19. STUDY ON CATALYTIC ACTIVITY OF MUCOR IN THE PROCESS OF CHITOSAN BIODEGRADATION

Katarzyna Struszczyk, Mirosława Szczęsna-Antczak, Tadeusz Antczak, Małgorzata Rzyska, Stanisław Bielecki

Institute of Technical Biochemistry, Technical University of Łódź
Stefanowskiego 4/10, 90-924 Łódź, Poland
e-mail: tad45an@anack.p.lodz.pl

Henryk Struszczyk

Institute of Chemical Fibres
M. Skłodowskiej-Curie 19/27, 90-570 Łódź, Poland

1. Introduction

Owing to the captivating biological activity, chitosan and chitosan oligomers (CHOS) have attracted much attention. Their properties are exploited in the agriculture, medicine, pharmaceutical and food industries. CHOS obtained by enzymatic degradation of chitosan display improved antimicrobial and antiviral activities relative to the high-molecular-weight biopolymer [1, 2].

Several enzymes catalyze hydrolytic cleavage of chitosan to the biologically active oligomers. Chitosanases synthesized by bacteria and fungi are most widely applied for this purpose. *Mucorales* fungi, which contain significant amounts of chitin and chitosan in cell walls, can be used as a source of these biopolymers and enzymes involved in their degradation. *Mucor rouxii* ranks among these filamentous fungi, which are known producers of chitin, chitosan and chitin-digesting enzymes such as chitinases, chitin deacetylases and chitosanases [3, 4].

This work provides evidence that the *in situ* immobilized preparations of Mucor circinelloides chitosanase can be used for biodegradation of chitosan.

2. Materials and methods

2.1. Microorganism

Mucor circinelloides strain was obtained from the microbial culture collection of the Institute of Technical Biochemistry (IBT) of the Technical University of Lodz. Strain of *Mucor* was cultivated for 72 h at 30 °C and agitation rate of 180 rpm in liquid medium

containing: 3.7% (w/v) corn steep liquor, 2.7% (v/v) olive oil, 0.1% (w/v) glucose and 0.1 – 0.5% (w/v) chitosan. The initial pH of medium was 4.7. Mycelia of *M. circinelloides* was harvested by filtration, washed three times with acetone, air-dried at room temperature and used as the *in situ* immobilized enzyme preparations.

2.2. Chitosan

Physicochemical properties of chitosan preparations applied for the studies are shown in Table 1.

Table 1. *Physicochemical properties of commercial chitosan preparations.*

Sample	Manufacturer	DD,	Mv,	WRV,	Moisture content,	Heavy metal content,
		%	kDa	%	%	%
India I	Chemopol, Complex Pvt. Ltd, Tada, India	66.2	409	108	1.2	-
Vanson 01-CISF-1847	Vanson, Redmont, USA	78.9	121	101	-	-
Vanson 01-CISC-1653	Vanson, Redmont, USA	79.8	405	106	0.6	0.02
India P	Chemopol, Complex Pvt. Ltd, Tada, India	83.3	433	100	-	-

Abbreviations: *DD – degree of deacetylation, Mv – average molecular weight, WRV – water retention value.*

2.3. Enzymatic degradation of chitosan

The efficiency of enzymatic digestion of chitosan by chitosanase produced by the examined *Mucor* strain was determined on the basis of the rise in reducing sugars contents during this process and a decrease in the average molecular weight of biopolymer digests.

Reaction mixtures contained 0.02 g of chitosan (2% chitosan in 2% acetic acid) and 0.02 g of enzymatic preparation in 1 cm^3 of 0.1 M Tris-HCl buffer (pH of reaction mixtures ranged from 4.1 to 6.0). The mixtures were incubated for 6 to 72 h at temperatures ranging from 25 °C to 55 °C. Controls to the samples were incubated under the same conditions. One of them has contained 0.02 g enzymatic preparation in 2 cm^3 of 0.1 M Tris-HCl buffer, and the second one has contained 0.02 g of chitosan in 2% acetic acid (substrate and enzyme were incubated separately). The enzymatic reaction was terminated by heating the supernatant for 5 min in a boiling water bath followed by centrifugation at 12 000 rpm for 5 min.

