Peptidase Profiles of *Pseudomonas fluorescens*: Identification and Properties

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ABSTRACT

The cell-associated peptidase profiles of 12 strains of *Pseudomonas fluorescens* (ATCC 948 and 11 related biotypes) were examined. Employing Analytab system API ZYM, a general, strong peptidase activity was detected using L-lysyl-, L-pyrrolidonyl-, L-arginyl-, L-alanyl-, and glycyl-glycyl-β-naphthylamides as substrates. Conversely, L-tyrosyl-, L-phenylalanyl-, L-histidyl-, L-prolyl-, γ-L-glutamyl-β-naphthylamides substrates were hydrolyzed by only a few strains. The peptidases were active, therefore, on substrates responsible for the bitter taste in dairy products. Properties of hydrolytic systems showed no significant changes in the enzymatic profiles when cells were grown on different fermentation media. Enzyme activity was relatively stable during refrigerated (5°C) and frozen (–18°C) storage. The peptidases of *P. fluorescens* ATCC 948, considered as reference, and strain 22 were identified on Pro-β-naphthylamides by Michaelis constant values of .528 and .394 m*M*, respectively, and by different optimal pH and temperature activity on Leu- and Pro-β-naphthylamides. The peptidase activity on Gly-Phe-β-naphthylamide in *P. fluorescens* 30 had optimal values at pH 7.50 and 45°C. These results confirm the relations defined in the enzymatic identification phase and suggest the presence of any analogous peptidases in the biovars of *P. fluorescens* considered.

(Key words: bitter taste, peptidases, *Pseudomonas fluorescens*)

Abbreviation key: βNA = β-naphthylamide, NB = nutrient broth, PMS7 = pyruvate mineral salts, TSB = tryptic soy broth.

INTRODUCTION

The possible role of microbial peptidases has been considered in peptide utilization and transport (29), enzymatic ripening of cheese (3), protein synthesis (5), recognition, degradation of protein fragments, incorrectly folded intracellular proteins (27), and bioactive peptide synthesis from milk proteins (23). The specific role of peptides in dairy biotechnology has been related to the characteristic taste developed during cheese ripening and to the shelf-life of pasteurized and UHT milk. The combined activities of enzymes such as chymosin, endoproteinases, endopeptidases, and exopeptidases produced by starter cultures result in extensive proteolysis of caseins (26). Degradation of the caseins (especially β-casein) can cause bitterness in cheese because of bitter peptides (13, 30). In this respect, lactic acid starter cultures can be potentially "bitter" or "not bitter", based on their ability to form bitter peptides that can be sequentially degraded by peptidase activity (9, 26, 31). Psychrotrophic microorganisms (mainly *Pseudomonas fluorescens*) can also produce extracellular proteases that can withstand HTST and UHT treatments (6, 16), thus hydrolyzing milk proteins, causing gelation or off-flavor during the storage of milk products (1, 15, 18).

Because of limited information related to intracellular or cell-associated aminopeptidases and endopeptidases of psychrotrophs and to their importance (14), the goal of this study was to identify the peptidase profiles of individual strains of *P. fluorescens* and to examine the properties of cell-associated peptidases on synthetic substrates.

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MATERIALS AND METHODS

Strains and Culture Conditions

Pseudomonas fluorescens ATCC 948 and 11 related strains [strains 2, 4, 22, 30, 31, 39, 40, 43, 44, 45, and 47 identified by API 20 NE (API ZYM, Biomerieux, La Balme les Grottes, France) and supplementary assays] from the Collection of the Institute of Dairy Microbiology of Perugia (Perugia, Italy) were used biweekly. Strains were maintained on nutrient broth (NB) (Difco, Detroit, MI) agar slants at 5°C between use.

Preparation of Cell-Associated Peptidases

Strains were propagated in NB medium. Pyruvate mineral salts (PMS7) (21) and tryptic soy broth (TSB) (Difco) media were used only when specified. Cultures (100 ml in 500-ml Erlenmeyer flasks) were shaken at 160 rpm for 48 h at 28°C. At the stationary phase, the cells were harvested by centrifugation at 3000 × *g* for 30 min, then washed three times with K-phosphate buffer (pH = 7.0, .05 *M*), and concentrated in the same buffer (.1 *M*) at one-half of the original volume, corresponding to the O.D. = 3.60 (absorbance at 620 nm) (log 9.30 cfu/ml). The cellular concentration was determined by plating on NB agar at 28°C for 72 h.

Analytab System

The API ZYM kits for peptidase profiles (AP I, II, III) were obtained from API Laboratory Products Ltd. (Biomerieux). Each of the 30 microcupules of the gallery containing 29 dehydrated chromogenic enzyme substrates was inoculated with two drops (.25 µl) of the cellular suspension, corresponding to point 6 of the McFarland scale. The strips were incubated for 5 h at 28°C (different strains were tested in duplicate or triplicate). Hydrolytic action of the respective enzymes on naphthyl-derivatized substrates results in the release of β-naphthol, which was detected by reagents A and B supplied with the system. Activity was measured by comparing the color developed in 5 min with the color chart provided and expressed on an arbitrary scale from 0 (no activity) to 5 (maximum activity). Means and standard deviations were obtained using only those strains showing activity between 1 and 5 (excluding 0).

