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Food Chemistry 86 (2004) 385–389
Food Chemistry
www.elsevier.com/locate/foodchem

Effects of curing agents and the stability of a glutaminase from *Debaryomyces* spp.

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Received 23 May 2003; received in revised form 8 September 2003; accepted 8 September 2003

Abstract

The stability of a glutaminase from *Debaryomyces* spp. CECT 11815 was studied. The enzyme showed poor stability at acid pH (half-life below 10 h at pH 5.5). Better stability was observed at pH 7.5–8.5; however, some variability was found, depending on the type of buffer used. The thermal stability of the glutaminase was rather poor at 37 °C (half-life of 7.4 h) but increased at lower temperatures, reaching a half-life of 323.2 h at 4 °C. EDTA and ethylene glycol extended the enzyme half-life while L-Asp, DTT, glycerol and several salts had the opposite effect. In addition, the influence of curing agents on glutaminase activity was investigated. Nitrate, nitrite, glucose and ascorbic acid had no significant effect on enzyme activity at the levels typically used in the processing of dry-cured sausages, while 1 M NaCl inhibited nearly 55% of the glutaminase activity.

Keywords: Glutaminase; *Debaryomyces*; Enzyme stability; Yeast; Curing agents

1. Introduction

The ingredients and the chemical and biochemical changes that occur during the fermentation and ripening processes have a decissive influence on the flavour of dry-cured sausages (Dainty & Blom, 1995). The compounds implicated in flavour generation arise from many sources, such as spices, sugar metabolism, lipolysis and lipid oxidation, proteolysis and amino acid degradation (Toldrá, Sanz, & Flores, 2001).

Yeasts can tolerate the low water activity of dry-cured sausages and can enhance the flavour of these products due to their action on lipids and proteins which are precusors of specific metabolic compounds. Moreover, these microorganisms contribute to sausage colour development as a result of their oxygen consumption and their catalase activity, which is also beneficial in delaying rancidity (Cook, 1995; Leistner, 1995; Lücke & Hechelmann, 1987; Miteva, Kirova, Gadjeva, & Radeva, 1986).

Free amino acids can be converted into flavour compounds through the action of both muscle and microbial enzymes. The action of muscle enzymes has been widely reported (Molly et al., 1997; Toldrá, 1998). However, microbial enzymes are particularly significant because, depending on the microorganisms inoculated, different flavours can be obtained. Among the various amino acid degradation pathways, deamidation of both glutamine and asparagine has an important role from the point of view of sausage production, especially the deamidation of glutamine, since hydrolysis of the glutamine amide group produces ammonia, an acidity neutraliser, and glutamate, a flavour enhancer. This reaction is catalysed by the enzyme known as glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2).

In a previous paper (Durá, Flores, & Toldrá, 2002), the purification and characterisation of a glutaminase from *Debaryomyces* spp. CECT 11815 was described. This enzyme could have a significant impact on fermented sausage sensory quality if the yeast strain is used as starter culture, but also if the enzyme is directly added to the sausage mixture. However, this last possibility

Abbreviations: DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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doi:10.1016/j.foodchem.2003.09.012

could be restricted, depending on the enzyme stability under the severe conditions that take place during sausage ripening and on inhibition, due to the presence of curing agents in the meat mixture. Therefore, the objective of this work was to study the stability and the effect of curing agents on *Debaryomyces* spp. glutaminase.

2. Materials and methods

2.1. Glutaminase

Growth conditions of *Debaryomyces* spp. CECT 11815 and enzyme purification were reported by Durá et al. (2002). The glutaminase employed in this investigation was obtained by gel filtration chromatography; it was eluted in 20 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl, 0.5 mM EDTA and 10% (v/v) ethylene glycol. The enzyme was purified 250-fold, contained 70 μg of protein/ml and showed a specific activity of 1 μmol of L-Glu/min/mg at 37 °C.

2.2. Glutaminase activity

The standard assay mixture for glutaminase activity was performed by incubation, at 37 °C for 30 min, with 10 mM L-Gln and 50 mM Tris–HCl, pH 8.5. The reaction was stopped by adding acetic acid up to a 0.2 M final concentration and the L-Glu released from L-Gln was followed by the method of Gella and Pascual (1982), as described by Durá et al. (2002).

