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Factors affecting the activity of enzymes involved in peptide and amino acid catabolism in non-starter lactic acid bacteria isolated from Cheddar cheese

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Abstract

Peptidolytic and aminotransferase enzymes involved in amino acid release and catabolism were formed constitutively by cheese lactobacilli. Activities in controlled batch and continuous cultures of *Lactobacillus rhamnosus* F3 were affected by the rate and stage of growth and declined, under aerobic and anaerobic conditions, in the stationary growth phase. Enzyme formation was repressed by glucose and activity in cell lysates was affected by the nitrogen source. Activity was retained when cell integrity was maintained at pH 5.0–6.5 and at salinities of 5 g 100 mL^{-1}. Aminotransferase activity in lysates of two *Lb. paracasei* strains was maximal at, or close to, pH 6.0 and 30°C but was detectable under simulated cheese conditions. The activity response to increasing salinity differed with leucine and phenylalanine as substrates. Aromatic, branched-chain and sulphur-containing amino acids were the most effective substrates for glutamate formation with α-ketoglutarate as acceptor, and although other keto acids functioned as acceptors for leucine and phenylalanine transamination activities were highest with α-ketoglutarate. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Amino acid release from peptides formed by proteinase action on casein and the subsequent bacterial breakdown of the amino acids are critical events leading to flavour development during cheese ripening. The free amino acids can contribute directly to the flavour of the cheese, but more importantly many volatile flavour compounds have structures that are consistent with their formation from amino acid precursors (Urbach, 1995). Although many of the lactic acid bacteria in cheese are auxotrophic and dependent on an effective protease system to release the amino acids essential for their growth (Kunji, Mierau, Hagting, Poolman, & Konings, 1996) the microorganisms are also able to generate energy from amino acid catabolism (Nakae & Elliott, 1965; Williams, Withers, & Banks, 2000; Williams, Noble, & Banks, 2001). These catabolic activities can result in the formation of a number of compounds that have the potential to impact on cheese flavour (Fox & Wallace, 1997; McSweeney & Sousa, 2000). Inactivation of the aminotransferase enzymes involved in the breakdown of amino acids by lactococci has been shown to reduce aroma formation during cheese ripening (Rijnen et al., 1999).

The lactic acid bacterial population of Cheddar cheese comprises the starter lactococci and the ultimately numerically dominant non-starter lactobacilli (Peterson & Marshall, 1990; Fox, McSweeney, & Lynch, 1998). Both bacterial groups are actively proteolytic and generate a wide range of peptides from casein (Kunji et al., 1996; Law & Haandrikman, 1997). At least 16 peptidases responsible for the conversion of the released peptides into free amino acids have been characterized from lactic acid bacteria (Christensen, Dudley, Pedersen, & Steele, 1999). The routes for the breakdown of the released amino acids may involve deamination, decarboxylation and desulphuration reactions (Hemme,

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Bouillanne, Metro, & Desmazeaud, 1982). Information currently available on the enzymic mechanisms involved in amino acid catabolism and the formation of sulphur compounds by LAB has been summarized recently (Christensen, Dudley, Pedersen, & Steele, 1999; Weimer, Seefeldt, & Dias, 1999) and it is now recognized that in all lactic acid bacteria amino acid degradation to aroma compounds occurs via a pathway that is initiated by a transamination reaction (Yvon & Rijnen, 2001).

The non-starter population of Cheddar cheese is dominated by mesophilic *Lactobacillus* spp. (Williams & Banks, 1997) and the inclusion of lactobacilli as adjunct cultures is often associated with elevated levels of free amino acids and enhanced flavour intensities (Broome, Krause, & Hickey, 1990; Lane & Fox, 1996; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996; Lynch, Muir, Banks, McSweeney, & Fox, 1999). Although the majority of the NSLAB are actively peptidolytic (Williams & Banks, 1997; Williams, Felipe, & Banks, 1998) the non-starter lactobacilli do not necessarily increase the extent of primary proteolysis during ripening (Lane & Fox, 1996; Lynch et al., 1996). The implication, therefore, is that the effects of NSLAB on flavour intensity occur as a consequence of increased amino acid release and turnover. Recent investigations have confirmed that the non-starter lactobacilli possess amino acid catabolizing enzyme (AACE) activities (Dias & Weimer, 1998; Groot & De Bont, 1998; Gummalla & Broadbent, 1999, 2001; Williams et al., 2001) although these enzymes as yet are not as well characterized as the AACE produced by starter lactococci (Christensen et al., 1999; Weimer et al., 1999; Yvon & Rijnen, 2001). Additionally, there is a paucity of information available on factors that affect the occurrence and activity of NSLAB amino acid-metabolizing enzymes. The regulation of expression of the component enzymic activities of the proteolytic system of the starter lactococci has likewise received little attention although it appears that proteinase formation can be regulated by the exogenous supply of peptides and amino acids (Exterkate, 1985; Laan, Bolhuis, Poolman, Abee, & Konings, 1993; Meijer, Marugg, & Hugenholtz, 1996). Amino acid availability can also affect the biosynthesis of the aminotransferase in lactococci (Rijnen, Bonneau, & Yvon, 1999). Peptidase formation by *Lactobacillus* spp. has also been shown to be dependent on the composition of the medium (El Soda, Bergère, & Desmazeaud, 1978; Abo-Elnaga & Plapp, 1987; Atlan, Laloi, & Portalier, 1989; Hébert, Raya, & de Giori, 2000) but no information is available on factors affecting the production of enzymes involved in amino acid breakdown.

In view of the fact that the release and turnover of amino acids by lactobacilli in cheese curd during ripening has implications for flavour development, a study was undertaken to investigate factors that affect the activity of the peptidase and aminotransferase enzymes that initiate amino acid release and the initial stage in their subsequent conversion to aroma and flavour compounds. Studies were conducted under controlled conditions in batch and continuous cultures with non-starter *Lactobacillus* spp. isolated from Cheddar cheese to examine the effects of nutritional and environmental factors on amino acid metabolizing enzyme activities. An understanding of factors that regulate these activities may ultimately enable the development of strategies to more precisely control flavour formation in fermented food products.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used had all been isolated from Cheddar cheese and had been assigned to the species level on the basis of morphological characteristics and carbohydrate utilization profiles determined with the Biomerieux (Basingstoke, UK) API 50CHL system (Tammam, Williams, Noble, & Lloyd, 2000; Williams et al., 2001). All cultures were maintained at 4°C on MRS medium (Oxoid Ltd., Basingstoke) and were subcultured at regular intervals to maintain viability.

