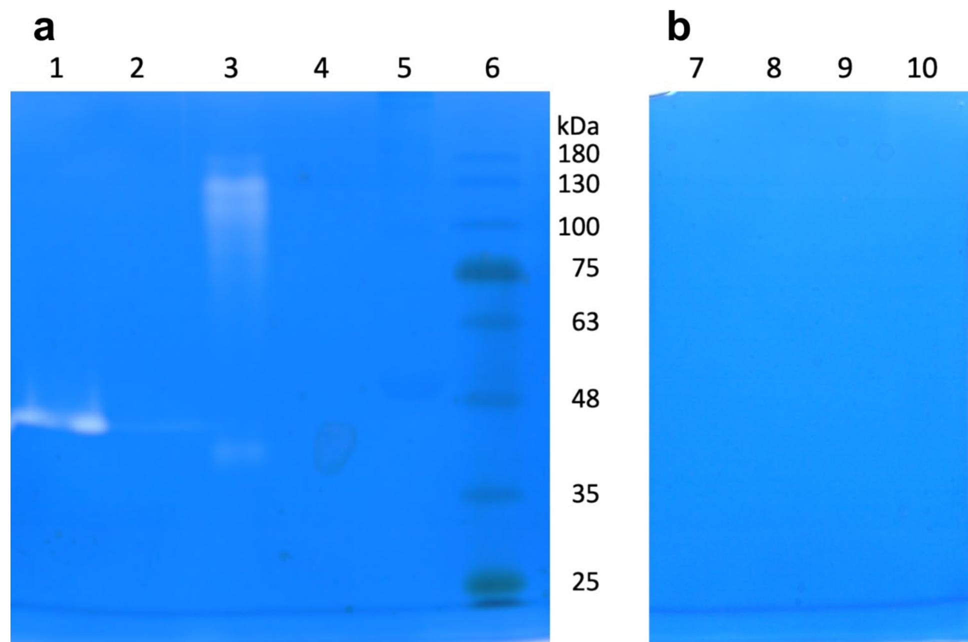

Fig. 3 Zymogram on SDS–polyacrylamide gel (11%) co-polymerized with 0.1% (w/v) gelatin. Following electrophoresis, the gel was cut into two strips which were washed and developed either without additives (**a**) or with 10 mM 1,10 phenanthroline (**b**). Lane1 and 8: AU13PE (5 µg protein); lane 3 and 10: thermolysin as positive control (2.5 µg protein); lane 5: bovine serum albumin as negative control (15 µg protein); lane 6: molecular weight marker. In lanes 2, 4, 7 and 9, no sample was loaded. Clear zones, where the gelatin in the gel has been degraded by proteolytically active bands, are visualized against a dark blue background after staining the gel with Coomassie Brilliant Blue

1,10 phenanthroline (lane 8, Fig. 3b). Similarly, the proteolytic activity of thermolysin (lane 10, Fig. 3b) was also fully inhibited by the metalloprotease inhibitor.

Characterization of the AU13PE

To evaluate the potential of AU13PE for industrial detergent applications, we investigated its operational properties, including temperature and pH activity profiles.

Temperature-activity profile and thermostability Results show that AU13PE remains active over a wide range of temperatures (15 to 50 ºC) with maximum activity at 40 ºC (Fig. 4a). At 60 °C (optimum temperature of many mesophilic enzymes) the activity of AU13PE dropped abruptly. Thus, AU13PE showed a characteristic activity-temperature profile of psychrophilic enzymes [30]. The proteolytic activity was highly stable (residual activity above 79.9% ± 1.5) after 5 h of incubation at pH 6.0, at 25 or 40 ºC (Fig. 4b).

pH-activity profile The AU13PE was active over a wide pH range (4.5 to 10.0) with a maximum at pH 6.0, at 30 ºC (Fig. 5). The lowest values of activity were obtained at pHs 9.5 and 10, where it showed 67.7% ± 4.5 and 59.3% ± 6.9 activity. The AU13PE retained over 86.5% ± 3.8 of the proteolytic activity after incubation at a pH range from 5.0 to 10.0 for 180 min, both at 25 and 40 ºC.

