

Protein degradation and amino acid metabolism by propionibacteria

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Summary — This article reports the existing knowledge about the proteolytic system of *Propionibacterium* and its ability to degrade amino acids. *Propionibacterium* contains at least 2 weak proteinases, 1 cell wall-associated and 1 intracellular or membrane-bound. A wide variety of peptidases, such as amino peptidases, proline iminopeptidase, proline imidopeptidase, X-prolyl-dipeptidyl-amino-peptidase, endopeptidases and carboxypeptidase, has been described and characterized. A wide variety of amino acids, especially aspartic acid, alanine, serine and glycine, were easily degraded by *Propionibacterium*, but large strain and species variations were observed.

***Propionibacterium* / proteinase / peptidase / amino acid**

Résumé — **Dégradation protéique et métabolisme des acides aminés par les bactéries propioniques.** Cet article fait l'état des connaissances actuelles sur le système protéolytique des bactéries propioniques, et sur sa capacité à dégrader des acides aminés. *Propionibacterium* contient au moins deux protéinases, l'une associée à la paroi cellulaire et l'autre cytoplasmique ou membranaire. Une vaste variété de peptidases ont été décrites et caractérisées, telles que aminopeptidases, proline imino-peptidase, proline imidopeptidase, X-prolyl-dipeptidyl-amino-peptidase, endopeptidases et carboxy-peptidase. Une grande variété d'acides aminés, en particulier l'acide aspartique, l'alanine, la sérine et la glycine, pouvaient être facilement dégradés par *Propionibacterium*, mais de grandes variations dues à la souche et à l'espèce étudiée ont été observées.

***Propionibacterium* / protéinase / peptidase / acide aminé**

INTRODUCTION

The dairy propionibacteria are essential for eye formation and the development of the characteristic sweet flavour in Emmenthaler and in Swiss-type cheeses (Sherman, 1921). Propionic and acetic acid and other

volatile compounds have been implicated as providing part of the flavour complex in addition to amino acids, particularly proline and small peptides that are assumed to contribute to the sweet, nutty flavour (Langsrud and Reinbold, 1973; Biede and Hammond, 1979a, b). However, propionibacteria grow

in synergism with thermophilic lactic starter bacteria in Emmentaler cheese; thus, it is difficult to distinguish their individual contributions to proteolysis and the release of peptides and amino acids.

Very few reports on the proteolytic activity of propionibacteria appeared in the literature before the 1970s. Virtanen (1923) observed a slow growth and weak proteolysis by propionibacteria in milk at 37°C over a 2-month incubation period. However, the addition of peptone to milk increased the growth rate and the number of cells. Next, Berger *et al* (1938) found an intracellular tripeptidase in *Propionibacterium pentosaceum*. Klimovski *et al* (1965) concluded in their study on cheese ripening that dairy propionibacteria did not contribute significantly to proteolysis. However, Searles *et al* (1970) observed proteolytic activity of propionibacteria.

PROTEIN DEGRADATION

This was the background when we started work on protein degradation by propionibacteria in 1970. An important fact was that 1 amino acid, proline, was found in larger amounts in Swiss-type cheeses than in any other cheese, and that this amino acid could be important for the sweet, nutty flavour. Propionibacteria were shown to produce large amounts of proline from media containing casein hydrolysates, but there were large strain differences (Langsrud *et al*, 1977, 1978). Proline was mainly produced by peptidase action, and proline biosynthesis was of little significance. Although some proline was produced by proteolysis of casein, the rate was much slower than by peptidase action on casein hydrolysates. The release of proline from peptides coincided with autolysis of the propionibacteria (Østlie *et al*, 1995). A complex mixture of peptidase activities were observed using different assay substrates, and PAG elec-

trophoresis of ultrasonic extracts from several *Propionibacterium* strains followed by zymogram detection showed 7 to 8 peptidase bands (Floberghagen *et al*, 1978; Østlie *et al*, 1995). The strains showed distinct differences in specificity, but all strains showed high activity towards substrates containing L-proline at the amino end. Proteinase activity of cell-free extracts was also detected by a zymogram technique with alpha-lactalbumin and cytochrome c as substrates (Ward, 1976), and 6 to 8 bands were observed.

The following discussion of recent results will first address the proteinase or caseinolytic activity of propionibacteria and then the peptidase activities.

