

Characterization of the exopeptidase activity existing in *Theobroma cacao* L. during germination

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ABSTRACT

The present work reports exopeptidase activity existing in cacao (*Theobroma cacao* L.) during germination. Aminopeptidase (APE), carboxypeptidase (CP) and Xaa-Prolyl dipeptidyl aminopeptidase (Xaa-Pro-DAP) membrane-bound enzymes have been identified. The Xaa-Pro-DAP enzyme (E.C. 3.4.14.5) had not been previously detected in germinating cacao seeds. Xaa-Pro-DAP was partially purified and characterized, and the highest activity was found after 10 days of germination. Xaa-Pro-DAP was isolated by precipitation with 40% ammonium sulfate and partially purified with two chromatographic steps. The enzyme had a relative molecular weight of 80 kDa as determined by Native-PAGE and was, able to use Ala-Pro-4 μ β NA as substrate. In the presence of SDS, this enzyme did not show activity because it must be in a trimer to be functional. Its activity was inhibited 44% by the chelating agent EDTA and 48% by the serine peptidase inhibitor PMSF at 0.1 mM, indicating that the partially purified Xaa-Pro-DAP is a serine metallopeptidase. The cations Cu²⁺ and Cd²⁺ caused 44% and 67% inhibition, respectively, while the other divalent cations tested had no significant effect on the activity of the partially purified enzyme. The enzyme showed a high specificity for Ala-Pro-pNA as a proteolytic substrate.

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1. Introduction

Peptidases carry out a large number of vital processes in plants, such as the proteolysis of proteins providing free amino acids. During the germination process, the biological activity of these peptidases increases due to the activation of pre-existing enzymes and the synthesis of new enzymatic molecules [1]. In dicotyledon seeds, such as cacao, degradation of reserve proteins produces an accumulation of free amino acids in cotyledons [2]. *Theobroma cacao* seeds contain albumins, globulins, prolamins and glutelins, albumins being the main protein fraction [3,4]. *T. cacao* L. has been described as a plant whose seed contains a vicilin-class globulin (but not legume-class) as storage protein [5]. The precursors of the cacao aroma are derived from these storage proteins by the proteolytic process that takes place during fermentation [6]. Hansen et al. [7] reported endopeptidase, aminopeptidase and carboxypeptidase activity in cacao seeds during fermentation. In ripe seeds that had not been germinated, the predominant proteolytic activity is attributed to aspartyl peptidase. This activity is found at very high levels in cacao seeds compared to the levels detected in seeds from other plants [8].

Xaa-Prolyl dipeptidyl aminopeptidase (dipeptidyl aminopeptidase IV) (Pep-XP, PepX; Xaa-Pro-DAP, EC 3.4.14.5) was first identified in rat liver [9] and was subsequently purified from various mammalian tissues and from lactic acid bacteria such as *Lactococcus* and *Lactobacillus* [10,11]. Xaa-Pro-DAP activity has also been reported in insects, yeast and plants [12–14]. The function of Xaa-Pro-DAP is not clearly established in plants, although its activity has been detected in poppy [13,14], gherkin seedlings [15], ginseng sprouts [16], and barley [17], and is suggested to play a role in the mobilization and/or utilization of storage proteins during germination. The present work contributes to the characterization of the exopeptidase activities of cocoa. Our aim was to isolate and characterize its exopeptidases, specifically the dipeptidyl aminopeptidase (Xaa-Pro-DAP) activity, present during the germination process of *T. cacao* L.

2. Materials and methods

2.1. Seed material and germination

T. cacao seeds of the criollo genotype were obtained from state of Tabasco, Mexico. For germination, seeds from which the testa had been removed were kept in wet agrolite at 25 °C over a 12 h period of light/darkness and the germinated seeds were collected every 24 h for 15 days after imbibition [18].

2.2. Acetone dry powder

Dry cacao powder (DCP) was obtained according to the method described by Hansen et al. [7]. Fat was removed from the powder by adding 10 ml/g 100% hexane

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at 4 °C and stirring constantly for 2 h. Finally, the solvent was eliminated from the sample and it was dried at 30 °C. To remove the polyphenols, the defatted seed powder was extracted five times with 80% (v/v) aqueous acetone and then three times with 100% acetone. The suspensions were centrifuged at 4 °C for 5 min at 5000 × g and 20,000 × g for the last three extractions. Finally, the solvent was evaporated from the resulting paste at room temperature. It was ground again until a homogeneous appearance was achieved. The dry powder, which was white/slightly yellow, was stored at –20 °C until analysis.

2.3. Enzyme extract

The enzyme extract was obtained from 15 mg of DCP and 30 mg of polyvinylpyrrolidone (PVPP) in 900 µl of 0.1 M phosphate buffer (pH 7.0) containing 1% Triton X-100. The mixture was stirred for 30 min at 37 °C and then centrifuged at 20,000 × g for 10 min at 4 °C.