Effect of inhibitors The AU13PE was significantly inhibited by EDTA (74.2% ± 18.2) (Fig. 6) and fully inhibited by 1,10 phenanthroline (98.5% ± 0.5), suggesting the presence of at least one zinc-dependent metalloprotease, consistent with its potential identification as an epralysin. In contrast, PMSF, iodoacetamide, or pepstatin did not significantly affect the proteolytic activity. This result suggests that there are no serine, cysteine, or aspartic proteases in AU13PE.

AU13PE compatibility with detergents

To further investigate the potential of AU13PE as a detergent additive, we evaluated its compatibility with key detergent components as well as with commercial laundry detergents.

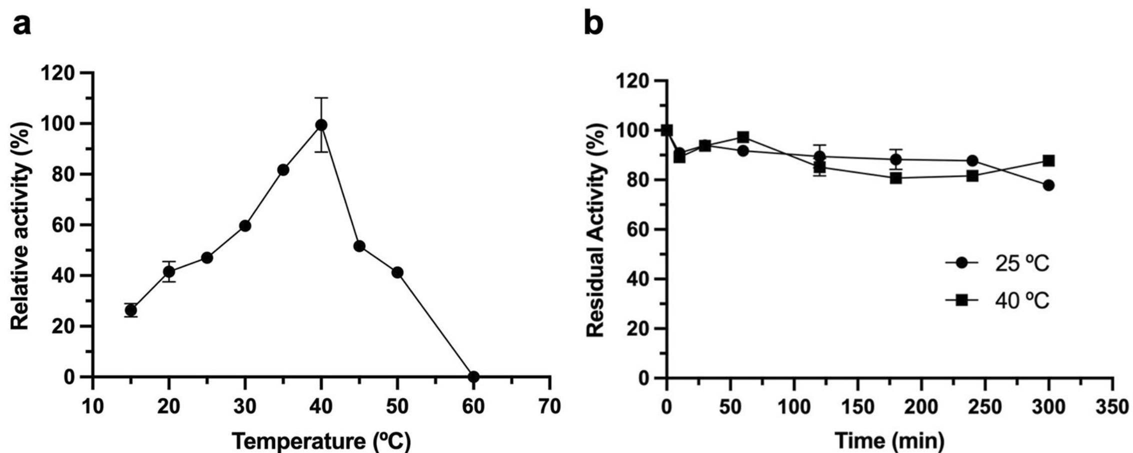

Fig. 4 (**a**) Temperature-activity profile of the AU13PE. The assays were performed at pH 6.0. (**b**) Thermostability of AU13PE. The standard deviation of each independent measurement is represented by vertical bars

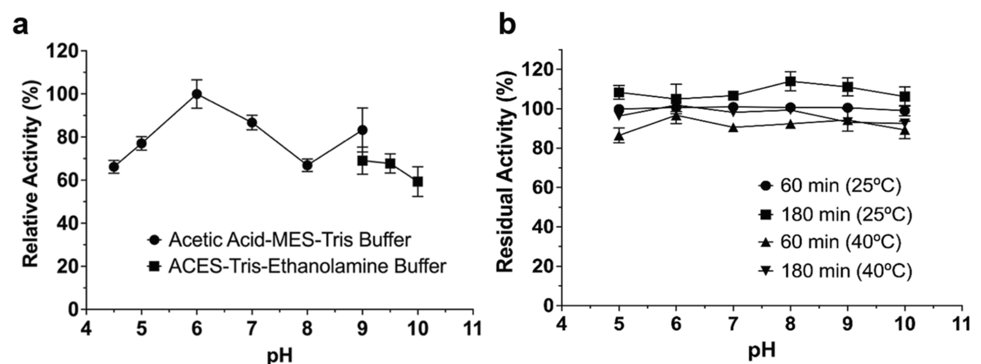

Fig. 5 pH-activity profile of the AU13PE. The assays were performed at 30 ºC. The standard deviation of each independent measurement is represented by vertical bars. In some cases, the deviation is smaller than the symbol