2.3. Glutaminase stability

The thermal stability and the effect of pH and several substances on glutaminase stability were assayed by the measurement of the remaining glutaminase activity in the standard assay mixture. Two replicates (samples + controls) were measured for each experimental point. The activity was expressed as percentage of the activity remaining at each time interval using, as control, the value obtained at zero time.

2.3.1. Effect of pH on glutaminase stability

The enzyme was concentrated up to half its initial volume by centrifugation in an Ultrafree-4 filter with a Biomax-30 membrane (Millipore, Bedford, MA, USA), at the same time changing the buffer to 0.1 M NaCl solution, containing 0.5 mM EDTA and 10% (v/v) ethylene glycol. Aliquots of the resulting solution were mixed with equal volumes of several buffers (acetate, citrate, Bis Tris Propane and Tris) at 40 mM concentration, containing 0.1 M NaCl and 10% (v/v) ethylene glycol. The buffer solutions had previously been adjusted to different pH values, ranging from 4.4 to 8.5. The mixtures were incubated at 25 °C, and aliquots were taken at different time intervals and glutaminase activity rapidly assayed.

2.3.2. Thermal stability of glutaminase

The glutaminase was incubated at several temperatures (−20, 4, 15, 25 and 37 °C), and at −20 °C in the presence of 40% ethylene glycol, keeping pH at 7.5. Aliquots were taken at different time intervals and glutaminase activity assayed.

2.3.3. Effect of different substances on glutaminase stability

The enzyme was concentrated by centrifugation in an Ultrafree-4 filter with a Biomax-30 membrane, at the same time changing the buffer to 20 mM Tris–HCl, pH 7.5. Aliquots of the resulting solution were mixed with equal volumes of buffer containing the following substances: 10 mM EDTA, 2 mM DTT, 2 mM L-Asp, 20% (v/v) glycerol, 20% (v/v) ethylene glycol or different salts (NaCl, KCl, Na_2SO_4 and K_2SO_4) at 0.2 M concentration. The mixtures were incubated at 25 °C, and aliquots were taken for glutaminase activity assay. 0.05 mM EDTA and/or 1% (v/v) ethylene glycol were added to the standard assay mixture of the samples that did not contain any EDTA and/or ethylene glycol in order to make glutaminase detection possible.

2.4. Effects of curing agents on glutaminase activity

The glutaminase was centrifuged in an Ultrafree-4 filter with a Biomax-30 membrane, in order to change the buffer to 20 mM Tris–HCl, pH 7.5, containing 0.5 mM EDTA and 10% (v/v) ethylene glycol. Aliquots of the resulting solution were incubated in the standard assay mixture in the presence of 0.05–1.00 M NaCl, 100 and 300 ppm KNO_3, 100 and 300 ppm $NaNO_2$, 0.2–1.0% (w/v) glucose and 100–500 ppm ascorbic acid. The activity was measured by the standard assay method, except for the measurements in the presence of ascorbic acid. In this case, a strong interference was observed in the redox reactions on which the method is based. So, L-Glu generation was analysed as phenylthiocarbamyl derivative by reverse-phase HPLC with a previous deproteinisation of the samples with acetonitrile (Aristoy & Toldrá, 1991). Two replicates (samples + controls) were measured for each experimental point.

3. Results and discussion

3.1. Glutaminase stability

3.1.1. General

Lack of stability is a common feature of many glutaminases, especially those from mammals (Haser,

Shapiro, & Curthoys, 1985; Heini, Gebhardt, Brecht, & Mecke, 1987; Patel & McGivan, 1984), although some microbial enzymes also show high unstability (Huerta-Saquero, Calderón, Arreguin, Calderón-Flores, & Durán, 2001; Prusiner, Davis, & Stadtman, 1976). The *Debaryomyces* spp. enzyme shares this characteristic.