2.4. Analytical methods

2.4.1. Determination of saccharification degree of chitosan

The saccharification degree of chitosan [%] was calculated on the basis of the increase in reducing sugars contents during enzymatic degradation of polymer (under the conditions described in chapter 2.3.). The concentration of reducing sugars was estimated by Somogyi-Nelson method, using glucosamine as a standard [5].

2.4.2. Determination of hydrolytic lipase activity

The hydrolytic activity of lipase from *Mucor circinelloides* was assayed using olive oil as substrate [6]. The reaction substrate was 20% oil emulsion in 2% solution of polyvinyl alcohol in phosphate buffer at pH 7.5 in 1:1 ratio. The hydrolysis was carried out for 30 min, using a shaker (120 rpm) at the temperature 37 °C. In order to stop the reaction, ethyl alcohol was added. The amount of acid liberated during olive oil hydrolysis was determined by titration with 0.05 M NaOH up to pH 10.0.

The hydrolytic activity of lipase was expressed as an amount of enzyme (lipase – containing dried mycelium) necessary to release 1 μmol of fatty acids per second under the conditions described above [μkat] .

2.4.3. Determination of chitosan average molecular weight

The average molecular weight of chitosan was determined according to the viscometric method using an aq. solution of 0.2 M acetic acid, 0.1 M sodium chloride and 4 M urea, computed from the Mark-Houwink equation, using the constants $K = 8.93 \times 10^{-4}$ and $a = 0.71$. [7]. The viscosity measurement were taken at a temperature of 25 °C using an Ubbelohde's viscometer (instrument constant $K \approx 0.01$ mm$^2 \cdot$s^{-2}) [8].

3. Results and discussion

M. circinelloides is known producer of intracellular lipases [9, 10]. Therefore also lipolytic activity was assayed during the studies on effects of different chitosan concentrations in culture medium on Mucor strain growth and total activity of chitosan-degrading enzymes. Owing to reports on chitosan degradation by preparations of lipases [11, 12], the correlation between these two different, intracellular enzymatic activities in examined samples of mycelia of Mucor strain was also evaluated.

3.1. Effects of chitosan contents in culture medium on growth of M. circinelloides and activity of lipases and chitosanases

The first step of studies included estimation of:

- the chitosan-degrading activity of examined strain of *M. circinelloides*,
- effects of chitosan concentrations in culture medium on growth and activity of lipases and chitosanases synthesized by the examined fungal strain.

The influence of chitosan contents in culture medium of *M. circinelloides* on activity of lipases and chitosanases is shown in Figure 1.

Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio - 1:1 (w/w), pH 4.4, 37 °C, 24 h. Chitosan manufactured by Vanson 01-CISF-1847. Conditions of olive oil hydrolysis are described in chapter 2.4.2.

The composition of liquid medium used for cultures of *M. circinelloides* was optimized for lipase biosynthesis [10]. In presented studies, this medium was additionally supplemented with chitosan. Our assays showed that enrichment of culture medium with chi-

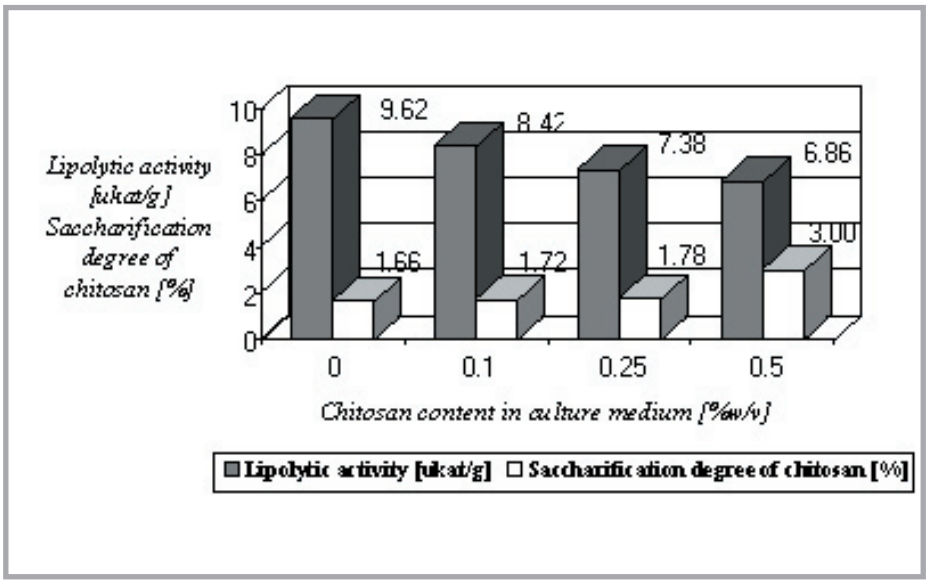

Figure 1. *The influence of chitosan contents in culture medium of M. circinelloides on activity of mycelium-bound chitosanolytic and lipolytic enzymes.*