Uncontrolled batch cultures were grown for 48 h at 30°C in shaken flasks (50 rev min^{-1}) in MRS broth, modified to contain 0.2 g 100 mL^{-1} glucose. The effect of the nature of the nitrogen source on enzyme activity was determined in duplicated shake flask cultures (50 rev min^{-1}). The cultures were grown at 30°C for 48 h in a defined medium with either casein, casamino acids, lactalbumin hydrolysate (all 1 g 100 mL^{-1}) or an amino acid mixture as the nitrogen source. The filter sterilized amino acid mix used contained the 20 amino acids alanine, arginine, aspartic acid, citrulline, cysteine, glycine, glutamic acid, glutamine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, valine (all 2.5 mM), leucine, tryptophan (1.25 mM) and 0.025 mM tyrosine. It was confirmed by HPLC analysis that the hydrolysates contained peptide nitrogen; the free amino acid profiles of the casamino acids and lactalbumin hydrolysate were different but represented a similar proportion of the dry matter (49.4 and 50.9 g 100 g^{-1}, respectively). Batch cultures were also obtained under controlled conditions using a Bioflo III laboratory fermenter (New Brunswick Scientific, Hatfield, UK).

Continuous (chemostat) cultures were prepared using an LH 500 series modular fermenter (LH Engineering, Stoke Poges). All cultures were maintained at 30°C and were continuously gassed with N_2 (50 mL min^{-1}) to maintain anaerobic conditions; aerobic conditions were achieved with an aeration rate of 500 mL min^{-1}. The

defined medium (pH 6.2) contained ($g\,L^{-1}$) casamino acids, 10; yeast extract, 5; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.025; $MnSO_4 \cdot 4H_2O$, 0.05; EDTA, 0.1; and, with the exception of when it was an experimental variable, glucose, 0.2. Batch cultures were harvested after 24 h, unless indicated otherwise, and the chemostat cultures were recovered when the steady state had been maintained during a period in which the volume of growth medium added had equalled 4 times the culture volume. Duplicate cultures were prepared for all of the growth conditions examined.

2.2. Cell lysate preparation

The cells were harvested by centrifugation (26,000*g* for 30 min at 4°C) and the cell pellet was washed once with, and then resuspended in, cold (4°C) 0.1 M 2-(*N*-morpholino) ethanesulphonic acid (MES) buffer (pH 7). Washed cell suspensions were disrupted at 4°C by 8×30 s periods of sonication with an amplitude setting of 10 μm using an MSE 150 Soniprep ultrasonic disintegrator (probe tip diameter 9.5 mm, MSE Scientific Equipment, Crawley). Unbroken cells were removed by centrifugation (10,000*g* for 15 min at 4°C) and separate aliquots (0.5 mL) of the lysate were stored at –70°C prior to assay.

2.3. Enzyme assay procedures

Aminopeptidase and dipeptidyl peptidase activities were determined using the appropriate *p*-nitroanilide substrate (0.5 mM in 0.1 M MES, pH 7). The *p*-nitroaniline released after incubation at 30°C for a maximum period of 60 min was determined spectrophotometrically at 405 nm using known standards (Williams & Banks, 1997).

Aminotransferase activities were measured by determining glutamate formation from the substrate amino acid in the presence of α-ketoglutaric acid (Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997). The incubation mix contained 100 mM tris buffer (pH 8), pyridoxal phosphate (0.1 mM), amino acid substrate (5 mM) and neutralized α-ketoglutarate (5 mM). Glutamic acid formation during the incubation period (60 min at 30°C) was determined enzymically with glutamate dehydrogenase (Williams et al., 2001). The keto acid specificity was examined using a range of potential acceptors. Aminotransferase activity was determined by monitoring the formation of the appropriate amino acid by RP-HPLC. Amino acids were extracted with chloroform:methanol (2:1), derivatized with phenylisothiocyanate and analysed on an automated Spectra Physics HPLC using a Tosohaas TSK-Gel ODS-80TS column, as described previously by Banks et al. (2001).

Assays were performed in duplicate and appropriate control incubations performed to determine the extent of endogenous and non-enzymic conversions. The protein content of the cell-free lysates was determined by the dye-binding method of Bradford (1976).

3. Results and discussion

3.1. Factors affecting aminotransferase activity in cell-free extracts of Lb.paracasei

Aminotransferase activity is present in non-starter lactobacilli (Williams et al., 2001) but despite the potential importance of this population in amino acid catabolism during cheese maturation, the principal characteristics of NSLAB aminotransferase activity have not been described previously. A preliminary characterization of aminotransferase activity in unfractionated lysates of 2 strains of *Lb. paracasei* was, therefore, undertaken. The isolates, initially recovered from Cheddar cheese that had been matured for 36 months (Tammam et al., 2000), were grown for 48 h at 30°C in modified MRS broth before harvesting and lysate preparation. Triplicate enzyme preparations were examined.

3.1.1. Effect of pH and temperature

Leucine/α-ketoglutarate, phenylalanine/α-ketoglutarate and methionine/α-ketoglutarate aminotransferase activities (LeuAT, PheAT and MetAT, respectively) were maximal at, or close to, pH 6 and 30°C in lysates of both *Lb. paracasei* CI9 and *Lb. paracasei* CI3. Activity against the 3 amino acids was retained in both strains at pH 5, which is a value similar to that in ripening Cheddar cheese. The mean residual aminotransferase activities, determined with Leu, Phe and Met as substrates, at pH 5 and at 30°C, as a percentage of their maximal activity at pH 6 and 30°C, were $72.3 \pm 3.5\%$, $83.6 \pm 9.1\%$ and $68.4 \pm 16.4\%$, respectively. The corresponding relative specific activity values at the ripening temperature of 10°C and at pH 6, as compared to those determined at 30°C, were $33.5 \pm 9.9\%$, $26.2 \pm 2.3\%$ and $21.4 \pm 7.2\%$, respectively. These data indicate that NSLAB aminotransferase released as a consequence of cell lysis could potentially retain activity at the pH and temperature that appertain in ripening Cheddar curd although additional studies would be required to establish the stability of enzymes released into the cheese matrix.

The pH and temperature optima of the *Lb. paracasei* aminotransferase are lower than those determined with purified preparations from *Lactococcus lactis* strains in which values of pH 6.5–8.0 and 35–40°C were determined (Yvon et al., 1997; Yvon, Chambellon, Bolotin, & Roudot-Algaron, 2000). However, in one *Lac. lactis* subsp. *cremoris* strain the temperature optimum was even higher at 45–50°C (Engels et al., 2000). The residual activity at 10°C of the aminotransferase of

these starter lactococci was, at 10–25% (Yvon et al., 1997, 2000), broadly similar to that of the two non-starter *Lb. paracasei* strains reported in this study. However, at cheese curd pH values the activity decline of the *Lb. paracasei* aminotransferase with Leu or Phe as substrate (28% and 16%, respectively) was less than the one that was observed with the lactococcal preparations (70–80% and 40%, respectively, Yvon et al., 1997; Engels et al., 2000). Thus, residual activity originating from lactobacilli and lactococci may potentially contribute to amino acid turnover under cheese conditions.

