THE ACTION OF VARIOUS LYSOZYMES ON CHITOPENTAOSE*

Danièle CHARLEMAGNE** and Pierre JOLLÈS
Laboratory of Biochemistry, University of Paris VI, 96 Bd. Raspail, Paris 6, France

Received 3 March 1972

1. Introduction

In the course of our comparative study concerning lysozymes of various origins and following our recent data concerning the importance of pH and ionic strength (I) on the lysis of *Micrococcus lysodeikticus* cells by these enzymes [1], we tried to determine the influence of these two factors on the digestion of chitopentaose. The degradation products obtained after the reaction with 3 bird egg-white, human milk and turnip lysozymes under the optimum conditions were separated by gel filtration and quantitatively determined.

2. Material and methods

Hen egg-white lysozyme was purchased from Worthington. Duck egg-white II [2], goose egg-white [3] and human milk [4] lysozymes were chromatographically pure samples prepared in this laboratory. Turnip lysozyme was purified by gel filtration on Sephadex G-100 [5]. Chitobiose, chitotriose, chitotetraose and chitopentaose were isolated from acid hydrolysates of chitin according to the procedure of Bedell and Johnson [6]. Bio-Gel P-4 was obtained from Touzart and Matignon (Paris).

The digestion of the GlcNAc oligosaccharides was carried out at 37° for different time intervals (from 0.25 to 16 hr) in ammonium acetate solutions of different ionic strengths and pHs. The enzyme/substrate ratios (the enzyme being expressed as hen lysozyme) were 1/10 for mammalian lysozymes and 1/1000 for the turnip lysozyme. The enzymic digest was deep-frozen and lyophilized. The reaction products obtained at different pHs and ionic strengths were identified by paper chromatography [7] or by gel filtration. The sample was taken up in a minimum of water, put on Whatman no. 1 paper and developed in descending chromatography according to the procedure of Sharon [8].

The reaction mixture was also submitted to gel filtration on a column (250 × 0.9 cm) of Bio-Gel P-4 (fig. 1) which was equilibrated with water. Usually the hydrolysate obtained from 3 mg chitopentaose taken up in 0.5 ml water was put onto the column; the eluting solvent was water and 1.5 ml fractions were collected. Their optical density was determined at 220 nm; under these conditions, it was proportional to the sugar concentration; however GlcNAc was eluted together with ammonium acetate and was determined according to the procedure of Dygert [9]. The recovery of the polymers of GlcNAc in the course of the gel filtration was 100%. The fractions corresponding to a peak were pooled and the nature of the sugar was verified by paper chromatography.

Possible transglycosylation products were characterized after Bio-Gel P-4 filtration by their elution volume as the corresponding peaks were eluted before chitopentaose; they were also submitted, as the other sugars, to paper chromatography (R_f of chitohexaose: 0.03; R_f of higher polymers: 0).

3. Results

3.1. *The digestion of chitopentaose by different lysozymes: determination of the optimum conditions.*

* 85th communication on lysozymes.
** This paper is based on a part of the thesis submitted by D.C. in partial fulfillment of the requirements for the degree "Docteur es Sciences", Paris (AO 4511).

The enzymic digestions were carried out for 1 and 3 hr, at 37°, at three different ionic strengths I (0.025; 0.05; 0.1) and six different pHs (4.0; 4.7; 5.5; 6.7; 8.0; 8.5). The chromatograms were quite similar for the 3 lysozymes from vertebrates (hen, duck II and human milk) and all the possible degradation products (chitotetraose, chitotriose, chitobiose and GlcNAc) were observed. Their highest amount was obtained at $I = 0.05$ and at pH 4.7: at pH 4 as well as at higher pHs (6.7; 8.0; 8.5), their yield decreased. A small amount of transglycosylation products was characterized only at optimum conditions and after a hydrolysis of 0.5 or 1 hr.

The behaviour of the turnip lysozyme was different. It was less sensitive to I (between 0.025 and 0.1), but at pH values higher than 6.7 its activity diminished. Here again, we found that the optimum conditions were, at 37°, pH 4.7, $I = 0.05$; after 1 hr nearly all the chitopentaose was digested; the main reaction products were chitobiose and chitotriose; transglycosylation products were not characterized.

