ELSEVIER

International Journal of Food Microbiology 76 (2002) 117–126

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Purification and characterisation of a glutaminase from *Debaryomyces* spp.

M.A. Durá, M. Flores, F. Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjassot, Valencia, Spain

Received 26 October 2001; accepted 27 December 2001

Abstract

A glutaminase was purified from the cell-free extract of *Debaryomyces* spp. CECT 11815 by protamine sulphate treatment and several chromatographic procedures including anion exchange chromatography and gel filtration. The purified enzyme consisted of two subunits, with molecular masses of 65 and 50 kDa, respectively. Activity was optimal at 40 °C and pH 8.5, and the K_m value for L-glutamine was 4.5 mM. The glutaminase exhibited activity against L-γ-Glu-methyl ester, L-γ-Glu-hydrazide, and L-albiziin, while L-asparagine, CBZ-L-Gln, CBZ-L-Gln-Gly, glutathione, L-γ-Glu-pNA and L-γ-Glu-AMC were not hydrolysed. The enzyme was not affected by PMSF, DTT and EDTA. However, the enzyme was inhibited by sulfhydryl group reagents, DON, L-albizziin, L-asparagine and high concentrations of L-glutamine and ammonium, while L-aspartate did not affect the activity. Phosphate and acetate did not produce any significant effect on the glutaminase activity, but it was slightly stimulated by lactate and borate.

Keywords: Glutaminase; *Debaryomyces*; Enzyme purification; Yeast

1. Introduction

Yeast microbiota present in meat products has been scarcely studied. However, the use of yeasts helps the development of the typical sausage flavour through its high lipolytic activity (Larpent-Gourgaud et al., 1993; Sorensen and Samuelsen, 1996; Sorensen, 1997). Furthermore, yeasts are also able to increase the ammonium content and reduce the amount of lactic and acetic acids, with the concomitant suppression of the acid taste (Miteva et al., 1986; Gehlen et al., 1991). The yeast microbiota in meat products are *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, *Debaryomyces* and *Torulopsis* (Dillon and Board, 1991). In Spanish dry-fermented sausages (salchichón), the most abundant genus is *Debaryomyces*, followed by *Rhodotorula*, *Candida*, *Pichia*, *Yarrowia* and *Trichosporon* (Santos-Mendoça, 2000). However, the composition and development of the mycoflora are dependent on the nature of the product, the processing time and the ripening conditions (Toledo et al., 1997; Ordoñez et al., 1999).

Abbreviations: CBZ, carbobenzyloxy; pNA, *p*-nitroanilide; AMC, 7-amido-4-methyl coumarin; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DON, 6-diazo-5-oxo-L-norleucine; INT, iodonitrotetrazolium chloride; NEM, *N*-ethylmaleimide.

* Corresponding author. Tel.: +34-96-3900022; fax: +34-96-3636301.

E-mail address: ftoldra@iata.csic.es (F. Toldrá).

0168-1605/02/$ - see front matter
PII: S0168-1605(02)00024-7

Essentially, yeasts degrade nitrogenous compounds to one or two final products: ammonium and/or glutamate (Henschke and Jiranek, 1993). So, glutamine is hydrolysed to glutamate and ammonium, being this reaction, catalysed by the enzyme called glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2), which has been detected in a large number of bacteria, moulds and yeasts (Imada et al., 1973). This enzyme has been purified from mammals (Smith and Watford, 1988) and several bacteria and moulds (Moriguchi et al., 1994; Lu et al., 1996; Huerta-Saquero et al., 2001). In yeasts, only two studies have purified and characterised a glutaminase enzyme from *Cryptococcus albidus* (Iwasa et al., 1987) and a γ-glutamylarylamidase-glutaminase from *Saccharomyces cerevisiae* (Penninckx and Jaspers, 1985). Also, the physiological role of glutaminase activity using desalted cell extracts from *S. cerevisiae* has been studied by Soberón and González (1987).

The addition of starter cultures containing glutaminase activity to fermented sausages is desirable because this enzyme is able to act on L-glutamine, present in relatively high amounts in the fresh mix, generating ammonium, as a neutraliser of acidity, and L-glutamate, as a flavour enhancer. The object of this work was to focus on the purification and characterisation of glutaminase from *Debaryomyces* spp. in order to better understand its role on flavour generation during the processing of dry-fermented sausages.

2. Experimental procedures

2.1. Yeast strain and growth conditions

Debaryomyces spp. CECT 11815 was originally isolated from the indigenous flora of dry-fermented sausages (Santos-Mendoça, 2000). The organism was routinely cultured at 27 °C, without agitation, in a medium containing (in g/l): malt extract, 3; yeast extract, 3; L-aspartic acid, 15. The medium pH was adjusted to 6.7 with NaOH before autoclaving.

2.2. Preparation of cell-free extract

The microorganism was grown in 250-ml Erlenmeyer flasks with 100 ml of medium. Following one subculturing, it was inoculated at 1.5×10^6 cfu/ml and incubated at 27 °C for 5 days. Cells were harvested by centrifugation (8000 × *g*, 10 min, 4 °C), washed once in water, and suspended in 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM EDTA at a ratio of 1 ml of buffer per gram of cells (wet weight). The suspensions were maintained at −80 °C until used.

