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NOTE TECHNIQUE

Peptide hydrolases from the *Thermobacterium* group of Lactobacilli

III. Characterization of the intracellular exopeptidases

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Résumé

Les peptides hydrolases de lactobacilles du groupe Thermobacterium
III. Caractérisation des exopeptidases intracellulaires

A partir des broyats cellulaires de *Lactobacillus helveticus, L. bulgaricus* et *L. Lactis* des activités dipeptidasiques et aminopeptidasiques intracellulaires ont été séparées par chromatographie et leurs propriétés générales déterminées.

La température optimum des aminopeptidases était de 35 ou de 40° C, celle de toutes les dipeptidases de 35° C à l'exception de celle de *L. helveticus* qui était de 30° C. Le pH optimum des différentes activités était proche de la neutralité et variait selon les enzymes de pH 7,0 à 6,0.

Certaines de ces activités n'étaient pas inhibées par l'éthylène diamine tétracétique. Celles qui y sont sensibles étaient réactivées par les ions cobalt ou magnésium. La plupart de ces enzymes, sauf une dipeptidase de *L. bulgaricus,* montraient aussi une sensibilité au parahydroxymercuribenzoate et au phénylméthylsulfonyl fluorure laissant supposer l'intervention de groupes sulfhydryles ou séryles dans leur site actif.

Mots clés : Exopeptidase intracellulaire - Dipeptidase - Aminopeptidase - *Lactobacillus helveticus* - *L. bulgaricus* - *L. lactis* - *Thermobacterium* - Peptide hydrolase.

Introduction

Some lactobacilli from the *Thermobacterium* group play an important role in the dairy industries and more particularly *L. bulgaricus* which is used in the

manufacture of yoghurt and many other fermented milks ; this microorganism is also used as starter for a large variety of cheeses. *L. helveticus* is almost as important as *L. bulgaricus* since it is used in the starter cultures for swiss cheeses as well as many italian cheese varieties. *L. lactis* is probably the less utilized among the three species but, increase interest is given to this organism nowadays as a cheese starter.

Since proteolysis is one of the main activities leading to better texture and flavour formation during cheese ripening, a research program was developed in our laboratories for a better understanding of the peptide hydrolase system of these technologically important microorganisms.

In previous communications, we described the peptide hydrolase system of *L. bulgaricus, L. helveticus, L. lactis* and *L. acidophilus* (EL SODA and DESMAZEAUD, 1982). It was shown that each *Bacterium* had aminopeptidase activity towards various aminoacid β-naphthylamide and dipeptides. The four species also showed true dipeptidase activities on a large number of dipeptides.

Intracellular enzymes from these organisms also hydrolysed α_{s1}- and β-casein fractions as well as α-lactalbumin and β-lactoglobulin. An investigation on the influence of some physiological factors on enzyme production (EZZAT *et al.*, 1982) revealed that pH and temperature modulate the proteolytic activity of *L. bulgaricus, L. helveticus* and *L. lactis*. Optimal conditions for peptidase production by *Thermobacteria* in cultures with free running pH were found to be : cells harvest at the beginning of the stationary phase after growth at $\pm$ 5° C of their optimal growth temperature.

More recently (EZZAT *et al.*, 1984) it was shown that *L. bulgaricus, L. lactis* and *L. helveticus* exhibit cell bound proteinase activity. The enzymes are produced when the cells are grown in either skim milk or a peptide rich media. Production of the cell wall proteinases was usuallly highest during exponential growth.

The present work aimed at the characterization of the partially purified intracellular peptidases from *L. helveticus, L. bulgaricus* and *L. lactis* in order to obtain a clearer picture on the general properties of their enzymes for a better understanding of their possible role during cheese ripening and fermented milks making.

I. Materials and methods

Cultivation of the microorganisms and preparation of the crude cell free extract

The following strains were used for this study : *L. helveticus* CNRZ 303, *L. bulgaricus* CNRZ 369 and *L. lactis* CNRZ 250.

