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Purification and properties of an arginyl aminopeptidase from *Debaryomyces hansenii*

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Abstract

A metallo arginyl aminopeptidase (EC 3.4.11.6) activated by Co^{2+} was isolated from *Debaryomyces hansenii* CECT 12487. The enzyme was purified after precipitation with protamine sulphate, followed by a weak anion exchange chromatography, gel filtration chromatography and a strong anion exchange chromatography. The arginyl aminopeptidase (AAP) was purified 337 folds, with a 18% recovery. The AAP appeared to be a dimer with a molecular mass of 101 kDa. The enzyme was active in the pH range from 6 to 9. The optimal activity was detected at pH 7.0 and at 37 °C. AAP activity was inhibited by typical aminopeptidase inhibitors (puromycin and bestatin), reducing agents (DTT), chelating agents (EDTA, EGTA and phenantroline) and sulphydryl groups reagents (iodoacetate). Ca^{2+}, Mn^{2+} and Co^{2+} activated the enzyme, while Cu^{2+}, Cd^{2+}, Hg^{2+} and Mg^{2+} inhibited it. The K_m values calculated for Arg-AMC (7-amido-4-methylcoumarin) and Leu-AMC were 0.071 and 0.094 mM, respectively. The enzyme showed maximum specificity for basic amino acids (Arg and Lys), but was also able to hydrolyze non-charged amino acids (Leu, Met and Ala) and, at a minor rate, aromatic amino acids (Phe and Tyr). AAP showed higher activity when an acid residue was located at the C-terminal position of dipeptides.

The described purification of an arginyl aminopeptidase from the yeast *D. hansenii* can contribute to the lack of knowledge about the exopeptidase activity in one of the yeasts more frequently isolated in sausage and to understand its role during the ripening of a fermented sausage.

Keywords: Arginyl aminopeptidase; Peptidases; Proteases; *Debaryomyces hansenii*

1. Introduction

Dry-fermented sausages constitute one of the most typical Mediterranean food products. A large number of biochemical reactions take place during the fermentation and ripening of dry sausages. Lipolysis and proteolysis constitute two major groups of reactions induced by endogenus enzymes from the meat and fat and by enzymes from microorganisms growing in the mince (Verplaetse et al., 1992). In this sense, proteolysis leads to an increment in protein fragments, peptides and amino acids. These compounds, together with their derived compounds, give specific taste to the sausage. Proteolytic system of lactic acid bacteria (LAB) has been deeply studied and numerous exopeptidases have been characterized (Montel et al., 1992; Sanz and Toldrá, 1997; Fadda et al., 1999a,b; Sanz et al., 1999; Sanz and Toldrá, 2002). It is believed that lactic acid bacteria are the most impor-

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tant group of microorganisms affecting sausage changes, since LAB lead the fermentation and grow at the highest levels. Moreover, Micrococcoci, yeasts and moulds are also important components of the sausage microflora and have been proved to take part in proteolysis (Grazia et al., 1986; Leistner, 1986; Geisen et al., 1992; Chen and Guo, 1992; Bermell et al., 1992; Montel et al., 1992; Selgas et al., 1993; Hammes et al., 1995; Trigueros et al., 1995; Toledo et al., 1997; Santos-Mendoza, 2000). *Debaryomyces hansenii* is the most frequent yeast isolated from fermented sausages (Encinas et al., 2000; Santos-Mendoza, 2000), with its peroxidase activity, hydrolytic action against lipids and contribution to aroma formation being well known (Gehlen et al., 1991; Bjorn-Sorensen and Samuelsen, 1996; Olensen and Stahnke, 2000). The strain *D. hansenii* 12487 has been proved to be active against sarcoplasmic muscle proteins (Santos et al., 2001). On the whole, microorganisms take part mainly in the degradation of protein fragments generating peptides and free amino acids (Molly et al., 1997). Thus, the priority research must be focused on microbial exopeptidases as it will allow a better understanding of the biochemical pathways of these nitrogen compounds.

The exoproteolytic system of *Saccharomyces cerevisiae* has been deeply studied. It is basically integrated by the cytosolic proteasome, some vacuolar exopeptidases (carboxypeptidases Y and S, aminopeptidases Y and I and dipeptidyl aminopeptidase B) and two exopeptidases of the secretory pathway (carboxypeptidase and dipeptidyl aminopeptidase A) (Jones, 1983, 1991; Jones et al., 1997).