Cells were disrupted at 4 °C by homogenisation of the suspension with an equal volume of glass beads in a Bead-Beater model 1107900 (BioSpec Products, Bartlesville, USA). Two 1-min bursts with an intermediary pause of 1 min were employed. The extract was diluted with an equal volume of buffer, previously used to wash the glass beads. Unbroken cells were removed by centrifugation (8000 × *g*, 10 min, 4 °C), and the supernatant was considered as the cell-free extract.

2.3. Glutaminase activity

The glutaminase activity was routinely assayed by detecting the L-glutamate generated, or, in a few cases, the ammonia generated in the reaction when an interfering substance was present in the reaction medium.

2.3.1. Determination of L-glutamate

This assay was used throughout the purification procedure and in most of the assays. The enzyme preparation was incubated at 37 °C for 30–60 min with 10 mM L-Gln and 50 mM Tris–HCl, pH 8.5 as standard assay medium. The reaction was stopped by adding acetic acid up to a 0.2 M final concentration. The L-glutamate released from L-glutamine was followed in the final mixture by means of two coupled reactions involving glutamate dehydrogenase and the phenazine methosulphate-catalyzed reduction of iodonitrotetrazolium chloride (INT) by NADH (Gella and Pascual, 1982). The amount of INT-formazan produced was measured at 490 nm in a multiwell plate reader, ELX 800 (Bio-Tek Instruments, Winooski, VT, USA).

2.3.2. Determination of ammonia

The enzyme reaction was done in the standard assay medium and conditions as described above, but in this case, ammonium formation was coupled with the oxidation of NADH via glutamate dehydrogenase (Bergmeyer and Beutler, 1985). The change in absorbance at 340 nm was followed in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Three replicates were measured for each experimental point. One unit of glutaminase activity (U) was defined as the amount of the enzyme that generates 1 nmol of product (either L-glutamate or ammonium) per minute at 37 °C.

2.4. Enzyme purification

2.4.1. Protamine sulphate treatment

The cell-free extract was centrifuged (17,000 × *g*, 60 min, 4 °C) in order to remove cell debris and most of the membranes. A 2.5% (w/v) solution of protamine sulphate up to a final concentration of 0.1 mg protamine sulphate/mg protein was added to the supernatant (soluble extract) with continuous stirring at 4 °C for 20 min. The mixture was centrifuged (10,000 × *g*, 10 min, 4 °C) and the supernatant frozen at −20 °C. Then, it was thawed at 37 °C and centrifuged again (10,000 × *g*, 10 min, 4 °C).

2.4.2. Weak anion exchange chromatography

The chromatographic separation was done in a FPLC system (Pharmacia). The supernatant was filtered through a 0.22-μm membrane filter and injected in a Biosep-DEAE-P (75 × 7.80 mm) column (Phenomenex, Torrance, USA), previously equilibrated with 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA. The column was eluted at 1 ml/min for 15 min with the equilibration buffer and then, with a linear NaCl gradient from 0 to 0.25 M in 40 min. Fractions of 1 ml were collected and those with glutaminase activity were pooled and concentrated up to 1–2 ml by centrifugation in an Ultrafree 30 K NMWL membrane filter (Millipore, Bedford, USA).

2.4.3. Gel filtration chromatography

Concentrated fractions were applied to a Sephacryl S-200 HR (89 × 1.6 cm) column (Pharmacia) previously equilibrated with 20 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl, 0.5 mM EDTA and 10% ethylene glycol. The column was run at a flow rate of 10 ml/h and 2 ml fractions were collected. Fractions containing glutaminase activity were pooled.

2.4.4. Strong anion exchange chromatography

The chromatographic separation was carried out in a biocompatible (titanium) 1050 Hewlett-Packard liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a variable wavelength UV detector (280 nm). The active fractions obtained from the previous step were applied to a 1-ml Resource Q anion exchange column (Pharmacia) previously equilibrated with 20 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl, 0.5 mM EDTA and 10% ethylene glycol. The column was eluted at 1 ml/min with the equilibration buffer during 10 min and then, with a linear NaCl gradient from 0.1 to 0.35 M during 20 min. Fractions of 0.5 ml were collected and assayed for glutaminase activity. The active fractions were applied to the same column but equilibrated with 20 mM Tris–HCl, pH 8.5, containing 0.15 M NaCl, 0.5 mM EDTA and 10% ethylene glycol. The column was eluted at 1 ml/min for 5 min with the equilibration buffer and then, with a linear NaCl gradient from 0.15 to 0.35 M during 20 min. Eluted fractions of 0.5 ml were collected and assayed for glutaminase activity.

2.5. Determination of protein concentration

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The fractions eluted from the chromatographic systems were also monitored at 280 nm.

2.6. Electrophoresis

The purification was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels and staining with Coomassie blue R-250 (Laemmli, 1970) or silver (Merril et al., 1981). Native PAGE was carried out using 8% polyacrylamide gels. The electrophoresis buffer was 90 mM Tris, 80 mM boric acid, 2.5 mM EDTA, pH 8.4 (Aledo et al., 1993).

