SYNTHETIC SUBSTITUTE LYSOZYMES*

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Received September 28, 1967

Elegant X'Ray studies on hen egg-white lysozyme (Blake et al., 1965) and on lysozyme-inhibitor complexes (Johnson and Phillips, 1965) have led to the location of all its 129 amino acid residues and the identification of the amino acid residues involved in its active site. These studies have also permitted the formulation of a mechanism for the β-(1-4)glucosaminidase activity of this enzyme (Phillips, 1966). The most important features of this mechanism are the protonation of the glycosidic oxygen atom at the point of fission of the polysaccharide chain by the unionised γ-carboxyl of the glutamic acid residue 35 and the stabilization of the carbonium ion formed after bond fission by the carboxylate ion of aspartic acid residue 52. This situation is made possible by the location of glutamic acid residue 35 and aspartic acid residue 52 in hydrophobic and hydrophyllic regions of the enzyme molecule respectively. We now report the synthesis of polypeptides having carboxyl functions in hydrophobic as well as in hydrophyllic regions and the ability of these polypeptides to degrade the cell wall of Micrococcus lysodeikticus in a manner similar to egg-white lysozyme.

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*Communication No.1213 from the Central Drug Research Institute.
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For the synthesis of such polypeptides, the N-carboxyanhydride of a hydrophobic amino acid was copolymerised with γ-benzyl-L-glutamate N-carboxyanhydride in two stages. During the first stage, the hydrophobic amino acid N-carboxyanhydride was the predominant component and in the second stage, γ-benzyl-L-glutamate N-carboxyanhydride was in excess. Subsequent removal of the benzyl groups liberated carboxylic functions in both hydrophobic and hydrophyllic regions of the synthesised polypeptides. The synthesis of two such polymers is described.

Polypeptide PG

L-Phenyl alanine N-carboxyanhydride (Sela and Berger, 1955) (1.71 g.) and γ-benzyl-L-glutamate N-carboxyanhydride (Blout and Karlson, 1956) (0.263 g.) were dissolved in dry dioxane (100 ml.) and polymerisation initiated with triethylamine (0.007 ml.). After 3 hr. at 20°, a solution of L-phenylalanine N-carboxyanhydride (0.19 g.) and γ-benzyl-L-glutamate N-carboxyanhydride (2.367 g.) in dry dioxane (100 ml.) was added to the polymerisation mixture which was allowed to stand for a further 48 hr. at 20°. The viscous solution was then concentrated to half its volume and poured with stirring into chilled ethanol (1000 ml.) to yield a fibrous polymer (2.48 g.).

The crude polymer (2.0 g.) was treated with 98% formic acid (2 X 50 ml.) for a period of 4 hr. with occasional stirring and the insoluble material collected, washed with ethanol and dried (1.82 g.).

For debenzylation, the polymer so obtained (1.5 g.) was suspended in dry liquid ammonia (180 ml.) and sodium pellets added until the solution retained a blue colour for 15 min. Excess sodium was neutralised with ammonium acetate, ammonia

allowed to evaporate and the residue treated with water. The insoluble peptide was collected by filteration, triturated with N HCl, washed repeatedly with water and dried. This product (800 mg.) was purified by dissolving in chloroform (240 ml.), filtering and precipitating the peptide by pouring this solution into ethanol (1500 ml.). Peptide PG (680 mg.) was collected by filteration.

Mean molecular weight*, 63,650; Phe:Glu :: 1:0.3

Polypeptide GcG

Copolymerisation was carried out in two stages as in the case of PG with γ-cholesteryl-L-glutamate N-carboxyanhydride (M.M. Dhar and K.L. Agarwal, 1964) and γ-benzyl-L-glutamate N-carboxyanhydride. The polymer so obtained was debenzylated by repeated hydrogenolysis in the presence of Pd/C and acetic acid.

Mean molecular weight, 12,000; γ-cholesteryl glu: γ-benzyl glu:glu :: 1:0.067:0.66

Biological Activity

The lytic activity of polypeptides PG and GcG were established by their ability to decrease substantially the turbidity of suspensions of Micrococcus lysodeikticus (Kidwai and Krishna Murti, 1963). As both polypeptides are practically insoluble, it was necessary to use suspensions of the peptides prepared by dispersing the peptide in buffer (0.2 M Tris, pH 7.4) in a homogeniser. These suspensions were allowed to stand for 15 min., decanted and the residual solid dried and weighed

*Approximation based on U-V absorption of 2,4-dinitrophenyl derivatives.

to assess the amount of polymer suspended in the supernatant, aliquots of which were used for the various experiments.

Both polypeptides degrade the cell wall of Micrococcus lysodeikticus (Salton and Horne, 1951). On the basis of turbidity measurements, suspensions containing 3.0 mg./ml. of PG or 19.0 mg./ml.* of GcG had equivalent activity to a 0.1 mg./ml. solution of thrice crystallised egg-white lysozyme (Sigma Chemical Co.). The estimation of liberated reducing sugars (Park and Johnson, 1949) was complicated by the adherence of the cell wall fragments and ferric ferrocyanide to the polypeptide. Cell wall-PG suspensions were therefore treated with ferricyanide and carbonate-cyanide reagents, centrifuged and the blue colour, developed in the supernatant with ferric iron solution, estimated. On the basis of these estimations, a 6 mg./ml. suspension of PG had the same activity as a 0.1 mg./ml. solution of crystalline lysozyme.

The glycopeptide formed on degradation of Micrococcus lysodeikticus cell wall by PG and by egg-white lysozyme were compared. These were indistinguishable on paper electrophoresis (0.1 M sodium borate-HCl buffer, pH 6.5; 3.8 volts/cm for 18 hr.) and paper chromatography of their 6N HCl hydrolysate revealed the same ninhydrin-positive zones.

GcG is also of interest because of its ability to inhibit the growth of Salmonella typhi.

*Computed value

REFERENCES

Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R., Nature, 206, 757 (1965).
Blout, E.R. and Karlson, R.H., J.Amer.Chem.Soc., 78, 941 (1965).
Dhar, M.M. and Agarwal, K.L., Steroids, 3, 139 (1964).
Johnson, L.N. and Phillips, D.C., Nature, 206, 761 (1965).
Kidwai, A.M. and Krishna Murti, C.K., Indian J.Chem., 1, 177 (1963).
Park, J.T. and Johnson, M.J., J.Biol.Chem., 181, 149 (1949).
Phillips, D.C., Scientific American, 215, 78 (1966).
Salton, M.J.R. and Horne, R.W., Biochim.Biophys.Acta, 7, 177 (1951).
Sela, M. and Berger, A., J.Amer.Chem.Soc., 77, 1893 (1955).