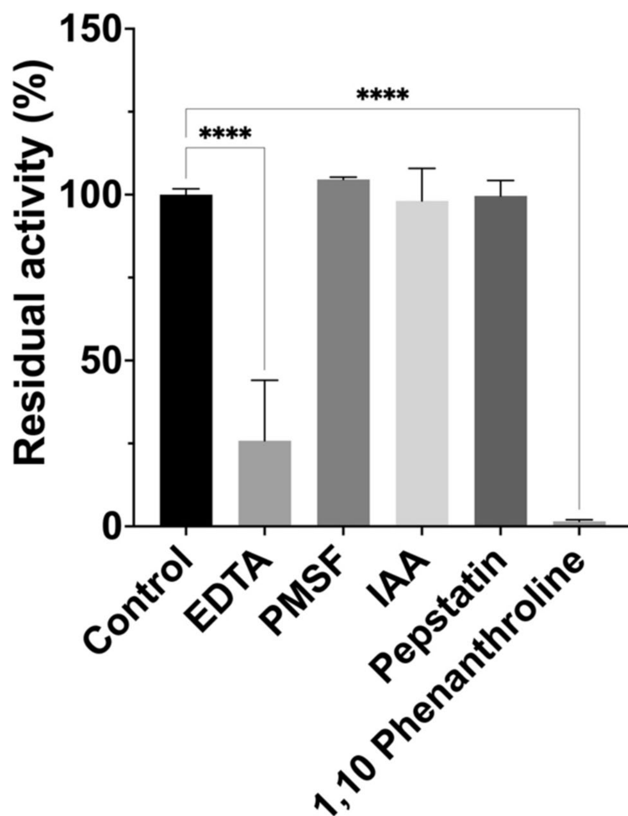

Fig. 6 Effect of inhibitors on AU13PE activity. The final concentrations of inhibitors in the pre-incubation mixture with AU13PE were: 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µM Iodoacetamide (IAA), 1 mM pepstatin, and 10 mM 1,10 phenanthroline

Effect of detergent components on proteolytic activity Surfactants and oxidizing agents are present in commercial laundry detergents, so it is essential to analyze how they affect the enzymatic activity of AU13PE under study. The results of these assays are shown in Fig. 7. We observed an increase in activity in the presence of all surfactants (Fig. 7a and b), even at the highest concentration tested (20%). A maximal activation effect was observed for 20% Triton X-100, causing a two-fold increase in the activity of AU13PE (Fig. 7b). The effect of hydrogen peroxide on the proteolytic activity was dependent on the oxidant concentration. We observed an increase in activity (40.4% ± 8.1 and 27.7% ± 7.3) in the presence of the oxidant at concentrations between 3 and 10%. However, a clear decrease in activity was observed at higher concentrations (Fig. 7c).

Evaluation of AU13PE as laundry detergent additive To evaluate the potential use of AU13PE as a detergent additive, its enzymatic activity was tested in the presence of different commercial laundry detergents, as described in Materials and Methods. As shown in Fig. 8, AU13PE showed high stability and compatibility with a wide range of laundry detergents. The proteolytic activity was not affected or significantly increased when tested within Ariel® (35.0% ± 0.4) and Nevex Matic® (51.8% ± 6.1) (Fig. 8a).

Stability studies indicated that AU13PE retained above 80% of its maximal activity after 1 h at 25 ºC in the presence of 0.5% Nevex Matic® (85.4% ± 2.6) and Persil® (89.9% ± 1.0). However, AU13PE showed moderate stability in the presence of Nevex Vivere® (72.6% ± 1.5), Skip Black® (70.9% ± 0.1), and Skip White® (59.3% ± 2.6).