However, the enzymes involved in the proteolytic pathway in *D. hansenii* have not been identified yet. Thus, the present work is focused on the purification and characterization of an arginyl aminopeptidase (AAP) from *D. hansenii* CECT 12487, which contributes to the knowledge of the proteolytic system of this halotolerant species.

2. Material and methods

2.1. Yeast strain and growth conditions

D. hansenii CECT 12487 was isolated from the natural microflora and selected because it shows interesting biochemical abilities, like high rates for lactic acid consumption and ammonia generation (Santos-Mendoza, 2000), good development in a sausage manufacture process and activity against sarcoplasmic muscle proteins (Santos et al., 2001). *D. hansenii* CECT 12487 was routinely grown in malt extract agar (Scharlau Microbiology, Barcelona, Spain) at 27 °C for 48 h and stored at −80 °C in 15% glycerol. For purification purposes, the microorganism was grown in 1.17% (w/v) Yeast Carbon Base (Bacto®, DIFCO, Detroit, USA) plus 0.1% (w/v) urea as nitrogen source. A pre-culture of 120 ml was inoculated with the microorganism and grown at 27 °C, with shaking at 110 rpm in an orbital incubator, for 2 days. After that, the pre-culture was inoculated to a 400-ml fresh medium and grown at the same conditions for 5 days.

2.2. Preparation of cell free extract

Cells were harvested at 4080 × *g*, at 4 °C, for 10 min and resuspended in 20 mM sodium phosphate, pH 7.5. Then, cells were disrupted in a bead beater (Biospec Products, Washington, NC, USA) after adding an equivalent volume of glass beads (0.5 mm diameter, Sigma, St. Louis, MO, USA) and applying three shakings for 30 s, with 2 min intervals on ice. Glass beads, non-broken cells and debris were separated by centrifugation (27 000 × *g*, for 20 min, at 4 °C). The supernatant was centrifuged (27 000 × *g*, for 15 min, at 4 °C) to obtain the cell extract.

2.3. Enzyme assay

Arginyl aminopeptidase was measured by adding 50 μl of enzyme extract to 250 μl of McIlvaine buffer (0.1 M citric acid, 0.2 M disodium phosphate) pH 7.0, containing 0.12 mM L-arginine-7-amido-4-methylcoumarin (pro-AMC). The reaction mixture was incubated at 37 °C, for 1 min. Fluorescence was measured in a multiscan fluorometer (Fluoroskan II, Labsystem, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. Three replicates were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 μmol of substrate hydrolyzed per hour at 37 °C.

2.4. Enzyme purification

2.4.1. Protamine sulfate fractionation

A concentration of 0.3 g protamine sulfate/g protein was slowly added to the cell-free extract under slight stirring, for 10 min, at 5 °C. Afterwards, the solution was centrifuged (27 000 × *g*, for 11 min at 5 °C) and the supernatant submitted to further purification steps.

2.4.2. Weak anion exchange chromatography

The supernatant was injected into a HiPrep™ 16/10 DEAE column (Amershan Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted by applying an initial gradient from 0 to 50 mM NaCl in 25 mM Tris–HCl, pH 7.5 in 10 min and then from 50 to 200 mM NaCl in the same buffer, in 20 min. The flow rate was 4 ml/min and fractions of 4 ml were collected. The three fractions with maximum activity were pooled and concentrated using a filter device Biomax 10 K NMWL membrane (Millipore, Bedford, MA, USA).

2.4.3. Gel filtration chromatography

The sample obtained after concentrating, was injected onto a Sephacryl S-200 HR column (100 by 1.5 cm, Amershan Pharmacia Biotech) previously equilibrated with 25 mM Tris–HCl, pH 7.5, containing 40 mM NaCl. The column was run at a flow rate of 10 ml/h. Fractions of 3 ml were collected and assayed for aminopeptidase activity. The three fractions containing the maximum activity against Arg-AMC were pooled for a further purification step.

2.4.4. Strong anion exchange chromatography

The pooled fractions were injected into a Resource™ Q column (1 ml, Amersham Pharmacia Biotech). Proteins were eluted by applying an initial isocratic step in 25 mM Tris–HCl, pH 7.5, for 4 min, followed by a 1 min linear gradient from 0 mM to 50 mM NaCl in the same buffer, and then to 150 mM NaCl for 25 min. The flow rate was 1 ml/min and fractions of 1 ml were collected.