3.1.2. Glutaminase stability at different pH values

The enzyme stability, when incubated at 25 °C and different pH values, is shown in Fig. 1. Both pH and buffer type influenced the glutaminase stability. *Debaryomyces* spp. enzyme displayed the longest half-life (136.6 h) at pH 7.5 when incubated in Tris buffer. The modification of the Tris buffer pH from 7.5 to 8.5 caused a 25% reduction of the enzyme half-life, while varying buffer type to Bis Tris Propane, and maintaining pH value at 7.5, produced a 36% decrease of glutaminase half-life. Moreover, the stability loss was higher when pH was reduced than when it was increased (Fig. 1), and severe consequences on the enzyme stability were observed at pH 5.5, although it maintained better stability when incubated in acetate than in citrate buffer (9.6 h vs 3.8 h, respectively). The half-life with the last buffer was also worse than Bis Tris Propane at pH 6.5 that was 39.1 h. Glutaminase instability at acid pH has frequently been observed and, in fact, most of these enzymes keep better activity at pH values in the range 7.0 to 9.0 (Lu, Yu, & Chou, 1996; Moriguchi, Sakai, Tateyama, Furuta, & Wakayama, 1994; Yano, Ito, Tomita, Kumagai, & Tochikura, 1988).

3.1.3. Glutaminase thermal stability

The glutaminase thermal stability results, when incubated at different temperatures are shown in Fig. 2. The enzyme was highly unstable at 37 °C, with a half-life as short as 7.4 h. However, as temperature decreased, a stabilising effect was observed, as reflected by higher half-life values, such as 135.8 h at 25 °C, 267.4 h at 15 °C

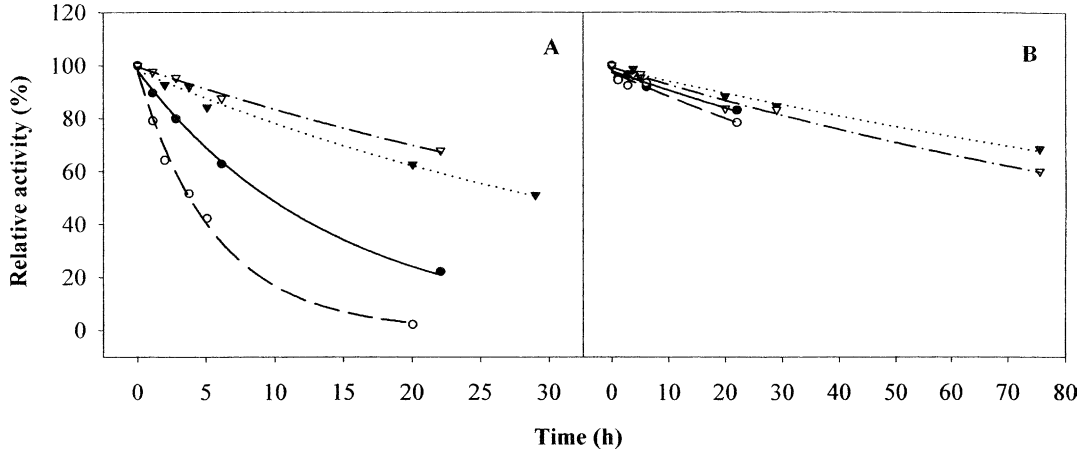

Fig. 1. Stability of *Debaryomyces* spp. glutaminase incubated at 25 °C and different pH values. (A) Acetate, pH 5.5 (●, —); citrate, pH 5.5 (○, – – –); citrate, pH 6.5 (▼, ·····); and Bis Tris Propane, pH 6.5 (▽, —·—). (B) Bis Tris Propane, pH 7.5 (●, —); Bis Tris Propane, pH 8.5 (○, – – –); Tris, pH 7.5 (▼, ·····); and Tris, pH 8.5 (▽, —·—).