3.1.2. Effect of salinity

The effects of increasing NaCl concentration (0–5 g $100\,mL^{-1}$) on the aminotransferase activity of *Lb. paracasei* CI3 and CI9 were amino acid substrate-dependent. The specific activity of PheAT and MetAT decreased as the salinity increased. The decrease of PheAT and MetAT activity in strain CI3 in the presence of 5 g $100\,mL^{-1}$ NaCl was 52.7% and 55.9%, respectively of the values determined in the absence of salt; the corresponding values for CI9 were 68.9% and 57.0%, respectively. The activities of the branched-chain and aromatic aminotransferases of *Lac. lactic* were also reduced by increasing salinity (Yvon et al., 1997, 2000; Gao & Steele, 1998; Engels et al., 2000), with activity losses in the range 20–40% occurring in the presence of 4 g $100\,mL^{-1}$ NaCl. The mean activity decrease of PheAT and MetAT of the CI3 and CI9 at this salinity level were slightly higher at $46.5 \pm 7.0\%$ and $53.9 \pm 3.9\%$, respectively. However, the specific activity of LeuAT increased in both *Lb. paracasei* strains and was maximal at a salinity of 4 g $100\,mL^{-1}$ NaCl. The temperature–activity profile of LeuAT in *Lb. paracasei* CI3 also differed slightly from those of MetAT and PheAT, which exhibited a less broad temperature optimum. These substrate-dependent differences in activity characteristics may be indicative of the involvement of more than one enzyme. In the lactococci, both a branched-chain and an aromatic aminotransferase exhibit some activity against methionine (Yvon et al., 1997, 2000; Gao & Steele, 1998; Engels et al., 2000), and although there are reports of the occurrence of aromatic and methionine aminotransferase activity in *Lactobacillus* spp. (Dias & Weimer, 1998; Groot & De Bont, 1998; Gummalla & Broadbent, 1999; Amarita, Requena, Taborda, Amigo, & Pelaez, 2001; Williams et al., 2001), confirmation that the lactobacilli, like the lactococci, may form more than one aminotransferase necessitates the purification and characterization of the individual enzymes for resolution. An aspartate aminotransferase has been purified from *Lb. murinus* (Rollan, Manca de Nadra, De Ruiz Holgado, & Oliver, 1988) but currently there is no other published information on the aminotransferases of lactobacilli.

3.1.3. Substrate and acceptor specificity

The amino acid/α-ketoglutarate aminotransferase activity in the lysates of *Lb. paracasei* CI3 and CI9 exhibited a wide amino acid substrate specificity as glutamate was produced from a wide range of amino acids (Fig. 1). Of the 20 amino acids tested, however, highest activities ($>10\,nmol\,mg^{-1}$ protein h^{-1}) were detected in both the strains with leucine, valine, phenylalanine, tyrosine and the sulphur-containing amino acids methionine and cysteine. These amino acids were also the most effective substrates for the purified branched-chain (Engels et al., 2000; Yvon et al., 2000) and aromatic amino acid aminotransferases (Yvon et al., 1997; Gao & Steele, 1998) of various *Lac. lactis* strains. Although aminotransferase activity was detectable with another 10 amino acids, including alanine, arginine, histidine, lysine and threonine, the levels were $<10\%$ of the activity detected with leucine as substrate. Strain differences in the activity profiles of the two NSLAB isolates were apparent with CI3 having higher activity against methionine and branched-chain amino acid substrates whereas the CI9 lysate was more active against phenylalanine, the aromatic amino acid aminotransferase substrate (Fig. 1).

When the lysates were incubated under cheese-like conditions (pH 5, 4 g $100\,mL^{-1}$ NaCl, 10°C) the activities in both strains with all amino acid substrates were markedly reduced as compared to the values determined at pH 8, 30°C and in the absence of NaCl (Fig. 1). The activities determined with amino acid substrates leucine, phenylalanine and methionine, (i.e. the most active aminotransferases) under cheese-like conditions were all reduced by $>85\%$ (Fig. 1). The activity loss was not as pronounced with some of the less effective amino acid substrates (e.g. arginine, histidine, threonine and tryptophan) where the decline in the initially lower activity was approximately 50%. However, under the simulated cheese-like conditions approximately 45% of the amino acid/α-ketoglutarate aminotransferase activities of CI9 and 30% of those in lysates of CI3 were $<10\%$ of the values measured at 30°C at neutral pH in the absence of added NaCl. The methionine aminotransferase activity in lysates of 3 strains of *Lac. lactis* and an isolate of *Lb. casei* was not detectable at pH 5.2 in the presence of 5 g $100\,mL^{-1}$ NaCl although only 25% of the activity was lost in a preparation of *Lb. helveticus* (Dias & Weimer, 1998). The activity of aminotransferases, in common with other hydrolytic enzymes (Weimer et al., 1997), is considerably lower under simulated cheese conditions and hence, the effects of enzymes released into the cheese matrix, as a consequence of cell lysis may be less significant during the maturation period than indicated by in vitro measurements made under optimal conditions. The role of enzymes released into the cheese