2.5. Determination of protein concentration

Protein concentration was determined by the BCA (bicinchoninic acid) method (Smith et al., 1985) with the BCA protein assay reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as standard. The eluted fractions from the chromatographic separations were also monitored at $\lambda = 280$ nm.

2.6. Electrophoresis

The purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% separating gels (Laemmli, 1970). Proteins were stained with Coomassie Brilliant Blue R-250 and silver to increase sensitivity. Broad range molecular mass standards were simultaneously run (Bio-Rad, Hercules, CA, USA).

2.7. Molecular mass determination

The molecular mass of the native enzyme was estimated by gel filtration using a Sephacryl S-200 column (Amershan Pharmacia Biotech) as previously described. The column was calibrated using the following standard proteins: β-amylase (200 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), carbonic anhidrase (29 kDa) and citocrom c (12.4 kDa). Blue dextran was used to estimate the void volume. The molecular mass of the enzyme in denaturing conditions was also determined by SDS-PAGE as described above.

2.8. Effect of pH and temperature

The arginyl aminopeptidase activity was assayed against Arg-AMC in the pH range from 3 to 11, at 0.5 pH units intervals, using the following buffers: from pH 3.0 to 8.0, McIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate), from pH 8.0 to 10.0, Clark and Lub's borate buffer (0.1 M boric acid in 0.1 M KCl, 0.1 N NaOH) and from pH 10.0 to 11.0, Sorensen's glycine II buffer (0.1 M glycine in 0.1 N NaCl, 0.1 N NaOH). The results were expressed as a percentage of the activity obtained at optimum pH.

The effect of temperature was determined in the range 5–55 °C. The substrate solution (250 μl) was previously equilibrated at each temperature and, then, the reaction was initiated by the addition of the purified enzyme (50 μl). After incubation for different times according to each temperature, the reaction was stopped by addition of 100 μl of 0.6 M acetic acid.

The results were expressed as a percentage of the activity obtained at optimum temperature.

2.9. Effect of chemical agents on the activity

The activity of the purified enzyme was assayed in the presence of different chemical agents to identify possible inhibitors or activators by the standard procedure. Leupeptin, puromycin, bestatin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) and pepstatin A were assayed at 0.05–0.5 mM; iodoacetate, 3,4-dichloroisocoumarin (3,4-DCI), phenylmethylsulfonylfluoride (PMSF) were assayed at 0.05–0.5–1 mM; chelating agents, EDTA and EGTA were assayed at 1–5–10 mM and Phenantroline at 0.1–1–5–10 mM; and the reducting agents dithiothreitol (DTT) and 2-mercaptoethanol were assayed at 1–5–10 mM. All reagents were purchased from Sigma.

The effect of the following divalent cations $CaCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$, $HgCl_2$, $MgCl_2$ and $ZnCl_2$ was also determined at 0.05–0.5 mM. These salts were purchased from Panreac (Barcelona, Spain).

2.10. Determination of kinetic parameters

The kinetic parameters of the purified enzyme were estimated by Lineweaver–Burk plots using Arg- and Leu-AMC at 0.005–0.2 mM substrate concentrations.

2.11. Substrate specificity

The activity was tested towards different fluorimetric substrates (Table 4) at 0.1 mM by the standard activity assay. The activity was also assayed against different peptides (Table 5). The reaction mixture consisted of 50 μl McIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate), pH 7.0, 25 μl 10 mM peptide solution and 30 μl of purified enzyme (samples) or of water (control). Two independent samples and controls were assayed for each peptide. The reaction was stopped adding 35 μl 0.6 M acetic acid after 45 min of incubation at 37 °C. The relative activity was determined by measuring the disappearance of substrate by capillary electrophoresis on a P/ACE™ MDQ CA system (Beckman Instruments, Fullerton, CA, USA). The sample was injected under pressure (0.5 psi) for 5 s into a 72-cm fused silica capillary (50 cm to detector). The separation was performed applying a voltage of 30 kV at 25 °C in 20 mM citric acid, pH 2.5. The peptides were detected by measuring the absorbance at 200 nm. The results were expressed as a relative hydrolysis rate given a value of 100% to the maximum activity that was for Arg–Asp.

