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CHITINOLYTIC ENZYMES OF THE GASTRIC MUCOSA OF *PERODICTICUS POTTO* (PRIMATE PROSIMIAN): PURIFICATION AND ENZYME SPECIFICITY

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Abstract—1. Enzymatic extracts of *Perodicticus potto* gastric mucosa showed chitinolytic activity together with lysozymic activity.

2. After purification by adsorption on colloidal chitin and gel chromatography, an enzymatic preparation with high chitinolytic activity was devoid of any lysozymic activity.

3. The gastric chitinolytic enzymes of *Perodicticus potto* are thus 'true' chitinases specific of the β-1,4-*N*-acetylglucosamine linkages in chitin molecule.

INTRODUCTION

Recent works have shown the existence of chitinolytic enzymes in the gastric mucosa and in the pancreas of certain vertebrates (Jeuniaux, 1963; Dandrifosse *et al.*, 1965). A chitinase (E.N. :3.2.1.14)* is defined by its capacity to hydrolyse chitin; the latter is a high linear polymer made up of *N*-acetylglucosamine units (N-AG) bound by β-1-4 linkages.

However, various authors have also demonstrated the chitinolytic activity of numerous lysozymes† (Berger & Weiser, 1957; Salton & Ghuysen, 1959; Wenzel *et al.*, 1961; Rupley & Gates, 1967; Baneryee *et al.*, 1973; Charlemagne & Jolles, 1972). According to these findings one would question the existence of a chitinase highly specific of chitin hydrolysis. Some of our previous research was oriented in such a manner as to define the enzymatic specificity of chitinases and lysozymes respectively. These results showed that gastric mucosal extracts of certain vertebrates presented simultaneously chitinolytic and lysozymic activities (Cornelius *et al.*, in press). Nevertheless, the relatively low lysozymic activity did not permit any final conclusions. The problem of enzymatic specificity would be best clarified by an attempt to extract and purify the enzymes responsible for the chitinolytic activity. Although the purification of different chitinolytic enzymes from Actinomycetes has been performed for two decades (Jeuniaux, 1958; Reynolds, 1954; Skujins *et al.*, 1970; Tiunova *et al.*, 1973), it is only very recently that such purifications have been realized in order to establish a clear distinction between chitinase and lysozyme. Such a study has only been performed with the chitinolytic enzymes of the haemolymph and cells cultures of an insect, *Periplaneta americana* (Bernier *et al.*, 1974).

The present paper deals with the purification of the gastric mucosa chitinolytic enzymes of a primate prosimian mammal, *Perodicticus potto edwarsi* and with the identification of chitinase and lysozyme properties of the purified enzymatic preparations.

* Poly-β-1,4-(2-acetamido-2 deoxy)-D-glucoside glycanohydrolase.

† E.N.3.2.1.17 *N*-acetylmuramide-glycanohydrolase.

METHODS

1. *Preparation of the enzymatic extracts*

The gastric mucosa carefully isolated was washed with a physiological solution. A portion of this mucosa, dried on filter paper, was weighed and then ground with a pestle and mortar containing washed sea sand. The mixture thus obtained was suspended in distilled water and the suspension (100 mg fresh tissue/ml) was allowed to settle overnight at 4°C, before centrifugation. The supernatant which contained the chitinolytic enzymes was kept at −20°C until purification time.

2. *Estimation of the chitinase and lysozyme activities*

The chitinolytic activity was measured by the method of Jeuniaux (1963), modified in order to estimate the activity of enzymatic solutions devoid of chitobiase activity (Jeuniaux, 1966). The chitinase activity is expressed in μg of *N*-acetylglucosamine liberated $\times$ hr^{-1} $\times$ g^{-1} of fresh tissue. The specific chitinolytic activity is given in chitinase units/mg proteins (C.U./mg prot.).

It is considered that 20 C.U. correspond to an enzymatic activity liberating the equivalent of 220 μg *N*-acetylglucosamine/hr from 4 mg of native chitin at pH 5.2 and 37°C.

The protein concentration was estimated by the Folin-Ciocalteu biuret-phenol method modified by Lowry *et al.*

(1951). Crystallized beef serum albumine was used to establish the 'standard' curves.

To determine the lysozymic activity, the quantity of disaccharides formed during the enzymatic hydrolysis of *Micrococcus lysodeikticus* bacterial walls is followed (Cornelius *et al.*, in press).

The specific activity is expressed in nM of disaccharide liberated $\times$ hr^{-1} $\times$ mg^{-1} protein.