tosan did not inhibit the growth of the strain of filamentous fungi. We found that the rise in chitosan concentration resulted in the increase in chitosanolytic activity and reduced the activity of lipases in mycelia (Figure 1).

3.2. Determination of optimal conditions for chitosan biodegradation

The highest saccharification degree of chitosan was observed at 37 °C and pH 5.5 during 12 h of polymer degradation. The influence of temperature and pH on activity of chitosanolytic enzymes from *M. circinelloides* is shown in Figure 2 and 3, respectively. The dependence of the rate of chitosan saccharification by *M. circinelloides* chitosanases on time is shown in Figure 4.

The optimum conditions for enzymatic degradation of chitosan by enzymes produced by examined strain of *M. circinelloides* were found to be similar to that determined for chitosanases synthesized by yeast strains, like Fusarium solani f. sp. phaseoil (40 °C, pH 5.6 [13]) and *Mucor rouxii* (40 °C, pH 6.1 [4]).

3.3. Substrate specificity

Chitosan-degrading enzymes produced by species of Mucor showed the high specificity for biopolymer with the high degree of deacetylation (DD). Most chitosanases are endo-acting enzymes and their activity stringently depends on DD of the substrate [14].

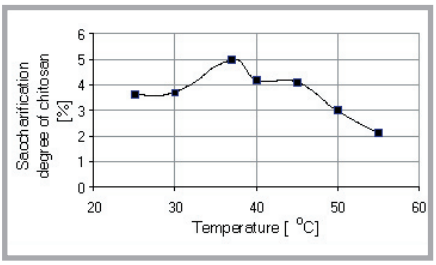

Figure 2. *The degree of chitosan saccharification by M. circinelloides chitosanases as a function of temperature. Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio - 1:1 (w/w), pH 4.4, 24 h. Chitosan manufactured by Vanson 01-CISF-1847.*

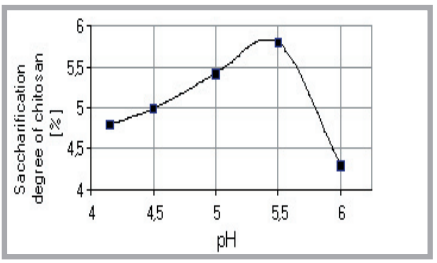

Figure 3. *The effect of pH on degree of chitosan saccharification by chitosanases of M. circinelloides. Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio - 1:1 (w/w), 37 °C, 24 h. Chitosan manufactured by Vanson 01-CISF-1847.*

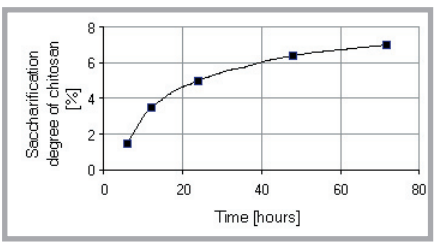

Figure 4. *Changes in degree of chitosan saccharification catalyzed by M. circinelloides chitosanases vs. time. Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio - 1:1 (w/w), 37 °C, pH 4.4. Chitosan manufactured by Vanson 01-CISF-1847.*

The effect of DD and Mv of chitosan on activity of chitosanolytic enzymes is shown in Table 2.

Table 2. *The influence of DD and Mv of chitosan on activity of M. circinelloides chitosanases. Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio - 1: 1 (w/w), pH 4.4, 37 °C, 24 h.*

Sample	DD, %	Mv, kDa	Saccharification degree of chitosan, %
India I	66.2	409.0	4.4
Vanson 01-CISF-1847	78.9	121.0	5.0
Vanson 01-CISC- 1653	79.8	406.0	5.3
India P	83.3	433.0	5.5

DD – degree of deacetylation, Mv – average molecular weight.

