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Studies on the Substrate Specificity of Egg White Lysozyme. II. Mode of the Enzymatic Action on Partially-*O*-carboxymethylated Chitin

Tsuyoshi MIYAZAKI and Yoshio MATSUSHIMA

Department of Chemistry, Osaka University, College of Science, Toyonaka

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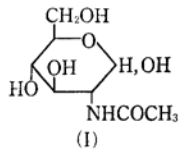
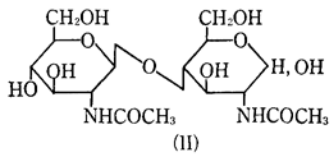
Partially-*O*-carboxymethylated chitin was found to be a soluble substrate of hen egg white lysozyme. The products of the lysozyme action were analyzed by gel filtration and by ion-exchange chromatography. The reducing ends of the saccharides produced by the enzyme were the *N*-acetyl-glucosamine residue or the 3-*O*-carboxymethyl-*N*-acetyl-glucosamine residue. 6-*O*-Carboxymethyl-*N*-acetyl-glucosamine never appeared as a reducing end. The *O*-substituted disaccharide detected in the reaction products was 6'-*O*-carboxymethyl-di-*N*-acetyl-chitobiose. The results of the reducing-end analyses and the finding that none of the monomers were formed except for the unsubstituted *N*-acetyl-glucosamine, while an *O*-substituted dimer was formed as is mentioned above, are in conformity with the conformation of the enzyme-substrate complex proposed by Blake *et al.* as a result of their X-ray crystallographic investigations.

An elaborate model of a three-dimensional structure of an ES-complex formed between chitin oligosaccharide and hen egg white lysozyme [EC 3.2.1.17.] was presented by Blake *at al.*[1] as a result of thier X-ray crystallographic investigations. When partially-*O*-carboxymethylated chitin is used instead of a natural substrate chitin, it is expected that the fairly bulky *O*-substitutent groups will not permit the substrate molecules to bind freely with the enzyme protein. The enzyme must avoid the steric hindrance exerted by the *O*-substituent groups on the substrate and select some suitable portions of the substrate in order to make an ES-complex. If this is the case, the orientation of the *O*-substituent groups in the enzymatic reaction products will afford information on the conformation of the binding site of the enzyme protein.

Partially-*O*-carboxymethylated chitin was reported by Hultin[2] to be a soluble substrate of chitinase. The present authors prepared the substance by the *N*-acetylation of the *O*-carboxymethylated chitosan reported by Okimasu.[3] This substance was found to be a substrate of hen egg white lysozyme[4] by the release of reducing power when it was incubated with the enzyme. Among the low-molecular-weight compounds produced by the enzyme, there were *N*-acetylglucosamine (I), di-*N*-acetylchitobiose (II), tri-*N*-acetylchitotriose (III), and 6'-*O*-carboxymethyl-di-*N*-acetylchitobiose (IV). None of the *O*-substituted monosaccharides were produced by the enzyme, and the reducing-end residues of the product saccharides were either unsubstituted or 3-*O*-substituted. These findings can be well interpreted when we take into account the conformation of the *O*-substituted groups of the substrate in the ES-complex.



(I)



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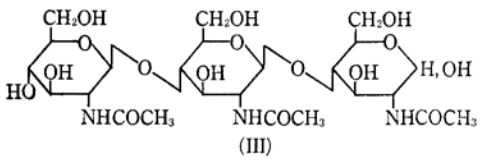

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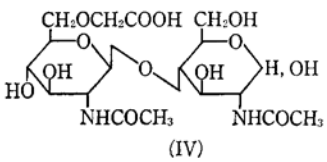

(IV)

1) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Proc. Roy. Soc.*, **167**, **B** 378. (1967).
2) E. Hultin, *Acta Chem. Scand.*, **9**, 192 (1955).
3) T. Okimasu, *J. Agr. Chem. Soc. Japan*, **32**, 303 (1958).
4) Y. Matsushima, S. Hara, T. Miyazaki and Y. Umemura, The 38th Annual Meetings of the Japanese Biochemical Society, October (1965).

Our results offer chemical evidence for the conformation of the active sites in the Phillips model[1] of the ES-complex of hen egg white lysozyme.

Experimental

The enzyme specimen used was a thrice recrystallized one purchased from Sigma Chemicals.

Abbreviations will be used for glucosamine: GlN, *N*-acetylglucosamine: GlNAc, glucosaminitol: GlNOH, carboxymethyl: CM.

