A RAPID MICROASSAY OF LYSOZYME ACTIVITY WITH REDUCED CHITIN OLIGOSACCHARIDES AS SUBSTRATE

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SUMMARY Penta- and hexa-N-acetylglucosamine were reduced with sodium borohydride. The reduced penta- and hexa-N-acetylglucosamine (R–5mer and R–6mer) were used as substrates for hen egg white lysozyme (HL) and for duck egg white lysozyme (DL). Accuracy of the enzyme assay, by measurement of reducing power, was high due to destruction of the inherent reducing terminal of the substrates.

The β-glucosaminidase activity of HL or DL in the range of 0.5 μg to 6 μg protein can be measured at 37 C, at pH 6.0 with a reaction time of 30 min with R–6mer as substrate. The limit of error in this method is less than 5%.

The rate of hydrolysis of R–6mer by DL was approximately 76% of that of HL.

INTRODUCTION

Hen egg white lysozyme (HL) induces lysis of certain gram positive bacteria. It also catalyses the hydrolysis of chitin (Berger and Weiser, 1957). Both bacteria and chitin are very large in size, so studies on the mechanism of enzymic action of HL have been difficult. Rupley (1964) succeeded in isolating chitin oligosaccharides by acid hydrolysis of chitin and subsequent separation of the oligosaccharides by charcoal-celite chromatography and these chemically well defined small substrates are more suitable for studies on the mechanism of action of HL (Chipman et al., 1967, Blake et al., 1967, Rupley et al., 1967a and 1967b).

One of the problems in the estimation of lysozyme activity using increase of reducing power of the substrate as a parameter is the reducing power of the reactants themselves. These oligosaccharides in particular have strong reducing power due to their reducing end group and thus in measurements high back ground are obtained. Therefore, penta- and hexa-N-acetylglucosamine were reduced by sodium borohydride and the N-acetylglucosamine of the reducing terminal was converted to N-acetylglucosaminitol.

Lysozyme activity was measured with reduced penta-N-acetylglucosamine (R–5mer) and reduced hexa-N-acetylglucosamine (R–6mer) as substrates.
Both reduced chitin oligosaccharides were hydrolyzed by lysozyme in the relatively short reaction period of 30 min at 37 C.

MATERIALS AND METHODS

1. *Chitin*

Chitin was purchased from Eastman Kodak and treated as described in our previous report (Fujio et al., 1968). The hydrolysis of chitin by concentrated hydrochloric acid and the separation of oligosaccharides were carried out essentially as described by Rupley (1964).

2. *Hen egg white lysozyme (HL)*

Six times recrystallized HL was purchased from Seikagaku-Kogyo Co. Ltd. and purified further by SE-Sephadex C–50 column chromatography (Fujio et al., 1968).

3. *Duck egg white lysozyme (DL)*

DL were purified by SE-Sephadex C–50 column chromatography in 0.2 M sodium phosphate buffer, pH 6.5, at 25 C as described in our previous reports (Imanishi et al., 1966 and Fujio et al., 1968).

4. *Sephadex*

Sephadex C–25, superfine, Pharmacia, was used for purification of chitin oligosaccharides or reduced chitin oligosaccharides.

5. *Measurement of reducing activity*

The method of Park and Johnson (1949) was used with the slight modification that 0.2 M instead of 0.05 N of H_2SO_4 was used to prepare the ferric iron solution.

6. *Ninhydrin reaction*

The reaction was performed by the method of Cocking and Yemm (1954).

7. *Quantitative determination of N-acetylglucosamine and N-acetylglucosaminitol.*

A given sample was hydrolysed with 4 N HCl at 105 C for 16 hours. The glucosamine and glucosaminitol contents of the reduced chitin oligosaccharides were estimated using a Yanagimoto automatic acid amino analyzer (Model LC–5) as described previously (Imanishi et al., 1966).