3. *Preparation of colloidal chitin*

Colloidal chitin was prepared as described by Jeuniaux (1966). It was obtained by solubilization of native chitin in cold 18 N H_2SO_4 at 0°C, followed by reprecipitation by 10 times dilution with distilled water.

4. *Steps of the purification process*

The purification process follows in general the principles of the method introduced by Jeuniaux (1959) and adapted by Dandrifosse (1975) to the gastric and pancreatic extracts of the frog, *Rana temporaria*. It is based on a specific property of the chitinolytic enzymes: their extremely high affinity for chitin, which other proteins do not have. The adsorption of these enzymes to colloidal chitin is the first step of the purification process. By enzymatic hydrolysis of the adsorbant, the adsorbed enzymes can be easily recovered. The following step consists of column chromatography on Biogel.

(a) *Adsorption on colloidal chitin.* Acidification of the enzymatic solution is obtained by adding slowly 0.02 N H_2SO_4 at 0°C until reaching pH 5. The precipitate obtained during the acidification is eliminated by centrifugation (5000 *g*, 10 min). Colloidal chitin is added to the supernatant to realise the adsorption of the chitinolytic enzymes. A maximum adsorption of the enzymes is obtained by 3 consecutive adsorptions using every time a minimal quantity of chitin (1 mg chitin/100 U.C.) in order to obtain a rapid subsequent elution. After slow magnetic stirring at room temperature for 90 min, the 3 suspensions were centrifuged (5000 *g*, 10 min). The 3 sediments of chitin were suspended separately in a buffer of pH 5 (citric acid 0.02 M Na_2 H PO_4 0.04 M). The resulting suspensions were stirred for 10 min, then centrifuged (5000 *g*, 10 min).

(b) *Elution of the enzymes by spontaneous digestion of the chitin.* The washed residues resuspended in the buffer were incubated at 37°C in presence of thymol. Every 3 hr the suspensions were centrifuged. The sediments were resuspended in buffer and incubated at 37°C until complete solubilisation of chitin is obtained. The supernatants were kept at −20°C.

(c) *Concentration of the enzymes by dialysis against Aquacide* II. All the supernatants with high chitinolytic activity were pooled and dialysed against Aquacide II (Calbiochem) at 4°C. The solutions obtained had approximately one volume of 1 ml.

(d) *Column chromatography on Biogel P* 60. The concentrated solutions were submitted to chromatography on a Biogel P 60 column (100 cm^3, Bio-Rad Lab.) equilibrated and eluted with a $(NH_4)_2$ SO_4 0.02 M solution. The eluted mixtures presenting a high chitinolytic activity were pooled and concentrated again by dialysis against Aquacide II at 4°C.

RESULTS AND DISCUSSION

(a) *Purification yield*

The purification of the chitinolytic enzymes of the gastric mucosa of *Perodicticus potto* is summarised in Table 1. Following acidification of the crude extract (C.E.), the chitinolytic enzymes of the acidified extract (A.E.) were adsorbed on colloidal chitin. The supernatants collected during the first 9 hr of the enzyme elution are pooled (fraction F1). The fraction F2 regroups the supernatants of the end of the elution

Table 1. Results of the chitinolytic enzymes purification of *Perodicticus potto* gastric mucosa

	Steps of purification					
	Acidification Adsorption on chitin		Enzymatic elution		Chromatography	
Fractions	C.E.	A.E.	F1	F2	F'1	F'2
Volume	38	34	2.45	2.5	3	1.5
Chitinolytic activity (C.U./ml)[a]	215,3	190	627	171.4	484.8	124
Recuperation (%)	100	79	20	5.4	18	2.2
Proteins[b] (mg/ml)	10	5	3.6	2.12	1	0.63
Specific activity (C.U./mg protein)	21.5	38	174.2	80.8	485	196.8
Lysozymic activity[c]	44	—	—	—	—	—

C.E., crude extract.
A.E., acidified extract.
F1–F2, concentrated solutions after adsorption on chitin and spontaneous hydrolysis of this adsorbant during the 9 first hours (F1) and during the 11 next hours (F2).
F'1–F'2, Chromatographied and dialysed fractions corresponding to F1 and F2.
[a] For explanation see Methods.
[b] Lowry *et al.* method.
[c] Expressed in nM of disaccharide liberated $\times$ hr^{-1} $\times$ mg^{-1} protein.

process. The final fractions F'1 and F'2 are obtained after chromatography and dialysis of F1 and F2. It clearly appears from Table 1 that most of the chitinolytic activity is eluted after the first nine hours of the incubation (fraction F1). A prolonged delay (F2) leads to the elution of about a quarter of the total adsorbed chitinase with a much lower specific activity.