3. Results

3.1. Purification of the arginyl aminopeptidase

The results of the purification of the AAP from the cell extract of *D. hansenii* are summarized in the Table 1. The precipitation of the cell extract with protamine resulted in a 25% protein reduction and DNA elimination as well. The separation thorugh the DEAE column achieved a 97% protein reduction in relation to the previous step. The maximum activity eluted from the DEAE column at 140–150 mM NaCl (see Fig. 1A). The gel filtration resulted in both purification and desalting (Figs. 1B and 2, lane 1). The final step was carried out by strong anion exchange chromatography with the enzyme eluting at 110 mM NaCl (see Figs. 1C and 2, lane 2). The whole purification process yielded 18.3% and a specificity increase of 337.5-fold.

Table 1
Purification of an arginyl aminopeptidase from *D. hansenii*

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	258.1	40.2	0.2	100.0	1
Supernatant after protamine precipitation	188.7	43.9	0.2	109.3	1.5
DEAE (weak anion exchange)	5.4	21.3	3.9	53.1	25.2
Sephacryl S-200 HR (gel filtration)	1.4	16.3	11.3	40.7	72.4
Resource Q (strong anion exchange)	0.1	7.3	52.5	18.3	337.5

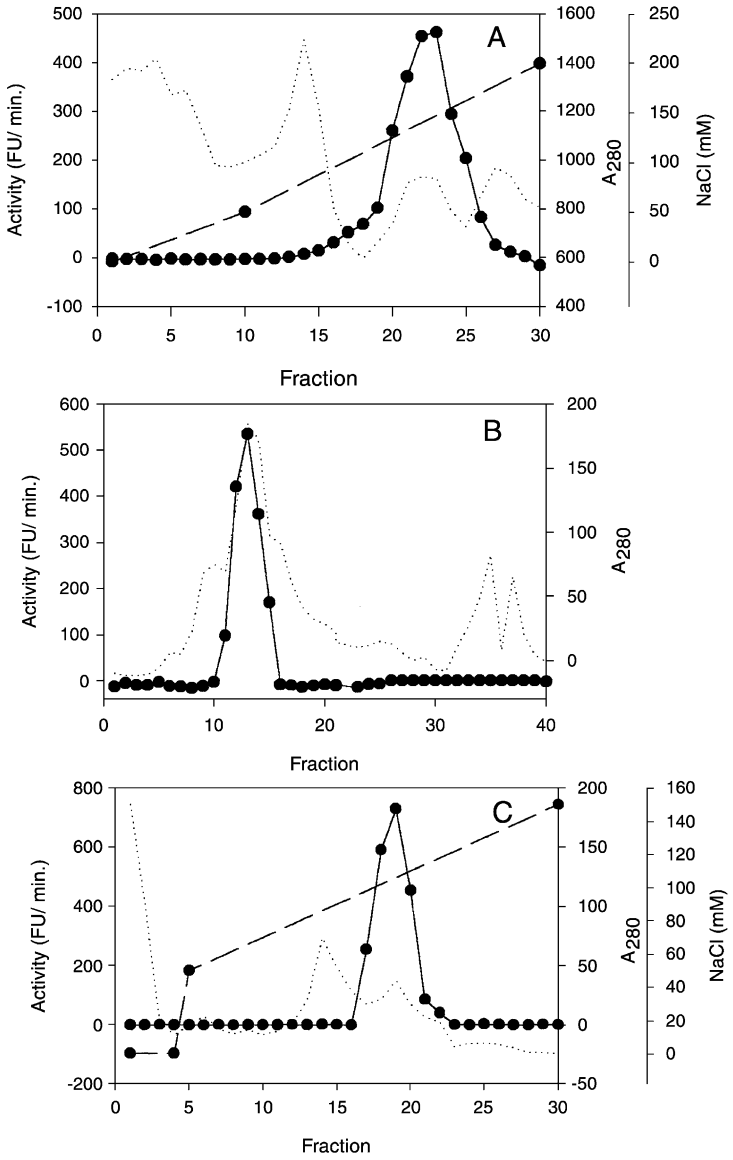

Fig. 1. Chromatograms of the different steps in the purification of *D. hansenii* arginyl aminopeptidase (AAP). (A) DEAE chromatography with NaCl gradient, (B) gel filtration in a Sephacryl S-200 column, (C) Resource Q chromatography with NaCl gradient. Protein was detected by measuring the absorbance at 280 nm (dotted line), arginyl aminopeptidase activity is expressed in fluorescence units (FU)/ min (solid line) and NaCl gradient (long dash line).