PROTEINASE ACTIVITY

Caseinolytic activity of the cell-free extract from propionibacteria was shown by El Soda *et al* (1992). Preliminary purification studies of the caseinolytic activity of the cytoplasm from *P freudenreichii* ATCC 9614 by ion exchange chromatography on Phast Q Sepharose separated 3 caseinolytic fractions (Pripp *et al*, 1995). Caseinolytic activity was also observed in the postcultivation medium. Localization studies of different proteolytic activities of *P freudenreichii* INF alpha and ATCC 9614 indicated caseinolytic activity with the cell wall and intracellularly (Sahlström *et al*, 1995b; Tobiassen *et al*, 1995b). Dupuis *et al* (1995) observed 2 types of proteolytic activities which could act on [¹⁴C] β-casein or [¹⁴C] α-casein. The first enzyme activity was tightly bound to the cell wall and acted preferentially on β-casein. The second enzyme, the free proteinase activity, was released at the stationary phase probably by autolysis or by excretion. Indications are that this enzyme may be located in the cell membrane. The cell wall associated proteinases from 2 strains of *P freudenreichii* were released

from the cells by washing with a Ca^{2+} -free buffer and assaying with tritiated casein as substrate (Sahlström *et al*, 1995b). The enzyme was purified and characterized. The proteinase(s) had a molecular mass of 41 kDa, pI 4.0, temperature optimum 51°C and pH optimum 7.0. The enzyme was inhibited by phenyl-methyl-sulfonyl fluoride (PMSF), which shows that the enzyme is a serine proteinase. EDTA and α -phenanthroline had no inhibitory effect (not a regular metalloenzyme), but the enzyme was activated by reducing agents.

PEPTIDASE ACTIVITY

Peptidase localization studies by Sahlström *et al* (1989) with dipeptides and tripeptides as substrates in a zymogram technique showed 1 peptidase band associated with the cell wall, 2 to 3 peptidase bands associated with the membrane and 6 to 7 intracellular bands for the 2 strains tested. Peptidase studies of extracts from different species and strains of propionibacteria showed that they contain aminopeptidases, proline iminopeptidase and proline imidopeptidase (Perez Chaia *et al*, 1990; El-Soda *et al*, 1992; Østlie *et al*, 1995). In addition, greater activities of leucine aminopeptidase and proline iminopeptidase were observed in strains of *P. freudenreichii* (Perez Chaia *et al*, 1990). Studies by Tobiassen *et al* (1995b) indicated that propionibacteria in addition to the previously mentioned enzymes also contain an X-prolyl-dipeptidyl-aminopeptidase, mainly intracellular, an intracellular endopeptidase with CBZ-Gly-Gly-Leu-pNA as substrate and an endopeptidase with activity towards methionine enkephalin which was about 20% associated with the cell wall. This and later work indicate the possible presence of more than 1 endopeptidase (Pripp *et al*, 1995).

These reports show a great variety of proteolytic enzymes in propionibacteria capable of hydrolyzing many different pep-

tides. This has been challenging for purification work to characterize individual enzymes and to clarify their role. The first publication was by Panon (1990), who isolated an intracellular proline iminopeptidase from *P. freudenreichii* 13673 by ion-exchange chromatography on Fractogel TSK DEAE 650 and gel filtration on Sephacryl S-100 HR. The enzyme had a molecular mass of 61 kDa. Optimum activity was observed at 40°C and pH 8.0. The proline iminopeptidase was a serine enzyme since it was inhibited by PMSF; divalent cations also inhibited this enzyme.

Sahlström *et al* (1989) reported the presence of a cell wall associated peptidase with an Rf value in PAGE of 57 and 0, respectively, in *P. freudenreichii* ATCC 9614 and INF alpha. These 2 peptidases were purified by anion exchange chromatography on Q-Sepharose and gel filtration in Superose 6B. Both enzymes appeared to be metalloenzymes since they were inhibited by EDTA and α -phenanthroline. The enzyme with Rf value 57 had a molecular mass of 134 kDa, pI 4.2, temperature optimum 42°C and pH optimum 8.6, and was also inhibited by iodoacetic acid and p-mercuribenzoate, which indicates that sulfhydryl groups are important for the activity. The enzyme with Rf value 0 had a molecular mass of 86 kDa, pI of 4.2, temperature optimum 60°C and pH optimum 10, and was inhibited by iodoacetic acid, but not by p-mercuribenzoate. Both enzymes were active towards N-benzoyl-Gly-Phe and N-benzoyl-Gly-Lys, indicating carboxypeptidase activity. Activity towards Leu-p-nitroanilide, Lys-p-NA and Ala-p-NA were absent or very low (Sahlström *et al*, 1995a).

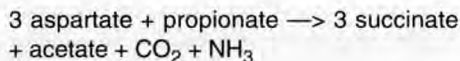
An endopeptidase activity with bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) as substrate was separated by Fast Q Sepharose (Pripp *et al*, 1995) and further purified by hydroxyapatite, a second anion exchange chromatography on mono Q followed by gel filtration on Superose 12. The

bradykinin-hydrolysing endopeptidase was a monomer of molecular mass 45 kDa, pI 5.2, optimum pH 7–8 and optimum temperature 50°C. The purified enzyme also hydrolyzed CBZ-Phe-Arg-7-amido-4-methylcoumarin (PAM) and methionin enkephalin, but was free from aminopeptidase activity. The enzyme showed no activity towards α s1 casein (Tobiassen *et al*, 1995a).