RESULTS

1. *Preparation and purification of chitin oligosaccharides*

The recovery of each chitin oligomer fraction varied with the batch of chitin used, although the conditions for acid hydrolysis were constant. Therefore, suitable conditions for acid hydrolysis must be determined for each batch of chitin to obtain predominantly a certain size of oligomer. An example of the charcoal-celite chromatography of one batch of HCl hydrolysate of chitin is shown in Fig. 1.
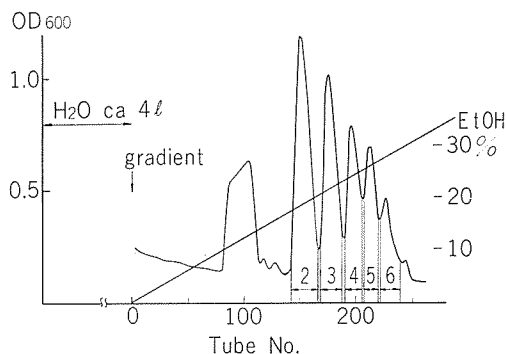

FIGURE 1. *Chromatography on charcoal-celite column of a partial hydrolysate.*
20g of chitin were hydrolyzed with 12 N HCl at 40 C for 70 minutes. Column size, 5×50 cm. Temperatur, 25 C. Fration size, 18 g. Linear grandient fram 0 to 30% ethanol in total volume of 4 liters. 15 μl of each fraction were used for Park and Johnson's method and OD values at 600 mμ were plotted. Numbers indicate those of fractions separated. Recoveries of Fr. 2, 3, 4, 5 and 6 were 1.2, 1.33, 1.28, 1.10 and 0.95 g, respectively,

After chromatography each fraction was concentrated in a rotatory evaporator using a vacuum oil-pump and a water bath at 28 C. Fractions were purified further by gel filtration on a Sephadex G–25 (superfine) column (3×150 cm) in 0.01N acetic acid at 25 C. The gel filtration pattern of fraction 5, which was eluted from the charcoal-celite column, is shown in Fig. 2 as an example.

The degree of polymerization in each purified oligomer preparation was estimated as follows; the preparation was first reduced by sodium borohydride and hydrolyzed with 4N HCl at 105 C for 16 hours. The resulting mixtures of glucosamine and glucosaminitol were analyzed in an automatic amino acid analyzer. The results are shown in Table 1.
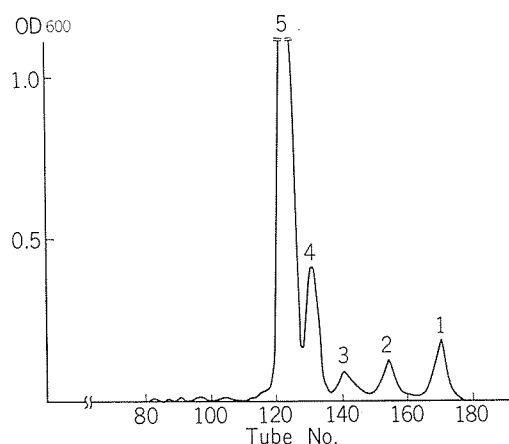
FIGURE 2. *Gel filtration pattern of chitin hydrolyzate Fr. 5 on Sephadex G–25 column.*

165 mg *of Fr. 5 were dissolved into 5 ml of deionized water and applied on a Sephandex G–25 column (3 × 153 cm). The column was eluted with 0.01 N acetic acid at the rate of 30 ml per hour at 25 C. Fraction of 5 g were collected. Samples of 50 μl from each tube were tested by the method of Park and Johnson. Number on the top of peaks represent the degree of polymerization.*

TABLE 1. *Analysis of the degree of polymerization of oligosaccharides fractions purified by gel filtration*

| | Reduction (%) | Observed μM | | GlcN : GlcNol |
		GlcN	GlcNol	
NAG	99.5	~0	0.9307	0 : 1
Fr. 2	100	0.3414	0.3530	0.97 : 1
Fr. 3	99.9	0.5489	0.2697	2.04 : 1
Fr. 4	99.8	0.6340	0.2145	2.96 : 1
Fr. 5	100	0.4226	0.1057	4.00 : 1

GlcN: glucosamine.   GlcNol: glucosaminototol. NAG: authentic sample.

Thus good separation of N-acetylglucosamine oligomers on the basis of their degree of polymerization can be achieved by gel filtration on a Saphadex G–25 (superfine) column.

