APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 837–839 Vol. 61, No. 2
0099-2240/95/$04.00+0

Purification and Characterization of a Dipeptidase from *Lactobacillus sake*

MARIE-CHRISTINE MONTEL,* MARIE-PIERRE SERONIE, RÉGINE TALON, AND MICHEL HÉBRAUD

Station de Recherches sur la Viande, Institut National de la Recherche Agronomique, Clermont-Theix, 63122 Saint-Genès Champanelle, France

Received 12 September 1994/Accepted 23 November 1994

A dipeptidase was purified from cell extracts of *Lactobacillus sake*. This compound was a monomer having a molecular weight of 50,000 and a pI of 4.7 and exhibited broad specificity against all dipeptides except those with proline or glycine at the N terminus. The enzyme was inhibited by EDTA or 1,10-phenanthroline but could be reactivated with $CoCl_2$ and $MnCl_2$.

Lactobacillus sake is the lactic acid bacterium that is most often inoculated as a starter culture in the production of sausages. Because of its metabolic activities, this bacterium has been implicated in the development of the color, texture, and flavor of sausages. During sausage ripening the proteolytic enzymes of *Lactobacillus sake* may be involved in an increase in the content of amino acids (7, 18), which may play a role in the development of aroma because amino acids or their metabolic products are important in the aroma of dry sausages (2, 15). There is no previously published information concerning the proteolytic system of *Lactobacillus sake*, in contrast to the numerous purification and characterization studies of endopeptidases and exopeptidases of the dairy lactic acid bacteria *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus delbrueckii* (reviewed in reference 17). In this paper we describe the purification and partial characterization of a *Lactobacillus sake* dipeptidase.

Lactobacillus sake L110, which was supplied by Texel (France), was grown in MRS broth at 30°C for 12 h. Cells were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C and were washed once with a saline solution. A solution containing 50 mg of dry material per ml of 20 mM Tris (pH 7.6) was sonicated for 10 min and then centrifuged at 30,000 × *g* for 30 min to obtain a cell extract.

During purification, hydrolysis of Leu-Gly was monitored in microtitration plates by the coupled enzyme reaction as described by Boven et al. (3), except that 2,2′-azino-di-3-ethylbenzthiazoline-sulfonate was used instead of *o*′-dianisidine. Protein contents were determined by the method of Bradford (4) with a protein assay kit (Bio-Rad, Munich, Germany). The cell extract was first applied to a Sephadex G-150 column (2.3 by 105 cm) and eluted with 20 mM Tris (pH 7.6). Fractions that hydrolyzed the dipeptide Leu-Gly and Leu–*p*-nitroanilide were loaded onto a DEAE column (1 by 20 cm) equilibrated with 20 mM Tris HCl (pH 7.6) buffer containing 140 mM NaCl. After elution with a linear gradient of 140 to 190 mM NaCl in the same buffer (total volume, 500 ml), the enzyme that hydrolyzed Leu-Gly was separated from the enzyme that hydrolyzed Leu–*p*-nitroanilide. Thus, *Lactobacillus sake* L110 synthesized at least two intracellular peptidases. Fractions that hydrolyzed Leu-Gly were concentrated with a filtration unit (type PM 25 cone; Amicon Corp.). The concentrate was then injected into a high-performance liquid chromatography column (mono Q) equilibrated with 20 mM Tris HCl (pH 7.6) containing 200 mM NaCl and was eluted with a linear gradient of 200 to 400 mM NaCl in the same buffer at a flow rate of 1 ml/min. The enzyme, which eluted from the mono Q column at an concentration of 250 mM, was purified 126-fold from the cell extract, and the yield was 5%. The purity of the enzyme was confirmed by native polyacrylamide gel electrophoresis (5); the resulting gel was silver stained for protein with a kit (Amersham, Buckinghamshire, United Kingdom). The peptidase activity on the gel was detected by the procedure of Lewis and Harris (14), using 2,2′-azino-di-3-ethylbenzthiazolinesulfonate instead of *o*′-dianisidine. The purified enzyme produced a single band, which was active against Leu-Gly.