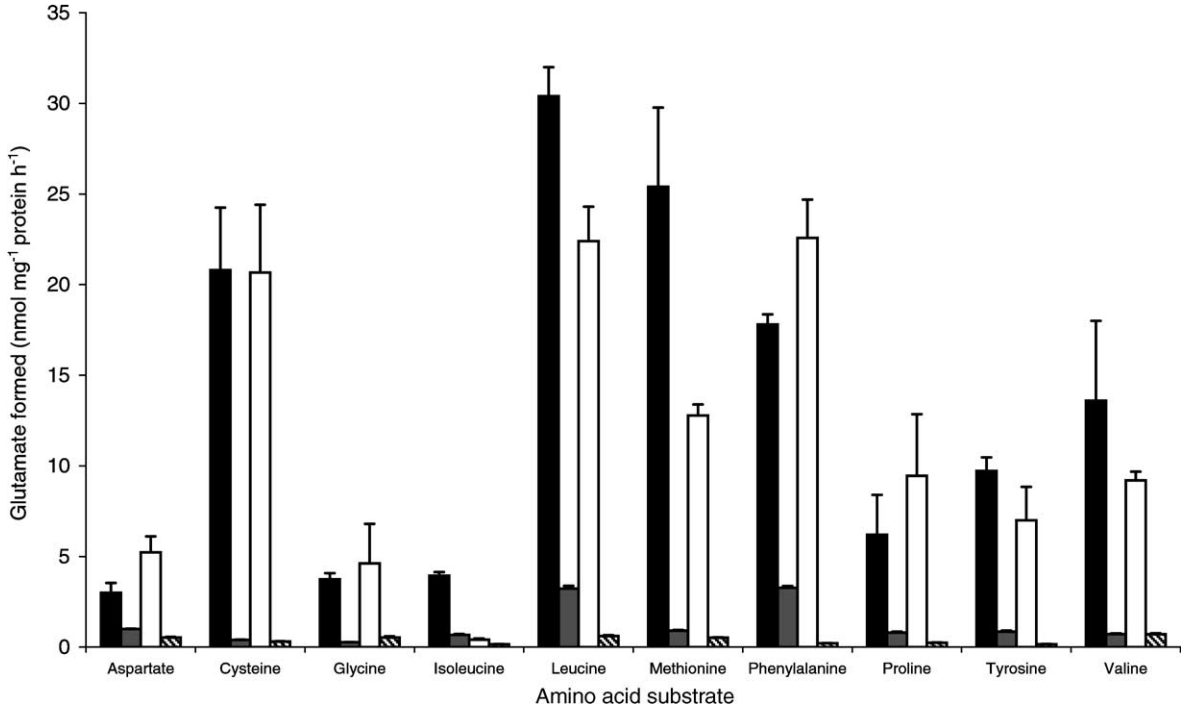

Fig. 1. Specific activity of amino acid/α-ketoglutarate aminotransferase with different amino acid substrates in lysates of *Lactobacillus paracasei* CI3 (■ ■) and *Lb. paracasei* CI9 (□ ▨) determined under optimal assay (■ □) and simulated cheese (■ ▨) conditions. The mean specific activities for triplicate determinations ± standard deviations are presented.

matrix during maturation has yet to be established conclusively.

It was confirmed that, in addition to having a wide substrate specificity, some of the aminotransferase activity of *Lb. paracasei* CI3 was retained, under optimised assay conditions, with keto acids other than α-ketoglutarate. Activity in triplicate lysate preparations was monitored by HPLC quantification of the amino acid transamination products formed from a range of keto acid acceptors. Although both the leucine and phenylalanine aminotransferases were able to function with other acceptors, as are the branched-chain and aromatic aminotranferases of *Lac. lactis* (Yvon et al., 1997, 2000; Roudot-Algaron & Yvon, 1998; Engels et al., 2000), the efficiency of the reaction was reduced considerably. Ketoisovalerate (KIV), ketoisocaproate (KIC) and hydroxyphenylpyruvate (HPP) maintained 28%, 46% and 55%, respectively, of the α-ketoglutarate-mediated activity of phenylalanine aminotransferase in *Lb. paracasei* CI3 lysates. The activity, however, was reduced by in excess of 80% with ketomethylvalerate in comparison with α-ketoglutarate, whilst the activity with ketomethiolbutyrate, oxaloacetate, ketopropionate and hydroxypyruvate as alternative keto acid acceptors was reduced by >90%. The loss in leucine aminotransferase activity with alternative keto acid acceptors was generally 60–80% although α-ketopropionate, oxaloacetate and ketomethiolbutyrate were particularly ineffective as acceptors with activity losses exceeding 90%. The KIV, KIC and HPP also functioned as effective alternative acceptors in the corresponding transamination reactions in *Lac. lactis* (Yvon et al., 1997, 2000). However, in contrast to the lactococcal enzymes (Yvon et al., 1997, 2000) methylthio-2-ketobutyrate was not an effective acceptor for the leucine and phenylalanine aminotransferases in the lysate of the *Lb. paracasei* CI3.

Aminotransferase activity with either leucine or phenylalanine as substrate was detected with all the acceptors in incubations conducted under simulated cheese conditions [pH 5, 4 g 100 mL^{-1} NaCl, 10°C]. The residual activities, relative to those under standard assay conditions averaged 26.3 ± 13.4% and ranged from 14.9% (β-hydroxypyruvate) to 50% (ketomethylthiolbutyrate and α-ketopropionate) with leucine as the donor amino acid. The equivalent values with phenylalanine as substrate were 38.3 ± 21.2% with values ranging from 12.5% (α-ketomethylvalerate) to 100% (β-hydroxypyruvate). It is evident, therefore, that there is the potential for limited aminotransferase-mediated amino acid turnover to occur in the cheese matrix during maturation. Various α-keto acids have been detected in Cheddar cheese (Ney & Wirotama, 1971) and these may serve as acceptors for subsequent transamination reactions. There is evidence, however, that keto acid concentrations are limiting in the cheese matrix as amino acid catabolism and the formation of aroma compounds during cheese ripening can be

enhanced by addition of α-ketoglutarate to the curd at the initial production stage (Yvon, Berthelot, & Gripon, 1998; Banks et al., 2001).

3.2. Factors affecting peptidase and aminotransferase occurrence and activity

The effects of nutritional and environmental factors on the occurrence and activity of 6 aminopeptidases, Gly.Pro dipeptidyl peptidase and amino acid/α-ketoglutaric acid aminotransferase were investigated using *Lb. rhamnosus* F3, a non-starter strain, initially isolated from Cheddar cheese (Williams et al., 2001). This isolate was selected as it was shown in preliminary experiments to possess higher levels of enzyme activity than the other NSLAB strains monitored. The effects of nutritional and other growth conditions were examined in duplicate controlled batch and continuous (chemostat) cultures in a defined medium containing 1 g $100\,mL^{-1}$ casamino acids as the nitrogen source. Under steady state conditions in a chemostat the dilution rate (D) has the same value as the growth rate, and since all parameters are controlled the effects of a single variable can be examined independently.

3.2.1. Growth stage and growth rate

The aminopeptidase activities monitored in *Lb. rhamnosus* F3 were detectable at various stages in the growth cycle of cultures grown under aerobic or anaerobic conditions (Fig. 2a). Activities were maintained throughout the exponential phase of growth and into the early stages of the stationary growth phase (5–24 h) but declined in older cultures. Peptide hydrolase activities in *Lb. casei* and the thermophilic species *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* were also expressed throughout the active growth phase and were maximal in the late logarithmic phase (Ezzat, El Soda, Desmazeaud, & Ismail, 1982; Habibi-Najafi & Lee, 1994; Vesanto, Peltoniemi, Purtsi, Steele, & Palva, 1996). Aminopeptidase activities were present in controlled anaerobic chemostat cultures of *Lb. rhamnosus* F3 at dilution rates (D) ranging from 0.03 to $0.2\,h^{-1}$, but were highest in the most rapidly growing cultures ($D = 0.2\,h^{-1}$) with a generation time of 3.45 h (Fig. 2a). Arginyl aminopeptidase and prolyl iminopeptidase activities were similarly affected by the growth status. Aminopeptidase activities in this particular NSLAB strain are thus influenced by both the rate and stage of growth. It is possible, therefore, at the low growth rates typically encountered in the cheese matrix enzyme activities would be sub-maximal although this requires experimental confirmation. Activities were, however, not markedly different after growth under aerobic or anaerobic conditions. Nakae and Elliott (1965) observed that total volatile fatty acid formation from casein hydrolysate by lactobacilli isolated from Cheddar cheese was similar under air and a nitrogen atmosphere.