3.4. Reduction of average molecular weight of chitosan digests

The decrease in chitosan solution viscosity resulted from the random cleavage of biopolymer chains. The decrease in average molecular weight of products of chitosan hydrolysis catalyzed by chitosanases of the examined *Mucor* strain vs. time is shown in Figure 5.

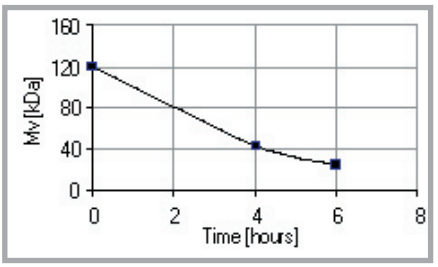

Figure 5. *The decrease in average molecular weight of products of chitosan digestion by M. circinelloides chitosanases vs. time. Conditions of chitosan hydrolysis: enzymatic preparation (air-dried mycelium):chitosan ratio – 1:1 (w/w), 37 °C, pH 4.4. Chitosan manufactured by Vanson 01-CISF-1847.*

The chitosan-digesting enzymes produced by *M. circinelloides* strain displayed the activity of endo-hydrolases because they rapidly reduced an average molecular weight of chitosan digestion products (Figure 5) and released only small amounts of reducing sugars from the polymer (10 mg/g of chitosan).

4. Conclusion

Presented results of preliminary researches indicate that the examined strain of *M. circinelloides* is effective producer of chitosan-degrading enzymes. Supplementation of culture medium of this strain with chitosan did not inhibit its growth and significantly increased activity of chitosanases and reduced the activity of lipases.

Optimum conditions for chitosan degradation by the *Mucor* enzymes, described in this paper, were similar to that reported for other enzymes isolated from various yeast strains. Enzymes produced by species of *Mucor* showed high specificity for chitosan, particularly for the biopolymer having the relatively high DD. The significant drop in average molecular weight of chitosan hydrolysis products and the low content of reducing sugars in the reaction mixtures could provided evidence that the endo-hydrolases dominated in preparations of chitosanases of the examined *Mucor* strain.

5. References

1. **Hirano S., Nagano N.:** Agric. Biol. Chem., 53, 1989, 3065 - 3066.
2. **Shahidi F., Arachchi J.:** Trends in Food Science & Technology, 10, 1999, 37 - 51.
3. **Synowiecki J.:** Food Chemistry, 60, 1997, 605 - 610.
4. **Kołodziejska I., Malesa-Ciećwierz M., Górna E., Wojtasz-Pająk A.:** Chitin Enzymology, vol 2, Muzzarelli R. A. A. (ed.), Atec Edizioni, Italy, 1996, 415 - 426.
5. **Wood T. M, Bhat K. M.:** Methods in Enzymology, Wood T. M., Kellogg S. T. (ed), 160, 1988, 87 - 111.
6. **Chmiel A., Zarzycki R., Modrzejewska Z., Antczak T., Szczęsna-Antczak M.**: Progress on Chemistry and Application of Chitin and its Derivatives, Struszczyk H. (ed.), 2002, 51 - 61.
7. **Roberts G. A. F.**: Chitin Chemistry, 1992, 106 - 110.
8. Standard Procedure GLP SPR/BB/5 (IWCh).
9. **Szczęsna-Antczak M., Antczak T., Rzyska M., Bielecki S.:** Journal of Molecular Catalysis B: Enzymatic, 19-20, 2002, 261 - 268.
10. **Antczak T., Graczyk J., Szczęsna-Antczak M., Bielecki S.:** Journal of Molecular Catalysis B: Enzymatic, 19-20, 2002, 287 - 294.
11. **Pantaleone D., Yalpani M.:** Carbohydr. Res., 237, 1992, 325 - 332.
12. **Muzzarelli R. A. A., Xia W., Tomasetti M.:** Enzyme Microb. Technol., 17, 1995, 541 - 545.
13. **Shimosaka M., Kumehara M., Zhang X., Nogawa M., Okazaki M.:** Ferment. Bioeng., 82, 1996, 426 - 431,
14. **Somashekar D., Joseph R.:** *Bioresource Technology, 55, 1996, 35 - 45.*