Chemicals and Substrates

In further trials, the following substrates were used: H-Pro-β-naphthylamide (βNA), H-Lys-βNA, H-Arg-βNA, H-Leu-βNA, H-Ser-Tyr-βNA, and H-Gly-Phe-βNA (BACHEM Feinchemikalien AG Products, CH-4416 Bubendorf, Switzerland). The choice of these aminoacyl and dipeptidyl substrates was related to their importance to bitter taste development (7, 24, 25) and to the specific activity of the API ZYM peptidase profiles.

Peptidase Activity

Cell-associated peptidase activity of each strain (.5 ml of cellular suspension) was determined in the presence of .33 m*M* aminoacyl- or dipeptidyl-βNA (.5 ml) by the method of Goldberg and Rutenburg (11) after 90 min at 37°C. Using the molar coefficient of extinction 10.1 m*M*/cm to naphthylamine, one unit of the peptidase activity (nanokatal = nkat) was defined as the quantity of enzyme that hydrolyzes 1 nmol of substrate/s. The specific activity was defined as the unit of the peptidase activity per microgram of protein used in the assays. The Michaelis constants and maximum velocities (37°C) were recorded by the plot representation of Hanes (12). Protein concentration was determined according to the method of Lowry et al. (17) with crystalline bovine serum albumin as standard.

Characterization of Peptidase Activity

Some strains of *P. fluorescens* screened by Analytab System and, sequentially, by βNA substrates were analyzed for the influence of the following parameters: type of cell storage (refrigeration at 5°C and freezing at –18°C for 24 to 168 h); composition of the fermentation medium (NB, TSB, PMS7); substrate concentration (.11 to 1.10 m*M* H-Pro-βNA); and pH (4.50 to 5.50 K-phthalate, 5.50 to 8.00 K-phosphate, 8.00 to 9.50 Na-tetraborate buffer .1 *M*) and temperature (5 to 55°C) of reaction.

RESULTS AND DISCUSSION

Peptidase Profiles of *Pseudomonas fluorescens* Strains

Peptidase profiles of *Pseudomonas fluorescens* ATCC 948 and the other strains

are shown in Figures 1 and 2. The strains showed a certain variability in the specific activities and, at the same time, similar activities toward defined types of substrates. L-Lysyl-, L-pyrrolidonyl-, L-arginyl-, L-alanyl-, and glycyl-glycyl-βNA (Figure 1) were strongly and rapidly hydrolyzed by all strains tested (range of means of activity, 3.66 to 4.83, and percentage of producing strains, 83.33 to 100.00). S-Benzyl-L-cysteyl, N-benzoyl-L-leucyl (Figure 1), L-isoleucyl, L-threonyl, and L-tryptophane (Figure 2) were hydrolyzed slowly by all strains (range of means of activity, 1.00 to 2.00, and percentage of producing strains, 16.66 to 58.33). These observations indicate that the profile of peptidase activity is a characteristic of *P. fluorescens* species. Only a few strains, probably because of the physiological variability of the microbial tested pool, showed higher peptidase activity against L-tyrosyl, L-phenylalanyl, L-histidyl (Figure 1), L-prolyl, and L-glutamyl (Figure 2) (SD .71 to 1.41). On the basis of the peptidase profile related to high activity, three strains (PF, 22, 30) were selected for further trials.

