

## General properties and substrate specificity of an intracellular soluble dipeptidase from *Streptococcus diacetilactis*

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**Summary.** Dipeptidase activity from *S. diacetilactis* was isolated and the main activity, Dipeptidase I, characterized as to substrate specificity, requirements for activity and molecular size. Optimum Dipeptidase I activity was recorded at pH 7.5-8.0 and molecular weight on gel filtration was estimated to be 51,000 daltons ; it was most stable at temperatures under 50 °C and at pH 7.0-8.0. This activity was totally inactivated by 0.01 mM EDTA, then reactivated by metal ions (Co<sup>++</sup> and Zn<sup>++</sup>). This enzyme constitutes a true dipeptidase as it has no tripeptidase or arylamidase activities and does not hydrolyse whole proteins. Studies of substrate specificity show that the enzyme preferentially hydrolyses methionyl-dipeptides (Met-Ala > Met-Val > Met-Ile). The amino-acid composition of Dipeptidase I is comparable with one dipeptidase from *Mycobacterium phlei* or one renal dipeptidase.

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### Introduction.

Lactic acid streptococci show complex and variable nutritional requirements. Minimal requirements may involve amino-acids, peptides, purines, pyrimidines, vitamins and occasionally fatty acids and elevated CO<sub>2</sub> tension (Deibel and Seeley, 1974). *Streptococcus diacetilactis* in particular requires arginine or phenylalanine (Reiter and Oram, 1962). Therefore, growth of lactic acid streptococci in milk can generally be stimulated by enzymatic protein hydrolysates (Garvie and Mabbitt, 1956 ; Speck *et al.*, 1958). The present work realised in our laboratory constitutes part of a more general study in progress to determine the mechanism of peptidic stimulation of lactic streptococcus growth in milk. Since these peptides are a source of amino-acids (Desmazeaud and Hermier, 1972, 1973), their hydrolysis by intracellular proteolytic enzymes must be understood.

On the other hand, little attention has been paid to peptidase activities in streptococcus group N (Exterkate, 1975 ; Mou *et al.*, 1975 ; Sorhaug and Solberg, 1973), and no true dipeptidase has been purified from *S. diacetilactis* although this type of enzyme was characterized from *S. thermophilus* (Rabier and Desmazeaud, 1973), *Escherichia coli* (Brown, 1973 ; Patterson *et al.*, 1973), *Mycobacterium phlei* (Plancot and Han, 1972) *Neurospora crassa* (Johnson and Brown, 1974) or eukaryotic cells (Rene and Campbell,

1969 ; Sjöström *et al.*, 1973). We isolated a dipeptidasic activity in a previous work (Desmazeaud and Zevaco, 1976) dealing with purification of proteases from a cellular extract of *S. diacetilactis*. The present paper describes the isolation of two dipeptidases and the general properties of the main dipeptidase. Its dipeptide-substrate specificity is also determined.

## Materials and methods.

*Chemicals and substrates.* All chemicals employed were of analytical grade and purchased from Sigma ; DFP (Calbiochem), LL-dipeptides-substrates (Cyclo), Sephadex G-100 and Blue-Dextran 2000 (Pharmacia) excepted. Leu-Leu, Phe-Gly and Met-Leu were used as main substrates for monitoring dipeptidase activity throughout the purification procedure and studying properties.

*Organism.* *Streptococcus diacetilactis* strain CNRZ 267. This bacterium was maintained by subculture on sterile skim-milk and preserved by freezing at  $-30^{\circ}\text{C}$ .

*Cell culture.* 35 g of moist cells were obtained in a fermenter from 7.5 liters of a previously described culture medium of papain-hydrolysed milk (Valles and Mocquot, 1968) with a pH maintained at a constant 6.5 value. The bacteria were collected at the end of the exponential growth phase as preliminary results had shown general peptidase activity was maximum at that culture time.

### *Measurement of protein and enzyme activities.*

*Proteins :* Protein content was determined according to Layne (1957) and Lowry *et al.* (1951) ; bovine serum albumine was used as a standard.

*Proteolytic activity* was determined at  $37^{\circ}\text{C}$  by measuring liberated  $\alpha$ -amino groups by oxidized-insulin hydrolysis after ninhydrin reaction according to Moore and Stein (1954). The reaction mixture contained 0.8 ml substrate at 0.03 p. 100 concentration in 0.1 M sodium phosphate buffer, pH 7.0, and 0.2 ml of a suitable dilution of enzyme preparation. Incubation time was 30 min.