The Enzymatic Action on Partially-*O*-carboxymethylated Chitin. An example of the time course of the enzymatic reaction is shown in Fig. 1. The pH-activity curve is shown in Fig. 2. The molar composition of the substrate is: 56.7% GlNAc, 3.2%

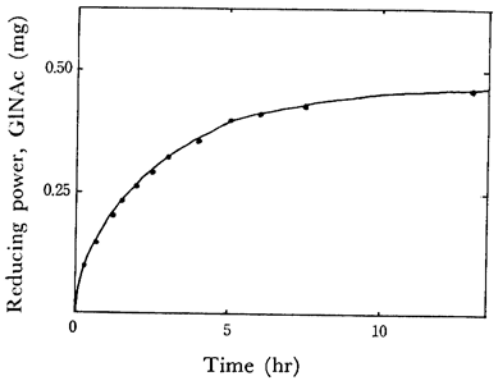

Fig. 1. Incubation mixture: Five mg of the substrate in 1 m*l* of water, 0.5 m*l* of 0.4 M acetate buffer (pH 5.6) and 0.1 mg of the enzyme in 0.5 m*l* of water. Incubation temperature: 37°C. Reducing power measurement: Somogyi-Nelson method.[5]

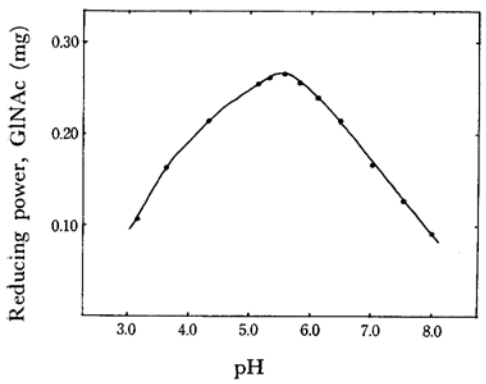

Fig. 2. Incubation mixture: Five mg of the substrate in 1 m*l* of water, 0.5 m*l* of 0.4 M acetate buffer (pH 3.3—5.8) or phosphate buffer (pH 6.0—8.0) and 0.1 mg of the enzyme in 0.5 m*l* of water. Incubation temperature: 37°C. Incubation time: 2 hr. Reducing power measurement: Somogyi-Nelson method.

5) "Methods in Carbohydrate Chem.," Vol. 1, Academic Press, New York and London (1962), p. 368.

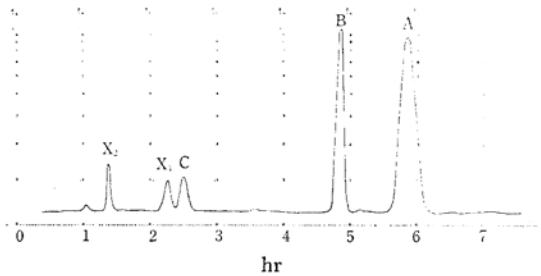

Fig. 3a. The composition of the substrate. The specimen was hydrolyzed in 4 N hydrochloric acid at 105°C for 4 hr. The hydrolysate was evaporated *in vacuo*, and 1 mg of the dried residue was placed on a column of Hitachi Autoanalyzer KLA-3. Elution was performed with 0.2 M citrate buffer (pH 3.11—4.25). The peaks shown are: GlN(A), 6-*O*-CM-GlN(B), 3-*O*-CM-GlN(C) and unknown substances (X_1, X_2). The peaks were identified by using standard specimens.[6]

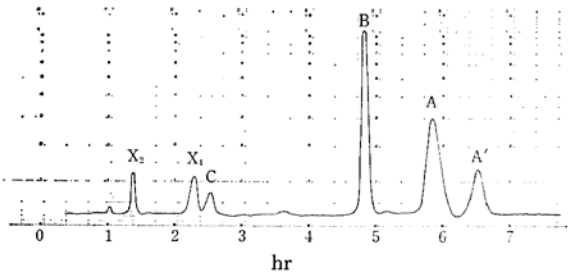

Fig. 3b. The composition of the enzymatic reaction products which was reduced with borohydride before hydrolysis. The peaks shown are: GlNOH(A'), GlN(A), 6-*O*-CM-GlN(B), 3-*O*-CM-GlN(C) and unknown substances (X_1, X_2). The peak of 3-*O*-CM-GlNOH was not recognizable due to a low ninhydrin color yield of the compound, and due to its close vicinity to the peak X_1. The retention time for the peak of 6-*O*-CM-GlNOH, which did not appear, is 4.58 hr.

