PURIFICATION AND CHARACTERIZATION OF A SECRETED COLLAGENASE FROM THE PHYTOPATHOGENIC BACTERIUM *Clavibacter michiganense* subsp *michiganense*

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SUMMARY: *Clavibacter michiganense* is a pathogenic coryneform bacterium which is pathogen for a variety of plants of agricultural importance such as tomato, potato, and maize.

In this paper, the properties of a purified extracellular collagenase from *C. michiganense* subsp *michiganense* are presented. The purification steps include ammonium sulfate precipitation, DEAE cellulose chromatography and Sephadex G200 chromatography. The purified collagenase migrate with a Mr = 105 000 upon sodium dodecyl sulfate-polyacrylamide electrophoresis. The enzyme displays a broad pH activity profile in the neutral to basic range. It is inhibited by histidine, cysteine, EDTA and 1,10-phenathroline suggesting that it is a metalloprotease like the other known collagenases. The inhibition data show many properties similar to those of other known bacterial collagenases particularly the enzyme from *Corynebacterium rathayii*. This new collagenase is able to degrade collagen and gliadins extracted from wheat indicating that the enzyme could be necessary for the bacterium to multiply into seeds from which it is frequently isolated.

INTRODUCTION: The synthesis of collagenase by the phytopathogenic bacterium *Corynebacterium rathayii* was recently described by LABADIE (1990). The production by a phytopathogenic bacterium of a protease with a narrow specificity for collagen and its degradation or denaturation products is questionning because these substrates are only isolated from animals. As an hypothesis LABADIE (1990) proposed that this enzyme could degrade proteins from plants sharing sequence homologies with collagen. In order to strengthen this hypothesis it was decided to search for other bacteria that could also produce similar enzymes and to test this new enzyme against plant proteins that have similarities with collagen. *Clavibacter michiganense* is a bacterium which frequently degrades gelatin (DAVIES et al, 1984) the product of the thermal denaturation of collagen. Hence, it was decided to identify the specificity of the proteolytic enzyme that is produced by *C. michiganense* subsp *michiganense* strains (DAVIES et al, 1984). After the demonstration that *C. rathayii* and *C. michiganense* belong to different species, production and purification of the extracellular gelatinase were carried out as for the *C. rathayii* collagenase.

This new enzyme is degrading the Pz-peptide(Pz-Pro-Leu-Gly-Pro-DArg) generally used for studying bacterial collagenase and high molecular collagen as well. More over degradation of gliadins is also observed, indicating that the collagenase could be at least necessary to destroy some of the major proteins that are present inside the seeds of plants where *C. michiganense* is frequently isolated from.

MATERIAL AND METHODS

Bacteria :*Corynebacterium rathayii* (CRZV1) was taken from our laboratory's collection. *Clavibacter michiganense* subsp *michiganense* (10 strains) was a gift of Mr Luisetti, French National Collection of Phytopathogenic Bacteria (INRA d'Angers, France). Hybridizing the DNA of *C. rathayii* with that of *C. michiganense* 1715 was carried out according to STACKEBRANDT and KANDLER(1979). 11% homologies between these DNA showed that these bacteria belong to different species.

Culture media : With respect to the production of collagenase, an appropriate medium was prepared as previously described(LABADIE, 1990). Briefly, Ficin (crude extract from Nutritionals Biochemical Corporation, USA) was added to Gelatin (Difco) 10g/l, at the same enzyme substrate ratio(1/100, w/w) and allowed to stay at 37°C for 6 hours. After that time, yeast extract (Merck) 2.5g/l, was added and 50ml of this medium was introduced into conical flasks of 300ml.

After autoclaving, the flasks were individually inoculated with the different *C. michiganense* strains and agitated 18 hours at 30°C in a rotary shaker.

Collagenolytic activity: The cultures of the different bacteria were centrifuged 30 min at 10000 g and the supernatant precipitated with ammonium sulfate (60% saturation). After a 18 hours dialysis against 0.05 M Tris buffer pH 7.4 $CaCl_2$ 4mM, the collagenolytic activity of these crude extracts was estimated according to LABADIE and MONTEL (1982) with the peptide Phenyl azo benzyl oxy Pro-Leu-Gly-Pro-DArg (Pz-peptide) as substrate.

Purification of the collagenase: It was carried out as for the *Corynebacterium rathayii* enzyme (previously, *Empedobacter collagenolyticum*)(LABADIE and MONTEL, 1982).