*Dipeptidase activity* was determined at  $37^{\circ}\text{C}$  by measuring liberated L-amino-acids from various dipeptide substrates after ninhydrin reaction. The reaction mixture contained 0.8 ml substrate (0.5 mM) in 0.1 M sodium phosphate buffer, pH 7.0, and 0.2 ml of a suitable dilution of enzyme preparation. Incubation time was 20 or 60 min.

*Tripeptidase activity* was determined with the same procedure on different tripeptides.

A unit of dipeptidase activity is defined as 1 micromole of substrate hydrolyzed/min at  $37^{\circ}\text{C}$  and specific activity is defined as enzyme units/min per mg of protein. Michaelis constants and maximum velocities ( $37^{\circ}\text{C}$ ) were recorded by means of an extrapolation of the linear portion of Lineweaver-Burk plots. Maximum velocities  $V_m$  were converted into molecular activities,  $k_{cat} = V_m/E$  (mole of substrate hydrolyzed at  $37^{\circ}\text{C}$  per sec per mole of enzyme), assuming a molecular weight of 51 000.

*Aminopeptidase activity* was assayed at  $37^{\circ}\text{C}$  on Leu-p-nitroanilide substrate according to Roncari and Zuber (1969) and on amino-acid-B-naphthylamides according to Miller and Mc Kinnon (1974).

Carboxypeptidase activity was assayed at 37 °C on Z-Gly-AA<sub>2</sub> (AA<sub>2</sub> = Arg, Leu or Phe) and Z-Glu-Tyr after ninhydrin reaction according to Morihara *et al.* (1968).

#### *Purification procedures.*

*Step 1 = Preparation of the cellular extract and elimination of nucleic acids.* 35 g of cells were crushed in a Manton-Gaulin homogenizer, type 15 M/8TA (APV-France) under a pressure of 8,000 P. S. I. The cell suspension was then centrifuged at 1,000 g for 1 h at 4 °C. The supernatant was preserved and the nucleic acids hydrolyzed by the addition of ribonuclease and deoxyribonuclease according to previously described methods (Desmazeaud and Zevaco, 1976).

*Step 2 = 1st chromatography on DEAE-cellulose.* After Sepharose 6B gel chromatography, the 800 mg of protein extract in 0.05 M sodium phosphate buffer was adsorbed on a DEAE-cellulose column equilibrated with the same buffer. The column was then washed with the buffer and the proteins eluted with a sodium phosphate buffer gradient according to the previously described methods (Desmazeaud and Zevaco, 1976).

*Step 3 = 2nd chromatography on DEAE-cellulose.* The 122 mg of the protein extract (previously described) in 5 mM Tris-HCl buffer (pH 7.5) was adsorbed on a DEAE-cellulose (DE 32, Whatman) column (2.5 × 35 cm) equilibrated with the same buffer. The column was then washed with 200 ml of the buffer and the proteins eluted with a linear gradient of NaCl (0 to 0.5 M) in the buffer. The flow rate was 40 ml/h (fraction = 10 ml). Proteolytic activity was determined by oxidized-insulin hydrolysis, and dipeptidase activity by Leu-Leu hydrolysis. In both cases the liberated  $\alpha$ -amino group from the substrates was measured by ninhydrin reaction.

*Step 4 = Chromatography on Agarose- $\epsilon$ -amino caproyl-D-Trp-methylester.* After being concentrated 10 times on a Diaflo UM-10 membrane (Amicon) Dipeptidases I and II, previously extracted (Step 3), were purified apart on hydrophobic chromatography gel : Agarose- $\epsilon$ -aminocaproyl-D-tryptophan methyl ester purchased from Miles-Yeda. This gel was equilibrated with 6.6 mM sodium phosphate buffer at pH 7.5, then one dipeptidase was adsorbed on the gel. The 2 × 9.5 cm column was washed with the buffer and the proteins eluted with sodium phosphate buffer gradients from 0.02 M to 0.05 M at pH 7.5 ; flow rate was 15 ml/h (fraction = 4 ml).

*Polyacrylamide gel electrophoresis.* Polyacrylamide gels containing 7 p. 100 acrylamide were prepared at pH 9.1 according to the method of Ornstein (1964) and Davis (1964) ; proteins were stained with Coomassie blue according to Chrambach *et al.* (1967). Dipeptidase activity on other gels was localized by the procedure of Lewis and Harris (1967).

*Molecular weight determination.* Molecular weight of Dipeptidase I was estimated by the method of Andrews (1964) with a Sephadex G-100 column (92 × 2 cm). It was equilibrated with 50 mM sodium phosphate at pH 7.5. Proteins were eluted with the same buffer and flow rate was 15 ml/h.