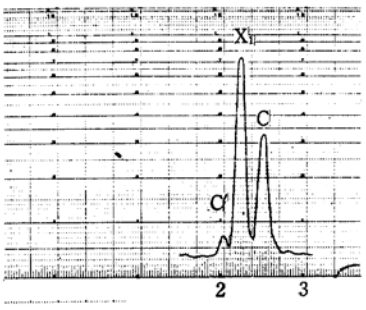

Fig. 3c. The pattern of the early region of Fig. 3b magnified by charging ten times amount (10 mg) of the specimen. The peaks shown are: 3-*O*-CM-GlN(C), an unknown substance (X_1) and 3-*O*-CM-GlNOH(C').

6) T. Miyazaki and Y. Matsushima, This Bulletin, in press.

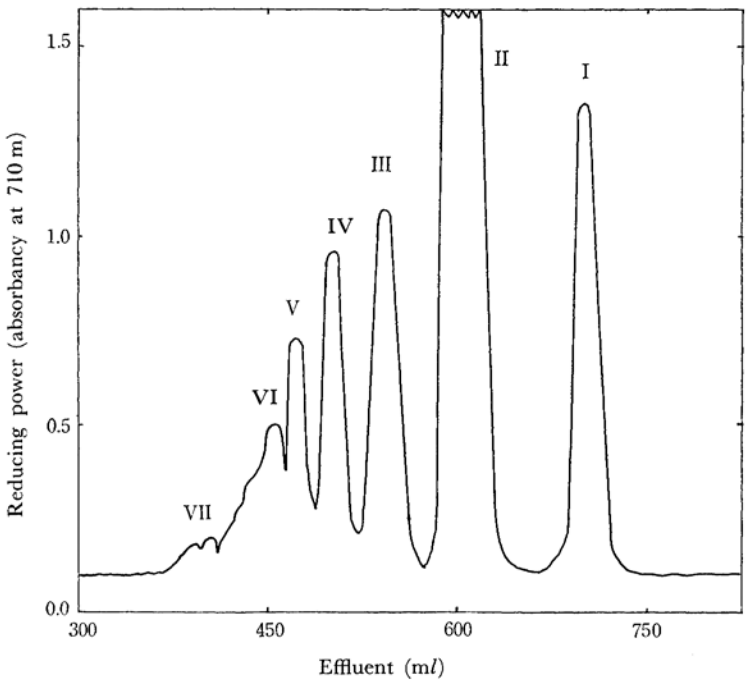

Fig. 4. The fractions of each 3 m*l* were collected. An aliquot (0.4 m*l*) was used for the reducing power measurement by Somogyi-Nelson method. Elution velocity: 4.5 m*l*/hr.

3-*O*-CM-GlNAc, 27.2% 6-*O*-CM-GlNAc, plus unknown components.

Analyses of the Reducing-end Residues Produced by the Enzyme. The whole of the enzymatic reaction mixture was reduced by sodium borohydride, and the mixture was hydrolyzed with hydrochloric acid. The acid hydrolysate contained monosaccharides and sugar alcohols, and the structure (3-*O*-, 6-*O*-, or unsubstituted) of the latter revealed what had been the reducing ends of the oligosaccharides produced by the enzyme. The chromatographic pattern of the acid hydrolysate, after the borohydride reduction, of the enzymatic reaction mixture is shown in Fig. 3(a—c) in comparison with the acid hydrolysate of the subsubstrate.

Fractionation by Dialysis and Gel Filtration of the Enzymatic Reaction Products. Three grams of the substrate were digested by 60 mg of the enzyme in a 100 m*l* of acetate buffer solution (pH 5.6) for 48 hr at 37°C. Sixty milligrams of the enzyme in 25 m*l* of water were then added to the mixture, and the incubation was continued for 24 hr more. The mixture was then dialyzed against 2000 m*l* of deionized water for 24 hr. The dialysis was repeated three times. The dialysate was then concentrated to 200 m*l* *in vacuo*, and the residual solution was passed through a column of Amberlite IR-120 (H^+ form, 3×15 cm) in order to eliminate the sodium ion of the buffer solution. The lyophilization of the solution gave 1.87 g of a mixture of saccharides. An aliquot (260 mg) of the mixture was placed on a column of Sephadex G-15 (3×160 cm) and eluted with 0.1 N acetic acid. The chromatographic pattern obtained is shown in Fig. 4.