2.7. Molecular mass determination

The molecular mass of the native enzyme was estimated by gel filtration as previously described. The column was calibrated using the following standard proteins: β-amylase (200 kDa; Sigma, St. Louis, MO, USA), aldolase (158 kDa; Roche, Mannheim, Germany), bovine serum albumin (68 kDa; Roche), carbonic anhydrase (29 kDa; Sigma), and cytochrome *c* (12.4 kDa; Sigma), and, finally, blue dextran was

used to estimate the void volume. Protein was monitored at 280 nm, blue dextran at 615 nm, and cytochrome *c* at 411 nm. The molecular mass of the enzyme in denaturing conditions was also determined by SDS-PAGE as described above using standard proteins from Bio-Rad (Hercules, CA, USA).

2.8. Optimal pH and temperature

The optimal pH was determined at 37 °C in the range from 5.0 to 10.0, using the following buffers: 50 mM citrate–NaOH, pH 5.0–6.5; 50 mM Bis Tris Propane–HCl, pH 6.5–9.5; 50 mM Tris–HCl, pH 7.5–9.0; and 50 mM glycine–NaOH, pH 9.0–10.0. The effect of temperature on the activity was determined in the range from 5 to 65 °C using the standard assay medium. The L-glutamate produced was determined and the activity expressed as a percentage of the activity at optimum pH and temperature, respectively.

2.9. Substrate specificity

The relative activity of the purified glutaminase was assayed through the standard assay against L-γ-Glu-hydrazide (10 mM) (Sigma) and glutathione (10 mM). The relative activity against CBZ-L-Gln (Aldrich, Steinheim, Germany), CBZ-L-Gln-Gly (Sigma), L-Asn and L-albizziin (Aldrich) was determined in the standard medium at a concentration of 10 mM but with detection of ammonia. The assay against L-γ-Glu-pNA (5 mM) and L-γ-Glu-AMC (1 mM) (Sigma) was done in the standard assay medium. The release of pNA was measured at 405 nm and the production of AMC in a multiscan fluorometer (Fluoroskan II, Labsystems, Helsinki, Finland) at 355 and 460 nm as excitation and emission wavelengths, respectively. The hydrolysis of L-γ-Glu-methyl ester (10 mM) (Sigma) was determined by amino acid analysis in a reverse-phase HPLC system (Aristoy and Toldrá, 1991).

2.10. Determination of kinetic parameters

The kinetic parameters of the glutaminase against L-glutamine were estimated by the Lineweaver–Burk plot using 0.5 to 10 mM of substrate. The activity was determined at 15-min intervals in the standard assay.

2.11. Enzyme assays

The study of other enzyme activities that can interfere in the glutaminase assay was done as follows: hydroxylaminolysis activity was assayed by determining the L-γ-Glu-hydroxamate produced described by Prusiner et al. (1976). The colour developed was measured at 490 nm in a multiwell plate reader, ELX 800 (Bio-Tek Instruments). Carbamoyl-phosphate synthetase activity was determined by trapping the carbamoyl-phosphate produced as hydroxyurea after reaction with hydroxylamine (Ingraham and Abdelal, 1978; Kaseman and Meister, 1985). Transglutaminase activity was assayed using hydroxylamine as the acceptor substrate with CBZ-L-Gln-Gly and the product measured as a coloured complex (Folk and Chung, 1985). The rates of formation of GMP from XMP (GMP synthethase activity) and CTP from UTP (CTP synthetase activity) were determined by a continuous spectrophotometric assay at 290 and 291 nm, respectively (Zalkin, 1985a,b). Glutamate syntase activity was measured in an assay solution containing 50 mM Tris–HCl, pH 8.5, 10 mM L-glutamine, 1 mM 2-oxoglutarate, 0.2 mM NADH and enzyme preparation. The mixture was incubated at room temperature and the decrease in absorbance at 340 nm was followed. Asparagine synthetase activity was measured in an assay solution containing 50 mM Tris–HCl, pH 8.5, 10 mM L-glutamine, 10 mM L-aspartate, 10 mM ATP and 5 mM magnesium chloride. The synthesis of asparagine was analysed as phenylthiocarbamyl amino acid derivative by reverse-phase HPLC with a previous deproteinization with acetonitrile (Aristoy and Toldrá, 1991).

Three replicates (samples + controls) were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 nmol of product/min at 37 °C.

2.12. Effect of inhibition/activation substances on the activity

The effect of potential inhibitors/activators on the glutaminase activity was tested: PMSF (0.1–0.5 mM), NEM (1–5 mM), iodoacetate (1–5 mM), DTT (0.2–1 mM), EDTA (1–5 mM), DON (0.02–0.1 mM), L-albizziin (2–10 mM), L-asparagine (2–10 mM), L-glutamine (10–50 mM), L-glutamate (10–50 mM),

ammonium chloride (10–50 mM), L-aspartate (10–50 mM), phosphate (5–20 mM), acetate (5–20 mM), lactate (5–20 mM), ATP (5–20 mM) and borate (5–20 mM). The activity was measured by the standard assay in the presence of the chemical agents except in the case of the assay with L-glutamate in which the generated ammonium was determined. Controls with the absence of the chemical compound were simultaneously run.