The methods described by EL SODA *et al.* (1978a) were used for the cultivation of the cells and the preparation of the crude cell free extract, with however some modifications : cells desintegration was also carried out using alumina powder (Sigma type 305) instead of the Vibrogen cell mill.

Assay of proteolytic and peptidase activities

Dipeptidase activity was determined by measuring after ninhydrin reaction the hydrolysis of Alanyl-Histidine substrate while aminopeptidase determination was carried out by measuring the extent of hydrolysis of L-leucine-paranitroanilide (Sigma). These methods were described by EL SODA and DESMAZEAUD (1982). The activities were tested into a pH or temperature range varying from 5 or 20° C to 8 or 50° C, respectively.

Isolation procedure

Gel filtration on Sepharose 6B (Pharmacia) was accomplished as previously described by EL SODA *et al.* (1978b).

Partially separated enzymatically active fractions obtained after gel filtration were pooled concentrated on a Sartorius membrane filter (collodion bag SM 13200). The concentrated fractions were then applied to a DEAE-Sephacel ion exchange column (K10/20 Pharmacia). The flow rate was 30 ml/hr 5 ml fractions were collected. A potassium phosphate buffer gradient was applied for the elution.

II. Results

Partial isolation of the activities was accomplished after gel filtration on Sepharose 6B and ion exchange chromatography on DEAE-Sephacel (fig. 1). Due to the instability of the activities in these fractions during chromatographies, it is not possible to calculate precise yield of these purifications. The isolated fractions for each activity were pooled as indicated on figure 1, and used for the determination of the optimum temperature and optimum pH ; the influence of proteinase inhibitors and activators was also considered.

As far as enzyme characterization is concerned, it is possible to notice that the optimum temperature for aminopeptidase activities was either 35 or 40° C for the different lactobacilli (table 1).

All the dipeptidase activities showed an optimum temperature of 35° C with the exception of *L. helveticus* which showed maximum hydrolysis of Ala-His at 30° C.

In overall, the isolated peptidase fractions had near neutral pH optima.

The actions of the proteinase inhibitors ethylenediamine tetraacetic acid (EDTA), parahydroxymercurybenzoate (pHMB), phenylmethylsulfonyl fluoride (pMSF) showed significant differences between the different activities (table 1). Contrary to most lactic acid bacteria aminopeptidases, the aminopeptidase isolated from *L. helveticus* as well as aminopeptidase I from *L. bulgaricus* are not inhibited by the metal chelator EDTA at a concentration of $1x10^{-3}$M. These enzymes are however inhibited by pHMB and to a higher extent by the serine proteinase inhibitor pMSF.

On the other hand, the two aminopeptidase activities of *L. lactis* as well as aminopeptidase II from *L. bulgaricus* are inhibited by the metal chelators EDTA and reactivated by divalent cations.