Leucine, phenylalanine and methionine aminotransferase activities of *Lb. rhamnosus* F3 were also affected by the rate and stage of growth of the culture but again were not influenced by the presence or absence of oxygen in the gas phase (Fig. 2b). Enzyme activity was detected throughout the growth cycle but was maximal after 24 h at the end of the period of active growth. In chemostat cultures, activities were lower in the slowest and most rapidly growing cultures (dilution rates 0.03 and $0.2\,h^{-1}$, respectively) being highest in cultures with a controlled generation time of 6.9 or 11.5 h (Fig. 2b). In view of the fact that enzyme activity was influenced by the growth status of the microorganism, subsequent controlled batch studies were all monitored after 24 h growth under anaerobic conditions.

3.2.2. Effect of carbohydrate

The specific activities of the aminopeptidases monitored in *Lb. rhamnosus* F3 were reduced in cultures in which the initial glucose concentration was 5 g $100\,mL^{-1}$ (Fig. 3a). The level of arginyl, lysyl and phenylalanyl aminopeptidase was also lower in cultures containing 2 g $100\,mL^{-1}$ glucose; however, the inclusion of 0.2 g $100\,mL^{-1}$glucose did not affect the specific activities determined after 24 h growth at a controlled pH of 6.2. Enzyme activities were maintained with lactose as the carbohydrate source (Fig. 3a) indicating that aminopeptidase synthesis by *Lb. rhamnosus* F3 was subject to control by glucose repression. The effects on Gly.Pro dipeptidyl peptidase activity were broadly similar (Fig. 3a). The control of prolidase activity, another enzyme involved in the breakdown of proline-containing peptides, in *Lb. delbrueckii* subsp. *bulgaricus* is also subject to catabolite regulation by carbohydrates (Lamarque et al., 2001). There are several reports that peptidase activity of lactobacilli is lower in cells grown in MRS broth as compared to the levels in cells grown in skim milk (Abo-Elnaga & Plapp, 1987; Habibi-Najafi & Lee, 1994; Choi, Laleye, Amantea, & Simard, 1995; Gilbert, Blanc, Frot-Coutaz, Portalier, & Atlan, 1997). Cells grown in MRS would be subjected to the effects of glucose repression whereas those in skim milk which contains lactose would not be similarly controlled. It is probable, therefore, that glucose repression of peptidase synthesis in cheese lactobacilli is not uncommon.

The effects of the presence of glucose on the aminotransferase activity of *Lb. rhamnosus* F3 were more pronounced. Activities in cells grown on lactose or in the absence of glucose were up to 5-fold higher than the levels in cells grown in the presence of glucose (initial concentration 0.2–5 g $100\,mL^{-1}$) (Fig. 3b). The specific activity of methionine aminotransferase measured in lysates of *Lb. casei* and *Lb. plantarum* was increased when the assay mix contained 0.3 g $100\,mL^{-1}$ glucose