3. Results and discussion

There is scarce published scientific literature on yeast glutaminases. Evidence has been presented that *S. cerevisiae* has two glutaminase activities, a readily extractable form (glutaminase B) and a membrane-bound enzyme (glutaminase A) (Soberón and González, 1987), but till now, only two yeast glutaminases have been purified: one from *C. albidus* (Iwasa et al., 1987) and the γ-glutamylarylamidase-glutaminase from *S. cerevisiae* (Penninckx and Jaspers, 1985). In this paper, the purification of a glutaminase from *Debaryomyces* spp. is reported. This glutaminase is very different from the two yeast glutaminases previously reported although it shares many properties with glutaminases from mammalian and microbial origin.

3.1. Purification of glutaminase

The results of the glutaminase purification from the cell-free extract of *Debaryomyces* spp. CECT 11815 are summarised in Table 1. A 2607-fold purification with 2.4% in yield was achieved. The first chromatographic step, on a Biosep-DEAE-P column, gave a high glutaminase activity peak, eluted at 0.11 M NaCl, and two lower peaks, eluted at 0.13 M and 0.15 M NaCl (see Fig. 1A). Gel filtration chromatography of the pooled fractions from the main peak resulted in a single peak (see Fig. 1B) with a 254-fold purification and 28.1% recovery. The glutaminase activity eluted at 0.28 M NaCl in the first strong anion exchange chromatography at pH 7.5 (see Fig. 1C). Finally, the partial purified enzyme was submitted to a second strong anion exchange step, at pH 8.5, and the purified enzyme eluted at 0.29 M NaCl (see Fig. 1D). The purification was rather difficult because the enzyme showed high instability, especially during the gel filtration and subsequent steps. The addition of 10% ethylene glycol and the reduction of the EDTA concentration in the buffers were necessary to avoid substantial activity losses. The lack of stability is a common feature in most of the glutaminases, especially those from mammalian sources (Heini et al., 1987) and from several microbial sources (Prusiner et al., 1976; Huerta-Saquero et al., 2001).

3.2. Purity and molecular mass

The purified glutaminase gave only one band on native PAGE (Fig. 2), but it showed two bands on SDS-PAGE corresponding to two subunits of approximately 65 and 50 KDa, respectively (Fig. 3). The molecular mass of the native enzyme determined by gel filtration was 105 kDa, confirming the presence of two subunits. This structure has not been described previously for a microbial glutaminase. The molecular mass of the glutaminase produced by *C. albidus* is 187 kDa (Iwasa et al., 1987), and 14.8 kDa for the γ-

Table 1
Purification of the glutaminase from *Debaryomyces* spp. CECT 11815

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	264	1104	4.2	100.0	1
Soluble extract	106.5	1046	9.8	94.8	2.4
Protamine sulphate treatment	74.3	1039	14	94.1	3.3
Weak anion exchange	2.4	811	337	73.4	80.4
Gel filtration	0.29	310	1065	28.1	254
Strong anion exchange, pH 7.5	0.027	147	5386	13.3	1287
Strong anion exchange, pH 8.5	0.002	26	10917	2.4	2607