Characterization of Peptidase Activity

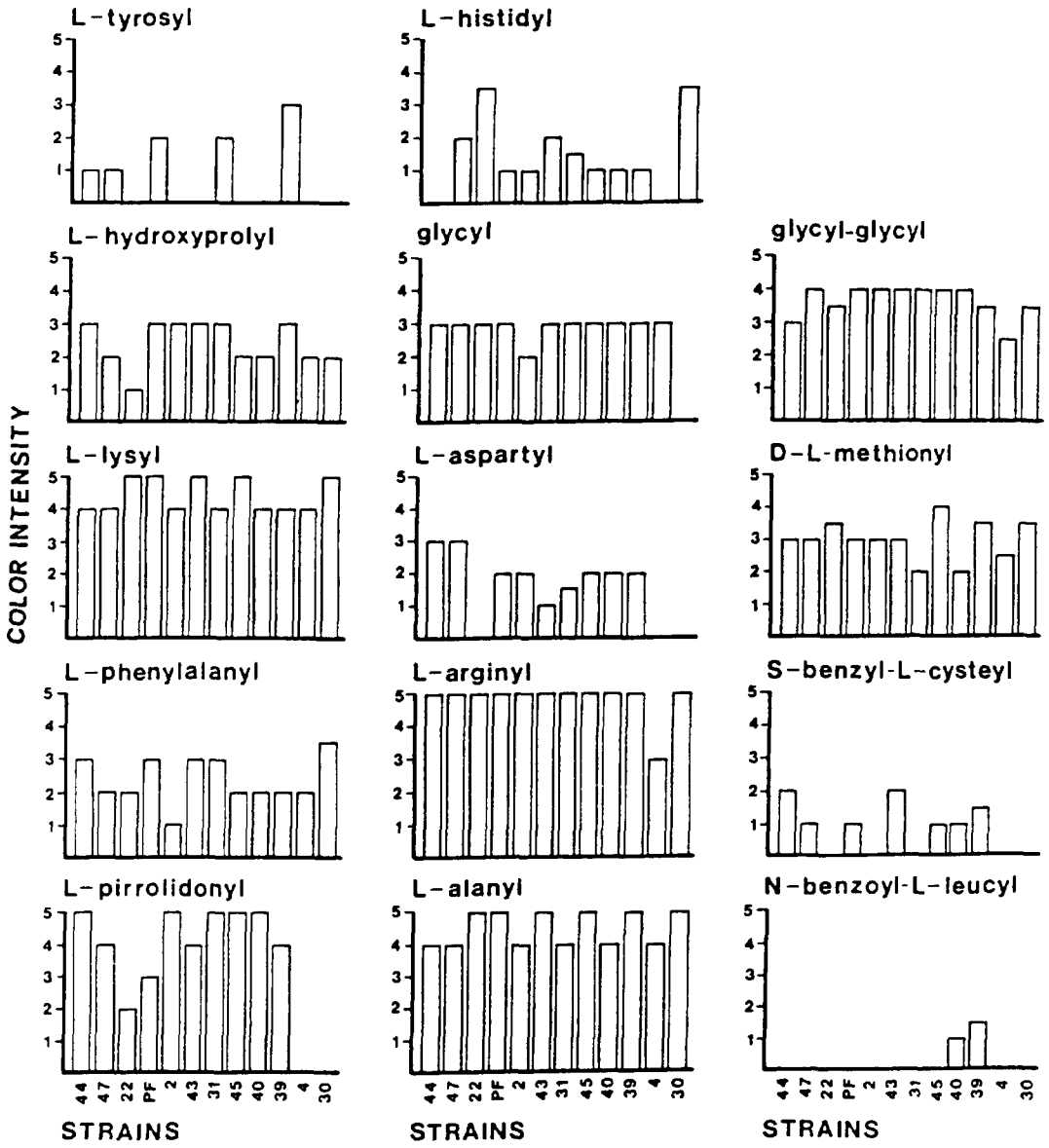
Six substrate βNA (H-Pro-, H-Lys-, H-Arg-, H-Leu-, H-Ser-Tyr-, and H-Gly-Phe-βNA) were tested for hydrolysis by enzymes in the cellular pool of strains PF, 22, and 30. The first two exhibit higher peptidase activity (Table 1) on H-Lys-βNA (19.94 and 14.21 nkat), H-Leu-βNA (15.28 and 10.46 nkat), and H-Pro-βNA (14.70 and 10.02 nkat, respectively); difference of specific activity was small for these substrates, and, therefore, enzymatic behavior was similar. In contrast, strain 30 had the lowest enzyme activity related to the same substrates (Table 1) but had the highest activity when H-Gly-Phe-βNA was the substrate (17.58 nkat).

There is no difference in peptidase activity for H-Pro- and H-Gly-Phe-βNA for any strains in relation to the mineral (PMS7) or organic (NB, TSB) N source of the fermentation medium, which might have affected the enzymatic synthesis of *Pseudomonas* bacteria (19, 31) (Table 2). The hydrolysis of H-Leu-βNA was related to the fermentation medium. Maximum activity was observed in TSB medium for all strains, suggesting that not all the studied enzymes were constitutive.

Peptidase activity was highest in the presence of fresh cells and then stabilized after 96 h during storage (5°C) and frozen (–18°C) conditions (Figure 3). However, after 168 h, the reduction in the rate of hydrolysis corresponded to less than 31% of maximum activity, which is in agreement with the psychrotrophic physiology of *P. fluorescens* strains and, particularly, with their proteolytic enzyme behavior (33).