2.4. Determination of enzymatic activity

The Xaa-Pro-DAP activity was measured by using Alanyl-proline-*p*-nitroanilide (Ala-Pro-pNA; Bachem, Bubendorf, Switzerland) as substrate. The reaction mixture included 250 µl 0.1 M sodium phosphate buffer (pH 7.0) containing 1% Triton X-100; 120 µl distilled water, 30 µl substrate solution (10 mM in water), and 100 µl enzyme extract. After 30 min of incubation at 37 °C, the reaction was stopped with 400 µl ZnSO₄ (5% w/v) and 100 µl Ba(OH)₂ (7.5% w/v), followed by centrifugation at 15,000 × g for 10 min. The absorbance of the *p*-nitroaniline liberated by the reaction was measured at 405 nm. One unit of Xaa-Pro-DAP (U) was defined as the amount of enzyme needed to liberate 1 µmol of *p*-nitroaniline from the substrate per minute under the standard assay conditions.

2.5. Protein concentration

Protein concentration was determined over the course of enzyme purification by the method of Folin-Lowry modified by Markwell et al. [19], using bovine serum albumin as a standard and measuring the absorbance at 280 nm.

2.6. Enzyme purification

2.6.1. Ammonium sulfate fractionation

The supernatant obtained in 2.3 was precipitated at 40% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 17,000 × g for 20 min at 4 °C and the pellet obtained was dissolved in 20 mM sodium phosphate buffer (pH 7.0) and dialyzed for 24 h at 4 °C with the same buffer. The protein concentration and Xaa-Pro-DAP activity were then determined.

2.6.2. Ion exchange chromatography

The protein fraction obtained by ammonium sulfate ammonia precipitation was applied to a High Q Macro-Prep® column (1.5 cm × 18 cm, Biorad, Hercules, CA 94547, USA) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.0). Fractions of 2 ml were collected at a flow rate of 0.2 ml/min with the same buffer; the elution was performed with a linear gradient of 0–1.0 M NaCl in 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.2 ml/min, monitored at 280 nm (FPLC-Econo Pump, Biorad, Hercules, CA 94547, USA). The protein concentration and activity against Ala-Pro-pNA were measured. Fractions with the highest enzyme activity were pooled, dialyzed against 20 mM sodium phosphate buffer (pH 7.0) at 4 °C, and concentrated by ultrafiltration with a 10 kDa molecular weight cutoff membrane (Amicon® Millipore).

2.6.3. Hydrophobic interaction chromatography

The active fractions from the High Q Macro-Prep were adjusted to 1.5 M ammonium sulfate and loaded onto an Octyl-Sepharose CL-4B column (1.5 cm × 18 cm, GE Healthcare SE-751 25 Uppsala, Sweden), previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1.5 M ammonium sulfate. The bound proteins were eluted with a decreasing linear gradient of 1.5–0 M of ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0), at a flow rate of 0.2 ml/min and fraction volume of 2 ml, monitored at 280 nm (FPLC-Econo Pump, Biorad, Hercules, CA, USA). Fractions with the highest enzyme activity were pooled, dialyzed against 20 mM sodium phosphate buffer (pH 7.0) at 4 °C, concentrated by ultrafiltration and used to characterize the enzyme. All the purification steps were conducted at 4 °C.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE

SDS-PAGE was performed with 4% stacking and 12% separating gels according to Laemmli [20]. The gels were stained with the Silver Stain Plus (Biorad, USA) commercial kit. For the estimation of molecular weight after electrophoresis, were used one standard set (Amersham Pharmacia Biotech). Native-PAGE was performed by the method described by Benoist and Schwencke [21] and Degraeve and Martial-Gros [22] with slight modifications. After electrophoresis, the gel was incubated with 0.1 M sodium phosphate buffer (pH 7.0), the substrate Ala-Pro-4µβNA (10 mM dissolved in 30% DMSO) and a fresh mixture of 2 ml Fast Garnet GBC (11 mg/ml in

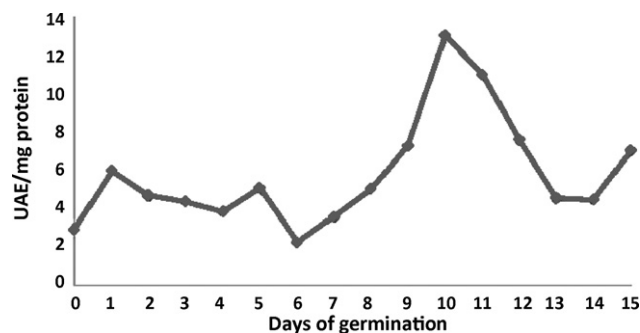


Fig. 1. Changes in the specific activity of Xaa-Pro-DAP during the germination process of cacao seeds of the criollo white almond genotype. Levels are reported as specific enzymatic activity in U/mg of protein.