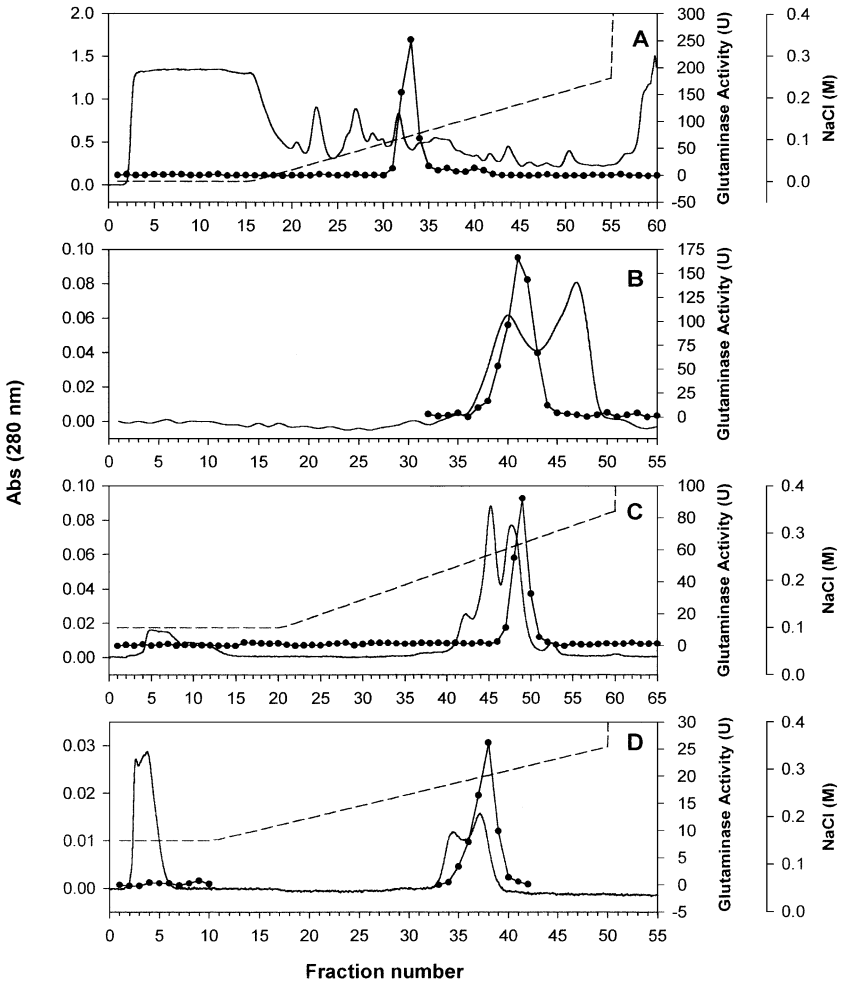

Fig. 1. Chromatographic purification of glutaminase from *Debaryomyces* spp. after protamine sulphate treatment. (A) Weak anion exchange, (B) gel filtration, (C) first strong anion exchange at pH 7.5 (0.1–0.35 M NaCl gradient) and (D) second strong anion exchange at pH 8.5 (0.15–0.35 M NaCl gradient). NaCl linear gradient (– – –), absorbance 280 nm (—), glutaminase activity (●).

glutamylarylamidase-glutaminase from *S. cerevisiae* (Penninckx and Jaspers, 1985).

3.3. Optimal pH and temperature

The enzyme was active in the pH range from 5.5 to 10 with an optimum at pH 8.5 (Fig. 4). The enzyme retained 20% of the maximal activity when assayed at pH 6.0, although at pH 9.5 about 27% of the optimal activity was retained. This optimum pH is higher than the values reported for other yeast glutaminases. Iwasa et al. (1987) observed that *C. albidus* enzyme showed its optimum pH at 6.0, while glutaminases A and B from *S. cerevisiae* have their pH optima at 7.5 and 8.1, respectively (Soberón and González, 1987), and the γ-glutamylarylamidase-glutaminase from *S. cerevisiae* acts optimally at pH 7.0 (Penninckx and Jaspers, 1985).

The optimal temperature was 40 °C (data not shown). The enzyme was rapidly inactivated at 65 °C, while about 8% of the optimal activity remained at 5 °C. There are several glutaminases from microbial sources that display optimum activity at temperatures above 40 °C such as the glutaminase of *C. albidus* with an optimum at 70 °C (Iwasa et al., 1987), this being a thermal resistant enzyme.

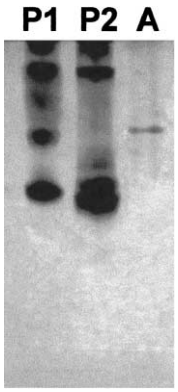

Fig. 2. Native PAGE of purified glutaminase after silver staining. (P1) Standards: catalase (240 kDa), aldolase (158 kDa), BSA (68 kDa) and carbonic anhydrase (29 kDa). (P2) Standards: β-amylase (200 kDa), aldolase (158 kDa) and ovoalbumin (45 kDa). (A) Glutaminase active fraction from the second strong anion exchange chromatography.

3.4. Substrate specificity

The specificity of the glutaminase for several compounds is shown in Table 2. L-Glutamine was the substrate hydrolysed at the highest rate. Other substrates also hydrolysed but at lower rates were L-γ-Glu-methyl ester, L-γ-Glu-hydrazide, and L-albizziin, while the enzyme did not show any activity against CBZ-L-Gln, CBZ-L-Gln-Gly, L-Asn, glutathione, L-γ-Glu-pNA nor L-γ-Glu-AMC. The activity on these substrates suggests that the presence of the α-amino and α-carboxyl groups is necessary for hydrolysis and that the size of the substrate is important for enzyme activity. Moreover, the group hydrolysed from the γ-glutamyl moiety has to be small. This substrate specificity differs from that of *C. albidus* enzyme, which hydrolyses CBZ-glutamine (Iwasa et al., 1987) and γ-glutamylarylamidase-glutaminase from *S. cerevisiae*, which does not act over L-asparagine nor glutathione, but hydrolyses L-γ-Glu-p NA (Penninckx and Jaspers, 1985).

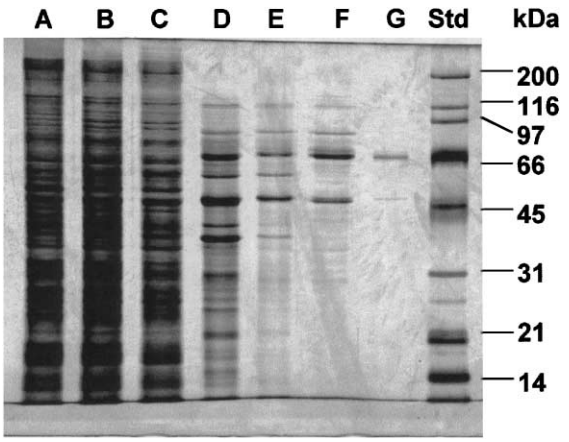

Fig. 3. Ten percent SDS-PAGE of the purification process stained with silver. (A) Cell-free extract, (B) soluble extract, (C) thawed extract of protamine treatment, (D) weak anion exchange, (E) gel filtration, (F) first strong anion exchange at pH 7.5, (G) second strong anion exchange at pH 8.5 and (Std) standards.

