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NOVEL FUNGAL COLLAGENASE FROM *ASPERGILLUS ORYZAE*

Daniela BĂLAN, Florentina ISRAEL-ROMING, Petruta Călina CORNEA, Evelina GHERGHINA, Gabriela LUȚĂ, Florentina MATEI, Mihai CURTAŞU

University of Agronomical Sciences and Veterinary Medicine Bucharest, Faculty of Biotechnologies, 59 Marasti Blvd., 011464, Bucharest, Romania, www.usamv.ro

Corresponding author Florentina ISRAEL-ROMING: florentinarom@yahoo.com

Abstract
The industrial processing of leather has a high environmental impact due to the heavy use of polluting chemicals in the tanning process. Microorganisms can be used in order to degrade natural fibers like fur, leather and cotton because they synthesized enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, have a number of industrial applications in fur and leather industry and, moreover, are nontoxic and eco-friendly. This paper reports the purification and characterization of a fungal collagenase produced by an Aspergillus strain. Aspergillus oryzae Mi 156 and Aspergillus ochraceus Mi 153 were tested for their capacity to degrade native collagen. Enzymatic activity and protein content were determined using spectrophotometrical methods. The best results regarding collagenolytic activity were obtained for fungal strain Mi 156. The enzyme was separated by ammonium sulphate precipitation and analyzed for its optimum pH and temperature. The effect of some activators and inhibitors on the enzymatic activity was also tested. The results indicated 8 − 8.3 as optimum pH and 35 − 40 ^{o}C as optimum temperature. When using Zn^{2+} as activator the collagenase activity was about 29 % higher, while for Co^{2+} as inhibitor, the activity dropped down about 14 %.

Key words*: activators, Aspergillus oryzae, characterization, collagenase, inhibitors.*

INTRODUCTION

The industrial processing of leather has a high environmental impact due to the heavy use of polluting chemicals in the tanning process. Microorganisms can be used in order to degrade natural fibers like fur, leather and cotton because they synthesized enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, registered an increasing use for industrial applications in fur and leather industry because they are nontoxic and eco-friendly.

Collagenases are endopeptidases that digest native collagen in the triple helix region. Collagen is the major fibrous component of animal extracellular connective tissue. Unlike animal collagenases that split collagen in its native triple-helical conformation, bacterial collagenase is unique because it can degrade both water-insoluble native collagens and water-soluble denatured ones. It can attack almost all collagen types, and is able to make multiple cleavages within triple helical regions (Worthington C., 1988).

Different authors reported as collagenase-producing strains *Bacillus* and *Aspergillus*: *B. subtilis* (Tran and Nagano, 2002; Okamoto, 2001; Rui et al., 2009), *B. licheniformis* (Baehaki et. al, 2012), *A. fumigatus* (Reichard U. et al, 1990).

This paper reports the separation and characterization of a fungal collagenase produced by some *Aspergilus* strain. The separation of the enzyme was done by precipitation with ammonium sulphate salt in a variety of saturated degree. The characterization of purified enzyme includes: determination of optimum pH and temperature and study of the effect of some activators and inhibitors on the enzymatic activity. Enzymatic activity and protein content were determined using spectrophotometrical methods.

MATERIALS AND METHODS

Microorganism and growth conditions. The fungal strains *Aspergillus oryzae* Mi 156 and *Aspergillus ochraceus* Mi 153 were obtained from the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest. The inoculation was done in a basal salt

medium supplemented with 0.1% glucose and 1% sheep leather meal. Proteolitic enzymes were produced in 500 ml flasks, kept at 27°C, agitated at 150 rpm, for 15 days. The culture was centrifuged at 6000 rpm, at 4°C, for 20 minutes.

Enzymatic assay of collagenase, based on some classical refferences (Moore S., Stein W.H., 1948; Mandl I. *et al*., 1953), was made as following: after collagenase catalytically promote hydrolysis of collagen, the degree of proteolysis is measured by color development with ninhydrin (absorbance was measured at 570 nm). The enzymatic activity of collagenase was measured in U/ml (*unit definition*: one unit liberates peptides from collagen equivalent in ninhydrin colour to 1 μmol of leucine in 2 hours at pH 7.4 and at 37 °C).

Separation of collagenolytic enzyme

The enzyme was separated by ammonium sulphate precipitation using 90% $(NH_4)_2SO_4$ saturated degree. The obtained precipitate was dissolved in 50 ml 0.05 M phosphate buffer and dialyzed against distillated water for 18 hours, at 8°C, with agitation.

Effects of pH and temperature

The pH and temperature effect on the enzymatic activity were studied in order to characterize the collagenase biosynthesized by selected strain. For this purpose measurements of enzymatic reaction rate were made at pH between 6.8 – 7.9 using TES buffer and between 8.0 – 8.6 with Tris-HCl buffer. The tested temperature range was 25°C – 45°C.

Effects of various activators and inhibitors

Effects of activators on the enzyme were investigated by adding 0,36 M $ZnCl_2$ solution in the mixture reaction in order to test the ability of Zn^{2+} ion to activate the activity of collagenase biosynthesized by selected strain. 10mM EDTA and $CoCl_2$ solutions were used as inhibitors of collagenolytic activity.

The protein concentration was determined by Lowry method, which is based on the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Lowry O.H. et al., 1951).

RESULTS AND DISCUSSIONS

The fungal strains *Aspergillus oryzae* Mi 156 and *Aspergillus ochraceus* Mi 153 were tested regarding the collagenolytic activity (table 1).

Table 1. Collagenolytic activity of enzymatic preparate sinthesized by fungal strains

Fungal strain	Soluble protein (mg/ml)	Collagenase	
		Enzymatic activity (U/ml)	Specific enzymatic activity (U/mg prot)
Mi 153	0.021	14.56	693.33
Mi 156	0.020	15.86	780.0

The best results were obtained for fungal strain Mi 156, which was selected in order to obtain and characterize the enzymatic preparate synthesized by cultivation in culture medium containing sheep leather as carbon source.