Effect of metal salts and proteases inhibitors on the collagenolytic activity : This effect was carried out according to

HANADA et al (1973) with the synthetic peptide (PZ-Pro-Leu-Gly-Pro-D-Arg) as substrate.

Molecular weight determination of the collagenase and gel electrophoresis: The molecular weight of the purified enzyme was determined after SDS-polyacrylamide gel electrophoresis and after gel filtration.

RESULTS

Production of collagenases by *Clavibacter michiganense* strains : Most of the strains studied(8 from 10) produced enzymes capable of degrading the Pz-peptide (data not shown). It appeared however that synthesis of the collagenolytic enzymes varied from one strain to another. The strain which produced the greatest quantities of enzyme was chosen for that study.

Purification of *Clavibacter michiganense* collagenase : DEAE cellulose chromatography and Gel filtration on Sephadex G200 allow the complete purification of the collagenase.

Effect of pH and temperature on the activity : Figure 1 illustrates the effects of pH and temperature on the collagenolytic activity using the Pz-peptide as substrate. The enzyme showed a rather broad range of optimum activity between pH 7.0 and pH 9.0. Activity is rapidly affected at pH lower than pH 7.0. Maximum activity is observed at 30°C. Above 40°C the collagenolytic activity decreases rapidly.

Molecular weight determination of the *Clavibacter michiganense* collagenase: After an SDS PAGE the enzyme appeared homogenous, its molecular weight is of 105 KD (Fig 2). This molecular weight is similar to that observed by gel filtration chromatography (100 KD). As only one band is observed without SDS (data not shown) the enzyme is certainly monomeric.

Effects of protease inhibitors and metal salts on the collagenolytic activity: Table 1 shows the effect of various divalent metal salts on the reaction rate of the enzymes. Ca, Mn salts accelerates the hydrolytic reaction. Conversely, Fe, Hg, Cu, Zn, Pb salts inhibited the reaction. DFP, pCMB exerted no effect on enzyme activity, β−mercaptoethanol slightly inhibits the collagenase. The chelating agents 1,10-phenanthroline and EDTA exerted a strong inhibition. Among the other agents which could influence the activity of the collagenase, urea have not any effect. On the contrary cysteine and histidine are inhibitors.

DISCUSSION

The present paper describes for the first time the isolation and the characterization of a collagenase produced by the phytopathogenic bacterium *Clavibacter michiganense.* Many properties of this new collagenase are in good agreement with those of the collagenases of *C. rathayii.*, and those of other bacteria, *Streptomyces* sp A8,(CHAKRABORTY and CHANDRA, 1984), *Vibrio alginolyticus* (LECROISEY and KEIL, 1979), *Clostridium histolyticum* (ANGLETON and Van HART, 1988). Particularly, they are all inhibited by EDTA, heavy metals, activated by Ca^{2+}. Besides, general properties which are common to the enzyme studied in this work and the other collagenases it appeared that the *Clavibacter* enzyme has a molecular weight which is higher than most of the collagenases from other sources particularly the higher form from *C. rathayii* with Mr=72000 (LABADIE et al , 1991). The enzyme of *C. michiganense* seems close to one of the 6 collagenase from *Clostridium. histolyticum.*(ANGLETON and Van HART, 1988).

One of the most interesting feature of the collagenase from *C. michiganense* is its activity on ß and γ gliadins(data not shown) which are slowly destroyed. As *Clavibacter michiganense* is frequently isolated fror seeds of plants (tomatoes for the strains used in that study) where these proteins could represent up to 10% of the protein content (POPINEAU Y and POPINEAU F, 1985), it is likely that the collagenase from this bacterium is necessary to initiate its growth. For other bacterial species belonging to the *Clavibacter* genus, synthesis of collagenases could exist and could also be necessary for the multiplication into seeds of *Graminaceae* or other plants where these bacteria are isolated from. Work is in progress to answer to these questions and also to determine whether other substrates such as extensins, plant glycoproteins which share homologies with collagen (LAMPORT, 1980) and which contain hydroxyproline, are degraded by collagenases. Apart from this work, it will be interesting to know whether these enzymes are more gliadinases (or extensinases) than true collagenases. To do so it will be necessary to identify the peptide bonds which are preferentially attacked by the enzyme from *C. michiganense* in order to compare these enzymes to the best known collagenases from *Vibrio alginolyticus* (LECROISEY and KEIL, 1979) and *Clostridium histolyticum* (ANGLETON and Van HART, 1988)