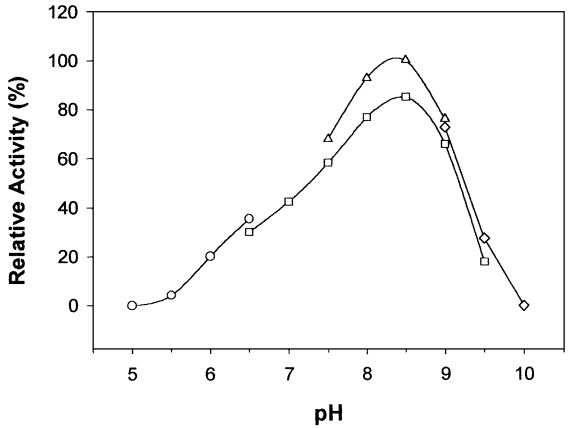

Fig. 4. Effect of pH on glutaminase activity. (○) citrate–NaOH buffer, (□) Bis Tris propane–HCl buffer, (△) Tris–HCL buffer, (◇) glycine–NaOH buffer.

On the other hand, the enzyme did not catalyse hydroxylaminolysis reactions with L-glutamine nor CBZ-L-Gln-Gly as substrates, so that it cannot be considered a transglutaminase because it is unable to generate L-γ-Glu-hydroxamate. Moreover, the enzyme did not show carbamoyl-phosphate synthetase, CTP

Table 2
Activity of the purified glutaminase from *Debaryomyces* spp. CECT 11815 on several substrates

Substrate	Relative activity (%)	Substrate	Relative activity (%)
L-Glutamine	100.0	CBZ-L-Gln	0.0
L-γ-Glu-methyl ester	48.7	CBZ-L-Gln-Gly	0.2
L-γ-Glu-hydrazide	18.4	glutathione (oxidised)	0.0
L-Albizziin	9.2	L-γ-Glu-p NA	0.0
L-Asparagine	0.0	L-γ-Glu-AMC	0.0

synthetase, glutamate synthase, asparagine synthetase nor GMP synthetase activities (results not shown).

3.5. Kinetic parameters

The K_m value for L-glutamine was 4.5 mM. This values is higher than the values displayed by the yeast glutaminase from *C. albidus* (Iwasa et al., 1987).

3.6. Effect of different substances on the activity

The effect of different substances on the glutaminase activity is shown in Table 3. The lack of clear inhibition by PMSF suggested that serine or threonine hydroxyl groups are not involved in the binding of substrate to the *Debaryomyces* spp. enzyme. However, the glutaminase-asparaginase of *Acinetobacter* is not affected by PMSF (Holcenberg, 1985), although it has a threonine residue in the catalytic site as the enzyme from *Pseudomonas* 7A (Holcenberg et al., 1978). The sulfhydryl group reagent NEM caused about 95% enzyme inhibition at 5 mM concentration while iodoacetate inhibited completely the enzyme activity at the same concentration. Inhibition by thiol group reagents is commonly reported for mammalian glutaminases (Sayre and Roberts, 1958) and several microbial glutaminases (Tower, 1967), indicating that sulfhydryl groups may be essential for the glutaminase activity. However, NEM and iodoacetate are not inhibitory in other bacterial glutaminases (Hartman, 1970).

It is noteworthy that a reducing agent such as DTT did not activate the *Debaryomyces* spp. enzyme as expected. The γ-glutamylarylamidase-glutaminase from *S. cerevisiae* is highly activated by protecting sulfhydryl group reagents (Penninckx and Jaspers, 1985). The presence of EDTA did not produce any significant change in activity. However, the presence

Table 3
Effect of different substances on the activity of the Glutaminase from *Debaryomyces* spp. CECT 11815

Substance	Concentration (mM)	Relative activity (%)	Substance	Concentration (mM)	Relative activity (%)
None	–	100.0	L-Glutamine	10	81.3
PMSF	0.1	99.8		20	59.8
	0.2	99.5		50	61.5
	0.5	95.0	NH_4Cl	10	82.1
NEM	1	11.9		20	67.1
	2	8.4		50	54.2
	5	4.9	L-Glutamate	10	103.6
Iodoacetate	1	1.2		20	98.6
	2	0.2		50	91.1
	5	0.0	Phosphate	5	99.5
DTT	0.2	102.2		10	103.1
	0.5	103.6		20	104.0
	1	101.8	Acetate	5	105.3
EDTA	0.2	104.0		10	100.5
	0.5	105.0		20	106.5
DON	0.02	69.0	Lactate	5	100.8
	0.05	49.1		10	108.4
	0.1	24.7		20	117.4
L-Albizziin	2	93.4	Borate	5	101.2
	5	86.8		10	107.0
	10	73.3		20	110.0
L-Asparagine	2	57.9	ATP	5	99.1
	5	36.8		10	96.4
	10	17.2		20	89.6
L-Aspartate	10	99.8			
	20	97.5			
	50	101.7			

of EDTA in the reaction mixture was necessary for enzyme activity since no activity was detected when EDTA was removed from the purification buffers (data not shown). Abe et al. (1974) reported a similar effect on a glutaminase from *Pseudomonas*. They concluded that contamination by traces of protease(s) for which metal ion(s) were essential might be the cause of loss of activity during incubation at 37 °C without EDTA. In general, glutaminase enzymes are not affected by EDTA (Hartman, 1970; Holcenberg, 1985).