3.2. *Quantitative study at pH 4.7, $I = 0.05$ as a function of time*

Chitopentaose was digested by the various lysozymes at pH 4.7, $I = 0.05$. The reaction products obtained after 0.5, 1, 4, 7 and 16 hr were quantitatively separated by gel filtration (fig. 1). The results are indicated in table 1 for the enzymes from vertebrates and in table 2 for the turnip lysozyme. In addition, in the case of duck II lysozyme, the products of digestion under less favorable conditions (pH 6.7, $I = 0.05$; pH 4.7, $I = 0.1$) were also examined quantitatively to see if they were the same as those produced under optimum conditions.

The 5 different lysozymes used throughout this research attacked chitopentaose in 3 different ways. The question of possible transfer reaction (transglycosylation products) is discussed in each case:

i) Goose lysozyme scarcely digested the substrate in the first hour. After 16 hr, 67% of chitopentaose remained undigested; only chitobiose and chitotriose were found among the reaction products. No transfer reaction was catalyzed by this enzyme.

ii) Hen and human milk lysozymes attacked chitopentaose in a similar manner; after 1 hr 46% of the substrate remained untouched and this quantity was reduced to 12% after 16 hr; chitotetraose, chitotriose, chitobiose and GlcNAc were obtained. The transglycosylation products characterized after the action of hen lysozyme corresponded at the best after 30 min to 12% of the total sugars (fig. 1). They were of course hydrolyzed, but considering their low proportion, the final result could not be very different from that obtained when only the hydrolysis products of chitopentaose were considered. With human milk lysozyme transglycosylation compounds could hardly be seen under similar experimental conditions.

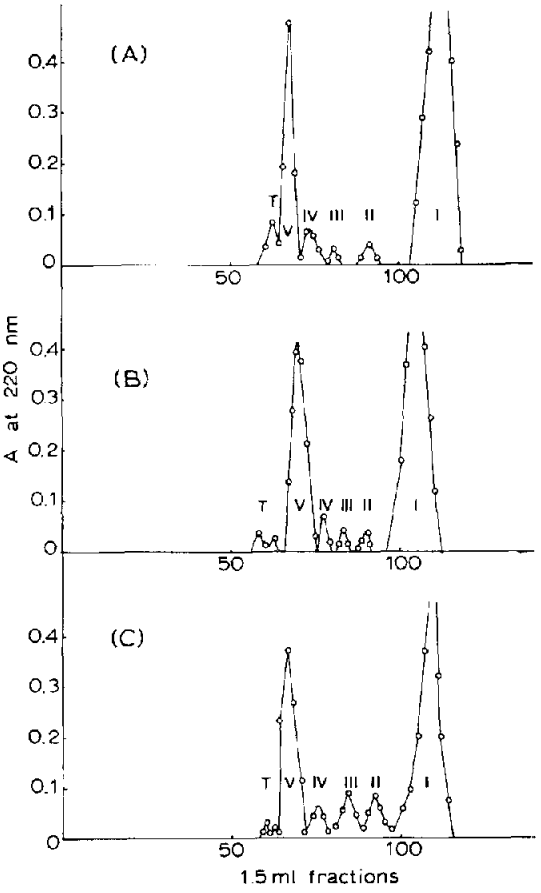

Fig. 1. Gel filtration on Bio-Gel P4 (250 × 0.9 cm) of the enzymic digest obtained by the action of hen (A), human milk (B) and duck II (C) lysozymes on chotipentaose. (E/S = 1/10; 0.5 hr; 37°; pH 4.7; $I = 0.05$). I = mixture of ammonium acetate and GlcNAc, II to V = $(GlcNAc)_2$ to $(GlcNAc)_5$. T = Transglycosylation products.

Duck II lysozyme differs from the two others in having a faster rate and in giving rise after 16 hr mainly to chitobiose and chitotriose. These latter polymers

Table 1
The action of different mammalian lysozymes on chitopentaose.