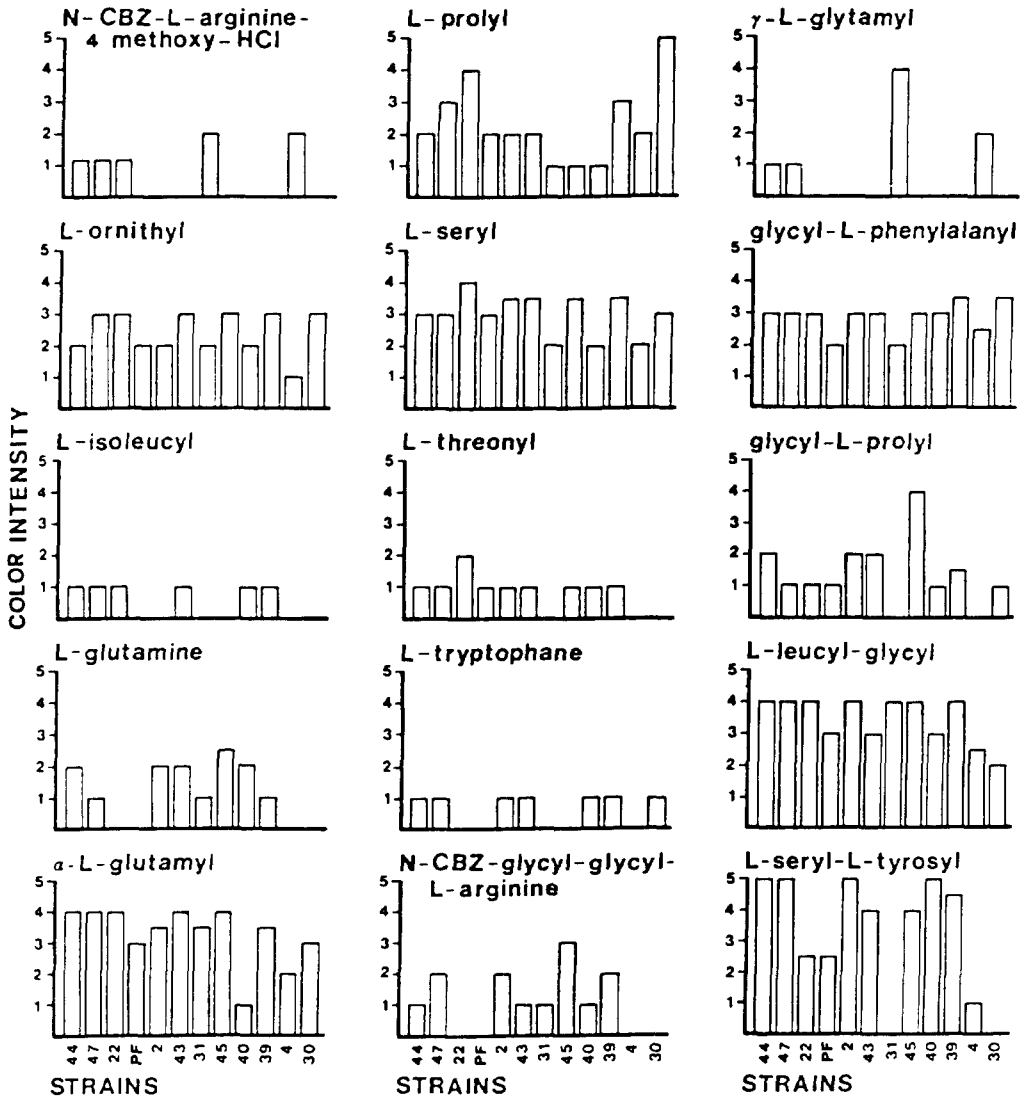
TABLE 1. Peptidase activities of *Pseudomonas fluorescens* (strains PF, 22, and 30) on different β-naphthylamide (βNA) substrates.

Strains	Total activity	Total protein	Specific activity	Strains	Total activity	Total protein	Specific activity
	(nkat)	(μg/.5 ml)	(nkat/μg)		(nkat)	(μg/.5 ml)	(nkat/ug)
	Lys-βNA				Arg-βNA		
PF	19.94	48	.41	PF	23.46	48	.48
22	14.21	45	.31	22	3.97	45	.08
30	6.49	51	.12	30	3.60	51	.07
	Leu-βNA				Pro-βNA		
PF	15.28	48	.31	PF	14.70	48	.30
22	10.46	45	.23	22	10.02	45	.22
30	5.15	51	.10	30	6.47	51	.12
	Gly-Phe-βNA				Ser-Tyr-βNA		
PF	9.00	48	.18	PF	9.90	48	.20
22	9.25	45	.20	22	3.34	45	.07
30	17.58	51	.34	30	6.63	51	.13



Enzyme	$\bar{X}$	SD	Percentage of strains producing	Enzyme	$\bar{X}$	SD	Percentage of strains producing
L-tyrosine arylamidase	1.80	.83	41.66	L-aspartate arylamidase	2.05	.63	75
L-hydroxyproline arylamidase	2.41	.66	100	L-arginine arylamidase	4.83	.57	100
L-lysine arylamidase	4.41	.51	100	L-alanine arylamidase	4.50	.52	100
L-phenylalanine arylamidase	2.37	.71	100	Glycyl-glycine arylamidase	3.66	.49	100
L-pyrrolidone arylamidase	4.24	1.03	83.33	Methionine arylamidase	3.00	.60	100
L-histidine arylamidase	1.75	1.00	83.33	S-benzyl-cysteine arylamidase	1.35	.47	53.33
L-glycine arylamidase	2.90	.30	91.66	N-benzoyl-leucine arylamidase	1.25	.35	16.66

Figure 1. Peptidase activities of *Pseudomonas fluorescens* (profiles and means). Color intensity: 0 = no activity to 5 = strong activity.