Inhibition of the activity by DON and L-albizziin, two L-glutamine analogs, is characteristic of glutamine requiring enzymes (Pinkus, 1977). DON exerted the strongest effect, since it suppressed about 75% of the enzyme activity at only 0.1 mM concentration. DON usually acts as a potent inhibitor of glutaminases (Penninckx and Jaspers, 1985; Smith and Watford, 1988). Unlike DON, which is an irreversible inhibitor, L-albizziin interferes with the utilisation of L-glutamine by the *Debaryomyces* spp. enzyme probably in a competitive and reversible manner since L-albizziin is also a substrate of the enzyme (Pinkus, 1977).

L-Asparagine produced about 80% enzyme inhibition at 10 mM concentration, although it was not a substrate, so that it probably acted as a non-competitive inhibitor. The $-NH_2$ moiety of the amide group seems to be the one responsible for the inhibition, since L-aspartate did not exert any effect on the enzyme activity. The substrate, L-glutamine, was inhibitory when it was added at the reaction mixture at high concentrations, and the same happened with the product ammonium, which inhibited near 50% of the enzyme activity at 50 mM concentration. However, the product L-glutamate was only slightly inhibitory at this concentration. Inhibition by ammonia has been reported for several glutaminases from mammalian (Sayre and Roberts, 1958) and microbial sources (Prusiner and Stadtman, 1976).

The effect of several anions was determined at concentrations between 5 and 20 mM. The presence of lactate and borate stimulated slightly the activity while phosphate and acetate did not produce any significant effect. The lack of activation by phosphate contrasts with the reported findings in mammalian glutaminases (Sayre and Roberts, 1958; Haser et al., 1985), for which phosphate is a clear activator as well as borate (Haser et al., 1985). Finally, the addition of 20 mM ATP caused an inhibition of about 10% of the enzyme activity. The γ-glutamylarylamidase-glutaminase from *S. cerevisiae* is not affected by ATP (Penninckx and Jaspers, 1985).

In summary, many glutaminases have been isolated and characterised from microbial and mammalian origins but only a few have been described from yeasts. This paper confirms the existence of a soluble glutaminase in *Debaryomyces* spp. with high substrate specificity, and composed by two distinct subunits. This glutaminase shows maximal activity at pH 8.5 and 40 °C but some activity can be found at the typical conditions of the processing of dry-fermented sausages. The contribution of this enzyme to the typical characteristics of dry-fermented sausages when *Debaryomyces* strain is used as a starter culture is the object of further research in our laboratory.

Acknowledgements

This work was supported by grant ALI98-0890 from Comisión Interministerial de Ciencia y Tecnología (CICyT, Spain). FPI/MCT scholarship to M.A. Durá is also acknowledged.

References

Abe, T., Takenaka, O., Inada, Y., 1974. Reinvestigation of the physicochemical and enzymic properties of L-glutaminase from *Pseudomonas*. Biochim. Biophys. Acta 358, 113–116.

Aledo, J.C., Gómez-Biedma, S., Segura, J.A., Molina, M., Núñez de Castro, I., Márquez, J., 1993. Native polyacrilamide gel electrophoresis of membrane proteins: glutaminase detection after in situ specific activity staining. Electrophoresis 14, 88–93.

Aristoy, M.C., Toldrá, F., 1991. Deproteinization techniques for HPLC amino acid analysis in fresh pork muscle and dry-cured ham. J. Agric. Food Chem. 39, 1792–1795.

Bergmeyer, H.U., Beutler, H.-O., 1985. Ammonia. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, 3rd edn., vol. VIII. Verlag Chemie, Weinheim, pp. 454–461.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Dillon, V.M., Board, R.G., 1991. Yeasts associated with red meats. J. Appl. Bacteriol. 71, 93–108.

Folk, J.E., Chung, S.I., 1985. Transglutaminases. Methods Enzymol. 113, 358–374.

Gehlen, K.H., Meisel, C., Fischer, A., Hammes, W.P., 1991. Influence of the yeast *Debaryomyces hansenii* on dry sausage fermentation. Proceedings of the 37th International Congress of Meat Science and Technology, Kulmbach, pp. 871–876.

Gella, F.J., Pascual, M.A., 1982. Assay of glutaminase activity by colorimetric determination of glutamate. Anal. Biochem. 127, 322–325.

Hartman, S., 1970. Glutaminase (*Escherichia coli*). Methods Enzymol. 17A (134), 941–945.

Haser, W.G., Shapiro, R.A., Curthoys, N.P., 1985. Comparison of the phosphate-dependent glutaminase obtained from rat brain and kidney. Biochem. J. 229, 399–408.

Heini, H.G., Gebhardt, R., Brecht, A., Mecke, D., 1987. Purification and characterization of rat liver glutaminase. Eur. J. Biochem. 162, 541–546.

Henschke, P.A., Jiranek, V., 1993. Yeast — metabolism of nitrogen compounds. In: Fleet, G.H. (Ed.), Wine Microbiol. Biotechnol., pp. 77–164, Hardwood Academic Publishers, Chur, Switzerland.

Holcenberg, J.S., 1985. Glutaminase from *Acinetobacter glutaminsificans*. Methods Enzymol. 113, 257–263.