Total protein extract (TPE) obtained by cultivation of *Aspergillus oryzae Mi156* strain was purified by precipitation with ammonium sulphate. The obtained precipitate, which is a protein fraction that contains most of collagenase, was redissolved in 0.05 M phosphate buffer, than the solution was dialysed for removing the ammonium sulphate. Both the centrifuged culture medium (TPE) and the protein fraction (PF) obtained by precipitation with ammonium sulphate were analyzed regarding the protein content and the collagenolytic activity.

The results (table 2) indicated a moderate efficiency of enzyme separation process by precipitation with ammonium sulphate, as the obtained purification factor of the enzyme was 1.48.

Table 2. Results of precipitation with ammonium sulphate

Mi 156	AES (μmol/mg proteins)	Purification factor
TPE	722	1
PF	1068	1.48

Effects of pH and temperature

Enzymatic activity depends on the thermodynamic activity of the hydrogen ions, that is solution pH. Most enzymes accomplish a maximum activity at certain pH value, namely optimum pH; the enzymatic activity decreases below and over this pH value.

Regarding the effect of temperature, generally the enzymatic activity increases as the temperature increases if the enzyme is stable and active during the respective temperature range.

The influence of pH on the activity of the collagenase (fig. 1a) was determined measuring the enzymatic activity by the previously described method at increasing values of pH (between 6.8 − 8.6). To test the influence of temperature on the activity of the collagenolytic enzyme, the enzyme was incubated in the reaction mixture at various temperatures (25-45°C) (fig. 1b). The remaining activity was measured as described previously.

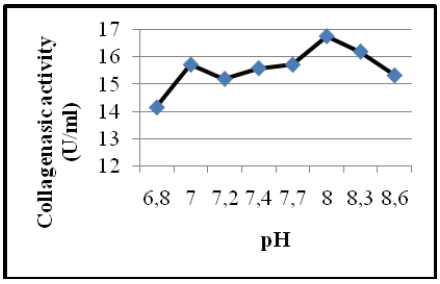

Figure 1a. Effects of pH on collagenolytic activity

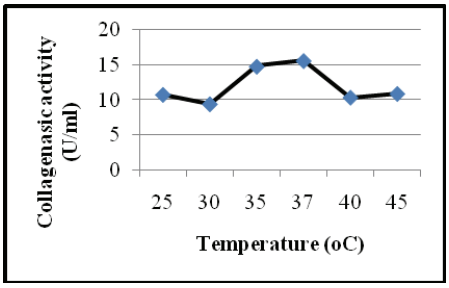

Figure 1b. Effects of temperature on collagenolytic activity

Experimental results (fig. 1a, 1b) indicated that the collagenase synthesized by the selected strain achieved maximum activity at pH 8.0 and 37°C.

Effects of various activators and inhibitors

An adequate activity of collagenases involves binding of the Ca^{2+} and Zn^{2+} ions, but only zinc ions are bounded in the catalytic situs, while calcium ions are necessary only for maintaining of the enzyme conformation (Lowry C.L. et al, 1992; Housley T.J. et al, 1993; Zhang Y. et al, 1997). Therefore, a $ZnCl_2$ solution was used in order to characterize the collagenolytic activity; calcium chloride is component of the buffer solution used in determinations.

Regarding the potential inhibitors, recent studies indicated that beside EDTA, well-known inhibitor for collagenases, also cobalt ions may inhibits this enzyme because they can be bounded by hystidin located in the catalytic situs instead zinc ions (Macartney H.W., Tschesche H., 1981).

For these reasons the sensitivity of the purified collagenase towards solution 0.36 M $ZnCl_2$ as activator and towards some inhibitors such as 10 mM EDTA and 1mM $CoCl_2$ was investigated. The activity was compared with that of the reaction that was free of the corresponding metal ions (control).

The obtained results (fig. 2) indicated that collagenolitic activity was significant increased in the presence of the tested Zn^{2+} concentration, the registered values beeing 30% higher compared to control activity.

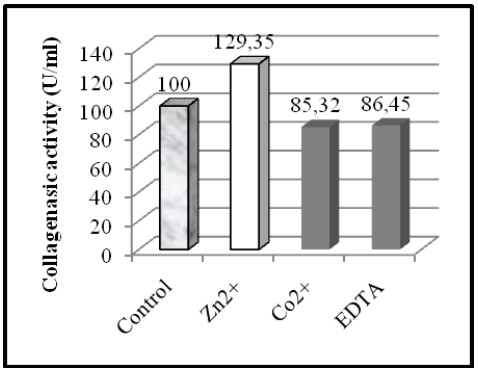

Figure 2. Effect of activators and inhibitors on collagenolytic activity

The values registered during the inhibition study revealed a decreasing of the collagenolytic activity both in the presence of EDTA and of Co^{2+} ions (fig. 2). When using

Co^{2+} as inhibitor, the activity of collagenase dropped down about 14 %.

CONCLUSIONS

The best results regarding the collagenolytic activity were obtained for fungal strain Mi 156 (15.86 enzymatic units/ml).
The purification factor of the enzyme was 1.48 after the precipitation with ammonium sulphate, which indicated a moderate efficiency of enzyme purification process.
Experimental results indicated that the collagenase synthesized by the selected strain achieves maximum activity at pH 8.0 and 37°C.
The collagenolitic activity was significant increased in the presence of Zn^{2+}, the registered values beeing with 30% higher compare to control activity.
As a result of using Co^{2+} as inhibitor, the activity of collagenase dropped down about 14 %.

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