Inhibitors or metal salts	Concentrations (mM)	Remaining activity (Substrate Pz-peptide) *Clavibacter* collagenase
None		100
o-Phenanthroline	1	5
EDTA	1	0
Cystéine	1	12
Urea	6M	92
Histidine	1M	10
2-Mercaptoethanol	1	60
PCMB	1	90
DFP	1	105
CaCL2	10	170
MnCL2	10	124
CuSO4	10	0
FeSO4	10	0
CoCL2	5	50
ZnCl 2	0,1	0

Table 1: Effects of proteases inhibitors and metal salts on the collagenolytic activity of the *Clavibacter michiganense* collagenase.
Inhibitors: To 0.2 ml of enzyme solution (ca 10µg of protein)was added 0.2ml of inhibitor dissolved in 0.05 mM Tris HCL pH 8.0 buffer to give the final concentration of inhibitors shown below. After preincubation for 30 min at 30°C 0.6ml of Pz-peptide was added to be followed by the standard activity assay. The remaining activity are shown as percentage of the initial activity.
Metal salts: The incubation mixture contained 0.1 ml of enzyme solution and 0.4 ml of each metal salt solution dissolved in 0.05 M Tri HCL bufferpH 8.0 to give the fixed concentration of each metal. Collagenolytic activities were estimated as for the inhibitors.

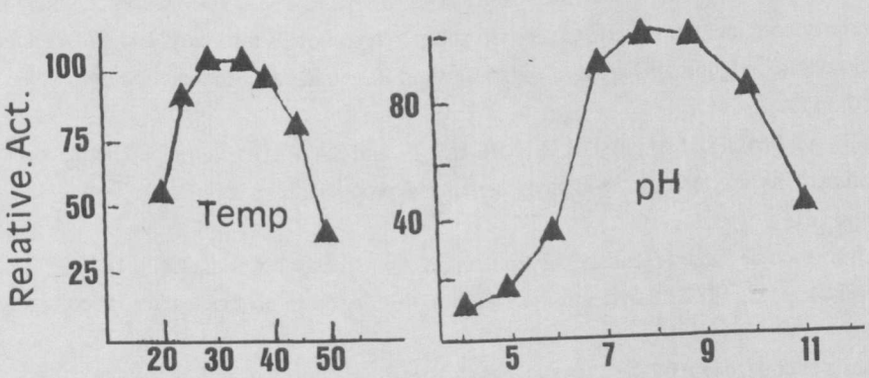

Fig 1 : Effect of temperature and pH on the activity of the *Clavibacter michiganense* collagenase. The reactions were performed with the Pz-peptide as substrate. For the temperature effects, enzyme solutions were incubate 20 min at various temperatures. All the data are expressed as percentage of activity at 30°C. For the pH effects, reactions were carried out in 10 mM buffer of various pH values. Buffers used : Acetate buffer (4-6), Veronal buffer (7-9), Borate buffer (10-11). The activities are expressed as percentages for the maximum activity of the assayed pHs.

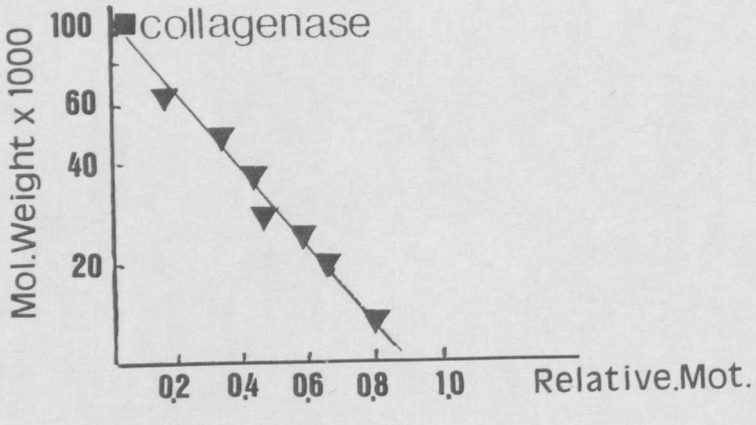

Fig 2 : Molecular weight determination of the *Clavibacter* collagenase. Protein markers were : Bovine albumine: 66000, Egg albumine : 45000, Glyceraldehyde-3-phosphate Dehydrogenase : 36000, Carbonic anhydrase : 29000, Trypsinogen : 24000. Trypsin inhibitor, soybean : 20100. α-Lactalbumin : 14200.

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