Holcenberg, J.S., Ericsson, L., Roberts, J., 1978. Amino acid sequence of the diazooxonorleucine binding site of *Acinetobacter* and *Pseudomonas* 7A glutaminase-asparaginase enzymes. Biochemistry 17, 411–417.

Huerta-Saquero, A., Calderón, J., Arreguin, R., Calderón-Flores, A., Durán, S., 2001. Overexpression and purification of *Rhizobium etli* glutaminase A by recombinant and conventional procedures. Protein Expression Purif. 21, 432–437.

Imada, A., Igarasi, S., Nakahama, K., Isono, M., 1973. Asparaginase and glutaminase activities of micro-organisms. J. Gen. Microbiol. 76, 85–99.

Ingraham, J.L., Abdelal, A.T.H., 1978. Carbamoyl-phosphate synthetase (glutamine): Salmonella. Methods Enzymol. L1, 29–35.

Iwasa, T., Fujii, M., Yokotsuka, T., 1987. Glutaminase produced by *Cryptococcus albidus* ATCC20293. Purification and some properties of the enzyme. Nippon Shoyu Kenkyusho Zasshi 13, 205–210.

Kaseman, D.S., Meister, A., 1985. Carbamyl phosphate synthetase (glutamine-utilizing) from *Escherichia coli*. Methods Enzymol. 113, 305–326.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.

Larpent-Gourgaud, M., Michaux, O.O., Callon, C., Brenot, O., Sirami, J., Bonnin, C., Boissonnet, B., 1993. Etude comparée des flores d'aromatisation du saucisson de fabrication industrielle ou artisanale. Viandes Prod. Carnés 14, 23–27.

Lu, L.M., Yu, R.C., Chou, C.C., 1996. Purification and some properties of glutaminase from *Actinomucor taiwanensis*, starter of sufu. J. Sci. Food Agric. 70, 509–514.

Merril, C.R., Goldman, D., Sedman, S.A., Ebert, M.H., 1981. Ultra sensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid. Science 211, 1437–1438.

Miteva, E., Kirova, E., Gadjeva, D., Radeva, M., 1986. Sensory aroma and taste profiles of raw-dried sausages manufactured with a lipolytically active yeast culture. Die Nahrung 30 (8), 829–832.

Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y., Wakayama, M., 1994. Isolation and characterization of salt-tolerant glutaminase from marine *Micrococcus luteus* K-3. J. Ferment. Bioeng. 77, 621–625.

Ordoñez, J.A., Hierro, E.M., Bruna, J.M., de la Hoz, L., 1999. Changes in the components of dry-fermented sausages during ripening. Crit. Rev. Food Sci. Nutr. 39, 329–367.

Penninckx, M.J., Jaspers, C.J., 1985. Caractérisation d'une activité γ-glutamylarylamidase-glutaminase chez *Saccharomyces cerevisiae*. Biochimie 67, 999–1006.

Pinkus, L.M., 1977. Glutamine binding sites. Methods Enzymol. XLVI (45), 414–427.

Prusiner, S., Stadtman, E.R., 1976. Regulation of glutaminase B in *Escherichia coli*: II. Modulation of activity by carboxylate and borate ions. J. Biol. Chem. 251, 3457–3462.

Prusiner, S., Davis, J.N., Stadtman, E.R., 1976. Regulation of glutaminase B in *Escherichia coli*: I. Purification, properties, and cold lability. J. Biol. Chem. 251, 3447–3456.

Santos-Mendoça, R.C., 2000. Aislamiento, selección y caracterización de levaduras de embutidos con vistas a su utilización como coadyuvante en el proceso de curado. PhD Thesis (in Spanish), University of Valencia, Spain.

Sayre, F.W., Roberts, E., 1958. Preparation and some properties of a phosphate-activated glutaminase from kidneys. J. Biol. Chem. 235, 1128–1134.

Smith, E.M., Watford, M., 1988. Rat hepatic glutaminase: purification and immunochemical characterization. Arch. Biochem. Biophys. 260, 740–751.

Soberón, M., González, A., 1987. Physiological role of glutaminase activity in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 133, 1–8.

Sorensen, B.B., 1997. Lipolysis of pork fat by the meat starter culture *Debaryomyces hansenii* at various environmental conditions. Int. J. Food Microbiol. 34, 187–193.

Sorensen, B.B., Samuelsen, H., 1996. The combined effects of environmental conditions on lipolysis of pork fat by lipases of the meat starter culture organisms *Staphylococcus xylosus* and *Debaryomyces hansenii*. Int. J. Food Microbiol. 32, 59–71.

Toledo, V.M., Selgas, M.D., Casas, M.C., Ordoñez, J.A., Garcia, M.L., 1997. Effect of selected mould strains on proteolysis in dry fermented sausages. Z. Lebensm.-Unters. Forsch. A 204, 385–390.

Tower, D.B., 1967. Enzymatic determination of glutamine and asparagine. Methods Enzymol. XI (8), 76–93.

Zalkin, H., 1985a. GMP synthetase. Methods Enzymol. 113, 273–278.

Zalkin, H., 1985b. CTP synthetase. Methods Enzymol. 113, 282–287.