3.2. Molecular mass and purity

The molecular mass of the native AAP, calculated by gel filtration, was 101 kDa. The SDS-PAGE analysis displayed two bands of approximately 63 and 56 kDa, respectively, indicating that it is a dimer.

3.3. Effect of pH and temperature on the activity

The enzyme was active, maintaining 75% of the maximum activity, in the range from pH 6.5 to 8.0, with an optimum at pH 7.0 (Fig. 3). The maximum activity was found to be at 37 °C (Fig. 3).

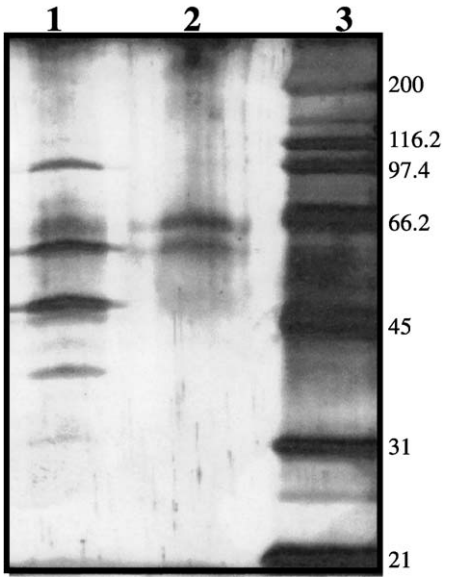

Fig. 2. Silver-stained SDS-PAGE of the purification for AAP from *D. hansenii*. (1) Sephacryl S-200, (2) Resource-Q and (3) standard proteins.

3.4. Effect of chemical agents

The effect of potential inhibitors or activators on AAP activity is shown in Tables 2 and 3. Neither the serin proteases inhibitors, 3,4-DCI and PMSF nor the aspartic protease inhibitor, pepstatin A, affected AAP activity (Table 2). PAP activity was not modified by the cystein protease inhibitors, leupeptin and E-64, but a total inhibition was observed in the presence of iodoacetate, a sulphydryl group reagent (Table 2). On the other hand, puromycin and bestatin, which are typical inhibitors of aminopeptidases, reduced the AAP activity to 21% and 5%, respectively, at 0.5 mM (Table 2). In addition, the chelating agents EDTA, EGTA and *o*-phenantroline, caused a great inhibition, specially *o*-phenantroline which was able to reduce the activity to 0% at 1 mM (Table 2). The reducing agent, DTT, also exerted an inhibition of 30% and 60% at 5 and 10 mM, respectively, but not β-mercaptoethanol.

In relation to the effects of different divalent cations, there were activators and inhibitors (Table 3). Cobalt, manganese and calcium activated the enzyme in a proportional way to the atom size—the bigger the atom, the more activation was observed (Table 3). On the other hand, the heavy metals, copper, cadmium and mercury inhibited the enzyme depending on the atom size as well—the bigger the atom, the more inhibition was observed. Only zinc, a heavy metal as well, did not affect the activity. Magnesium also exerted a clear inhibition on the AAP activity (see Table 3). The inhibition by chelating agents was proved to be reversible. In fact, when adding *o*-phenantroline plus $ZnCl_2$ or $CoCl_2$, no inhibition was observed (data not shown).

3.5. Kinetics parameters

The V_{max} and K_m values for AAP for Arg- and Leu-AMC were 0.357 and 0.3125 μmol min^{-1} mg^{-1} and 71 and 94 μM, respectively.

3.6. Substrate specificity

The relative activity of AAP was assayed against synthetic substrates and peptides (Tables 4 and 5, respectively). The enzyme showed maximum activity for basic amino acids (Arg and Lys), but was also able

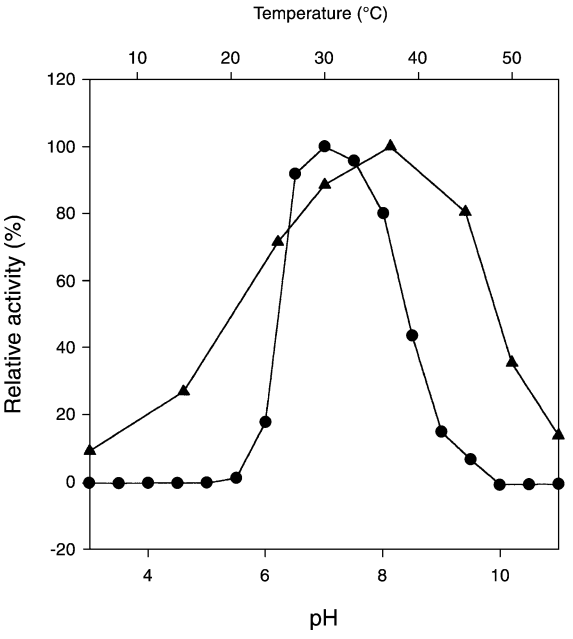

Fig. 3. Effect of pH and temperature on AAP from *D. hansenii*. pH (●) and temperature (▲).