Enzyme	$\bar{X}$	SD	Percentage of strains producing	Enzyme	$\bar{X}$	SD	Percentage of strains producing
N-CBZ-arginine-4-methoxy-arylamidase	1.40	.54	41.66	L-tryptophane arylamidase	1.00	0	58.33
L-ornithine arylamidase	2.41	.66	100	N-CBZ-glycyl-glycyl-argine arylamidase	1.62	.74	66.66
L-isoleucine arylamidase	1.00	0	50.00	γ-Glutamyltransferase	2.00	1.41	33.33
L-glutamine arylamidase	3.29	.94	100	Glycyl-phenylalanine arylamidase	2.87	.48	100
L-glutamate arylamidase	1.68	.59	66.66	Glycyl-proline arylamidase	1.55	.68	83.33
L-proline arylamidase	2.33	1.17	100	Leucyl-glycine arylamidase	3.41	.70	100
L-serine arylamidase	3.04	.75	100	L-seryl-tyrosine arylamidase	3.05	1.06	83.33
L-threonine arylamidase	1.11	.33	75				

Figure 2. Peptidase activities of *Pseudomonas fluorescens* (profiles and means). Color intensity: 0 = no activity to 5 = strong activity.

TABLE 2. Peptidase activities of *Pseudomonas fluorescens* (strains PF, 22, and 30) growing on different fermentation media (NB, PMS7, and TSB).[1]

Fermentation media	Total activity	Maximum activity	Total activity	Maximum activity	Total activity	Maximum activity
	NB		PMS7		TSB	
	(nkat)	(%)	(nkat)	(%)	(nkat)	(%)
	P. fluorescens ATCC 948					
Leu-βNA	14.42	67.25	14.23	66.37	21.44	100
Pro-βNA	14.25	100	13.94	97.82	13.18	92.48
Gly-Phe-βNA	9.61	98.16	9.04	92.33	9.79	100
	Strain 22					
Leu-βNA	10.50	61.04	11.25	64.00	20.39	100
Pro-βNA	10.26	100	9.83	91.82	9.13	97.52
Gly-Phe-βNA	9.88	89.49	10.32	93.47	100	11.04
	Strain 30					
Leu-βNA	5.59	76.68	4.79	65.70	7.29	100
Pro-βNA	5.72	100	5.37	93.88	5.08	88.81
Gly-Phe-βNA	16.88	100	16.12	95.49	16.55	98.04

[1]NB = Nutrient broth, PMS7 = pyruvate mineral salts, TSB = tryptic soy broth, βNA = β-Naphthylamide.

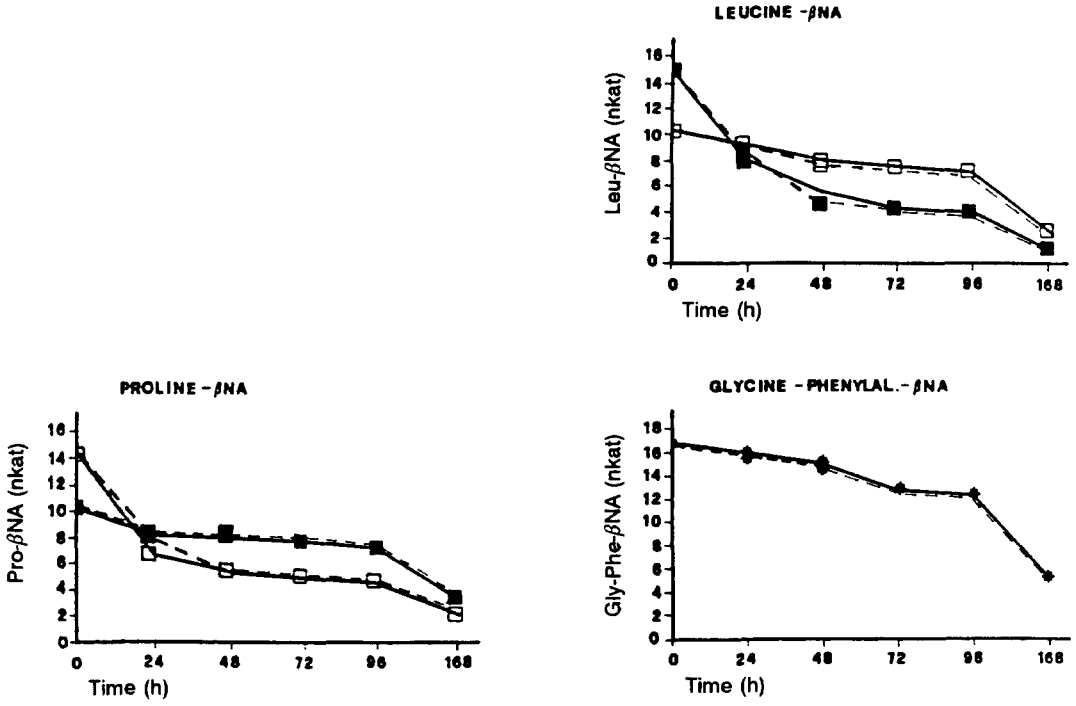


Figure 3. Peptidase activities of *Pseudomonas fluorescens* strains PF (■), 22 (□), and 30 (*) after storage under different refrigeration conditions (—) (5°C) and freezing (– –) (–18°C). βNA = β-Naphthylamide.