*Quantitative amino-acid composition.* Determination was carried out using a Beckman multichrome autoanalyzer after Dipeptidase I hydrolysis by tridistilled 5.6 N HCl in vacuum-sealed tubes for 24 h and 72 h at 115 °C.

## Results.

### *Isolation and purification of Dipeptidase I and II.*

Step 1. The 800 mg of crude extract obtained did not contain any titratable nucleic acids. It had dipeptidase activity, but did not hydrolyse amino-or-carboxypeptidase substrates (table 1).

TABLE 1  
*Purification of dipeptidases from S. diacetilactis*  
Dipeptidase activity was measured on Leu-Leu 0.5 mM at 37 °C  
for 20 mn in 0.1 M sodium phosphate buffer at pH 7.0

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield p. 100	Purifi- cation (fold)
<i>Step 1</i>						
a) Elimination of nucleic acid.	200	2 996	2 800	1.07	100	1
b) After Sepharose 6B.....	160	2 157	800	2.69	72	2.5
<i>Step 2</i>						
DEAE-cellulose (DE-23) chro- matography .....	280	1 501	122	12.30	50.1	11.5
<i>Step 3</i>						
DEAE-cellulose (DE-32) chro- matography						
Dipeptidase I.....	180	659	33	20	22	18.7
Dipeptidase II.....	240	359	5.5	65	12	61
<i>Step 4</i>						
Agarose-ε-amino-caproyl-D- Trp methyl ester chromato- graphy						
Dipeptidase I.....	170	420	4.7	89.36	14	83.5
Dipeptidase II.....	150	220	2.8	78.57	7.3	73.4

Step 2. The main protease, Protease A, was eluted at the beginning of the 0.2 M phosphate gradient. Dipeptidase activity overlapping another proteolytic fraction, Protease B, was also separated at this previously described buffer molarity (Desmazeaud and Zevaco, 1976). The active fractions, having dipeptidase activity and representing 122 mg of protein, were combined and dialysed against 5 mM Tris-HCl buffer at pH 7.5. Purification ratio was 11.5 fold and 50 per 100 initial units were collected (table 1).

Step 3. The main dipeptidase, Dipeptidase I, was eluted with a NaCl concentration of 0.22 M ; Protease B proteolytic activity still overlapped Dipeptidase I (fig. 1). Dipeptidase II was also separated with a NaCl concentration of 0.3 M. The fraction containing Dipeptidase I represented 33 mg of protein and 22 per 100 of initial units (table 1).

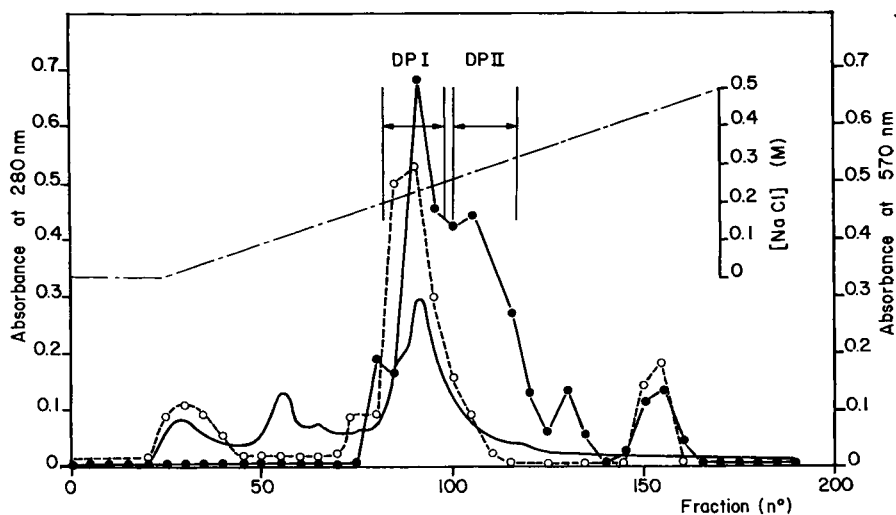


FIG. 1. — DEAE. — cellulose chromatography of dipeptidases from *S. diacetilactis*.

● — dipeptidase activity was determined by hydrolysis of Leu-Leu ; ○ — proteolytic activity (protease B) by hydrolysis of oxidized-insulin then in both cases by measuring the liberated  $\alpha$ -amino group (absorbance at 570 nm) as indicated in Materials and methods. Protein elution (—) After protein elution absorbance was measured at 280 nm. (· — · — ·) indicated buffer molarity.

Step 4. Hydrophobic chromatography separated Dipeptidase I and Protease B. Proteolytic activity was not adsorbed on the gel ; Dipeptidase I was thus eluted with 0.016 M phosphate gradient. Dipeptidase II was eluted with the 0.025 M phosphate gradient (fig. 2). Dipeptidase I and II represented 4.7 and 2.8 mg of protein, respectively (table 1).