Table 2
Effect of different chemical agents on the purified arginyl aminopeptidase

Chemical agent	Relative activity[a]											
	Concentration (mM)											
	0.05		0.1		0.5		1		5		10	
	μ	V.C	μ	V.C	μ	V.C	μ	V.C	μ	V.C	μ	V.C
Leupeptin	99	3	–[b]	–	99	2	–	–	–	–	–	–
Puromycin	60	3	–	–	21	5	–	–	–	–	–	–
Bestatin	21	6	–	–	5	1	–	–	–	–	–	–
E-64	95	1	–	–	110	3	–	–	–	–	–	–
Iodoacetate	102	13	–	–	59	7	0	0	–	–	–	–
3,4-DCI	99	2	–	–	96	3	90	5	–	–	–	–
PMSF	97	2	–	–	108	3	101	4	–	–	–	–
Pepstatin A	99	3	–	–	94	7	–	–	–	–	–	–
EDTA	–	–	–	–	–	–	94	5	74	3	69	7
EGTA	–	–	–	–	–	–	94	4	72	5	69	8
o-Phenantroline	–	–	57	1	–	–	0	0	0	0	0	0
DTT	–	–	–	–	–	–	98	2	70	12	44	20
βmercaptoethanol	–	–	–	–	–	–	101	1	108	7	114	5

[a] Expressed as a percentage of the activity obtained in the absence of any added chemical agent, which was given a value of 100%. (μ: average, and V.C: variation coefficient).
[b] (–) Non-determined.

to hydrolyze non-charged amino acids such as Leu, Met and Ala and, at a minor rate, aromatic amino acids such as Phe and Tyr (Table 4). The enzyme was unable to hydrolyze Glu, Val, Pro, Gly and Ser at the N-terminal position and did not release Arg from the C-terminal position (Table 4). The specificity against natural peptides showed a similar trend as that against synthetic substrates. The hydrolysis rate of Lys-, Leu- and Ala-AMC were 95, 71 and 27, respectively, and that of Lys–Ala, Leu–Ala and Ala–Ala were 93, 73 and 44, respectively (Tables 4 and 5). AAP showed the highest activity if a negatively charged amino acid residue was located at the C-terminal position of dipeptides (Arg–Asp, Table 5) and minimum activity

Table 3
Effect of different divalent cations on the purified arginyl aminopeptidase

Metal salt	Relative activity[a]			
	Concentration (mM)			
	0.05		0.5	
	μ	V.C	μ	V.C
$CaCl_2$	113	9	121	1
$MnCl_2$	115	7	122	2
$CoCl_2$	118	8	143	2
$CuCl_2$	110	4	89	10
$CdCl_2$	101	1	59	5
$HgCl_2$	10	2	0	6
$MgCl_2$	19	8	30	10
$ZnCl_2$	98	6	96	10

[a] Expressed as a percentage of the activity obtained in the absence of any added chemical agent, which was given a value of 100%. (μ: average, and V.C: variation coefficient).

Table 4
Relative activity of the purified arginyl aminopeptidase on various synthetic fluorescent substrates

Substrate	Relative activity[a] (%)	
	μ	V.C
Arg-AMC[b]	100	3
Lys-AMC	95	4
Leu-AMC	71	3
Met-AMC	67	3
Ala-AMC	27	3
Phe-AMC	27	5
Tyr-AMC	11	2
Glu-AMC	0	0
Val-AMC	0	0
Pro-AMC	0	0
Gly-AMC	0	0
Ser-AMC	0	0
N-benzoyl-Arg-AMC	0	0

[a] Expressed as a percentage of the activity obtained against Arg-AMC, which was taken as value 100%. (μ: average, and V.C: variation coefficient).
[b] AMC: Amino methyl coumarin.