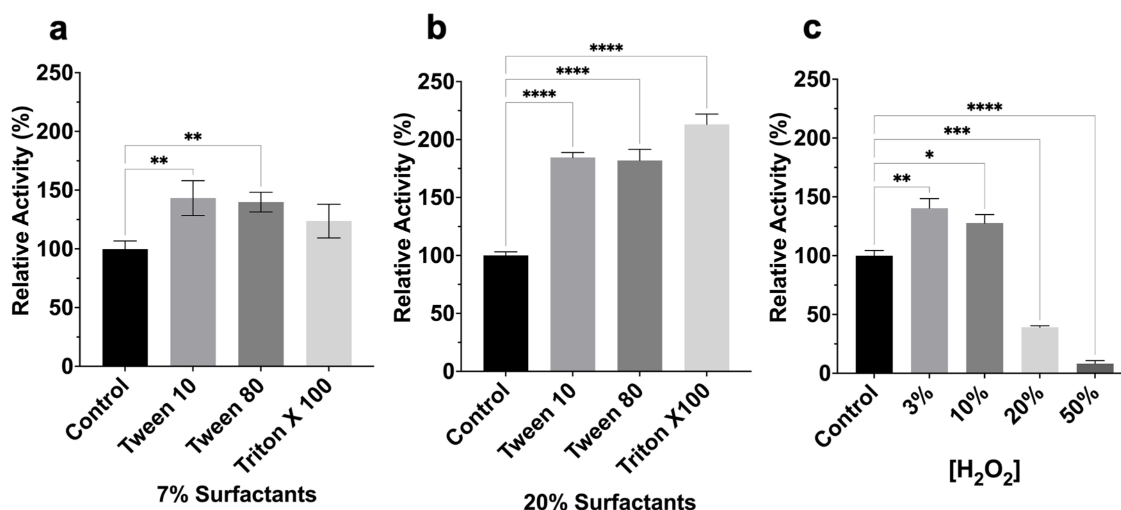

Fig. 7 Effect of detergent components on proteolytic activity. The activity of the AU13PE (0.05 U) was assayed at 40 °C for 30 min in the presence of: (**a**) different laboratory detergents at concentrations of 7% and (**b**) 20%; (**c**) H_2O_2 as oxidizing agent at concentrations of 3, 10, 20 and 50%. Results are expressed as the relative activity with respect to the control without surfactant or oxidant