50% DMSO) (Sigma Chemical, USA) at 37 °C until red or orange bands were observed, which indicate the presence of Xaa-Pro-DAP activity.

2.8. Dynamic light scattering analysis (DLS)

Analysis of the distribution of molecular size was carried out as a part of the protein characterization, using a Malvern Zetasizer Nano Series S-90 (Malvern Instruments, Malvern, England) instrument and analyzing the molecular diameters with the equipment software. Molecular diameter was determined over a temperature interval of 25–80 °C, with gradual increases of 10 °C.

2.9. Substrate specificity

The activity of the Xaa-Pro-DAP against *p*-nitroanilide substrates was determined according to the standard conditions described for the enzyme assay. The following substrates were used in the analysis: Arg-Pro-pNA, Gly-Pro-pNA, Ala-Ala-Pro-pNA, and Ala-pNA at both 1 and 10 mM. The lysyl and leucyl aminopeptidase activities were tested with Lys-pNA and Leu-pNA and the carboxypeptidase-type activity was tested with Bz-Tyr-pNA.

2.10. Effects of inhibitors and reducing agents on enzyme activity

The effects of inhibitors and reducing agents on enzyme activity were studied in both the enzyme extract and the partially purified enzyme, using 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), *p*-chloromercuribenzoate (PCMB), dithiothreitol (DTT), leupeptin, pepstatin, bestatine, diprotine, pefabloc, β-mercaptoethanol and cysteine at concentrations of 0.1 and 1.0 mM. The enzyme was pre-incubated with inhibitors for 30 min at 37 °C and the remaining enzyme activity was determined using Ala-Pro-pNA as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100% [23–25].

2.11. Effects of metal ions on enzyme activity

The effects of several metal ions on enzyme activity were also investigated in the enzyme extract and partially purified enzyme. The enzyme was pre-incubated with 0.1 and 1.0 mM metal ions at 37 °C for 30 min. The following metal ions were used: Ba²⁺, Zn²⁺, Cu²⁺, Co²⁺, Cd²⁺, Mg²⁺, Ca²⁺ and Na⁺. The Xaa-Pro-DAP activity was determined by the standard assay as described above using Ala-Pro-pNA as substrate. The activity of the enzyme without metallic ions was considered 100% [23,26].

3. Results and discussion

3.1. Germination

During the germination process, the level of Xaa-Pro-DAP enzymatic activity from day 0 to 8 did not change. After this point, it started to increase, reaching the highest activity values at 10–11 days with 13.03 UAE and 10.99 UAE and then decreasing in the days following germination (Fig. 1). These results may be related to the drastic changes in the protein vacuoles, as observed by electron microscopy, which took place after 10–13 days of germination. Protein vacuoles inflated and fused to form one central vacuole which appeared optically empty [6]. According to these results, Xaa-Pro-DAP together with other peptidases may play a role in the degradation of storage proteins during the germination of cacao