Lysozymes (origin)	Hydrolysis time (hr)	T*	Reaction products (%) $(GlcNAc)_5$	$(GlcNAc)_4$	$(GlcNAc)_3$	$(GlcNAc)_2$	GlcNAc
Hen egg-white	0.25	8	80	7	2	2	1
	0.5	12	65	13	3	3	4
	1.0	3	46	22	11	10	8
	4.0	0	13	32	17	22	16
	7.0	0	11	33	17	22	17
	16.0	0	12	27	19	27	15
Human Milk	0.25	1	78	5	4	6	6
	0.50	6	56	12	11	10	5
	1.0	1	45	10	17	17	11
	4.0	0	26	27	20	21	6
	7.0	0	20	32	23	24	1
	16.0	0	12	30	24	26	8
Duck II egg-white	0.25	2	70	10	3	3	2
	0.50	5	42	15	12	16	10
	1.0	2	22	18	30	21	8
	4.0	0	0	23	32	23	22
	16.0	0	0	14	39	41	6
Goose egg-white	0.5	0	94	0	3	2	1
	1.0	0	87	2	5	5	1
	4.0	0	74	2	10	12	2
	16.0	0	67	2	14	16	2
	Less favourable conditions.						
Duck II egg-white pH 6.7; $I = 0.05$	16	0	5	20	36	27	14
Duck II egg-white pH 4.7; $I = 0.1$	4.0	0	10	27	24	30	8
	16.0	0	0	24	31	38	7

* T = transglycosylation products.

Optimum conditions: E/S = 1/10; 37°; pH = 4.7; $I = 0.05$.

Table 2
The action of turnip lysozyme on chitopentaose and chitotetraose.

Substrate	Hydrolysis time (hr)	Reaction products (%)				
		$(GlcNAc)_5$	$(GlcNAc)_4$	$(GlcNAc)_3$	$(GlcNAc)_2$	GlcNAc
Chitopentaose	0.5	20	4	36	37	3
	1	12	5	38	40	6
	2	1	4	43	46	7
	7	0	0	45	49	6
Chitotetraose	0.25		78	5	17	0
	0.50		38	14	37	11
	2		10	18	67	5
	7		0	12	77	11

E/S = 1/1000; 37°; pH 4.7; I = 0.05.

seem to come only from chitopentaose; duck lysozyme as human milk lysozyme does not seem to provoke an appreciable transfer reaction; after 30 min the higher polymers represent less than 5% of the total sugars.

iii) Turnip lysozyme digested very quickly and completely chitopentaose at a concentration one hundred times lower than that employed for the enzymes from vertebrates. Chitobiose and chitotriose in equal amounts were characterized as reaction products. Transfer reactions could not be characterized. Thus turnip lysozyme resembles papaya lysozyme. Following Dahlquist et al. [10] the hydrolysis of chitotriose by this latter enzyme was more rapid than with the hen and human lysozymes but did not result in any detectable transglycosylation.

Table 2 reports also the results concerning the digestion of chitotetraose by turnip lysozyme; chitobiose (77%) and only small amounts of chitotriose (18%) and GlcNAc (11%) were obtained.

Acknowledgements

The partial financial support of the C.N.R.S. (ER 102), D.G.R.S.T. (grant 71 7 2630) and I.N.S.E.R.M. (Groupe de recherche U 116 and research grants 1971/2) are gratefully acknowledged.

References

[1] J. Saint-Blancard, P. Chuzel, Y. Mathieu, J. Perrot and P. Jollès, Biochim. Biophys. Acta 220 (1970) 300.
[2] J. Jollès, G. Spotorno and P. Jollès, Nature 208 (1965) 1204.
[3] A.-C. Dianoux and P. Jollès, Biochim. Biophys. Acta 133 (1967) 472.
[4] P. Jollès and J. Jollès, Nature 192 (1961) 1187.
[5] I. Bernier, E. van Leemputten, M. Horisberger, D.A. Bush and P. Jollès, FEBS Letters 14 (1971) 100.
[6] C. Bedell and L.N. Johnson, personal communication (1968).
[7] D. Charlemagne and P. Jollès, Bull. Soc. Chim. Biol. 49 (1967) 1103.
[8] N. Sharon and S. Seifter, J. Biol. Chem. 239 (1964) 2398 P.
[9] S. Dygert, L.H. Li, D. Florida and J.A. Thoma, Anal. Biochem. 13 (1965) 367.
[10] F.W. Dahlquist, C.L. Borders Jr., G. Jacobson and M.A. Raftery, Biochemistry 8 (1969) 694.