Hydrolysis of peptides was evaluated by measuring the release of amino acids by the cadmium-ninhydrin method described by Doi et al. (8). Hydrolysis of aminoacyl–*p*-nitroanilide was determined by the method of El Soda et al. (9). The enzyme exhibited no activity against aminoacyl–*p*-nitroanilide, tripeptides, and peptides with blocked terminal N residues. It exhibited activity only against dipeptides (Table 1). Therefore, the purified enzyme was a dipeptidase. The enzyme exhibited no activity against dipeptides containing N-terminal basic amino acids, proline, or glycine and exhibited greater activity against dipeptides with tyrosine and valine at the N terminus than against dipeptides composed of neutral amino acids. Similar results were obtained for *Lactococcus lactis* subsp. *cremoris* H 61 dipeptidase (13). The broad substrate specificity of the *Lactobacillus sake* enzyme is very similar to the specificities of the dipeptidases of *Lactococcus lactis* subsp. *cremoris* Wg 2 (3) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (19). The *Lactobacillus sake* enzyme differed from dipeptidases DPI and DPIII of *Lactobacillus plantarum* and DPI of *Lactobacillus casei* in that it exhibited no activity against Gly-X peptides (1, 9, 10).

The molecular weight and pI of the *Lactobacillus sake* enzyme were estimated by two-dimensional electrophoresis performed as described by Hebraud et al. (11). Isoelectric focusing was performed with a gel containing 5% carrier ampholytes (LKB) and ampholines (2 parts pH 5 to 7, 3 parts pH 3 to 10). The enzyme was monomeric with a single subunit (Fig. 1). Its molecular weight was estimated to be 50,000, a value which is similar to the values obtained for the peptidases of *Lactobacillus delbrueckii* subsp. *bulgaricus* (19), *Lactococcus lactis* subsp. *cremoris* Wg 2 (3), and *Streptococcus diacetylactis* (6); however, this molecular weight differs from the molecular weight of the dipeptidase of *Lactococcus lactis* subsp. *cremoris* H 61 (100,000) (12). The pI was estimated to be 4.7; this value

* Corresponding author.

TABLE 1. Substrate specificity of a dipeptidase from *Lactobacillus sake*[a]

Substrate	Relative activity (%)
Ala-Glu	8
Ala-Gly	61
Ala-Leu	219
Ala-Val	128
Arg-Leu	0
Gly-Leu	33
Gly-Phe	14
Gly-Pro	0
His-Leu	0
Leu-Gly	100[b]
Leu-Ala	311
Leu-Leu	169
Leu-Tyr	81[c]
Lys-Phe	33
Lys-Val	41
Met-Ala	355
Met-Gly	259
Phe-Leu	130
Pro-Gly	0
Pro-Leu	0
Val-Leu	56
Val-Tyr	168
Trp-Ala	0
Tyr-Ala	314[d]
Tyr-Leu	126
Ala-Leu-Ala	5
Ala-Leu-Leu	9
NCBZ-Ala[e]	0
NCBZ-Gly-Leu	0
NCBZ-Leu-Gly	5
Ala-*p*Na[f]	0
Arg-*p*Na	0
Leu-*p*Na	0
Met-*p*Na	0

[a] The activity against Leu-Gly was arbitrarily given a value of 100%. The amino acids released were determined by the cadmium-ninhydrin method of Doi et al. (8).
[b] The K_m value was 2.4 mM.
[c] The K_m value was 0.25 mM.
[d] The K_m value was 0.65 mM.
[e] NCBZ, *N*-carbobenzoxy.
[f] *p*Na, *para*-nitroanilide.

is similar to the value obtained for the dipeptidase of *Lactococcus lactis* subsp. *cremoris* Wg 2 (3).