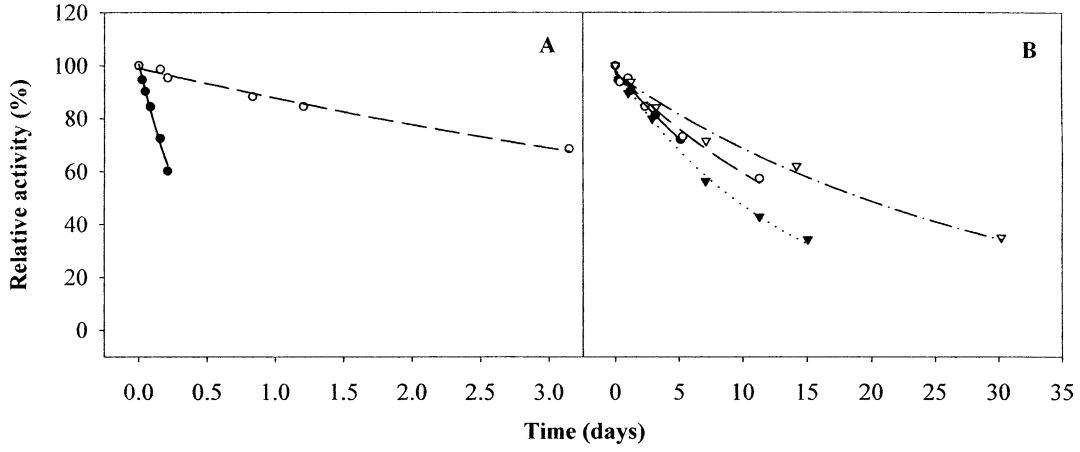

Fig. 2. Thermal stability of *Debaryomyces* spp. glutaminase incubated at pH 7.5. (A) 37 °C (●, —); 25 °C (○, – –). (B) 15 °C (●, —); 4 °C (○, – –); −20 °C (▼, ·····); −20 °C and 40% ethylene glycol (▽, —·—).

and 323.2 h at 4 °C. Among yeast glutaminases, *Cryptococcus albidus* enzyme and *Saccharomyces cerevisiae* glutaminase A were reported to be thermostable, but the B isozyme of this last yeast was thermolabile (Iwasa, Fujii, & Yokotsuka, 1987; Soberón & González, 1987). Similarly, *Rhizobium etli*, that contains two glutaminases, had the A form thermolabile and the B form thermoresistant (Durán et al., 1996). On the other hand, *Escherichia coli* glutaminase B lacked long-term stability at room temperature, although this is an atypical enzyme, since it is reversibly inactivated at temperatures below 24 °C (Prusiner et al., 1976).

The stability of *Debaryomyces* spp. glutaminase rapidly decreased at −20 °C (Fig. 2B). At this temperature, the enzyme showed a half-life of 220.9 h. However, the addition of 40% ethylene glycol, as anti-freezing agent, produced a stabilisation of the glutaminase, and its half-life increased up to 461.0 h, 43% more than at 4 °C. The negative influence of freezing on some glutaminase stabilities has been described (Holcenberg, 1985; Prusiner et al., 1976), although some of them are unaffected (Hartman, 1970; Ramadan, El Asmar, & Greenberg, 1964; Tower, 1967).

3.1.4. Glutaminase stability in the presence of different substances

The effects of several substances on glutaminase stability are shown in Table 1. The enzyme incubated at 25 °C in 20 mM Tris–HCl, pH 7.5, lost 50% of its initial activity within 43 h. The half-life was increased in the presence of 5 mM EDTA or by adding 10% ethylene glycol. These substances acted as stabilisers and extended the enzyme half-life to 55 and 60 h, respectively. On the other hand, the presence of 1 mM L-Asp and, especially 1 mM DTT and 10% glycerol, clearly shortened the half-lives by 10, 27 and 29 h, respectively. These facts do not agree with the stabilising effect of glycerol reported by Moriguchi et al. (1994) and Ramadan et al. (1964) for the glutaminases of *Pseudomonas* and *Micrococcus luteus*, respectively, nor with Prusiner et al. (1976) who reported that L-Asp stabilised *E. coli* glutaminase B, while EDTA and DTT had negative effects.