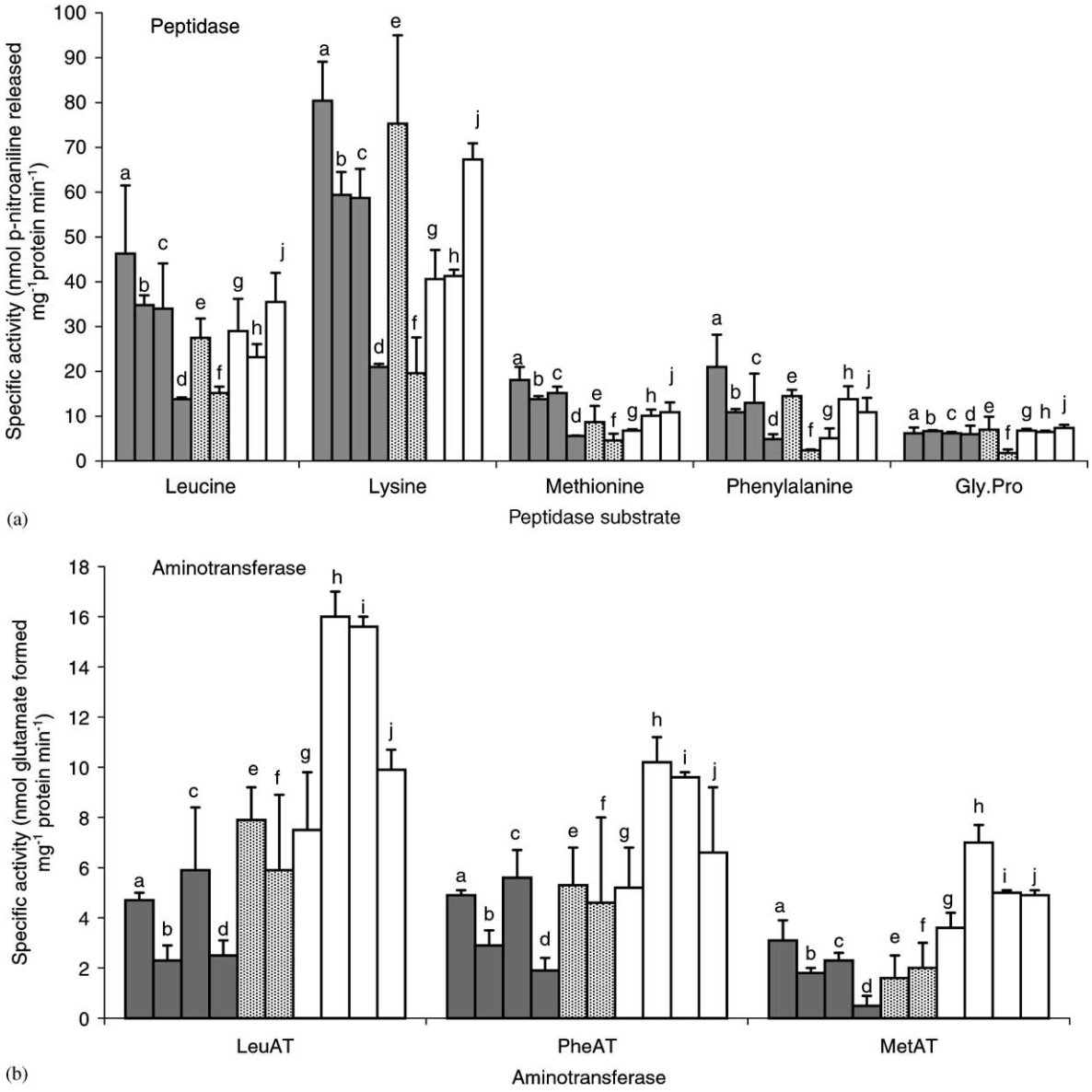

Fig. 2. The effect of the stage and rate of growth on the peptidase (Fig. 2a) and aminotransferase activity (Fig. 2b) of *Lb. rhamnosus* F3 grown under controlled conditions in batch culture aerobically (■), batch culture anaerobically (▩) and continuous chemostat cultures (□). Activities were monitored after: (a) 5 h, (b) 16 h, (c) 24 h, (d) 64 h in aerobic batch cultures, after (e) 24 h and (f) 64 h in anaerobic batch cultures, or after continuous culture at dilution rates (D) of (g) 0.03 h^{-1}, (h) 0.06 h^{-1}, (i) 0.1 h^{-1} and (j) 0.2 h^{-1}, respectively. Peptidase specific activities, determined using *p*-nitroanilide derivatives of the appropriate amino acid or peptide, are expressed as nmol *p*-nitroaniline released per mg of protein per min. Leucine aminotransferase (LeuAT), phenylalanine aminotransferase (PheAT) and methionine (MetAT) specific activities are presented as nmol glutamate formed per mg of protein per min. Specific activities are mean values determined using duplicate enzyme preparations; the standard deviations are indicated by error bars. Details of the enzyme assays are given in Section 2.

(Amarita et al., 2001). The effects observed in the *Lb. rhamnosus* F3 cultures are, therefore, unlikely to have arisen as a consequence of glucose inhibition of enzymic activity. The lower aminotransferase activities in strain F3 after growth, even in the presence of low levels of glucose, can be attributed to catabolite regulation by the carbohydrate.

3.2.3. *Effect of nitrogen source*

The effect of the nature of the nitrogen source on enzyme activity was determined with duplicated shake flask cultures (50 rev min^{-1}) of *Lb. rhamnosus* F3 and another non-starter isolate *Lb. paracasei* E7. The cultures were grown at 30°C for 48 h in the defined medium with either casein, casamino acids, lactalbumin hydrolysate (all 1 g 100 mL^{-1}) or an amino acid mixture as the nitrogen source.

The 6 aminopeptidase and the Gly.Pro dipeptidyl peptidase activities were formed constitutively by the 2 NSLAB isolates when grown on media containing either intact protein (casein), protein hydrolysates or free amino acids (Fig. 4a). There was a tendency for activity