The comparison of the specific activities of F'1 and F'2 with the crude extract, gives respectively a purification factor of 22.5 and 9.1. The final purification yield is approx 20%.

(b) *Electrophoretic analysis of 'purified' fractions*

1. *Determination of the number of protein fractions.* Electrophoresis on polyacrylamide gel slab was performed following the method of Akroyd (1967), modified by Perrie & Perry (1970) and by Leger & Focant (1973). The purified solutions F'1 and F'2 gave 2 major bands stainable by the Coomassie brilliant blue G250 (Serva). The bands contained approx 97% of the total protein measured in the gel by photodensitometry.

2. *Determination of the chitinolytic protein fractions.* Areas of unstained gel corresponding to the observed protein bands were cut out. These fragments were ground in a 2 ml $CaCl_2$ mM solution. The resulting extracts were centrifuged and the chitinolytic activity of the supernatant was measured. The 2 main protein bands both present a chitinolytic activity. These results are similar to these obtained by Dandrifosse (1975) with the frog gastric mucosa and pancreas. Due to difficulties in extraction of the enzymes from the gel, no quantitative data could be obtained.

3. *Purified gastric chitinolytic enzymes: estimation of mol. wt.* The purified solutions F'1 and F'2 were treated by sodium dodecylsulfate, then submitted to electrophoresis according to the method of Neville (1971) adapted to the polyacrylamide gel slabs (Leger & Focant, 1973).

The presence of 2 major bands confirms that the solutions F'1 and F'2 contained only 2 major proteins in appreciable quantities. The mol. wt of the chitinolytic enzymes was determined by the preceding method and by using purified proteins of known mol. wt as reference (ovalbumin, catalase, beef serumalbumin, β-galactosidase). The mol. wt was estimated at between 60,000 and 70,000. This value is higher than that found by Jeuniaux (1958) and Skujins *et al.* (1970) for chitinolytic enzymes of *Streptomyces sp.*, by Bernier *et al.* (1974) for *Periplaneta americana* and lower than that found by Dandrifosse (1975) for the gastric chitinase of *Rana temporaria.*

(c) *Enzymatic specificity of the purified preparations*

The comparison between the chitinolytic and lysozymic enzyme activities (Table 1) indicates that the purified solutions F'1 and F'2 present a net chitinolytic activity but do not show any neuraminidasic activity that characterizes the 'lysozyme type' enzymes.

In other words, the chitinolytic enzymes purified from the gastric mucosa of *Perodicticus potto* can be defined as true chitinases, hydrolysing specifically the endo-β-1,4-*N*-acetylglucosaminido-*N*-acetylglucosamine linkages of the chitin.

CONCLUSION

The extraction and purification of chitinolytic enzymes devoid of any lysozymic activity from the gastric mucosa of *Perodicticus potto* allowed us to conclude that these enzymes are true chitinases i.e. the enzymes of the β-1,4-*N*-acetylglucosamine linkages hydrolysis of the chitin molecule.

By generalizing this conclusion, it thus appears that the chitinolytic activity observed in the extracts of gastric mucosa in some vertebrates is due to the secretion of a true chitinase rather than the result of the chitinolytic activity of lysozyme.

SUMMARY

The purification of chitinolytic enzymes from the gastric mucosa of *Perodicticus potto* is described. The specificity and preliminary results concerning the properties of these digestive enzymes are reported.

1. The extracts of *Perodicticus potto* gastric mucosa showed a chitinolytic activity as well as a low lysozymic activity. The purification of the chitinolytic enzymes has been obtained by adsorption on pure colloidal chitin followed by elution by autolysis of the adsorbant and by column chromatography on Biogel.

2. The purified preparation was devoid of any lysozymic activity estimated on *Micrococcus lysodeikticus* walls but showed a high activity on native chitin.

3. It was concluded that these enzymes are 'true' chitinases i.e. they are specific of the hydrolysis of the β-1, 4-*N*-acetylglucosamine linkages in chitin molecule. By generalizing this conclusion, it thus appears that the chitinolytic activity observed in the extracts of gastric mucosa in some vertebrates is due to the secretion of a true chitinase rather than the result of the chitinolytic activity of lysozyme.

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