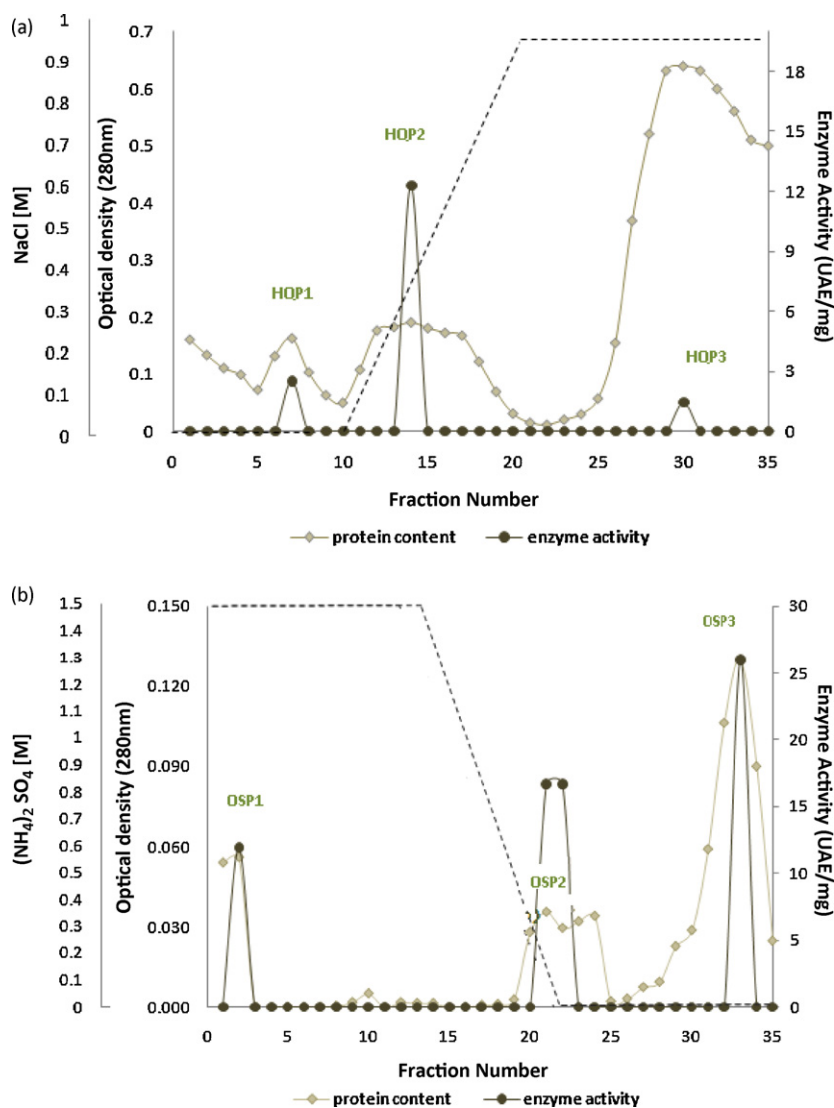


Fig. 2. Purification of the Xaa-Pro-DAP from the seeds of cacao *Theobroma cacao* germinated. (a) Ion exchange chromatography on HQ-Sepharose and (b) hydrophobic interaction chromatography on Octyl-Sepharose CL-4B.

seeds, taking part in the mobilization of the amino acids needed for the growth of the embryo. This last stage corresponds to the initiation of visible morphological changes, a function that has been attributed to the enzyme in other plants [13,14,16]. Therefore, the enzyme may be involved in the development of the plant and be expressed in other organs besides cotyledons.

3.2. Purification of the Xaa-Pro-DAP

The elution profile showed three peaks (HQP1, HQP2 and HQP3) containing the Xaa-Pro-DAP activity (Fig. 2a). The HQP1 peak (fractions 6–8) eluted before the saline gradient started. Elution of the HQP2 peak (fractions 10–13) was observed at 0.3 M NaCl, and therefore the fraction is weakly retained by the column, possi-

bly because of a low concentration of amino acid residues with a net negative charge. Finally, the HQP3 peak (fractions 30–32) was strongly retained in the column, eluting at 1 M NaCl. Peak 2 (HQP2) showed the highest enzymatic activity and was subjected to hydrophobic interaction chromatography on Octyl-Sepharose CL-4B, which gave three peaks containing the Xaa-Pro-DAP activity (Fig. 2b). The OSP1 peak (fractions 1–3) eluted at 1.5 M $(\text{NH}_4)_2\text{SO}_4$. The OSP2 peak (fractions 20–23) eluted at 0.25 M $(\text{NH}_4)_2\text{SO}_4$ and the OSP3 peak (fraction 33) eluted at 0 M $(\text{NH}_4)_2\text{SO}_4$. The last peak (OSP3) had the highest enzymatic activity. After the final purification step, the enzyme was purified 37.53-fold with a yield of 21.97% and a specific activity of 16.66 UAE/mg protein. The results of the purification procedure are summarized in Table 1.

Table 1
Purification of Xaa-Pro-DAP of cacao seeds *Theobroma cacao* L.

Purification stage	Total protein (mg)	Total activity (U ^a)	Specific activity (UAE/mg)	Purification factor	Yield (%)
Crude extract	9.7684	4.3376	0.4440	1.00	100.00
Ammonium sulfate precipitate (40%)	4.9118	4.1433	0.8435	1.89	95.00
Ion exchange with HQ-sepharose	0.1460	1.796	12.3013	27.70	41.50
Hydrophobic interaction with octyl-sepharose	0.0572	0.9533	16.6660	37.53	21.97

^a An enzyme unit is defined as the amount of enzyme needed to liberate 1 mol pNA/min, using Ala-Pro-pNA as substrate.

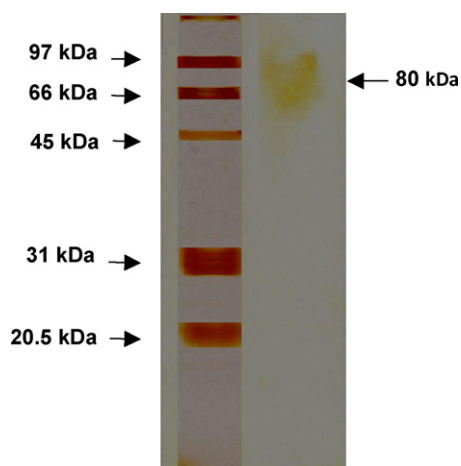


Fig. 3. Native-PAGE analysis of the enzymatic extract.