Analytical polyacrylamide gel electrophoresis indicated that purified Dipeptidase I (20  $\mu$ g) consisted of a single protein band (Experiment B ; fig. 3). A further test of preparation homogeneity (60  $\mu$ g) showed that purified Dipeptidase I, besides producing a main component corresponding to dipeptidase activity against various dipeptides (fig. 3. Experiments LL, LA, LG, ML, PG and HS), evidenced two minor bands (Experiment A ; fig. 3). The general properties of this enzyme were studied without further purification.

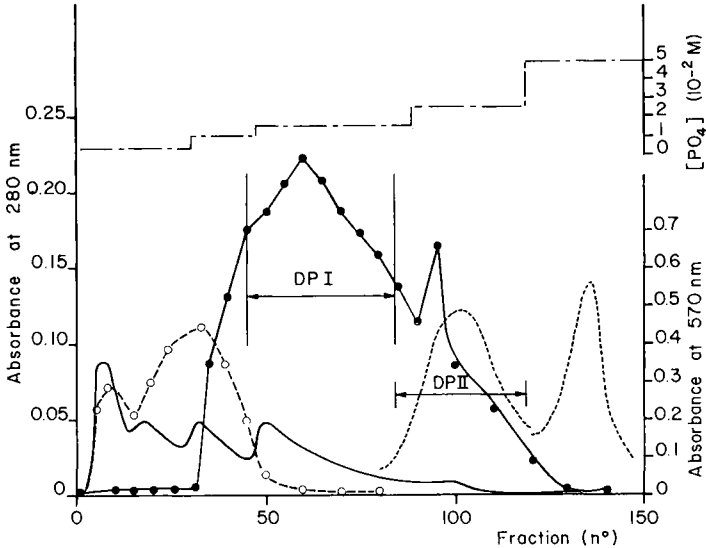


FIG. 2. — Chromatography of dipeptidase on Agarose- $\epsilon$ -aminocaproyl-D-Trp methylester.

- — ● and — — — — = dipeptidase activity was determined by hydrolysis of Leu-Leu (absorbance at 570 nm).
- — — — — ○ = proteolytic activity (absorbance at 570 nm).
- — — — = protein elution (absorbance at 280 nm).
- · · · · = buffer molarity.

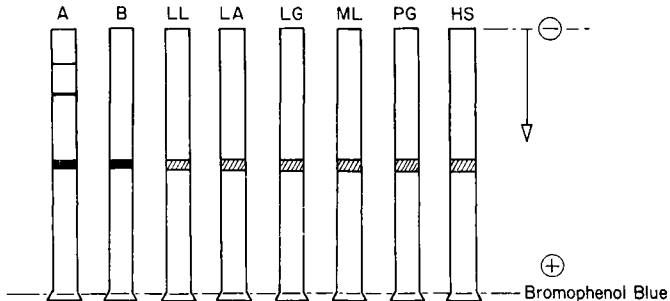


FIG. 3. — Polyacrylamide gel electrophoresis of Dipeptidase I.

A = experiment with 60  $\mu$ g of Dipeptidase I ; B = experiment with 20  $\mu$ g of Dipeptidase I. After electrophoresis at pH 9.1 the gels were stained with Coomassie blue. Other tubes with 60  $\mu$ g of enzyme displayed dipeptidase localization on gel with various dipeptide substrates (LL = Leu-Leu, LA = Leu-Ala, LG = Leu-Gly, ML = Met-Leu, PG = Phe-Gly and HS = His-Ser).

### General properties of dipeptidase I

**Molecular weight.** The apparent molecular weight of the dipeptidase was estimated with a Sephadex G-100 column to be 51 000 daltons, as compared to that of trypsin, pepsin and bovine serum-albumin.

*Effect of temperature.* The purified Dipeptidase I retained its activity after storage over several months at  $-20^{\circ}\text{C}$ . It could be kept at  $25^{\circ}\text{C}$  for some hours or at  $50^{\circ}\text{C}$  for 30 min without becoming inactive (fig. 4); it was slowly inactivated at  $55^{\circ}\text{C}$  and rapidly at higher temperatures. Fifty per cent of its activity was lost after 15 min at  $60^{\circ}\text{C}$  and 90 per cent after 10 min at  $65^{\circ}\text{C}$  (fig. 4).