The characteristics of the enzyme are summarized in Table 2. The dipeptidase of *Lactobacillus sake* is similar in many ways to the dipeptidases of *Lactococcus* strains and other *Lactobacillus* strains. Like most dipeptidases that have been characterized (1, 3, 6, 10, 12, 19), the dipeptidase of *Lactobacillus sake* is a metalloproteinase and Co^{2+} is an essential ion. $CoCl_2$ (1 mM) activates the dipeptidases of *Lactobacillus sake*, *Lactobacillus delbrueckii* subsp. *bulgaricus* (19), and *Lactococcus lactis* subsp. *cremoris* H 61 (12), whereas 1 mM $CoCl_2$ inhibits *Lactococcus lactis* subsp. *cremoris* Wg 2 dipeptidase (3). As observed for the dipeptidases of *Lactococcus lactis* subsp. *cremoris* Wg 2 (3) and H 61 (12), sulfydryl groups are involved in the active site. The strong inhibition by $CuCl_2$, $FeCl_2$, $ZnCl_2$, and $CaCl_2$ is similar to the inhibition reported previously for the dipeptidases of *Lactococcus lactis* subsp. *cremoris* H 61 (12) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (19).

The optimum pH values were 7.6 in phosphate buffer and 8 in Tris buffer; 20% of the optimum activity was observed at pH 5.7. The higher activity in phosphate buffer than in Tris buffer

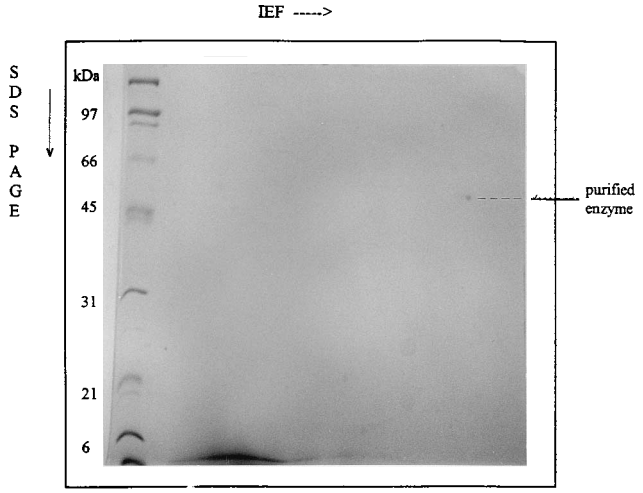

FIG. 1. Two-dimensional gel electrophoresis. Purified enzyme after mono Q chromatography (4.5 μg of protein was loaded) and standard proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The gel was stained with Coomassie blue. IEF, isoelectric focusing.

was due to the activation of the enzyme by potassium. The optimum temperature was between 40 and 45°C. At 15°C, 20% of the optimum activity was still found. Hence, the enzyme can be active under the conditions found in ripening sausages.

Because of its specificity, the dipeptidase of *Lactobacillus sake* could contribute to the increase in valine, methionine, and leucine contents observed in dry sausages (7). More relevant information about the contribution of this enzyme will be obtained by studying the release of amino acids from sausages inoculated with a wild strain or a dipeptidase-negative mutant

TABLE 2. Effects of divalent cations and various reagents on dipeptidase activity against Leu-Gly[a]

Reagent and/or conditions	Concn (mM)	Relative activity
Control		100
PMSF[b]	1.0	102
EDTA	0.1	4
	1.0	0
1,10-Phenanthroline	1.0	0
*p*CMB[c]	1	0
Ca^{2+}	1.0	13
Co^{2+}	1.0	264
Cu^{2+}	1.0	0
Fe^{2+}	1.0	4
Li^{2+}	1.0	121
Mg^{2+}	1.0	174
Mn^{2+}	1.0	74
Zn^{2+}	1.0	1.0
K^+	1.0	168
Na^+	1.0	175
Inactivated by 0.1 mM EDTA and reactivated by:		
Ca^{2+}	1.0	11
Co^{2+}	0.1	133
	1.0	156
Mg^{2+}	1.0	1
Mn^{2+}	1.0	83

[a] The amino acids released were determined by the cadmium-ninhydrin method of Doi et al. (8).
[b] PMSF, phenylmethylsulfonyl fluoride.
[c] *p*CMB, *para*-chloromercuribenzoate.

of *Lactobacillus sake*. As *Lactobacillus sake* requires amino acids for growth (16), it would also be interesting to determine the nutritional role of the dipeptidase by studying the physiological behavior of a dipeptidase-negative mutant.