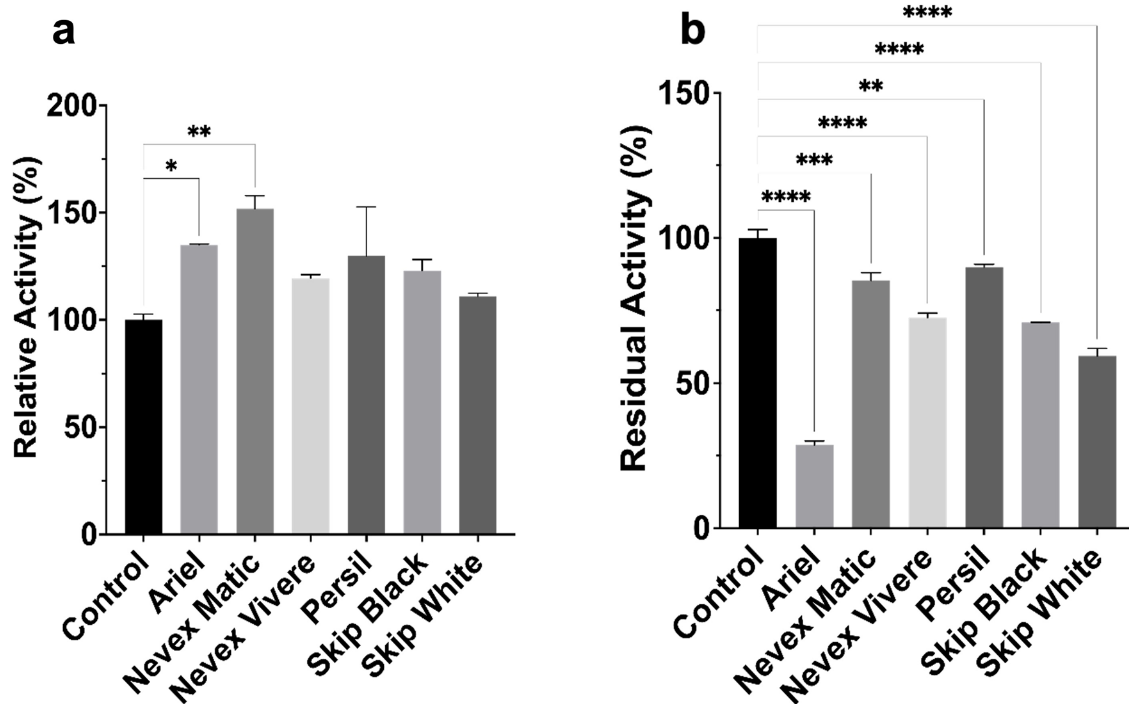

Fig. 8 Compatibility of the AU13PE with laundry detergents. **(a)** AU13PE activity (0.04 U) in the presence of different commercial detergents at a final concentration of 0.5%. The results are expressed as relative activity (%) with respect to the control without detergent. This test shows how well the proteolytic activity performs in the presence of common detergents. **(b)** Stability of the activity in the presence of detergents. The AU13PE (0.04 U) was incubated with the different detergents for 1 h at 25 °C and residual activity (%) was determined. The results are expressed as percentage of residual activity with respect to the control without detergent. These results show how stable the proteolytic activity is when exposed to detergents, indicating its potential use in cleaning or laundry applications

Worst performance was achieved with Ariel® (28.7% ± 1.4) (Fig. 8b).

Overall, *Flavobacterium* sp. AU13 produces at least a 48 kDa extracellular protease identified as an epralysin-like metalloprotease. The proteolytic milieu (AU13PE) showed maximal activity at 40 °C and pH 6.0, and it was compatible with some commercial laundry detergents. These findings underscore the potential of AU13PE as a highly effective and stable additive for laundry detergent formulations.

Discussion

Antarctica is a great source of psychrophilic and psychrotolerant organisms, that have adapted to living at low temperatures. Among these adaptations is the production of psychrophilic or cold-adapted enzymes [31] that can eventually be used for biotechnological purposes [32]. Herein we present the proteolytic potential of an Antarctic bacterium, previously identified as *Flavobacterium* sp. isolate AU13, which is a psychrotrophic extracellular protease-producing bacterium when growing at least between 4 °C and 18 °C, with an optimum growth temperature at 16 °C [21].

We found that AU13 produces an extracellular 48 kDa protein (Fig. 1), being the most abundant band in the SDS-PAGE analysis and exhibits gelatinolytic activity characteristic of a zinc-dependent metalloprotease, as shown in the zymogram (Fig. 3). This protein matches an epralysin produced by *Pseudomonas fluorescens* (Fig. 2). Although the peptide match suggests the presence of an epralysin, further validation through genome sequencing is necessary to confirm this identification.

Epralysins (MEROPS Database Identifier: M10.060) are alkaline metalloproteases belonging to the M10 metalloendoprotease family (M10B subfamily). These proteases are extracellular proteins, which usually have a nutritional role or degrade cell matrix proteins [33]. The bacterial epralysins (M10.060) reported so far are produced by *Pseudomonas* strains and belong to the serralysin M10B subfamily of proteases as found in MEROPS (version 12.4). However, there is a report about a psychrophilic *Flavobacterium* sp. strain YS-80–122 [34] that produces a psychrophilic alkaline metalloprotease that was identified as a member (M10.062) of the M10B subfamily [35]. Moreover, *Flavobacterium psychrophilum* [36], *Flavobacterium frigidimaris* ANT34-7 [37], and *Flavobacterium balustinum* P104 [38] produce metalloproteases different from epralysins. We searched for

epralysins produced by Arctic and Antarctic microbes, and to the best of our knowledge, this is the first report of an epralysin-producing Polar microbe. We found that AU13 cells produce and secrete at least one metalloprotease into the extracellular milieu (Figs. 1 and 3). This is not rare, since many Antarctic and non-Antarctic *Flavobacterium* strains produce extracellular proteases [38–42].