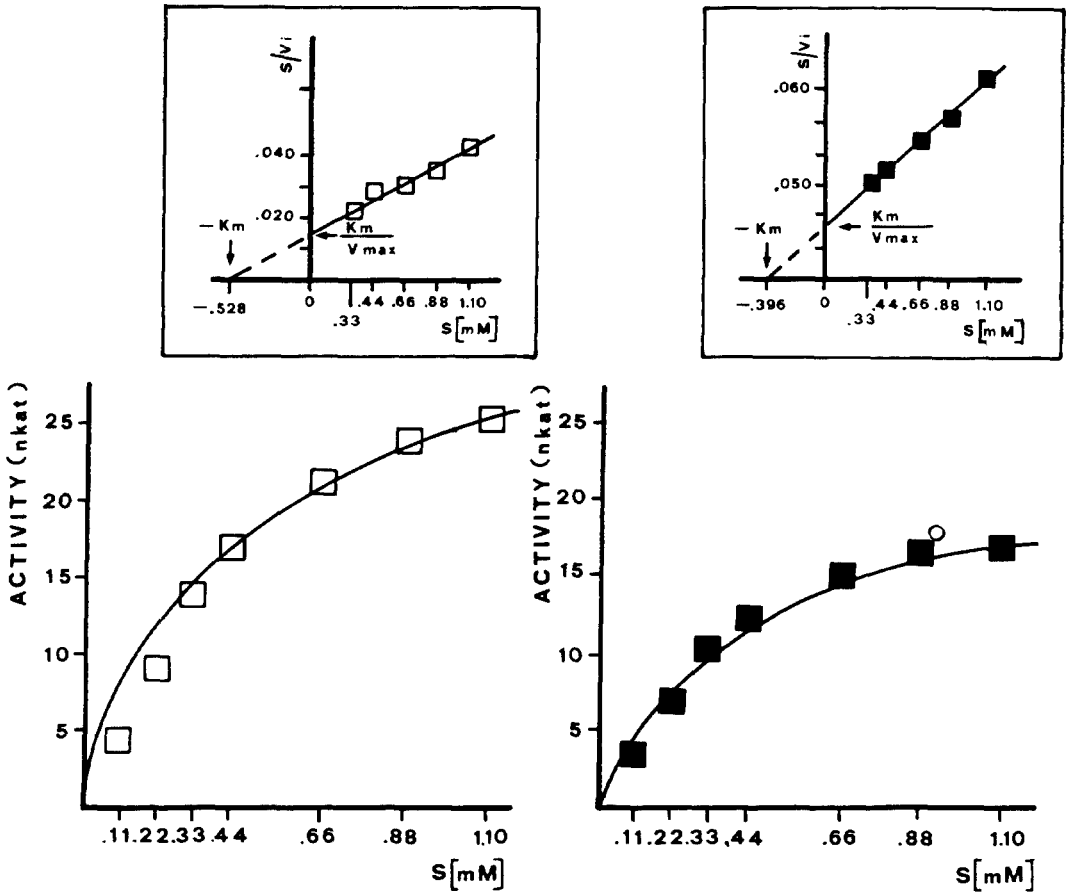


Figure 4. Effect of Pro-β-naphthylamide (βNA) concentration on peptidase activity of *Pseudomonas fluorescens* strains PF (■) and 22 (□) in the square representation of Hanes (12). K_m = Michaelis constant, V_{max} = maximum velocity, S/Vi = substrate/initial velocity, S = substrate.

The enzymatic activity of strains PF and 22 on H-Pro-βNA followed Michaelis-Menten kinetics (Figure 4). The maximum attainable activity was related, in both cases, to .33 m*M* of substrate. Substrate inhibition occurred at higher concentrations. Using the reciprocal plot of Hanes (12), the affinity for H-Pro-βNA (Michaelis constant = .528 and .394 m*M* for PF and 22, respectively) and the maximum apparent velocity (37.71 and 23.29 nkat in presence of log 9.39 cfu/ml) were determined for both microorganisms.

Strains PF and 22 exhibited a pH optimum of 7.50 with H-Leu-βNA as substrate (Figure 5). This enzyme was active over a wide pH range with residual activity (above 88%) at pH 6.50. In contrast, the hydrolysis of H-Pro-βNA increases at more alkaline pH (8.50) values and then rapidly decreases. *Pseudomonas fluorescens* 30, analyzed for specific activity on H-Gly-Phe-βNA, reached maximum activity at pH 7.50 and retained 68% of maximum activity at pH 6.50.