Table 5
Relative activity of the purified arginyl aminopeptidase on various dipeptides

Substrate	Relative activity[a] (%)	
	μ	V.C
Arg–Asp	100	6
Arg–Ala	98	5
Lys–Ala	93	9
Leu–Ala	73	4
Arg–Phe	62	1
Arg–Gly	45	3
Arg–Val	45	5
Ala–Ala	44	3
Arg–Leu	29	3
Arg–Lys	27	9
Pro–Gly	0	0
Val–Val	0	0

[a] Expressed as a percentage of the activity obtained against Arg–Asp, which was taken as value 100% (μ: average, and V.C: variation coefficient).

if a positively charged amino acid was at the same position (Arg–Lys, Table 5).

4. Discussion

This work constitutes the first evidence of the presence of an arginyl aminopeptidase in *D. hansenii*, although a similar aminopeptidase has been previously described in *S. cerevisiae* (Jones et al., 1997).

Until now, various aminopeptidases have been described in yeasts (Jones, 1983) in *S. cerevisiae*, but their classification is still confusing. There are three main aminopeptidases that are well characterized in yeast: a methionine aminopeptidase, API and APY or AAP. Methionine aminopeptidase is a cytosolic aminopeptidase that removes the N-terminal methionine of newly synthesized proteins and has a molecular mass 36 kDa (Chang et al., 1990). API is a typical leucine-aminopeptidase, located in the vacuole and has a high molecular mass, around 600 kDa (Metz and Röhm, 1976; Klionsky et al., 1992). APY cleaves basic residues and has a molecular mass between 75 and 100 kDa (Achstetter et al., 1982; Yasuhara et al., 1994). The AAP of *D. hansenii*, described in this paper, appears to be the cobalt-activated aminopeptidase (ApCo) described earlier by Achstetter et al. (1982) and also named ApY as described by Yasuhara et al. (1994) in *S. cerevisiae*. They have several common characteristics related to their activators, inhibitors and specificity. The ApY described by Nowak and Tsai (1988) has the same molecular mass and similar behavior in the presence of the activators and inhibitors but displays different substrate specificity showing preference for Leu rather than for Arg. This phenomenon could be explained by changes in the catalytic core which lead to different specificities, but maintaining the structure as a possible adaptation to the environment. The fact that AAP of *D. hansenii* has similar biochemical properties to that of *S. cerevisiae* suggests that AAP could be a common component of the exoproteolytic system of yeast, which is a metallo-aminopeptidase activated by Co^{2+} with preference for basic residues. The estimated molecular mass of the native enzyme (100.8 kDa) agrees with that of AAPs purified from *S. cerevisiae* by Achstetter et al. (1982) and Nowak and Tsai (1988), and in a minor extent, with that (70–75 kDa) purified by Yasuhara et al. (1994). The enzyme purified in this work seems to be integrated by two subunits of 63 and 56 kDa. The enzyme characterized by Nowak and Tsai (1988) and Yasuhara et al. (1994) were described as monomers.

Most of the purified AAP have their optimal pH between 6.5 and 8.0 (Sharma et al., 1989; Yamada et al., 1994; Fukasawa et al., 1996). The AAP of *D. hansenii* has its optimal pH at 7.0. The activity of AAP from *D. hansenii* was optimal at 37 °C in accordance with AAP of *Aspergillus parasiticus* (Sharma et al., 1989) and aminopeptidase Y (Nowak and Tsai, 1988).

On the basis of studies with various inhibitors, AAP of *D. hansenii* must be classified as a metalloenzyme. Thus, it shows inhibition by chelating agents and clear effects on the activity by some divalent cations (Tables 2 and 3). These results, together with the inhibition by aminopeptidase-specific inhibitors, bestatin and puromycin, show that this enzyme is a typical aminopeptidase. Similar results were obtained for the enzyme of *S. cerevisiae* (Achstetter et al., 1982; Nowak and Tsai, 1988; Yasuhara et al., 1994).

A cysteine might be involved in the catalysis since iodoacetate markedly reduces the activity of AAP of *D. hansenii*. The *p*-chloromercuribenzoate or *p*-hydroxymercuribenzoato also inhibited ApY (Yasuhara et al., 1994) and ApCo of *S. cerevisiae* (Achstetter et al., 1982). On the other hand, ApY was

activated by *p*-chloromercuribenzoato up to 55% (Nowak and Tsai, 1988).

Moreover, high concentrations of reducing agents such as DDT reduced the activity to 70% and 44%, respectively (Table 2), as found by Yasuhara et al. (1994). Once again, ApY described by Nowak and Tsai (1988) differs from the others and was not affected by reducing agents.