3.3. Determination of relative molecular weight and DLS analysis

The relative molecular weight of the native protein was estimated to be 80 kDa (Fig. 3). The enzyme shows no activity in presence of SDS, and therefore it must be in a polymeric state to be active (it is only active in the native state). This is due to the cooperative nature of the aggregation process and also because at some protein concentrations it is favorable for the molecule to aggregate because an increase in inter-molecular interactions between monomers results in a lower-energy state compared to the monomers alone. Through the study of the molecular dynamics in a solution of the enzymatic extract from a concentration of 0.09–0.019 mg/ml, it was observed that the monomer has a strong tendency to aggregate under native conditions (Fig. 4a). At 25 °C, a tetramer and a high molecular weight aggregate coexist (insert in Fig. 4a), the tetramer making up 97.3% of the population in the molecular-weight distribution. As the temperature increases, the tetramer breaks up into dimers with the presence of a polymer of very high molecular weight (showed in insert of Fig. 4a), which indicates the beginning of the denaturation process. At 40 °C, octamers and dimers are observed, while at 50 °C pentamers and monomers coexist, and finally at 80 °C only monomers of the protein are found. These monomers have, a diameter of 33.28 nm and a Pdl of 0.072, in the form of a mono-dispersed system (Fig. 4b). The partially purified enzyme at a concentration of 0.1416 mg/ml was a single species of 166 nm in molecular diameter (Fig. 4b).

3.4. Exopeptidase activities in *T. cacao* L.

The peptidases identified in our purification process were solubilized at neutral pH using 1% Triton X-100, indicating that they are membrane-bound enzymes. The difficulty in achieving the complete solubilization of cacao enzymes is probably one reason for the lack of available information on their properties. The results of testing the enzymatic activity on different substrates are shown in Table 2. Regarding the different substrates tested in the present work, the degree of hydrolysis at a concentration of 10 mM substrate was as follows, from the highest to lowest: Ala-pNA > Gly-Pro-pNA > Ala-Pro-pNA > Leu-pNA > Ala-Ala-Pro-pNA > Arg-Pro-pNA > Bz-Tyr-pNA > Lys-pNA. The highest activity toward substrates with X-Pro as the N-terminal sequence was found for Gly-Pro-pNA, followed by Ala-Pro-pNA, Ala-Ala-Pro-pNA and Arg-Pro-pNA. The relative activities toward these substrates showed that the N-terminal residue exerts an effect on the specificity of the enzyme with the following order of preference: glycine, alanine and arginine. Aminopeptidase activity (APE)

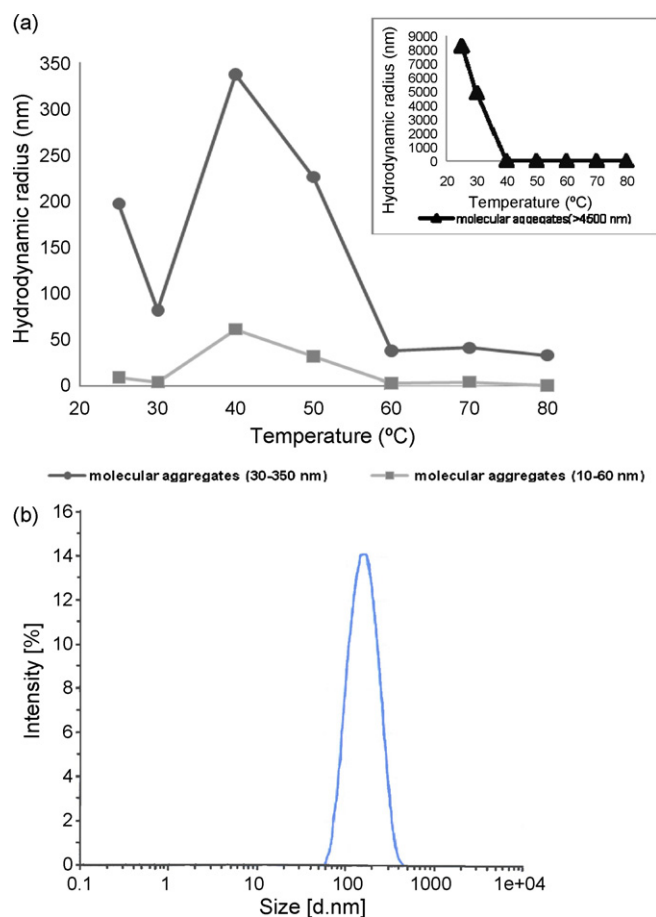


Fig. 4. Distribution of the molecular size during the thermal treatment of Xaa-Pro-DAP (a) and size distribution of Xaa-Pro-DAP in native conditions at 25 °C (b).

was also identified and the highest values were found for the substrates Ala-pNA followed by Leu-pNA, both at 10 mM. Non-specific aminopeptidase (Type N) (E.C. 3.4.11.1) is an aminopeptidase with wide substrate specificity that has been found in numerous strains

Table 2

Exopeptidase activity of *Theobroma cacao* towards various chromogenic substrates.