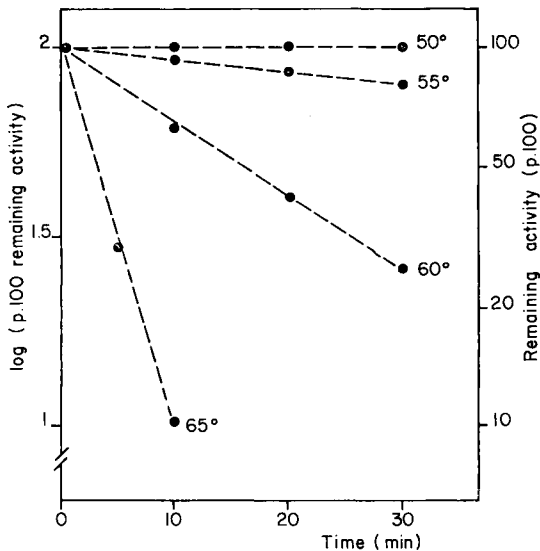


FIG. 4. — Thermal denaturation of Dipeptidase I.

Dipeptidase I ( $20\ \mu\text{g}$ ) was incubated at pH 7.0 in 10 mM sodium phosphate buffer. The remaining activity was estimated at pH 7.0 for 20 min at  $37^{\circ}\text{C}$  on Leu-Leu or Phe-Gly and expressed as a percentage of the control on a logarithmic plot.

*Effect of pH.* Maximum activity was obtained at pH 7.5-8.0 with three dipeptide substrates (fig. 5). Fifty or sixty per cent of this activity (substrate Met-Leu) was measured at pH 7.0 or 8.5, respectively. Dipeptidase I was quite stable over the pH range 7.0-8.0. Thirty-seven per cent of the activity was inactivated at pH 5.5 or pH 9.5 after 30 min at  $37^{\circ}\text{C}$  (fig. 5A).

*Effect of various inhibitors and metal-ion requirement.* Serine protease inhibitors such as DFP and PMSF showed no inhibition against Dipeptidase I. The sulfhydryl protease inhibitor pCMB 0.1 mM or mM reduced the activity to 80 and 0 per 100, respectively. Therefore, there is no seryl residue in the active site of Dipeptidase I. Sulfhydryl residues must be important to the activity, although the concentration of p-chloromercuribenzoate used was high. Sulfhydryl protein groups showed various reactions to pCMB.

Addition of  $10^{-3}$  mM EDTA to the reaction mixture reduced the hydrolysis rate of Leu-Leu or Met-Leu to 80 p. 100 as compared to the controls. Addition of 0.01 mM EDTA completely inhibited Dipeptidase I activity. This inhibition can be fully reversed

by addition of  $\text{Co}^{++}$  or  $\text{Zn}^{++}$ . Metal ions such as  $\text{Ca}^{++}$ ,  $\text{Mn}^{++}$  or  $\text{Mg}^{++}$  can reactivate Dipeptidase I to a certain extent (table 2). The results clearly indicate that the enzyme requires a divalent ion for its activity.

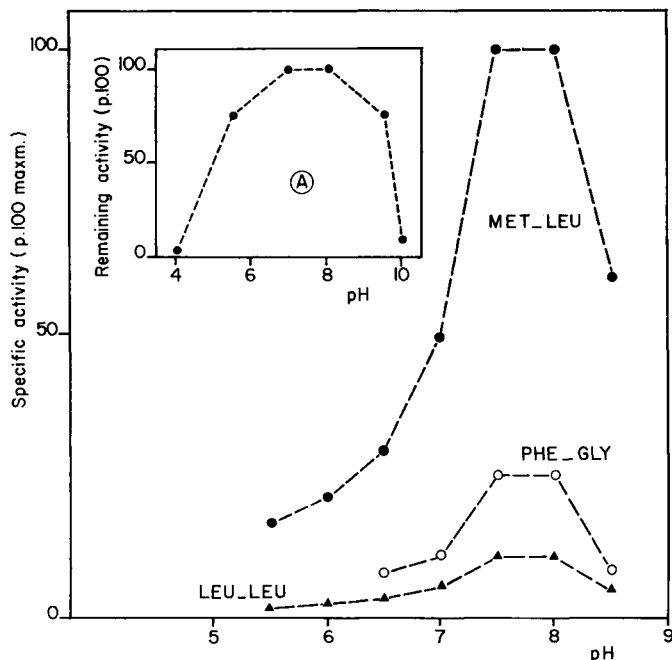


FIG. 5. — Activity and stability (Insert A) of Dipeptidase I as a function of pH.

Dipeptidase activity (20  $\mu\text{g}$ ) on Met-Leu, Phe-Gly and Leu-Leu (0.5 mM) was tested in pH range from 5.5 to 8.5 in 0.1 M sodium phosphate buffer. The activity was measured at 37 °C for 30 min and expressed as per cent of maximum specific activity on Met-Leu.