In our study, we tested a range of inhibitors targeting different catalytic types of proteases (Fig. 6). These results confirmed that AU13PE contains at least one metalloprotease, supporting that the 48 kDa protein could be an epralysin. While EDTA, a general metal chelator, did not result in complete inhibition of activity under the tested conditions, full inhibition was achieved using 1,10 phenanthroline, a chelating agent of high affinity for zinc ions. Despite this, we cannot exclude the possibility of other minor extracellular proteases in AU13PE, with different catalytic mechanisms. Further experiments, considering factors such as EDTA concentration or incubation time [43], may provide more insight. Nonetheless, the inhibition results with 1,10 phenanthroline support the presence of a zinc-dependent metalloprotease.

In general, epralysins can hydrolyze azocasein in a pH range of 7 and 9, and 30 and 37 °C [33], and we found that the AU13PE has its best hydrolytic activity on azocasein at 40 °C and, completely loses activity at 60 °C (Fig. 4a), supporting its psychrophilic nature [19]. Compared to psychrophilic proteases produced by *Flavobacterium* strains, such as *F. balustinum* [38] and *F. psychrophilum* [36] (optimum temperature at 40 °C and loss activity at 60 °C), we found that AU13PE shows a similar temperature behavior.

In addition, the AU13PE proteolytic activity was stable at 25 and 40 °C for 3 h (90% residual activity, Fig. 4b), like the purified protease produced by *F. balustinum,* which retains 60% activity after 1 h at 40 °C [38]. In contrast, the protease produced by *F. psychrophilum* completely lost activity after 1 h at 40 °C [36]. The range of thermal-stability of AU13PE proteolytic activity suggests its potential as a component during laundry.

The robustness of the proteolytic activity to pH variations would be a desirable feature in an enzyme with potential biotechnological use in the detergent industry since it would not be significantly inactivated at the pH at which conventional laundry washing is performed. The optimum pH of the protease should be close to that of a detergent solution [44]. Although the AU13PE presented an optimum pH of 6.0 (Fig. 5a), it had high activity at alkaline pH values (the relative activity remained above 50% in the pH range evaluated). Since the pH of laundry detergents is alkaline, proteases and other enzymes currently used in these detergents should show good activity at alkaline pH values [45]. When comparing the data with those from the proteases produced by *F. balustinum* P104 [38], *F. frigidimaris* ANT34-7 [37], and *F. psychrophilum* [36] (best activities at pH 8.0 and 30 °C, at pH range 7.0 to 10.0 and 25 ºC and, at pH 6.5 at 37 °C, respectively), AU13PE would show biochemical characteristics more likely related to the protease of *F. pychrophilum,* a psychrophilic protease. This suggests that AU13PE, as a psychrophilic protease preparation, could retain its activity at lower temperatures. This would be an advantageous feature for industrial processes that require energy-efficient enzyme use, such as detergents formulated for cold-water washing.

To further explore its potential for laundry applications, we also evaluated the compatibility of AU13PE with common components of commercial detergents (surfactants and bleaches). These assays are essential for selecting proteases as potential laundry detergent additives [46, 47]. All the surfactants tested enhanced the proteolytic activity (Fig. 7a and b). We think the detergent forms an interfacial area that favors enzymatic performance, as reported for plant proteases [48–50]. When the enzyme contacts with an interface, the dielectric environment at the protein surface is modified, and some electrostatic interactions are enhanced, probably favoring a rearrangement and reorientation of catalytic residues [51]. Even the authors tested different conditions, AU13PE showed better activity in the presence of surfactants as compared with the results reported by Farooq et al. [52], who were working with the psychrophilic metalloprotease produced by *Bacillus pumilus*. They reported, among other conditions, a total inactivation with 1% Triton X-100, meanwhile, we detected a two-fold increase in activity using 20% Triton X-100 (Fig. 7b).