The effect of the different cations is remarkable, Co^{2+} activated all ApYs of *S. cerevisiae* (Achstetter et al., 1982; Nowak and Tsai, 1988; Yasuhara et al., 1994) although the level of activation vary from 1.5-fold for AAP of *D. hansenni* to 11-fold (Achstetter et al., 1982; Nowak and Tsai, 1988; Yasuhara et al., 1994). Zn^{2+} is the only cation that does not affect AAP from *D. hansenii*, although it inhibited all ApYs of *S. cerevisiae* previously described (Achstetter et al., 1982; Nowak and Tsai, 1988; Yasuhara et al., 1994). Yasuhara et al. (1994) proved that Zn^{2+} is a constitutive compound of *S. cerevisiae* APY.

AAP was activated by Ca^{2+}, Mn^{2+} and Co^{2+}, and inhibited by Cu^{2+}, Cd^{2+}, Hg^{2+} and Mg^{2+}. Except for Mg^{2+}, the bigger the size of the atom, the more activation or inhibition was observed. ApY described by Achstetter et al. (1982) was activated by Cu^{2+}, Ni^{2+} and Ca^{2+} and inhibited by Hg^{2+} and Mn^{2+}, ApY described by Nowak and Tsai (1988) was activated by Hg^{2+} and inhibited by Cd^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Mo^{2+}, Ni^{2+} and ApY described by Yasuhara et al. (1994) was inhibited by Mn^{2+}, Cu^{2+} and Zn^{2+}.

AAP of *D. hansenii* releases preferently basic amino acids, Arg and Lys, in a minor rate non-polar amino acids such as Leu and Met and to a lesser extent aromatic amino acids such as Phe and Tyr (Table 4). The same results were reported by Achstetter et al. (1982) and Yasuhara et al. (1994), while the ApY preference was different (Nowak and Tsai, 1988). This one hydrolyze preferently non-polar amino acids, such as Leu, then basic amino acids, such as Lys, and finally aromatic ones (Phe and Tyr). Nevertheless, its molecular mass, activators and inhibitors are basically the same. Thus, it could be inferred a unique origin. The activity of AAP from *D. hansenii* against dipeptides showed that the highest activity takes place if a negatively charged amino acid is located at the C-terminal position of the dipeptides. On the basis of the special importance of the charge of the amino acid at the C-terminal position, it must be conclude that the net charge in the active core must have a very essential role in the catalytic mechanism typical of metallo-proteases, which is supported by the Zn content in the protein and the activation or inhibition described for the divalents cations and chelating agents.

The obtained K_m and V_{max} confirm the specificity for synthetic substrates and peptides that depends mainly on affinity constants. So the enzyme display higher affinity for basic amino acids rather than for non-polar ones as reflected in the K_m values for Arg- and Leu-AMC, which were 71 and 94 μM, respectively, while the V_{max} were similar in both cases.

S. cerevisiae can accumulate or degrade arginine to ornithine and further to proline or alternatively to urea which can be excreted to the external medium or act as precursor of allophanate, serine, threonine, tyrosine, pheylalnine and purines becoming one of the central amino acids in yeast nitrogen metabolism (Henschke and Jiranek, 1993). So, this AAP can be involved in the proteolytic pathway for the supply of arginine by degrading the peptides resulting from muscle proteins (Santos et al., 2001). Besides, AAP is the aminopeptidase that displays major activity in a crude cell extract (data no shown), so it could be one of the most important aminopeptidases of *D. hansenii.*

The knowledge of the proteolytic system will be very important to optimize food fermentations due to its importance in nutritional and physiological adaptations. In addition, the proteolytic changes in these processes lead to an increase of flavor molecules such the amino acids per se and their derived compounds.

The leakage of intracellular proteases from cells during the process could be an additional support to the activity for other proteolytic enzymes. Similar phenomena have been described during the baking of bread that causes hydrolysis of wheat proteins with a consequent loss of leaving power of the dough and during the storage of beer which results in a loss of beer foam (Dreyer et al., 1983; Ormrod et al., 1991).

Further studies should be carried out to purify and characterize other peptidases from *D. hansenii*, which would provide a more detailed information on its proteolytic system and its possible functions as well as differences in comparison with *S. cerevisiae*. Moreover, these studies will be profitable to predict the proteolytic changes that the yeast *D. hansenii* could carry out throughout the fermentation of sausages.

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