Insert A = Effect of pH on the stability of Dipeptidase I. The enzyme was incubated for 30 min at 37 °C in 10 mM sodium acetate (pH 5.5 to 8.5) and 10 mM sodium carbonate buffer (pH 8.5 to 10.0) adjusted to the desired pH. The remaining activity was estimated at pH 7.0 as indicated above and expressed as a percentage of the control.

**Substrate specificity.** The relative rates of 32 dipeptides hydrolysed by Dipeptidase I are summarized in table 3. Methionyl-alanyl appeared to be hydrolysed at the highest rate. All methionyl-dipeptides were the best substrates with one exception, Met-Glu, which was hydrolysed at a rate comparable to that of Leu-Gly or Phe-Gly (table 3). A wide range of dipeptides was poorly hydrolysed by Dipeptidase I. They contain N-terminal glycine, L-alanine or L-proline (table 3). Some N-terminal glycine or L-alanine dipeptides were not hydrolysed by Dipeptidase I (table 3). This purified enzyme must be classified as a true dipeptidase as it lacks activity against the tested tripeptides. Met-Leu-Gly in particular was not hydrolysed (table 3), though Met-Leu and Leu-Gly were good substrates; it evidenced no activity against aminoacyl-B-naphthylamides or Leu-p-nitroanilide (table 3). Experiments performed with com-



TABLE 2

*Dipeptidase 1 divalent cations requirement after inhibition for EDTA.*

The enzyme was pre-incubated for 15 min. at 37 °C with 0.01 mM EDTA in 50 mM TRIS-HCl buffer (pH 7.0) and the remaining dipeptidase activity measured on dipeptides at 37 °C for 15 min. in 50 mM TRIS-HCl buffer (pH 7.0) further incubated in divalent cation 1 mM solutions. Activity is expressed as a percentage of the control.

Addition of 1 mM cations after 0.01 mM EDTA inhibition	Dipeptidase activity as p. 100 of control
Co <sup>++</sup>	120
Zn <sup>++</sup>	105
Ca <sup>++</sup>	84
Mn <sup>++</sup>	65
Mg <sup>++</sup>	20

pounds having no free N-terminal amino group showed that the enzyme had no carboxypeptidase activity (table 3); these groups, and particularly N-terminal methionine, are necessary to hydrolyse the dipeptides effectively. Thus, Dipeptidase I from *S. diacetilactis* is a L-Methionyl-amino-acid hydrolase (EC. 3.4.13.12).

TABLE 3

*Substrate specificity of Dipeptidase I*

Dipeptide	Specific activity as p. 100 hydrolysis of Met-Leu	Dipeptide	Specific activity as p. 100 hydrolysis of Met-Leu
Met-Ala ....	164	Gly-Leu .....	0
Met-Ser .....	141	Gly-Trp .....	0
Met-Val ....	133	Gly-Pro .....	0
Met-His .....	120	Ala-Ala .....	0
Met-Ileu .....	107	Ala-Leu .....	0
Met-Leu ....	100	Ala-Phe .....	0
Leu-Gly ....	27	Ala-Met .....	0
Met-Glu ....	26	Ala-His .....	0
Phe-Gly ....	24	Ala-Asp .....	0
Tyr-Leu ....	21		
Leu-Ala ....	14	Other substrates	
His-Ser ....	13	Leu-p-nitroanilide .....	0
Lys-Tyr ....	12	Leu-β-naphthylamide ....	0
Leu-Leu ....	11	Arg-β-naphthylamide .....	0
Leu-Tyr ....	7	Ala-β-naphthylamide ....	0
His-His ....	7	Z-Gly-Arg .....	0
Gly-Phe ....	7	Z-Gly-Leu .....	0
Ala-Gly .....	5	Z-Gly-Phe .....	0
Ala-Phe ....	4	Z-Glu-Tyr .....	0
Gly-Tyr ....	3	Leu-Gly-Gly .....	0
Gly-Ala .....	2	Gly-Phe-Gly .....	0
Pro-Leu ....	1	Met-Leu-Gly .....	0
Pro-Gly ....	1		

The  $K_m$  and  $k_{cat}$  values for some of the methionyl dipeptides are shown in Table IV. The  $K_m$  values were similar for all substrates tested (though Met-Glu showed a poor

TABLE 4

*Kinetic constants for Dipeptidase U-catalysed hydrolysis of methionyldipeptides.*

Activity was determined at 37 °C by measuring liberated L-amino-acids from various dipeptides after ninhydrin reaction. The reaction mixture contained 0.8 ml substrate (from 0.05 mM to 0.5 mM) in 0.1 M sodium phosphate buffer, pH 7.0 and 0.2 ml of a suitable dilution of enzyme preparation. The incubation time was 10, 20 and 30 min. or 1, 2 and 3 h for Met-Glu.