The optimal temperature of hydrolysis by strains PF and 22 for H-Leu- and H-Pro-βNA respectively, was 35 and 45°C (Figure 6). In accord with the effect of pH, this suggests the presence of different enzymes for each substrate rather than a single enzyme capable of hydrolyzing several substrates. This observation is reinforced by the adaptability of the enzymatic activity to the different tempera-

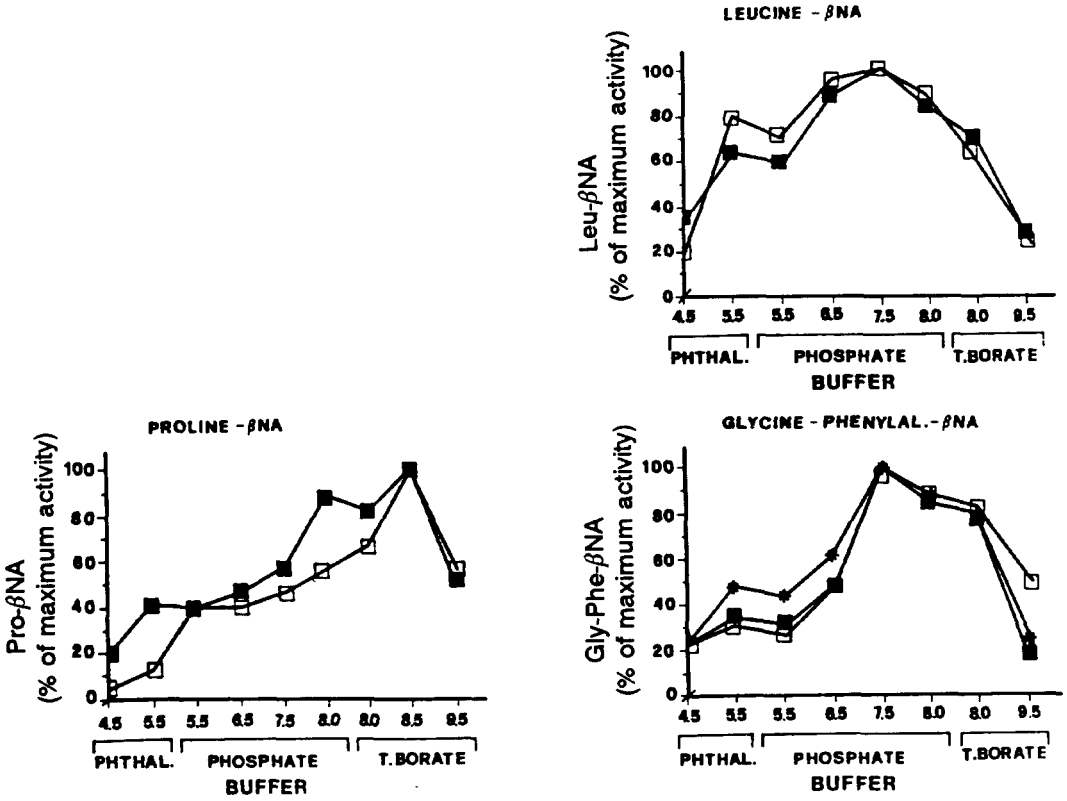


Figure 5. Effect of pH on the peptidase activity of *Pseudomonas fluorescens* strains PF (■) 22 (□), and 30 (*). PHTHAL = K-phthalate, BORATE = Na-tetraborate.

tures: the residual activity on H-Leu-βNA at 5°C was 22% and on H-Pro-βNA at 55°C was 40%. On H-Gly-Phe-βNA, strain 30 reached maximum activity at 45°C and was relatively stable at higher temperatures (68.12% of maximum activity at 55°C).

CONCLUSIONS

All strains of *P. fluorescens* tested were active on a wide variety of aminoacyl and dipeptidyl substrates. Hydrolysis of L-lysyl-, L-pyrrolidonyl-, L-arginyl-, L-alanyl-, and glycyl-glycyl-βNA was strong, and it is a general characteristic of *P. fluorescens* species. Other authors (2, 8) have used this system for identifying bacteria. This is in agreement with the study of McKellar (20), who demonstrated a wide variety of cell-associated enzymes and in relation to the initial cell concentration (14) that probably had a significant influence on the shelf-life of heat-treated milk. Peptidase activity on L-tyrosyl-, L-phenylalanyl-, L-histidyl-, L-prolyl-, and L-glutamyl-βNA was found in small number of strains, suggesting a minimum variability for the hydrolase profile related to the physiology of the species biovars.