2. *Preparation of reduced penta- and hexa-N-acetylglucosamine (R–5mer and R–6mer).*

Crude penta- or hexa-N-acetylglucosamine, which were eluted from the charcoal-celite column, were reduced with sodium borohydride as follows; NaHB$_4$ (1.5 g) was added to 150 mg of crude penta- or hexa-N-acetylglucosamine and the mixture was incubated for 16 hours at 0 C. The reaction was stopped by adjusting the pH to 3.0 with 50% (v/v) acetic acid. The reaction product was dried in a rotatory evaporator. To remove excess boric acid, methanol was added to the reaction mixture to esterify boric acid. The material was again dried in a rotatory evaporator connected to a vacuum oil-pump with a dry ice-actone trap.

The dried material was dissolved in a small volume of deionized water and applied to a Sephadex G–25 (superfine) column (3 × 150 cm) equilibrated with 0.01N acetic acid. It was eluted with the same solvent at 25 C. The elution pattern of the reduced hexa-N-
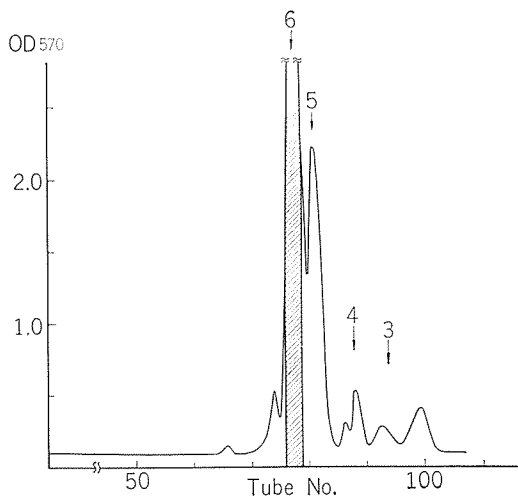

FIGURE 3. *Gel filtration pattern of reduced chitin oligosaccharide Fr. 6 on Sephadex G–25 column.*

185 mg *of reduced chitin oligosaccharide Fr. 6 (from a charcoal-celite column) were dissolved into 10 ml of deionized water and applied on the column. Column size, 3 × 151 cm. Temperature, 25 C. Elution rate 35 ml/hour. A sample of 100 μl from each tube was subjected to deacetylation with 3 N HCl at 100 C for 30 minutes. 1 ml of 4 N acetate buffer, pH 6.0, was added to each tube and the ninhydrin reaction was performed. Number on the top of peaks represent the degree of polymerization.*

acetylglucosamine fractions is shown in Fig. 3. Purified R–5mer and R–6mer were finally lyophilized and the reducing activities of both preparations were measured by the method of Park and Johnson (1949).

The results indicate that more than 99.5% of the reducing terminal N-acetylglucosamine of both preparations were converted to N-acetyl-glucosaminitol.

3. Time course of hydrolysis of R–5mer and R–6mer by HL.

To estimate the rates of hydrolysis of the R–5mer and the R–6mer by HL, the release of reducing groups as a function of time was measured quantitatively using Park and Johnson's method (1949). The results are plotted in Fig. 4.
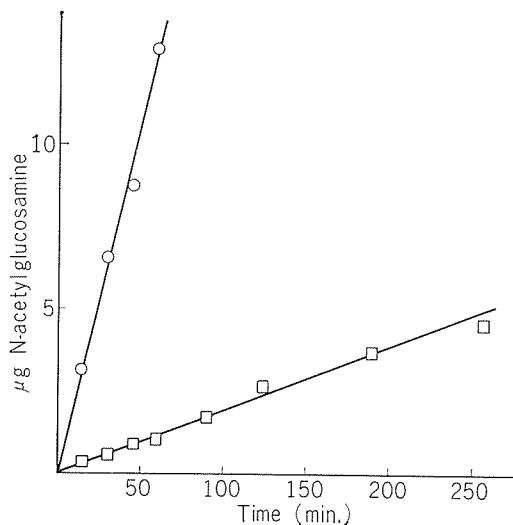

FIGURE 4. *Rates of hydrolysis of reduced penta- and hexa-N-acetylglucosamine as a function of time.*

HL, 9.2 μg per ml (6.4×10^{-4} M). *Substrate*, 800 μg per ml. *Temerature* 37 C. *Buffer*, 0.1 M NaCl *in* 0.025 M *sodium barbiturate*-HCl *buffer*, pH 6.0· *Sam-of* 500 μl *were tested by the method of Park and Johnson.*

☐ ----- ☐: R-5mer as substrate.
○ ----- ○: R-6mer as substrate.