Substrate ^a	Specific activity (UAE ^b /mg) (mean value ± S.D. (n = 3))	
	1 mM	10 mM
Enzymatic extract		
Ala-pNA	1.51 ± 0.022	2.35 ± 0.017
Gly-Pro-pNA	0.93 ± 0.003	1.91 ± 0.001
Ala-Pro-pNA	0.88 ± 0.030	1.73 ± 0.011
Leu-pNA	0.70 ± 0.016	1.62 ± 0.000
Ala-Ala-Pro-pNA	0.81 ± 0.001	1.30 ± 0.023
Arg-Pro-pNA	0.52 ± 0.009	1.14 ± 0.005
Bz-Tyr-pNA	0.59 ± 0.007	0.90 ± 0.060
Lys-pNA	0.90 ± 0.001	0.73 ± 0.009
Partial purified Xaa-Pro-DAP		
Ala-Pro-pNA	4.81 ± 0.001	24.06 ± 0.001
Gly-Pro-pNA	2.84 ± 0.002	6.41 ± 0.002
Arg-Pro-pNA	1.09 ± 0.002	4.37 ± 0.001
Ala-Ala-Pro-pNA	0.65 ± 0.001	2.18 ± 0.002
Leu-pNA	0.65 ± 0.001	1.33 ± 0.001
Bz-Tyr-pNA	0.241 ± 0.001	0.547 ± 0.003
Lys-pNA	n.d.	0.459 ± 0.002
Ala-pNA	n.d.	n.d.

n.d.: not detected.

^a pNA: *p*-Nitroanilide; Bz: benzoyl.

^b An enzyme unit is defined as the amount of enzyme needed to liberate 1 mol pNA/min.

of lactic acid bacteria. It is very active on Lys-*p*-NA, Leu-*p*-NA [27] and Ala-*p*-NA [25]. APE has been reported as the second most prevalent enzyme after aspartic peptidase (AP) (E.C. 3.4.23) in fermented cacao seeds. Therefore, the substrate Lys-*p*-NA was also tested, which had a lower degree of hydrolysis compared to Ala-*p*-NA and Leu-*p*-NA at 10 mM. Regarding Lys-*p*-NA, a higher activity at 1 mM was observed than at 10 mM, which suggests a substrate inhibition phenomenon against the APE enzyme. The substrate Bz-Tyr-*p*-NA was hydrolyzed at a higher proportion at 10 mM than at 1 mM, confirming the presence of carboxypeptidase activity (CP) (E.C. 3.4.17). A synergistic activity between aspartyl peptidase and carboxypeptidases has been reported during cacao fermentation [28]. In a similar manner, Xaa-Pro-DAP of *Lactobacillus sanfranciscensis* CB1 and non-specific aminopeptidase (Type N) showed a synergistic effect in boosting the degree of gluten hydrolysis [29]. Therefore, our results show that these enzymes work in a concerted manner during the germination process to degrade the storage proteins, a process required in the formation of seedlings. Xaa-Pro-DAP is important in cacao because cacao seeds contain a group of proteins that have proline in their structure (at concentrations of 0.72–1.97%), which are the natural substrates of the enzyme [30].

As shown in Table 2, the specificity of the partially purified Xaa-Pro-DAP of *T. cacao* was observed with -*p*-NA substrates. Maximum hydrolysis rates were obtained when proline was in the N-penultimate position. Among colorimetric substrates, Ala-Pro-*p*-NA was hydrolyzed at the highest rates, which indicates a high preference for the amino acid Ala adjacent to proline, followed by Gly-Pro-*p*-NA, Arg-Pro-*p*-NA and Ala-Ala-Pro-*p*-NA. The degree of hydrolysis increased in all the substrates with X-Pro as the N-terminal sequence compared with the extract, indicating higher Xaa-Pro-DAP activity due to the purification process, which also decreased the aminopeptidase and carboxypeptidase concentration. A similar substrate preference was reported for the enzyme purified from *L. sakei*, which hydrolyzed substrates with an X-Pro amino terminus almost exclusively [11] showing preference for Ala, Arg and Gly, in decreasing order [24].

Hydrolysis of the substrates Leu-*p*-NA (1 and 10 mM), Lys-*p*-NA and Bz-Tyr-*p*-NA (10 mM) was detected, indicating that leucine-aminopeptidase and carboxypeptidase enzymes were still present in the partially purified enzyme fractions, though their activities were quite low when compared with that of partially purified Xaa-Pro-DAP activity. Leu-*p*-NA maintained the hydrolysis level detected in the enzymatic extract, whereas Lys-*p*-NA and Bz-Tyr-*p*-NA decreased their activities to almost half of what was detected in the extract. The Ala-*p*-NA substrate was not hydrolyzed, indicating the absence of a non-specific aminopeptidase. It was clear however, that the Xaa-Pro-DAP activity increased almost 10 times compared to the activity found in the enzymatic extract over the course of the purification. Until now, there has been scarce information about the biochemical characteristics of aminopeptidases and carboxypeptidases present in cocoa.