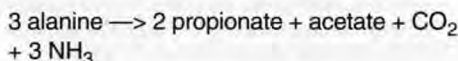
Even with the present relatively limited work on the proteolytic enzymes of dairy propionibacteria, they appear to be equipped with many of the enzymes necessary to produce amino acids and small peptides from (milk) proteins. The caseinolytic activity is so low that detection is possible only with the most sensitive methods. Thus, propionibacteria grow very slowly in milk; however, the addition of peptides in protein hydrolysates (eg Tryptone) stimulates for faster growth. The situation in Swiss-type cheeses is somewhat comparable because lactic acid bacteria, particularly some lactobacilli, are clearly more proteolytic than propionibacteria and should therefore contribute to amino-N availability during the early stages of proteolysis. Because the cell wall associated proteinase action of propionibacteria is not sufficient for effective growth, it may be conjectured that the enzyme has some other function, for instance, as an activator of proautolysin (Ghuysen and Shockman, 1973). In comparing it with the lactococci, most of the same proteolytic activities are found in both. However, detailed studies of individual enzymes also reveal interesting differences. The main endopeptidase from *P freudenreichii* ATCC 9614 was not inhibited by phosphoramidon and only slightly by β -casein f58-72 in contrast to the endopeptidase from *Lactococcus lactis* subsp *lactis* M 1363 (Stepaniak *et al*, 1995), which was strongly inhibited by both compounds. Another difference is that propionibacteria show much higher proline iminopeptidase activity than lactococci (Tobiassen *et al*, 1995b).

The cell wall associated peptidases from *P freudenreichii* ATCC 9614 and INF alpha both expressed carboxypeptidase activity (Sahlström *et al*, 1995a). Among the lactic acid bacteria, there is no report of carboxypeptidase activity in lactococci and only *Lactobacillus casei* has been shown to contain this activity (Pritchard and Coolbear, 1993).

Concerning the CO₂ production in Swiss-type cheeses, it exceeds the amount expected from lactate metabolism by propionibacteria. The difference may be explained by degradation of amino acids by propionibacteria or from growth of a secondary flora. Antila (1957) observed that many amino acids are catabolized by resting cells of propionibacteria. However, amino acid decarboxylase activity has not been reported for propionibacteria. Brendehaug and Langsrud (1985) studied the metabolism of amino acids in resting cell suspensions of *P freudenreichii* strains under aerobic and anaerobic conditions and observed a rapid degradation of aspartic acid, serine, glycine and alanine and a slower degradation of some of the other amino acids. Other species of propionibacteria showed slower or no degradation at all. Parallel with the degradation, increases were observed for ammonia, propionic and acetic acid and in CO₂. More detailed studies have been performed on the degradation of aspartic acid and alanine (Crow, 1986a, 1987; Rosner and Schink, 1990). The aspartase activity was already identified in propionibacteria by Virtanen and Tarnanen in 1932, and the enzyme was later isolated and characterized (Elfolk, 1953a, b). Observation showed that aspartic acid was the only amino acid that was metabolized during lactate fermentation in a complex medium (about 70%). In a defined medium, more than 90% was metabolized after lactate exhaustion. Aspartate was only metabolized in the presence of propionate, and then according to the equation suggested by Crow (1986a):



Alanine was only metabolized after exhaustion of lactate, which is also probably true for the other amino acids which are metabolized. Alanine dehydrogenase activity in propionibacteria was reported by Castberg and Morris (1978), and the enzyme was later studied by Crow (1987). Late degradation of alanine may be caused by the high content of pyruvate in propionibacteria from the lactate fermentation (Crow, 1986b) which inhibits the alanine dehydrogenase. After depletion of pyruvate, alanine was degraded according to the following equation:



To our present knowledge, dairy propionibacteria are endowed with proteolytic activities which apparently enable them to supply amino-N from proteins. Only a few of the enzymes have been purified and characterized, and the location of several enzymes needs to be determined. The properties of some of the enzymes differ from their counterparts in lactic acid bacteria.

The dairy propionibacteria are also capable of actively metabolizing some of the amino acids released. Details of these conversions should be the focus of future research. In order to better understand amino-N metabolism in propionibacteria, an important research area to investigate would be the transport system for amino acids and peptides. The protein/peptide hydrolysis and amino acid degradation provide amino acids (proline and other components) and CO_2 which contribute to the flavour and eye formation in Swiss-type cheeses.

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