The initial rate of hydrolysis of the R–6mer by HL was approximately 11 times that of the R–5mer. The difference is comparable with

that between the rates of hydrolysis of penta- and hexa-N-acetylglucosamine by HL reported Rupley et al. (1967a).

4. Rate of hydrolysis of R–6mer as a function of enzyme concentration.

The above experiment indicated that the R–6mer was hydrolyzed fairly rapidly. Thus studies were made on a suitable system for detection of the enzymic activity of a minute amount of lysozyme using a small substrate. Details of the procedure developed are as follows.

Various amounts of HL or DL (0.5 to 6 μg) in 300 μl of 0.1 M NaCl, 0.025 M sodium barbiturate-HCl buffer, pH 6.0, were incubated at 37 C for 30 min with 200 μl of substrate solution containing 480 μg of R–6mer (final concentration of substrate, 7.27×10^{-4} M).
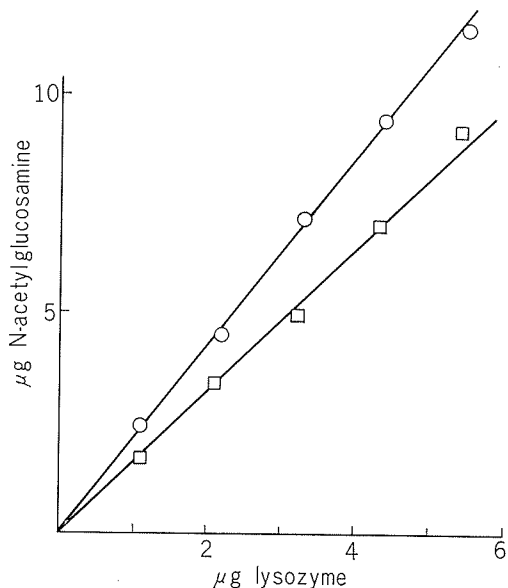

FIGURE 5. *Rates of hydrolysis of reduced hexa-N-acetylglucosamine by* HL *and* DL *as a function of enzyme concentration.*

Hydrolysis condition: 37 C *for* 30 min *at* pH 6.0. *Final concentration of subsrate;* 7.27×10^{-4} M. *Samples of* 0.5 ml *of reaction mixture tested by the method of Park and Johnson.*

○ ---- ○; HL, ☐   ☐ DL.;

The reaction was stopped by adding 1 ml of carbonate-cyanide solution and color was developed as described by Park and Johnson (1949). As a control, the enzyme solution was added to the substrate solution after the addition of carbonate-cyanide solution and then color was developed in the same way. Optical densities at 700 mμ were measured in a Zeiss spectrophotometer in cuvettes of 1 cm light path. The OD difference between the test and control tubes was a measure of enzymic activity. The results are shown in Fig. 5.

Hydrolysis of R–6mer by HL or DL increased linearly with the concentration of enzyme from 0 to 6 μg. The sensitivity of the enzyme assay using R–6mer as substrate was the same order as that using *M. lysodeikticus* as substrate (Shinka et al., 1962). The calculated error of the method using R–6mer as substrate was less than 5% when 1 μg to 6 μg of HL or DL was used.

The rate of hydrolysis of R–6mer by DL was approximately 76% of that of HL using the same amounts of enzyme.

DISCUSSION

For studies on the mechanism of neutralization of lysozyme activity by antibody (Imanish et al., 1968), a rapid microassay method for lysozyme activity with a small sized substrate is necessary. But no suitable substrate was available until Rupley (1964) succeeded in isolating chitin oligosaccharides. Rupley reported that hexa-N-acetylglucosamine can be hydrolyzed at almost the same rate as *M. lysodeikticus* by HL. Therefore, penta- and hexa-N-acetylglucosamine were prepared by the method of Rupley (1964) and their hydrolysis by HL was tested. However, when the increase in reducing power was used as a measure of enzymic activity, a very high blank value was obtained due to the reducng activity of the substrate itself, while the reducing power of the enzyme protein was weak.