The results obtained on substrates such as H-Pro-, H-Lys-, H-Arg-, H-Leu-, H-Ser-Tyr-, and H-Gly-Phe-βNA, identified by several authors (7, 24, 25) for their structural composition related to bitter taste in dairy products, confirmed the technological importance of specific peptidases of *Pseudomonas*. The relatively high activity of *P. fluorescens* ATCC 948 and other strains on structurally dissimilar aminoacyl substrate might suggest the possibil-

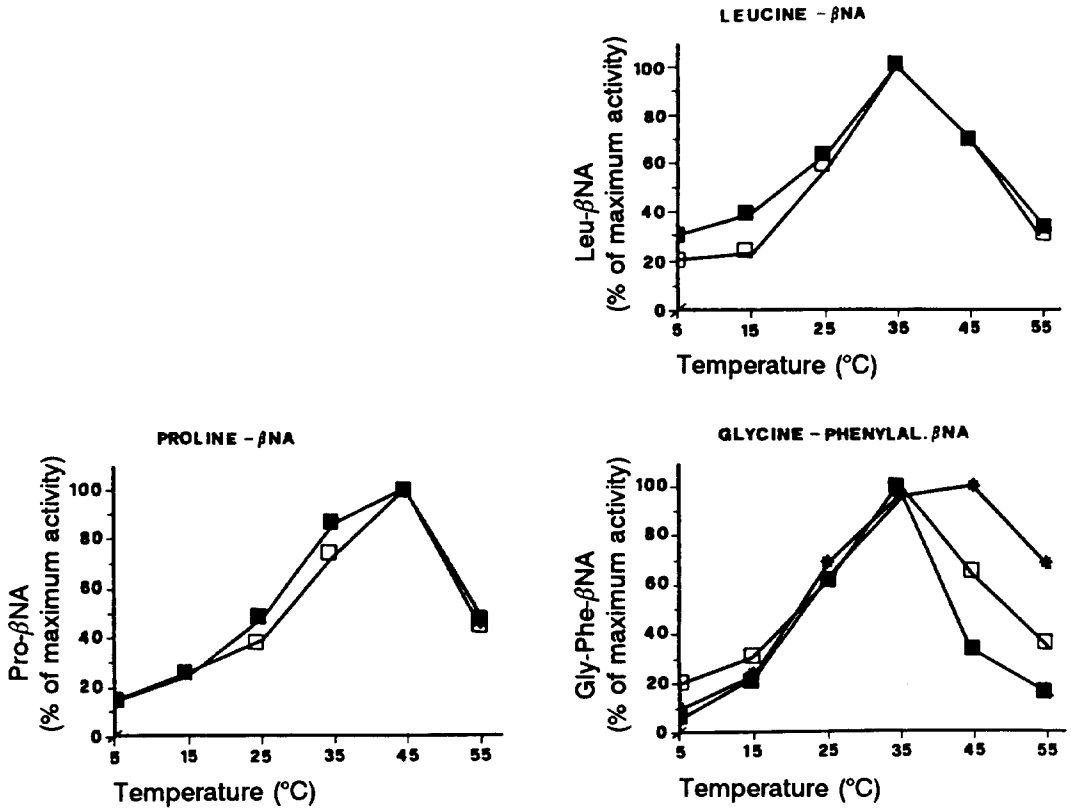


Figure 6. Effect of temperature on the peptidase activity of *Pseudomonas fluorescens* strains PF (■), 22 (□), and 30 (*).

ity that *P. fluorescens* has separate binding sites for these substrates, as reported by Young (34) for aminopeptidase of *Escherichia coli.*

Substrate hydrolysis profiles did not change significantly when cells were grown on BN, PMS7, or TSB, suggesting the presence of constitutive enzyme active for H-Pro- and H-Gly-Phe-βNA. However, an increase in hydrolase activity of H-Leu-βNA was observed when the fermentation medium was TSB. McKellar and Cholette (21) suggested that variability in enzyme concentrations may reflect changes in the nutritional state of the cells.

Peptidase activity of *P. fluorescens* strains was relatively stable to frozen and refrigerated storage of cells; this was in contrast to the results reported by Boquien et al. (4) for *Streptococcus cremoris* residual activity.

The pH and temperature profiles of the peptidases of the *P. fluorescens* strains showing a different optimal behavior on Pro-βNA and, as reported by Shamsuzzaman and McKellar (28), on Leu-βNA, confirmed the importance of using a purification technique or supplementary analysis to identify the presence of isolate or separate enzymes.

The results of this study indicate the presence of intracellular or cell-associated peptidase activity of *P. fluorescens* strains in addition to the proteases (10) and lipases (32) already widely studied. The peptidase was active against a large number of dipeptidyl and

aminoacyl substrates as a horizontal characteristic of the psychrotrophic species and vertically (through the species) as a minimum variable of the physiology tied to the biovariety of the bacterial species.

Finally, the peptidase specificity of *P. fluorescens* ATCC 948 and other strains show stability at pH conditions of milk and cheese ripening, at refrigeration, and at UHT and storage milk temperatures in which they might partially hydrolyze bitter peptides, which are characterized by the presence of a large number of hydrophobic amino acid residues.

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