REFERENCES

1. **Abo-Elnaga, I. G., and R. Plapp.** 1987. Peptidases of *Lactobacillus plantarum*. J. Basic Microbiol. **27:**123–130.
2. **Berdagué, J. L., P. Monteil, M. C. Montel, and R. Talon.** 1993. Effects of starter cultures on the formation of flavour compounds in dry sausage. Meat Sci. **35:**275–287.
3. **Boven, A., P. S. T. Tan, and W. N. Konings.** 1988. Purification and characterization of a dipeptidase from *Streptococcus cremoris* Wg 2. Appl. Environ. Microbiol. **54:**43–49.
4. **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. Chem. **72:**248–254.
5. **Davis, B. J.** 1964. Disc electrophoresis: method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121:**404–427.
6. **Desmazeaud, M., and C. Zevaco.** 1977. General properties and substrate specificity of an intracellular dipeptidase from *Streptococcus diacetylactis*. Ann. Biol. Anim. Biochim. Biophys. **17:**723–736.
7. **Dierick, N., P. Vandeckerkove, and D. Demeyer.** 1974. Changes in non protein nitrogen compounds during dry sausage ripening. J. Food Sci. **39:** 301–304.
8. **Doi, E., D. Shibata, and T. Matoba.** 1981. Modified colorimetric ninhydrin methods for peptidase assay. Anal. Biochem. **118:**173–184.
9. **El Soda, M., M. J. Desmazeaud, and J. L. Bergère.** 1978. Peptide hydrolases of *Lactobacillus casei*: isolation and general properties of various peptidase activities. Special Dairy Res. **45:**445–455.
10. **El Soda, M., H. Said, M. J. Desmazeaud, R. Mashaly, and A. Ismail.** 1983. The intracellular peptide-hydrolases of *Lactobacillus plantarum*. Comparison with *Lactobacillus casei*. Lait **63:**1–14.
11. **Hebraud, M., E. Dubois, P. Potier, and J. Labadie.** 1994. Effect of temperature on the protein levels in a psychrotrophic bacterium, *Pseudomonas fragi*. J. Bacteriol. **176:**4017–4024.
12. **Hwang, I. K., S. Kaminongawa, and K. Yamauchi.** 1981. Purification and properties of a dipeptidase from *Streptococcus cremoris* H 61. Agric. Biol. Chem. **45:**159–165.
13. **Hwang, I. K., S. Kaminongawa, and K. Yamauchi.** 1982. Kinetic properties of a dipeptidase from *Streptococcus cremoris*. Agric. Biol. Chem. **46:**3049–3053.
14. **Lewis, H. P., and H. Harris.** 1967. Human red cell peptidases. Nature (London) **215:**351–355.
15. **Maillet, J., and M. Henry.** 1960. Etude de quelques produits de la protéolyse et de la lipolyse dans le saucisson cru, p. 429–448. *In* VI^{e} Symposium Substances étrangères dans les aliments, Madrid.
16. **Montel, M. C., and J. Labadie.** 1984. Specific nutritional requirements of lactobacilli spp. from meat. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B **183:**1–7.
17. **Pritchard, G. G., and T. Coolbear.** 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. **12:**179–206.
18. **Sajber, C., R. Karakas, and P. Mitic.** 1971. Influences of some starter cultures upon the changes in proteins of "stajer" sausages during the fermentation, p. 744–757. *In* Proceedings of the 17th European Meeting on Meat Research Work, Bristol, England. vol. 2.
19. **Wohlarb, Y., and W. Bochelmann.** 1992. Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. **2:**345–361.