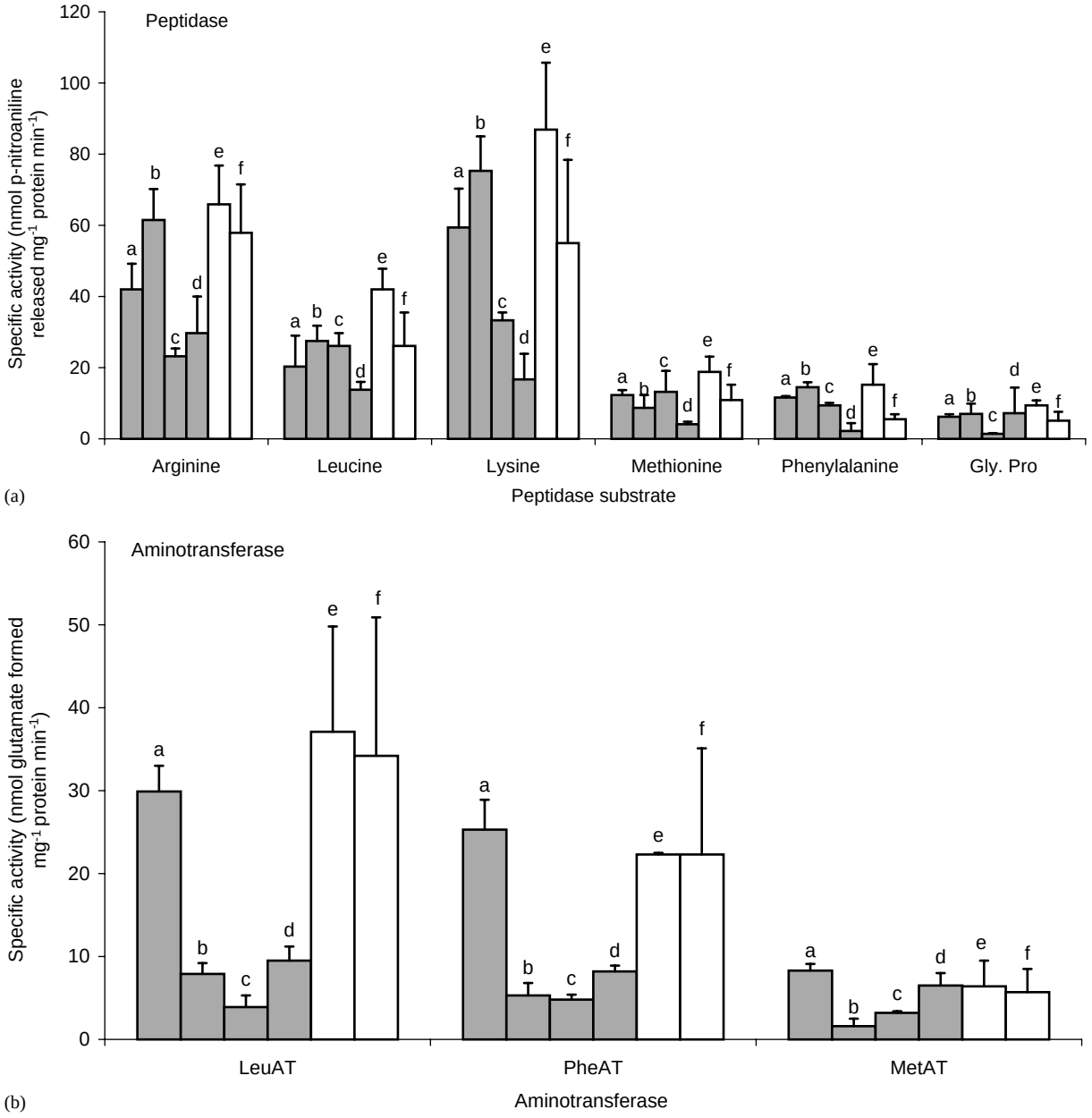

Fig. 3. The effect of glucose (■) or lactose (□) as the carbohydrate source in the growth medium on the peptidase (Fig. 3a) and aminotransferase activity (Fig. 3b) of *Lb. rhamnosus* F3 grown in controlled batch cultures. The initial glucose concentrations (g $100\,mL^{-1}$) used were: (a) 0, (b) 0.2, (c) 2.0 and (d) 5.0. The initial lactose concentration (g $100\,mL^{-1}$) was either (e) 0.2 or (f) 2.0. Peptidase specific activities, determined using *p*-nitroanilide derivatives of the appropriate amino acid or peptide, are expressed as nmol *p*-nitroaniline released per mg of protein per min. Leucine aminotransferase (LeuAT), phenylalanine aminotransferase (PheAT) and methionine (MetAT) specific activities are presented as nmol glutamate formed per mg of protein per min. Specific activities are mean values determined using duplicate enzyme preparations; standard deviations are indicated by error bars. Details of the enzyme assays are given in Section 2.

levels in the *Lb. rhamnosus* strain to be slightly elevated in cultures grown on amino acids and lower in cultures grown on an intact protein (casein) nitrogen source (Fig. 4a). There was a similar trend in activity levels in *Lb. paracasei* E7 with activities tending to be higher in amino acid grown cultures but in this strain, the activity level was maintained in cells grown on casein (data not shown). Aminopeptidase activity in *Lac. lactis* strain C27 was not affected by the nature of the nitrogen source in the growth medium (unpublished observations). The presence of peptides did not appear to be essential for aminopeptidase expression by the two non-starter isolates F3 and E7. The peptidase activity of other *Lactobacillus* spp. was likewise not affected by the peptide content of the medium (Morel et al., 1999; Hébert, Raya, & de Giori, 2000). In contrast, however,

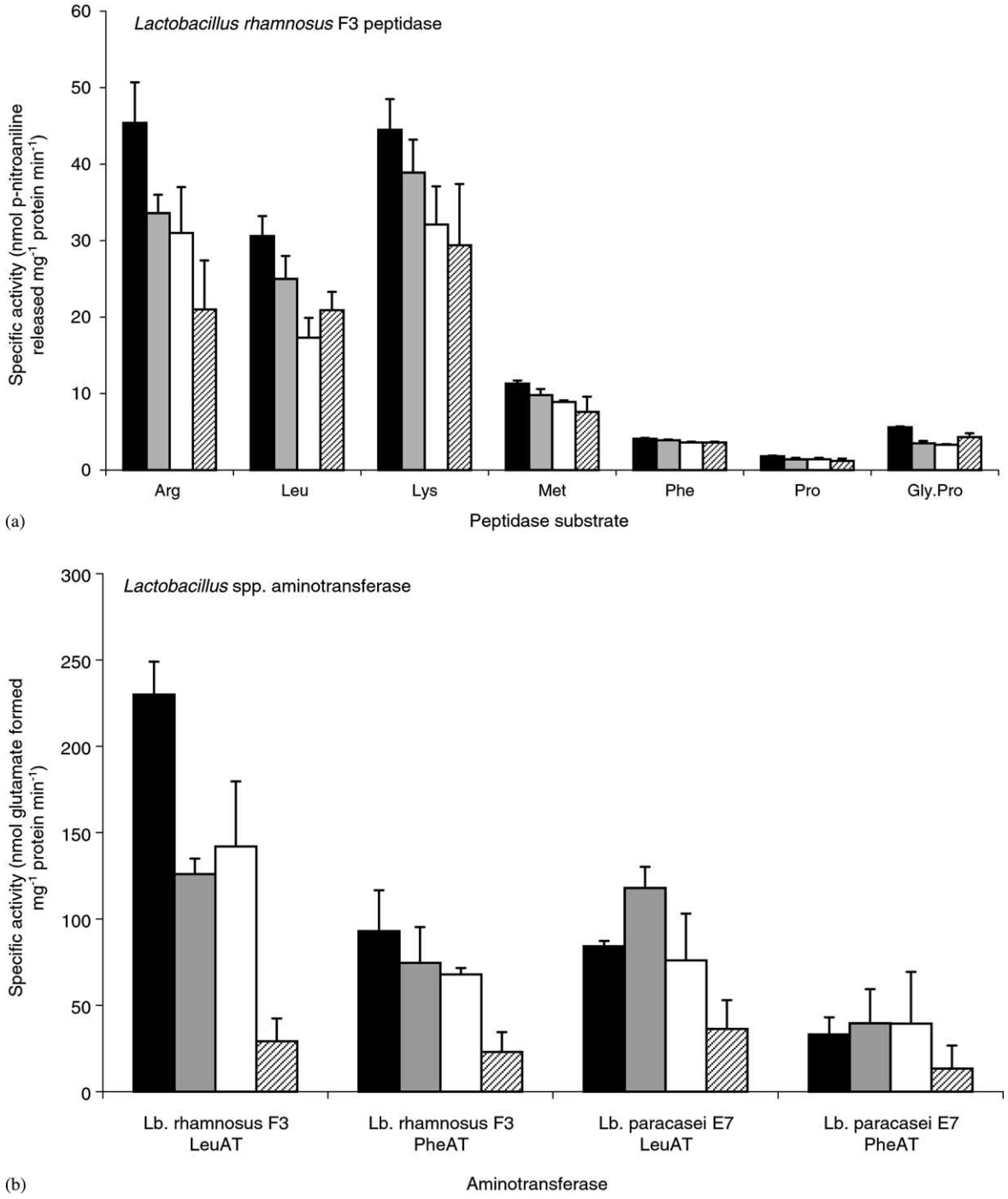

Fig. 4. The effect of the nitrogen source available in the growth medium on the peptidase activity of *Lb. rhamnosus* F3 (Fig. 4a) and aminotransferase activity of the non-starter lactobacilli *Lb. rhamnosus* F3 and *Lactobacillus paracasei* E7 (Fig. 4b). The nitrogen sources used were free amino acids (■), casamino acids (▩), lactalbumin hydrolysate (□), and casein (▨). Peptidase specific activities, determined using *p*-nitroanilide derivatives of the appropriate amino acid or peptide, are expressed as nmol *p*-nitroaniline released per mg of protein per min. Leucine aminotransferase (LeuAT), phenylalanine aminotransferase (PheAT) and methionine (MetAT) specific activities are presented as nmol glutamate formed per mg of protein per min. Specific activities are mean values determined using duplicate enzyme preparations; standard deviations are indicated by error bars. Details of the enzyme assays are given in Section 2.