Peptides	$K_m$ (mM)	$k_{cat}$ (sec <sup>-1</sup> )	$k_{cat}/K_m$ (sec <sup>-1</sup> × mM <sup>-1</sup> )
Met-Ala.....	0.33	34.6	104.8
Met-Val.....	0.34	35.3	104
Met-His.....	0.56	52.7	94.1
Met-Ile.....	0.39	36.5	93.5
Met-Ser.....	0.43	26.9	62.6
Met-Leu.....	0.68	26.3	38.7
Met-Glu.....	1.33	0.19	0.14

affinity), but the  $k_{cat} = V_M/E$  values varied more than 200-fold for Met-Glu. The « proteolytic coefficient » ( $= k_{cat}/K_m$ ) was similar for Met-Ala, Met-Val, Met-Ile and Met-His, and very weak for Met-Glu (table 4).

TABLE 5

*Amino-acid composition of Dipeptidase I from S. diacetilactis. Comparison with various dipeptidases*

Amino-acid	Dipeptidase from <i>S. diacetilactis</i> (51,000 daltons)	Dipeptidase from <i>Mycobacterium phlei</i> (45,000 daltons)	Renal dipeptidase (47,200 daltons)	Intestinal dipeptidase monomer (52,000 daltons)
Lysine.....	26	17	29	33
Histidine.....	10	8	10	12
Arginine.....	15	19	17	17
Aspartic acid.....	49	44	39	47
Threonine.....	19	24	22	20
Serine.....	26	21	28	28
Glutamic acid.....	56	43	43	51
Proline.....	18	20	21	22
Glycine.....	40	37	25	41
Alanine.....	35	47	32	32
Cysteine*.....	9*	3	17	8
Methionine.....	8	5	7	9
Valine.....	27	27	27	32
Isoleucine.....	20	20	15	25
Leucine.....	33	37	41	44
Tyrosine.....	14	13	12	17
Phenylalanine.....	16	15	18	16
Tryptophane.....		9	11	9

\* This value was determined by analysing the performic acid oxidation product of the Dipeptidase I.

**Amino-acid composition.** No significant differences were found between 24 and 72 h acid hydrolysis. Unfortunately we did not have enough protein for precise tryptophane determination. Dipeptidase I contained many aspartic acid, glutamic acid (or their amides) or glycine residues (table 5) and some methionine, histidine or cysteine residues.

## Discussion.

Until now, dipeptidase of lactic streptococci of the N-group and in particular that of *Streptococcus diacetilactis* has not been thoroughly studied, although its action has already been demonstrated in these bacteria (Mou *et al.*, 1975 ; Sorhaug and Solberg, 1973).

With an optimum pH of 7.5-8.0, *S. diacetilactis* dipeptidase is close to the dipeptidases of *S. thermophilus* (Rabier and Desmazeaud, 1973), *Neisseria catarrhalis* (Behal and Folds, 1967) *Escherichia coli* (Haley, 1968), as well as the dipeptidase of *Saccharomyces cerevisiae* var. *ellipsoideus* (Cordonnier, 1966), the pH optimum of brewer's yeast being slightly higher (Röhm, 1974).

*S. diacetilactis* dipeptidase, as most of the previously studied dipeptidases (Rabier and Desmazeaud, 1973 ; Brown, 1973 ; Johnson and Brown, 1974 ; Röhm, 1974), behaves like a metalloenzyme since EDTA is a potent inhibitor, and this inhibition can be totally or partially suppressed by cations,  $\text{Co}^{++}$  and  $\text{Zn}^{++}$ . Because of its relative sensitivity to sodium parachloromercuribenzoate it can be compared with dipeptidases of *S. thermophilus* (Rabier and Desmazeaud, 1973), *N. catarrhalis* (Behal and Folds, 1967) and *S. cerevisiae* var. *ellipsoideus* (Cordonnier, 1966).

The molecular weight of *S. diacetilactis* dipeptidase (51,000 daltons) is similar to that of *S. thermophilus* dipeptidase (Rabier and Desmazeaud, 1973) and slightly higher than that of *M. phlei* (Plancot and Han, 1972) or the renal dipeptidase (Campbell *et al.*, 1966) which have similar molecular weights of 45 000 and 47 000, respectively. The analogy with the two latter dipeptidases is confirmed by comparing their amino acid composition with that of *S. diacetilactis* dipeptidase after acid hydrolysis. The half values of a number of intestinal dipeptidase residues (molecular weight : 104 000 daltons) are also indicated in table 5, the two polypeptide chains constituting this dipeptidase being identical (monomers M.W = 52,000 daltons ; Noren and Sjöström, 1974). In all these enzymes there are many lysine, aspartic acid (or asparagine), glutamic acid (or glutamine), alanine or leucine residues and a smaller number of histidine, methionine or cysteine ones.