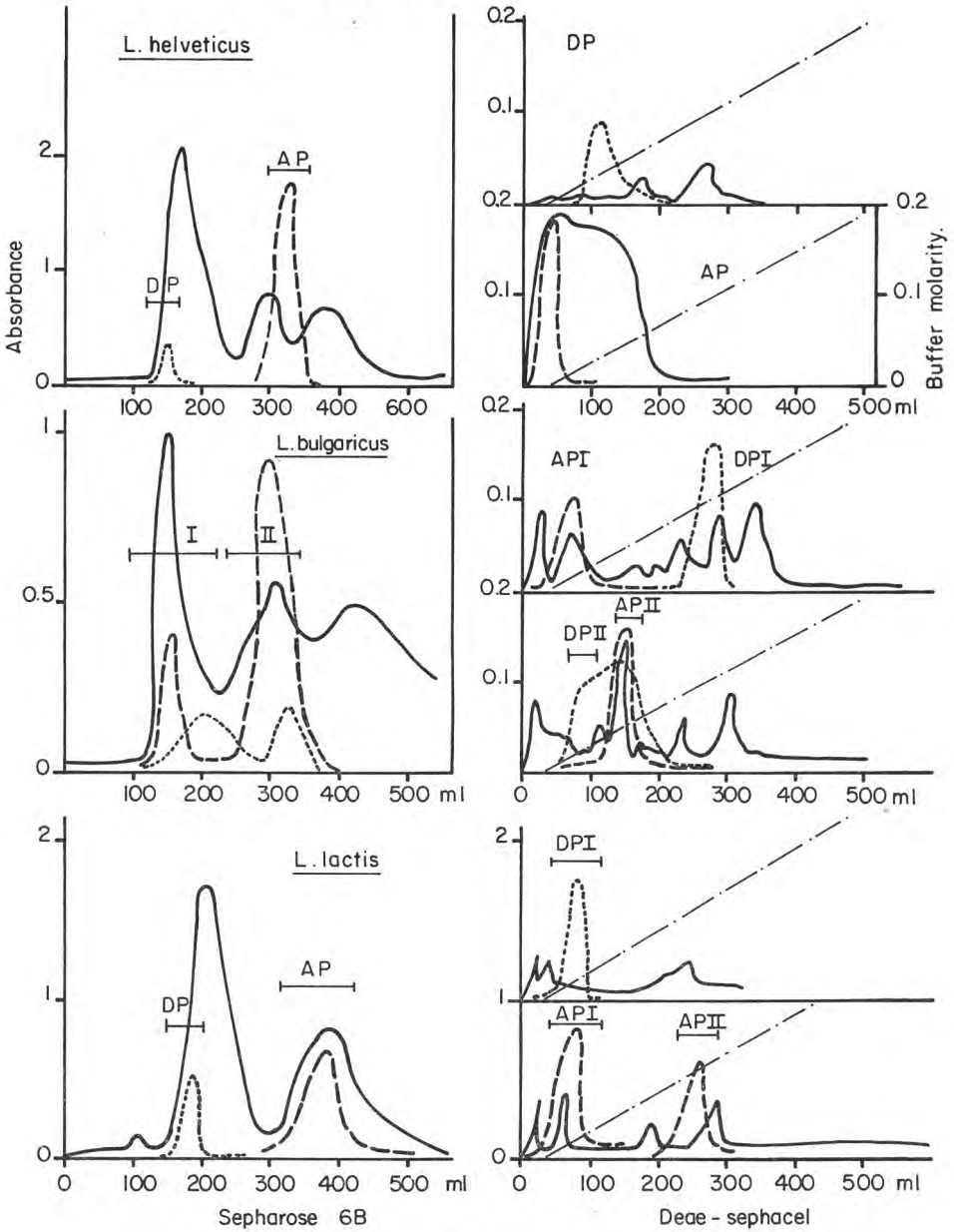

Fig. 1

Fractionation of various peptidase activities on Sepharose 6B and DEAE-Sephacel columns from crude cell-free extracts of L. helveticus, L. bulgaricus *and* L. lactis.

Fractionnement des différentes activités peptidasiques par chromatographies sur colonnes de gel Sepharose 6B et DEAE-Sephacel, à partir des broyats cellulaires de L. helveticus, L. bulgaricus *et* L. lactis.

..... *Dipeptidase activity (DP).*
Activité dipeptidasique (DP).
——— *Aminopeptidase activity (AP).*
Activité aminopeptidasique (AP).
—.—. *Phosphate buffer molarity (M).*
Molarité du gradient de phosphate (M).
——— *Protein concentration (280 nm absorbance).*
Concentration en protéines (absorbance à 280 nm).