3.5. Effect of inhibitors on enzyme activity

Table 3 shows the effects of inhibitors on the Xaa-Pro-DAP activity from the enzyme extract of *T. cacao*. The best inhibitors at 0.1 mM were PMSF, PCMB and pepstatin, which caused a decrease of 54, 40 and 39% of the maximum activity, respectively. Increases in inhibitory power directly proportional to concentration were only observed with leupeptatin (12–29%), diprotine (18–29%) and PCMB (40–49%). Among chemicals that react with sulfhydryl groups, β -mercaptoethanol, cysteine and dithiothreitol had no effect. Incubation with PCMB yielded a considerable inactivation of the Xaa-Pro-DAP activity. This indicates the possible involvement of functional sulfhydryl group(s) at or near the active site of the enzyme, which confirms the presence of a cysteine peptidase,

Table 3
Effect of inhibitors/activators on the exopeptidase activity in *Theobroma cacao* L.

Inhibitor ^a	Relative activity ^b (%)			
	Enzymatic extract		Partial purified Xaa-Pro-DAP	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM
Diprotine A	82	71	76	86
PMSF	46	68	52	83
EDTA	89	79	56	61
PCMB	60	51	115	99
Leupeptin	80	76	92	64
Pepstatin A	61	80	82	84
Bestatine	86	154	75	126
AESBF	102	112	91	97
Pefabloc	115	131	92	101
Cysteine	109	104	64	92
β -Mercaptoethanol	72	115	74	97
Dithiothreitol	85	109	62	70
Blank	100	100	100	100

^a PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoate; AESBF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride.

^b Expressed as a percentage of hydrolysis of Ala-Pro-*p*-NA in the absence of any added chemical agent, which was given a value of 100%.

which has been previously isolated [31]. These authors mention that it is very likely that it does not take part in the hydrolysis of storage proteins in ungerminated seeds. On the other hand, its activity increases during the germination process when the degradation of globular storage proteins occurs. Although all the Xaa-Pro-DAP originating from dairy lactic acid bacteria cited in the literature are considered serine enzymes, many authors have reported that sulfhydryl groups may also be important for the enzyme activity [32,33].

Regarding the specific inhibitors of serine peptidases tested such as leupeptatin, AESBF, pefabloc, and PMSF, only leupeptin and PMSF had an inhibitory effect. The presence of a carboxypeptidase has been reported in cacao, which has been characterized as a serine peptidase that acts on hydrophobic amino acids and is inhibited by PMSF. Differences observed among different inhibitors of serine peptidase show that the sulfonation of the protein is a more effective mechanism of inhibition than acylation. The Xaa-Pro-DAP activity was reduced by 39% by 0.1 mM pepstatin A, an inhibitor of aspartic peptidase, but this effect decreased at 1 mM, probably due to the inactivation of the aspartic peptidase present in the extract, reflecting less proteolytic action on Xaa-Pro-DAP by aspartic peptidase. It is known that the AP activity remains constant during the first days of germination and it does not decrease before the onset of globulin degradation [34]. The inhibitors of metallopeptidases analyzed in the present study showed inhibition in the range of 0–14% for bestatine, 18–29% for diprotin A, and 11–21% for EDTA. According to these results, the enzymatic extract of germinated *T. cacao* contains a cysteine metallopeptidase besides the carboxypeptidase and aminopeptidase. The carboxypeptidase and aminopeptidase were originally identified in cacao and other plants as metallopeptidases [35,36].

To determine the biochemical properties of the partially purified Xaa-Pro-DAP, the enzyme activity was measured in the presence of different enzyme inhibitors (Table 3). One of the enzyme inhibitors was the chelating agent EDTA (0.1 mM), which caused a 44% decrease from the original activity, indicating that metal ions are involved in enzyme function or stability. This means that the partially purified Xaa-Pro-DAP is a metalloenzyme. This result differs from the report of Xaa-Pro-DAP of *Lactobacillus helveticus* ITG LH1 [22], which was not inactivated by metal chelating agents such as EDTA or 1, 10-phenanthroline. The partially purified Xaa-Pro-DAP activity was reduced by 25% by 0.1 mM bestatine, an inhibitor

Table 4
Effect of metallic cations on the exopeptidase activity in *Theobroma cacao* L.

Metal salt	Relative activity ^a (%)			
	Enzymatic extract		Partially purified Xaa-Pro-DAP	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM
BaCl ₂	74	69	84	76
CoCl ₂	117	48	134	75
ZnCl ₂	70	59	77	85
CaCl ₂	104	49	96	82
CuCl ₂	74	52	63	56
MgCl ₂	89	115	72	98
CdCl ₂	97	55	98	33
NaCl	108	75	103	84

^a Expressed as a percentage of hydrolysis of Ala-Pro-pNA in the absence of any added metal salt, which was given a value of 100%.

of leucine-aminopeptidase, but this effect on activity decreased at 1 mM, probably due to the inactivation of the aminopeptidase present, reflecting less or none proteolytic action on Xaa-Pro-DAP by aminopeptidase. This enzyme was lightly inhibited by a dipeptidyl aminopeptidase inhibitor (diprotin A at 0.1 mM). Pepstatin A, which is an aspartic peptidase inhibitor, had no effect on its activity (Table 3). Under the same conditions, the enzyme was inhibited 48% and 36% by the serine peptidase inhibitor, PMSF at 0.1 mM and leupeptin at 1.0 mM. The effect of AEBSF and Pefabloc; other inhibitors of serine peptidase, was negligible (Table 3). The results from these serine peptidase inhibitors corroborate that Xaa-Pro-DAP is a serine peptidase.

