Note

Chemical Modification of
Haim, a Proteinaceous
 α -Amylase InhibitorMotoo ARAI, Naoki OOUCHI,
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The proteinaceous α -amylase inhibitor Haim inhibits animal α -amylase specifically and does not contain any carbohydrate as an integral part of the protein molecule.¹⁾ The inhibition by Haim of animal α -amylase involves protein-protein interaction. This paper presents evidence obtained by chemical modification about which amino acid residues are essential and which are not for the inhibitory activity of Haim.

The assay of Haim and preparation of Haim II were the same as described before.¹⁾

Chemical modification of the amino group of Haim using 2,4,6-trinitrobenzenesulfonic acid (TNBS) was by the method of Haynes *et al.*²⁾ The number of modified residues was calculated from the molar extinction coef-

TABLE I. CHEMICAL MODIFICATION OF CARBOXYL GROUPS IN HAIM WITH EDC AND Gly-OMe

Haim (1.25×10^{-4} M) was reacted with 1.0 M Gly-OMe-HCl and 0~100 mM EDC at 25°C and pH 4.8 under pH control for 1 hr. One ml of the reaction mixture was quenched with 1 ml of 1 M acetate buffer (pH 4.8) and dialysed against distilled water. The dialysate was lyophilized and hydrolyzed with 6 N HCl at 110°C for 24 hr, and subjected to amino acid analysis.

EDC (mM)	Number of residues modified (per mol of Haim)	Residual inhibition (%)
0	0	100
10	6.2	53
20	6.9	37
40	9.9	22
60	10.1	20
80	8.5	13
100	9.9	13

ficient³⁾ at 340 nm to be $14,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The amino group of Haim is an *N*-terminal amino acid residue. It was modified completely by TNBS with no loss of its inhibitory effects on hog pancreatic α -amylase (HPA). Chemical modification of the carboxyl groups of Haim with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and glycine methyl ester (Gly-OMe) was done by the method of Hoare *et al.*⁴⁾ The number of modified residues was calculated from additional covalently bound glycine, measured using an amino acid analyzer. About 10 carboxyl groups could be modified by EDC at concentrations of 40 mM or more, and inhibition remained at 13~22% (Table I). As the reaction was done at 10 mM EDC, about 6 carboxyl groups were modified, and residual inhibition was half the initial level. Modification of Haim with diethylpyrocarbonate (DEP) was done using the method of Rosemont,⁵⁾ and the number of modified histidine residues was calculated from the molar extinction coefficient at 240 nm to be $3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Only one residue in Haim was completely modified by DEP, and its 55% of the initial inhibition was retained. Haim was treated with 1,2-cyclohexanedione (CHD) using the method of Patthy *et al.*⁶⁾ and the number of modified residues cal-

TABLE II. MODIFICATION OF ARGININE RESIDUES IN HAIM WITH CHD

Haim (2.5×10^{-4} M in 0.25 M borate buffer, pH 8) was incubated with 20 or 200 mM CHD at 37°C for 1 hr in the dark. The modified Haim was separated from excess reagent on a Sephadex G-15 column (1.3×15 cm), the eluent being distilled water. The preparation was submitted to amino acid analysis.

CHD (mM)	Number of residues modified (per mol of Haim)	Residual inhibition (%)	Inhibition after treatment with hydroxylamine (%)
20	—	39	94
200	1.24	6	100

—, not determined.

TABLE III. NITRATION OF HAIM WITH TNM

Nitration of Haim (1.3×10^{-4} M) was done in 50 mM Tris-Cl⁻ buffer (pH 8), using 0.02 or 0.1% TNM. The reaction mixture was kept at 25°C for 1 hr and put on a Sephadex G-15 column (1.3×15 cm) equilibrated with the same buffer to remove reagents.

TNM (%)	Number of residues modified (per mol of Haim)	Residual inhibition (%)
0.02	0.62	25
0.1	1.25	1

TABLE IV. CHEMICAL MODIFICATION OF THE TRYPTOPHAN RESIDUE IN HAIM WITH NBS AND WITH MHNBS-Br

Oxidation with NBS was done in 50 mm acetate buffer, pH 5. The initial Haim concentration was 5×10^{-5} M, and 10 μ l of a 3 mM solution of NBS was added each time. Chemical modification with MHNBS-Br was done in 50 mm acetate buffer, pH 5.3. A 6.3×10^{-5} M solution of Haim was incubated with 30 mM MHNBS-Br at 30°C for 1 or 2 hr. The reaction mixture was put on a Sephadex G-15 column (1.3 $\times$ 15 cm) equilibrated with the same buffer.

NBS		MHNBS-Br	
Number of residues modified (per mol of Haim)	Residual inhibition (%)	Number of residues modified (per mol of Haim)	Residual inhibition (%)
0.63	8	0.86	15
0.89	1	1.1	0

culated from the amino acid analysis data. The results of this reaction with CHD are in Table II. When about one residue of the three arginine residues in Haim was modified, inhibition decreased strongly. After hydroxylamine treatment of Haim modified with CHD, the inhibitory activity of Haim was almost restored to the initial level. Nitration of Haim with tetranitromethane (TNM) was done by the procedure of Riordan *et al.*,⁷⁾ and the number of tyrosyl-bound nitro groups was determined by the method of Goto *et al.*⁸⁾ The results are shown in Table III. When one of four mol of tyrosine residues in Haim was modified with TNM, the inhibition of Haim decreased to below 10% of the control. Haim was incubated with *N*-bromosuccinimide (NBS) by the method of Spande *et al.*,⁹⁾ and calculation of the number of oxidized tryptophan residues was also done using their method. For chemical modification of Haim with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide (MHNBS-Br) and calculation of the number of modified tryptophan residues, we followed the procedure of Naik and Horton.¹⁰⁾ Results with NBS and MHNBS-Br are in Table IV. The tryptophan residue of Haim was almost completely modified by both procedures, and the inhibitory effects of Haim nearly vanished.

These results show that at least one each of arginine, tyrosine, and tryptophan residues are essential for inhibition by Haim of HPA. Wheat α -amylase inhibitors are inactivated by treatment with fluorodinitrobenzene or with nitrous acid.¹¹⁾ Amino group(s), methionine residue(s), and tyrosine residue(s) of the wheat amylase inhibitor AmI₂ are important for its inhibition.¹¹⁾ Chemical modification by TNBS of an α -amylase/trypsin inhibitor from the cereal grass ragi completely abolished amylase inhibition, but modification with CHD gave no detectable loss.¹²⁾ Vertesy *et al.*¹³⁾ reported that modification of amino groups in Tendamistat from *Streptomyces tendae* does not cause loss of inhibitory activity. These

results show clearly that functional groups essential to the activity of Haim are different from those in plant amylase inhibitors.

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