the aminopeptidase, but not the dipeptidase, activity of *Lb. casei* NCDO 151 (syn. *Lb. paracasei* subsp. *paracasei*) was higher after growth on casein in comparison with cultures grown with free amino acids as the nitrogen source (El Soda et al., 1978). The expression of some aminopeptidase and dipeptidase activities in *Lb. casei* and *Lb. bulgaricus* strains was induced when the medium was enriched with peptides

(Abo-Elnaga & Plapp, 1987; Atlan et al., 1989). It has also been reported that the regulation of proteinase synthesis was dependent on medium composition and that the activity in some, but not all, lactobacilli was markedly decreased in a peptide-containing medium (El Soda, Desmazeaud, Le Bars, & Zevaco, 1986; Gilbert et al., 1997; Hébert, Raya, & de Giori, 1997). Different components of the proteolytic system may, therefore, be regulated differently, as is evident from studies of protease activity in *Lactococcus* spp. (Marugg et al., 1995; Christensen et al., 1999). It is also possible that there are inter- and intra-species differences in the mechanisms of enzyme regulation. The peptidases monitored in the two non-starter isolates studied here were expressed constitutively although this evidently is not the situation in all other strains (El Soda et al., 1978; Atlan et al., 1989). The aminopeptidases have an important role in releasing amino acids from peptides generated by other proteolytic enzymes. The released amino acids are potential flavour precursors and it is, therefore, advantageous to utilize strains for cheesemaking in which aminopeptidases are expressed constitutively and not repressed by environmental conditions or the products of other peptide hydrolases.

Leucine and phenylalanine aminotransferase activities in *Lb. rhamnosus* F3 and *Lb. paracasei* E7 were at their lowest in cultures grown on casein (Fig. 4b). Free amino acid availability also enhanced aminotransferase activity levels of cultures of *Lb. rhamnosus* F3. This observation is indicative of the involvement of the aminotransferase in amino acid breakdown in these cheese isolates since their activity, and presumably biosynthesis, is higher when substrate amino acids are readily available in the medium. Rijnen et al. (1999) observed that the activity of a biosynthetic aminotransferase in *Lac. lactis* subsp. *cremoris* was negatively regulated by aromatic amino acid availability in that its activity was repressed by the presence of phenylalanine in the medium. Methionine aminotransferase in a different *Lac. lactis* subsp. *cremoris* strain was also repressed by increasing concentrations of methionine in the growth medium (Dias & Weimer, 1998). Amino acid availability would, therefore, seem to be a critical regulator of both catabolic and biosynthetic aminotransferase activity in lactococci and lactobacilli from cheese.

3.2.4. Effect of pH and NaCl concentration

The aminopeptidase, dipeptidyl peptidase and aminotransferase activity of *Lb. rhamnosus* F3 was not influenced in batch cultures grown at controlled pH values of pH 5.0, 5.5, 6.0 and 6.5. The mean specific activities $\pm$s.d. of duplicate cultures grown at the four pH values for arginyl, leucyl, lysyl, methionyl and phenylalanyl aminopeptidase were 56.9 ± 5.3, 31.3 ± 6.2, 56.3 ± 11.3, 8.0 ± 3.4 and 5.5 ± 4.7 nmol mg^{-1} protein min^{-1}, respectively. The corresponding values for proline iminopeptidase and Gly.Pro dipeptidyl peptidase were 1.6 ± 0.4 and 7.7 ± 0.6 nmol mg^{-1} protein min^{-1}, respectively. Aminotransferase activity was likewise not influenced by growth pH and mean specific activities over the 4 growth conditions for leucine, phenylalanine and methionine aminotransferase were 8.6 ± 0.6, 6.2 ± 1.3 and 3.6 ± 1.3 nmol glutamate formed per mg of protein per min, respectively.

The inclusion of 2 or 5 g 100 mL^{-1} NaCl in the growth medium did not markedly influence aminopeptidase, dipeptidyl peptidase and aminotransferase activities of *Lb. rhamnosus* F3. Thus, amino- and dipeptidyl peptidase specific activities (nmol per mg of protein per min) determined in duplicated cultures grown in the absence of NaCl and at two different salinities were 50.2 ± 8.9 (arginyl), 22.0 ± 3.9 (leucyl), 54.6 ± 16.6 (lysyl), 9.7 ± 0.9 (methionyl), 12.3 ± 3.6 (phenylalanyl), 1.8 ± 0.4 (prolyl) and 6.8 ± 0.8 (Gly.Pro), respectively. Aminotransferase activities (nmol glutamate formed per mg of protein per min) determined over the same range of salinities were 9.1 ± 1.4 (leucine), 9.0 ± 2.8 (phenylalanine) and 2.3 ± 0.7 (methionine). It was thus evident that when cell integrity of *Lb. rhamnosus* F3 was maintained in controlled batch cultures peptidase and aminotransferase activities were not influenced by culture salinity or pH over the ranges examined. This is in contrast to the pronounced effects of incubation salinity and pH on leucine and phenylalanine aminotransferase activity that were observed when cell lysates of *Lb. paracasei* CI3 and CI9 were assayed at different NaCl concentrations and pH values (Sections 3.1.1 and 3.1.2). Other groups have also reported that activities of aminopeptidase enzymes in lysates of some non-starter lactic acid bacteria were affected by reduced pH and increasing NaCl concentrations in the assay system (Weimer et al., 1997; Laan, Tan, Bruinenberg, Limsowtin, & Broome, 1998; Gobbetti et al., 1999). However, in the growth studies reported herein the activities of the peptidase and aminotransferase enzymes involved in amino acid release and turnover were maintained at, or close to, full activity under conditions typical of the cheese matrix provided that the cell structure and intracellular environment was maintained. It has already been demonstrated that the aminotransferase-mediated conversion of methionine to thiols proceeds more slowly under cheese-like conditions with extracts than with intact LAB cells (Dias & Weimer, 1998; Gao, Mooberry, & Steele, 1998). The effectiveness and stability of enzymes released as a consequence of cell lysis may, therefore, be less than those retained within viable cells. Flavour formation could, therefore, be diminished as a consequence of extensive cell lysis although the retention of activity in intact cells will be dependent on continued substrate uptake.

4. Conclusions

Aminopeptidase, dipeptidyl peptidase and aminotransferase enzymes were formed constitutively by the LAB examined. Activities were, however, affected by growth conditions but were maintained in cells, which retained their structural integrity under conditions in which activity in cell lysates was reduced. From the observations reported here and in other studies, it is apparent that the factors affecting the activity of amino acid metabolizing enzymes can be species or even strain specific. There is, thus, a need to establish that isolates selected for use as adjunct cultures to improve cheese flavour characteristics form the key enzymes constitutively and retain effective levels of activity under the conditions encountered in the cheese matrix during ripening.

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