Identification of the Fractions in Gel Filtration. *Fraction I.* On a paper chromatogram this fraction gave only one spot that corresponded to GlNAc, as is shown in Fig. 5. The identity was further confirmed by the use of an automatic amino-acid analyzer. The fraction, after it had been hydrolyzed with hydrochloric acid, gave a single peak which corresponded to GlN. After the borohydride reduction and acid hydrolysis, the fraction gave a single peak that corresponded to GlNOH. No trace of *O*-substituted saccharide was observed in this fraction. These results, when consolidated, are evidence for the fraction I being very

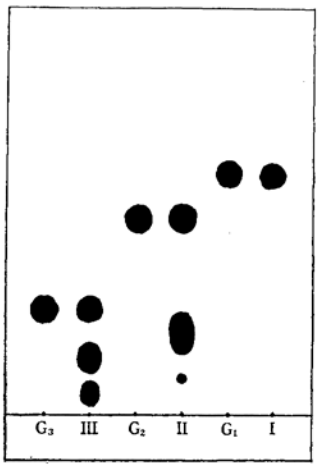

Fig. 5. Solvent system is *n*-butanol : pyridine : water = 6 : 4 : 3 (v/v). G_1: *N*-acetylglucosamine, G_2: di-*N*-acetylchitobiose, G_3: tri-*N*-acetylchitotriose.

Table 1. Molar ratio of the component sugars and sugar alcohols

	Neutral	Acidic
GlN	1.00	0.25
6-*O*-CM-GlN		1.18
GlNOH	1.00	1.00
3-*O*-CM-GlNOH		0.34

pure GlNAc. The recovery of the fraction was 0.118 g (referred to 3.0 g of the substrate).

Fraction II. This fraction was a mixture of disaccharides. As is shown in Fig. 5, the fraction contained at least three components, one of which corresponded to di-*N*-acetylchitobiose. By passing 20.2 mg of the fraction through a column of Dowex-1 (acetate form, 1.2×14 cm), it was resolved into a neutral and an acidic part. The component analyses of these two parts are shown in Table 1. The data for the neutral part demonstrate almost unequivocally the presence of di-*N*-acetylchitobiose in the enzymatic reaction products. The data for the acidic part can be interpreted as showing, along with the paper-chromatographic data, the presence of 6′-*O*-CM-di-*N*-acetylchitobiose (IV) and of 3-*O*-CM-di-*N*-acetylchitobiose, though the analytical values for the latter are not very reliable because of the low color yield in the ninhydrin reaction of 3-*O*-CM-GlNOH. The pattern in the cation-exchanger chromatography of the acidic part is shown in Fig. 6.

Fraction III. A spot corresponding to tri-*N*-acetylchitotriose is shown in Fig. 5. Further investigation of the fractions III—VII remains to be done.

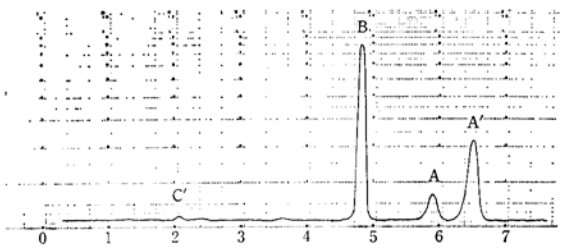

Fig. 6. The peaks are: GlNOH(A′), GlN(A), 6-*O*-CM-GlN(B) and 3-*O*-CM-GlNOH(C′).

Discussion

A three-dimensional model construction according to Blake *et al.*[7] reveales explicitly the orientation of the substituents on the substrate molecule bound to the active site (cleft) of the enzyme protein. The general features are presented in Table 2.

Table 2.

Substituent	Sub-sites A	B	C	D	E	F
3-*O*-	IN	OUT	IN	OUT	IN	OUT
6-*O*-	OUT	IN	OUT	IN	OUT	IN

The notation of the sub-sites is according to Blake *et al.*[1] "IN" means that a substituent group is directed toward inside of the "cleft", thus being ready to suffer steric hindrance, and "OUT" means contrary. Cleavage of a glycosidic bond of the substrate occurs on a residue which has been in the sub-site D. The reducing end of the saccharide chain is directed toward F.

The experimental results can be well interpreted when we take into account the general features shown in Table 2, and when the interpretation is made, as a first-order approach, only in terms of steric circumstances. The 6-*O*-substituted residue will not be able to fit into D because of its substituent group directed toward the inside of the cleft, and so this residue will not be able to be a reducing end of the various saccharides produced by the enzyme. A monosaccharide released by the enzyme must necessarily fit once into D, and once into E. Any *O*-substituted residue will suffer steric hindrance at either of these two subsites, and so can not be released as a monosaccharide. Any disaccharide to be released must fit once into C-D, and once into E-F. The disaccharides identified in the experiments meet this requirement. It seems that the C, D and E sub-sites do not have much room toward the inside of the cleft. The structure determination of the product oligosaccharides larger than disaccharide will afford information in a chemical sense about the "size" of the room of the sub-sites A, B and F. This task is now being undertaken.

This investigation was supported by U. S. Public Health Service Grant AI-04586-05. The authors are also grateful to Mr. S. Kasai for his assistance.

7) C. C. F. Blake, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Proc. Roy. Soc.*, **167, B,** 365 (1967).