TABLE I

General properties of the intracellular peptide hydrolase activities in L. helveticus, L. bulgaricus *and* L. lactis

Propriétés générales des activités peptide-hydrolases intracellulaires chez L. helveticus, L. bugaricus *et* L. lactis

	Opt. temp.	Opt. pH	% inhibition by EDTA pHMB pMSF 1×10^{-3} M			% reactivation by Co Mg DTT 5×10^{-3} M		
L. helveticus								
Aminopeptidase	35	7.0	0	60	82	—	—	96
Dipeptidase	30	6.0	0	100	100	—	—	90
L. bulgaricus								
Aminopeptidase I	35	6.5	0	22	44	—	—	—
Aminopeptidase II	40	6.0	100	64	79	80	100	95
Dipeptidase I	35	6.0	0	10	0	—	—	—
Dipeptidase II	35	6.5	0	100	100	—	—	60
L. lactis								
Aminopeptidase I	40	6.5	60	0	24	100	100	—
Aminopeptidase II	35	6.5	32	0	27	80	70	—
Dipeptidase I	35	6.0	100	53	100	80	90	95

— : Not determined.
EDTA : Ethylene diamine tetraacetic acid.
PHMB : Parahydroxymercuribenzoate.
PMSF : Phenylmethyl sulfonyl fluoride.
Mg : Magnesium chloride.
Co : Cobalt chloride.
DTT : Dithiothreitol.

Like many of the lactic acid bacteria described dipeptidases the Ala-His hydrolysing enzyme from *L. lactis* is strongly inhibited by EDTA and reactivated in the presence of the divalent cations cobalt and magnesium. On the other hand dipeptidase isolated from *L. helveticus* and dipeptidases I and II from *L. bulgaricus* are not affected by EDTA. SH-groups and serine seem to be important for the catalytic action of several dipeptidases which were either partially or totally inhibited in the presence of pHMB or pMSF.

III. Discussion - Conclusion

The optimum temperature for aminopeptidase activities from the different lactobacilli are comparable to those described by MOU *et al.* (1975) for *S. cre-*

moris ; DESMAZEAUD and ZEVACO (1979) for aminopeptidase I and II from *S. diacetylactis* and LAW (1979) for *S. lactis*. On the other hand, the aminopeptidases isolated from *L. helveticus, L. bulgaricus* and *L. lactis* show higher optimum temperature if compared the enzyme from *L. casei* (EL SODA *et al.*, 1978b) which showed maximum hydrolysis of LNA at 30° C and, lower temperature optima than the *L. lactis* cell bound aminopeptidase described by EGGIMANN and BACHMANN (1980).

All the isolated peptidase fractions had near neutral pH optima which is comparable to optimum pH obtained by many authors for dipeptidases and aminopeptidases isolated from other lactic acid bacteria (DESMAZEAUD, 1983). They however differ in that respect from the dipeptidase described by SORHAUG and KOLSTAD (1981) which showed an optimum pH of 10.3.

On the other hand, the two aminopeptidase activities of *L. lactis* as well as aminopeptidase II from *L. bulgaricus* are comparable to the aminopeptidase of *S. diacetylactis* (DESMAZEAUD and ZEVACO, 1979), *S. lactis* (LAW, 1979), *L. casei* (EL SODA *et al.*, 1978b), *L. lactis* (EGGIMANN and BACHMANN, 1980), *L. acidophilus* (MACHUGA and IVES, 1984) and *S. lactis* and *S. cremoris* (KAMINOGAWA *et al.*, 1984). All these enzymes are inhibited by the metal chelators EDTA and reactivated by divalent cations.

In conclusion, this study reveals that *L. helveticus, L. bulgaricus* and *L. lactis* possess a rather complex exopeptidase system composed of aminopeptidases. The enzymes are to a great extent comparable to previously described aminopeptidases and dipeptidases in lactic acid bacteria.

The presence of such a rich peptidase system confirms the important role of such bacteria as ripening agents and as cheese flavour producers.

The presence in *L. bulgaricus* of an active aminoacid producing systems also confirm the stimulatory role of this micro-organism in yoghurt cultures (TAMINE and ROBINSON, 1985).

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