Then, we analyzed the effect of hydrogen peroxide (H_2O_2), a common bleaching agent used for fabric whitening and brightening. The proteolytic activity of AU13PE increased in the presence of 3% and 10% H_2O_2; higher concentrations, such as 20 and 50%, led to a reduction in the proteolytic activity compared to the control without the oxidant (Fig. 7c). Similar findings were reported for other proteases; for instance, the activity of a protease produced by *Streptomyces olivochromogenes* also increased in the presence of this agent [53]. This phenomenon might be attributed to the oxidizing agent's capacity to hydrolyze azocasein, assisting in the hydrolysis of smaller fragments. This could explain the apparent increase of the activity to a certain limit, beyond which higher concentrations of this agent could alter the structure of the enzyme [53].

Once the effect of detergents and oxidants was determined, we studied the compatibility of AU13PE with various commercial laundry detergents. We chose conditions like those of a standard cold-water washing. The AU13PE maintained its full activity, and in some cases, its activity was even enhanced when combined with commercial detergents, at least in the tested conditions (Fig. 8a). The stability assay reveals that AU13PE was compatible with most

commercial detergents tested with a residual activity above 80%, for 1 h at 25 ºC. Results suggest that AU13PE could be used in the laundry industry. This performance was comparable to the mesophilic protease produced from *Bacillus* sp. RGR-14 (68 to 93% activity after incubation with 1% solutions of different commercial detergents for 1 h at 25 ºC) [54] and the serine protease from psychrophilic *Acinetobacter* sp. MN 12 (60 to 70% activity after incubation for 2 h with 1% commercial detergents) [55].

Among other psychrophilic bacteria, *B. pumilus,* isolated from Thajwas Glacier (Kashmir, India), produces a metalloprotease that showed lower compatibility with commercial laundry detergents [52], mainly compared with AU13PE; however, the authors tested different detergents and conditions. They found good stability only for two out of four commercial laundry detergents tested, retaining 67% and 74% residual activity for Ghari and Wheel, respectively, after pre-incubation with 0.5% detergent for 30 min at 20 ºC. In contrast, AU13PE was compatible with a broader range of commercial laundry detergents (five out of six) and, for a longer washing time (1 h) at room temperature (25 ºC) (Fig. 8b). Thus, AU13PE shows better performance compared with the psychrophilic metalloprotease produced by *B. pumilus*.

In summary, our results suggest that the Antarctic microbe *Flavobacterium* sp. AU13 produces at least an extracellular metalloprotease with biochemical features compatible with its use in biotechnological processes such as the detergent industry.

Further studies will focus on the genome sequencing of AU13 and the gene identification of extracellular proteases produced by this microbe. The use of an extracellular extract produced by a microbe growing at a low temperature is not feasible for the industry; thus, we will face the identification of the coding gene for the 48 kDa protein and other potential extracellular proteases, for their recombinant production, purification, and biochemical/biotechnological characterization. We think the detergent industry could benefit if we find a high-performance protease or a combination of proteases for a new laundry formulation release. Beyond detergents, this protease may also have broader industrial applications, such as in the in vitro hydrolysis of proteins to generate bioactive peptides, in the food industry for protein modification, or in cosmetic industry for skin care formulations. Its potential as a versatile biocatalyst makes it a promising candidate for a wide range of biotechnological applications.

Conclusion

The Antarctic microorganism *Flavobacterium* sp. AU13 produces a proteolytic extracellular extract with metalloprotease activity, probably an epralysin as determined by mass spectrometry analysis. AU13PE exhibits high activity at low temperatures and a broad pH range; it maintains activity in the presence of detergents and H_2O_2 and, is compatible with conventional laundry detergents. In summary, AU13PE could be an interesting candidate for searching proteases with industrial applications as a detergent additive.

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Authors contributions FL performed the experiments, data processing and contributed to write the manuscript; SCS contributed to design the studies and to manuscript revision; CV contributed to design the studies and wrote the manuscript. All authors read and approved the submitted version.

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Data availability GraphPad Prism version 9.5.0 **RRID:SCR_002798**; Mascot **RRID:SCR_014322;** NCBI Protein Database **RRID:SCR_003257.** *Flavobacterium* sp. AU13 is part of the bacterial Antarctic collection deposited in the Sección Bioquímica, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, Uruguay.

Declarations

Ethical approval No ethical approval is required for this study.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare that they have no conflict of interest.

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