Additionally, the reducing agents dithiothreitol, cysteine and β-mercaptoethanol as well as the sulfhydryl group reagent PCMB did not have any effect on its activity. These findings suggest that partially purified Xaa-Pro-DAP does not belong to the cysteine peptidase family. The cysteine peptidase activity identified in the enzymatic extract was not detected in semi-purified Xaa-Pro-DAP, likely due to its removal during the purifying process.

3.6. Effect of divalent cations on Xaa-Pro-DAP activity of *T. cacao* L.

Table 4 shows the effect of metal ions on the activity of Xaa-Pro-DAP in the enzymatic extract and partially purified enzyme.

Ca²⁺ and Mg²⁺ have been reported to increase the cysteine peptidase activity [37]; the absence of this effect on Xaa-Pro-DAP corroborates our conclusion that it is a serine peptidase rather than a cysteine peptidase. Co²⁺ and Ca²⁺ (1 mM) inhibited the enzyme activity by 52 and 51%, respectively, while the other divalent cations tested caused 31–48% inhibition. According to some authors, the cations Cu²⁺, Hg²⁺ and Zn²⁺ are strong inhibitors of the Xaa-Pro-DAP activity in bacteria such as *L. helveticus* CNRZ 32, *L. delbreckii* subsp. *bulgaricus* and *L. acidophilus*. In *Lactobacillus sakei* these cations had no significant inhibitory effect [24].

The Xaa-Pro-DAP activity decreases as the ionic strength increases by the effect of sodium chloride in the extract on *T. cacao*.

The activity of the partially purified enzyme was inhibited 44% and 67% by the presence of 1.0 mM Cu²⁺ and Cd²⁺, respectively. A similar inhibition by Cu²⁺ and Cd²⁺ was found for the Xaa-Pro-DAP of *L. sanfranciscensis* CB1 [11]. The other divalent cations tested had low or no effect on the partially purified enzyme (Table 4). Cobalt ions at 0.1 mM increased the enzyme activity by 34%, but this did not happen when the salt concentration was 1 mM. This effect is probably due to proteolytic cleavage of Xaa-Pro-DAP by the residual cobalt-activated carboxypeptidase and aminopeptidase. Some studies on the effect of cobalt on carboxypeptidases [38,39] and aminopeptidases [40,41] reported increases from 1.7 to 50 times the original activity using 4 μM to 1 mM CoCl₂. However, not every

aminopeptidase cited in the literature is considered to be cobalt-activated, and some authors have reported strong inhibition of a leucine-aminopeptidase by Co²⁺ at 10 mM [36,42].

Finally, the ionic strength difference caused by addition of 0.1 mM NaCl had no effect on the partially purified enzyme activity, while increasing the ionic strength decreased the enzymatic activity (Table 4).

The proteolytic system in the germinated *T. cacao* extract has been partially characterized, identifying lysine and leucine-aminopeptidase (APE), carboxypeptidase (CP) and Xaa-Prolyl dipeptidyl aminopeptidase (Xaa-Pro-DAP) membrane-bound enzymes. These peptidases may take part in the degradation of storage proteins during cacao germination in a synergistic manner. The Xaa-Pro-DAP activity detected in the enzymatic extract, with a relative molecular weight of 80 kDa, was active in the native state. The activity in the extract was sensitive to 0.1 mM PMSF, PCMB and pepstatin A, which inhibited activity by 54, 40 and 39%, respectively. All cations except Co²⁺, Ca²⁺ and Mg²⁺ decreased enzyme activity. The activation by reducing agents shows the presence of a cysteine metalloproteinase in the enzymatic extract of *T. cacao*. The partially purified Xaa-Pro-DAP enzyme showed a higher specificity for the Ala-Pro-pNA substrate but aminopeptidase and carboxypeptidase activities are still present. The activity of the partially purified enzyme was inhibited 44% by the chelating agent EDTA at 0.1 mM and 48% and 36% by the serine peptidase inhibitor, PMSF at 0.1 mM and leupeptin at 1.0 mM. The partially purified Xaa-Pro-DAP is a serine metalloproteinase. Because this Xaa-Pro-DAP has not been previously detected in cacao, the purification and characterization of this enzyme will help in the ongoing efforts to understand the proteolytic system of *T. cacao* and the role of these systems in the germination process.

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