Because of substrate specificity, *S. diacetilactis* dipeptidase is a L-methionyl-amino-acid hydrolase (EC. 3.4.13.12) analogous to that of *E. coli* (Brown, 1973) or *Neurospora crassa* (Johnson and Brown, 1974). For the substrates Met-Ala, Met-Ser or Met-Ile, the  $K_m$  values are very similar. However, the  $k_{cat}$  values measured for *S. diacetilactis* dipeptidase are much lower than those recorded for *E. coli* dipeptidase (Patterson *et al.*, 1973).

The specificity of *S. diacetilactis* dipeptidase towards  $\text{NH}_2$ -terminal methionine dipeptides shows the importance of the nature of the residues in obtaining good positioning of the substrate dipeptide. The active dipeptidase site must therefore be compos-

ed of two pockets of very accurate spatial configuration allowing adequate interaction between the amino acid residue in which the L- $\alpha$ -amino group is free ( $R_N$  pocket) and that of the amino acid with a free carboxyl group ( $R_C$  pocket) (Patterson *et al.*, 1973). L-methionyl-dipeptidase of *S. diacetilactis* is probably fitted with a deep, narrow  $R_N$  pocket adapted to a linear residue. This would account for the weak hydrolysis of Leu-x-type substrates as compared with Met-x-type substrates. Likewise, the absence of hydrolysis of some substrates with glycyl or alanyl residue into  $NH_2$ -terminal position could be the result of their inadequate positioning due to the lack of interaction with the  $R_N$ -pocket. Kinetic constants show the importance of residues having a free carboxylic group since the proteolytic coefficient ( $k_{cat}/K_M$ ) is 2.7 or 748 times lower with Met-Leu or Met-Glu, respectively, than with Met-Ala (or Met-Val).

*S. diacetilactis* methionyl-dipeptidase is thus different from the several dipeptidases exhibiting great affinity for dipeptides with  $NH_2$ -terminal-alanine or glycine (Patterson *et al.*, 1973 ; Plancot and Han, 1972 ; Noren *et al.*, 1973 ; Behal and Folds, 1967), proline (Akrawi and Bailey, 1976) or cysteine residue (Mc Corquodale, 1963).

The role of the dipeptidase in *S. diacetilactis* metabolism is unknown. However, in *M. crassa* (Johnson and Brown, 1974), it has been suggested that methionyl dipeptidases might be involved in the liberation of N-terminal methionine from the newly-initiated proteins. Intracellular dipeptidases, like those of *E. coli* (Simmonds, 1966) and many other bacteria (Payne, 1976), are probably also involved in the breakdown of peptides and proteins, thus participating in the general protein turn-over of the cell as well as in the utilization of the peptides as sources of amino-acids.

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**Résumé.** La purification, les principales propriétés et la spécificité d'action sur différents dipeptides, d'une dipeptidase intracellulaire de *S. diacetilactis*, sont décrites dans ce mémoire.

L'activité optimum de cette enzyme était obtenue entre pH 7,5 et 8,0 en utilisant la Met-Leu, la Phe-Gly ou la Leu-Leu comme substrat. Son poids moléculaire était estimé à 51 000 daltons par chromatographie sur gel Sephadex G-100. Cette dipeptidase était stable aux températures inférieures à 50 °C et dans la zone de pH 7,0-8,0. Elle était totalement inactivée par l'E. D. T. A. 0,01 mM, mais elle pouvait être réactivée par des cations tels que  $Co^{++}$  ou  $Zn^{++}$ .

Cette enzyme est une dipeptidase vraie car elle n'hydrolyse aucun substrat en dehors des dipeptides. En particulier, elle ne présente aucune activité envers les tripeptides ni les dérivés d'acide aminé, ni les protéines natives ou dénaturées. Les études de spécificité ont montré qu'elle hydrolyse préférentiellement les dipeptides ayant un résidu méthionyle N-terminal (Met-Ala > Met-Val > Met-Ileu). On peut donc la classer comme une enzyme de type méthionyle dipeptidase EC 3.4.13.12. Sa composition en acides aminés est assez comparable à celle obtenue pour une dipeptidase de *Mycobacterium phlei* ou celle d'une dipeptidase rénale.

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