Table 1
Effects of different substances on half-lives of *Debaryomyces* spp. glutaminase incubated at 25 °C and pH 7.5

Substance	Concentration	Half-life (h)
None	–	42.8
EDTA	5 mM	55.2
L-Asp	1 mM	32.9
Ethylene glycol	10% (v/v)	60.5
DTT	1 mM	15.7
Glycerol	10% (v/v)	13.6
K_2SO_4	0.1 M	17.3
Na_2SO_4	0.1 M	25.1
KCl	0.1 M	18.9
NaCl	0.1 M	28.4

Moreover, all the assayed salts, particularly the potassium salts, markedly decreased the glutaminase stability (Table 1). For instance, 0.1 M potassium sulphate reduced the half-life by approximately 60%. Regarding the anions, the calculated values suggest that sulphate affects the enzyme more negatively than chloride. On the other hand, Prusiner et al. (1976) observed that diverse anions stabilised *E. coli* glutaminase B, while Na^+ and K^+ did not produce any effect. Similarly, *Cr. albidus* enzyme was found to be salt-resistant (Iwasa et al., 1987) and *M. luteus* glutaminases increased their half-lives in the presence of NaCl (Moriguchi et al., 1994).

3.2. Effect of curing agents on glutaminase activity

The effects of potassium nitrate, sodium nitrite, glucose and ascorbic acid on glutaminase activity are shown in Table 2. These curing agents were assayed in the typical proportions used in sausage making. Nitrite, nitrate and glucose did not affect enzyme activity significantly. In contrast, increased concentrations of ascorbic acid produced a slight inhibition of the enzyme, diminishing its activity by about 10% in the presence of 500 ppm of ascorbic acid.

The effect of NaCl on glutaminase activity is displayed in Fig. 3. This curing agent exerted an important inhibitory effect, reducing the enzyme activity by approximately 35% at 0.5 M concentration, and nearly 55% at 1 M concentration. Among glutaminases, *Aspergillus oryzae* and *Actinomucor taiwanensis* enzymes were clearly inhibited by NaCl (Lu et al., 1996; Yano et al., 1988), while *M. luteus* glutaminases were activated by this salt (Moriguchi et al., 1994). Among yeast enzymes, *Cr. albidus* glutaminase was reported to be salt-resistant (Iwasa et al., 1987).

In summary, the expected action of *Debaryomyces* spp. glutaminase during dry-cured sausage processing would be restricted to the beginning of the fermentation stage, when temperature is about 20–25 °C, and pH is still around 6.0–5.5. The activity would rapidly decrease

Table 2
Effects of curing agents on *Debaryomyces* spp. glutaminase activity

Curing agent	Concentration	Relative activity (%)
Control	–	100
KNO_3	150 ppm	103
	300 ppm	101
$NaNO_2$	150 ppm	102
	300 ppm	100
Glucose	0.2%	103
	0.5%	104
	1%	105
Ascorbic acid	100 ppm	97.0
	200 ppm	92.8
	500 ppm	89.3

Results expressed as relative activity against control with absence of any curing agent.

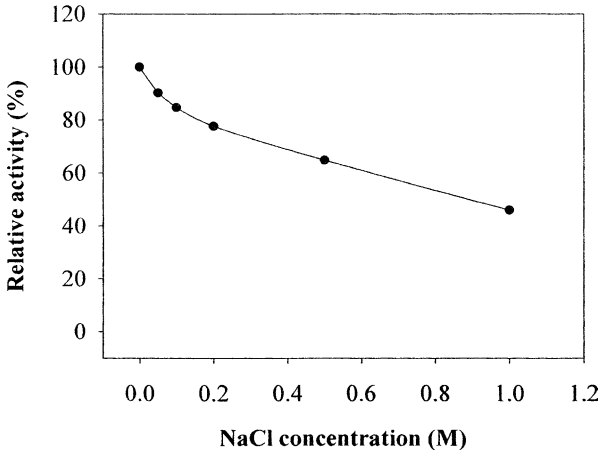

Fig. 3. Effect of sodium chloride on *Debaryomyces* spp. glutaminase activity.

during ripening and drying, especially due to the pH drop.

Acknowledgements

This work was supported by grant AGL2001-0500 from Comisión Interministerial de Ciencia y Tecnología (CICyT, Spain). FPI/MCyT scholarship to M.A. Durá is also acknowledged.

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