Thus errors of over 20% were obtained in estimations under certain circumstances, and it was especially difficult to measure lysozyme activity accurately in the presence of both antibody and substrate. Thus, we tested the possibility of using reduced penta- and hexa-N-acetylglucosamine in which the reducing termini of the oligosaccharides were converted to N-acetylglucosaminitol. We found that reduced products of hexa-N-acetylglucosamine were hydrolyzed by HL at a rate comparable to that of non-reduced hexa-N-acetylglucosamine. Moreover, reduction of chitin oligosaccharides completely abolishes the reducing activity of the substrate. Since the blank value due to the protein fraction in Park and Johnson's method was not so high as that of the substrate, it became possible to measure the lysozyme activity with an error of less than 5%. The sensitivity of our assay method is comparable with that of the turbidometric method using *M. lysodeikticus* as substrate (Shinka et al., 1962).

The Morgan-Elson test (Reissig et al., 1955) can also be used for estimation of lysozyme activity, but its sensitivity is not high, and it requires a reaction time of as long as 24 to 48 hours and more enzyme.

The rate of hydrolysis of R–6mer by DL is slower than that by HL. Charlemagne and Jollès (1967) also found tha penta-N-acetylglucosamine is hydrolyzed by DL more slowly than by HL at pH 4.7.

REFERENCES

BERGER, L. R. and R. S. WEISER. 1957. The β-glucosaminidase activity of egg-white lysozyme. Biochim. Biophys. Acta, 26: 517–521.

BLAKE, C. C. F., L. N. JOHNSON, G. A. MAIR, A. C. T. NORTH, D. C. PHILLIPS qnd V. R. SARMA. 1967. Crystallographic studies of the activity of hen egg-white lysozyme. Proc. Roy. Soc., B. 167: 378–388.

CHARLEMAGNE, D. et P. JOLLÈS. 1967. La spécificité de divers lysozymes vis-a-vis de substrats de faible poids moleculaire provenant de la chitine. Bull. Soc. Chim. Biol. 49: 1103–1113.

CHIPMAN, D. M., V. GRISARO and N. SHARON. 1967. The binding of oligosaccharides containing N-acetylglucosamine and N-acetylmuramic acid to lysozyme. J. Biol. Chem., 242: 4388–4394.

COCKING, E. C. and E. W. YEMM. 1954. Estimation of amino acids by ninhydrin. Biochem. J., 58: XII.

FUJIO, H., M. IMANISHI, K. NISHIOKA and T. AMANO. 1968. Antigenic structures of hen egg white lysozyme II. Significance of the N- and C- terminal region as an antigenic site. Biken J., 11: 207–218.

IMANISHI, M., S. SHINKA, N. MIYAGAWA, T. AMANO and A. TSUGITA. 1966. Amino acid composition of duck and turkey egg-white lysozymes. Biken J., 9: 107–114.

IMANISHI, M., H. FUJIO, M. SAKAKOSHI and T. AMANO 1968. Antigenic structures of hen egg white lysozyme III. Antibody specificity and neutralizing activity. 2nd symposium on immunochemistry, Tokyo, Abstract P. 45.

PARK, J. T. and M. J. JOHNSON. 1949. A submicrodetermination of glucose. J. Biol. Chem., 181: 149–151.

REISSIG, J. L., J. L. STROMINGER and L. F. LELOIR. 1959. A modified colorimetric method for the estimation of N-acetylamino sugers. J. Biol. Chem., 217: 959–

RUPLEY, J. A. 1964. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. Biochim. Biophys. Acta, 83: 245–255.

RUPLEY, J. A. and V. GATES. 1967a. Studies on the enzymic acitivity of lysozyme, II. The hydrolysis and transfer reactions of N-acetylglucosamine oligosaccharides. Proc. Nat. Acad. Sci., 57: 496–510.

RUPLEY, J. A., L. BUTLER, M. GERRING, F. J. HARTDEGEN and R. PECORARO. 1967b. Studies on the enzymic acitivity of lysozyme, III. The binding of saccharides. Proc. Nat. Acad. Sci., 57: 1088–1095.

SHINKA, S., M. IMANISHI, O. KUWAHARA, H. FUJIO and T. AMANO. 1962. Immunochemical studies on lysozyme II. On the non-neutralizing antibodies. Biken J., 5: 181–200.