Reaction of Trypsin with S-Methylisothiourea and with S-Methylglucosylisothiourea

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O-Methylisourea has long been used to introduce amidyl residue into amino acids and peptides. The reaction of *O*-methylisourea with proteins takes place only in ε-amino group of lysine, and homoarginine is produced by the hydrolysis of the product[1]. Hughes et al. have shown that in treating serum albumin with *O*-methylisourea, the more the value of Sakaguchi's reaction increased, the less amino nitrogen became[2]. Roche and his associates treated bovine serum albumin, casein, and ovalbumin etc. with *O*-methylisourea in concentrated ammonium hydroxide, and after the hydrolysis of the reaction products, they identified homoarginine by means of paper chromatography[3]. Lately Chervenka et al. demonstrated that all of the 13 lysines changed into homoarginine and no more changes were caused in the reaction of chymotrypsin with

1) R. M. Herriott, "Advances in Protein Chem.", III, Acad. Press Inc., New York (1947), p. 215.

2) W. L. Hughes, H. A. Saroff and A. L. Carney, *J. Am. Chem. Soc.*, **71**, 2476 (1949).

3) J. Roche, M. Mourgue and R. Baret, *Bull. Soc. chim. biol.*, **36**, 85 (1954).

O-methylisourea[4]. On the other hand, Schütte employed *S*-methylisothiourea instead of *O*-methylisourea[5]. Later on, this reaction was applied to the δ-amino group of ornithine[6].

By the application of a similar reaction, that is, the condensation reaction which liberates mercaptan from substituted isothiourea, Micheel et al. have treated case in and gelatin etc. with *S*-methylglucosylisothiourea. They supposed that the ε-amino group of lysine undergoes the reaction, since the amount of combined sugar runs parallelly only to the lysine content of protein[7].

It has already been illustrated that concerning the reaction of *S*-methylglucosylisothiourea upon histidine, the carbon of position 4 or 2 in the imidazole ring is more reactive than the α-amino group of amino acids or the ε-amino group of lysine[8].

Authors have undertaken the study of the reaction of *S*-methylglucosylisothiourea and trypsin as an enzyme, as it has already been suggested that histidine is an essential site of the enzymatic activity[9].

In respect to trypsin, various active and inactive derivatives have already been reported[10-16], but no work on the reaction of *S*-methylisothiourea and *S*-methylglucosylisothiourea has been reported yet.

The results showed that trypsin molecule[17] was combined with 6~7 mol. of glucosylamidyl residue, and yet the enzymatic activity was retained, but the autodigestion was remarkably depressed. The amidinated trypsin was more stable than the glucosylamidyl derivative. These facts described in this report were not owing to the mercaptan produced by the reaction, but evidently owing to glucosylamidination or amidination. The mercaptan produced during the reaction had no part in the stabilization of trypsin, which fact is consistent with the result obtained by using 2, 3-dimercapto-1-propanol[18].

Experimental

Amidination and Glucosylamidination of Trypsin. — One hundred milligrams of crystalline trypsin (Motida Pharm. Co.) was dissolved in 20 ml. of 1/15 M phosphate buffer, pH 8.0 (or Palitzsch's borate buffer) in the presence of 0.01 M calcium chloride[12,19]. The protein concentration of trypsin was determined spectrophotometrically, using values proposed by Green and Neurath[20]. Thus, enzyme solution was 0.695 mg./ml. Five milliliters of this enzyme solution was added to 3 ml. of aqueous solution of *S*-methylglucosylisothiourea hydroiodide (70 mg./ml.) and to the equal volume of *S*-methylisothiourea (50 mg./ml.) respectively. Then the reaction mixture was adjusted to pH 8 with a few ml. of 0.1 N sodium hydroxide, and covered with ether to dissolve mercaptan evolving. The solutions were allowed to stand at 10°C.

Determination of Combined Glucosylamidyl Residue.—To 1 ml. of the solution described above, which was taken out at certain time intervals, 5 ml. of 0.4 M solution of trichloroacetic acid (TCA) was added, and the mixture was warmed for a while at 50°C. The enzyme derivative precipitated was collected by centrifuge, then washed several times with TCA in order to remove the sugar compound which did not react. As for the precipitate, the

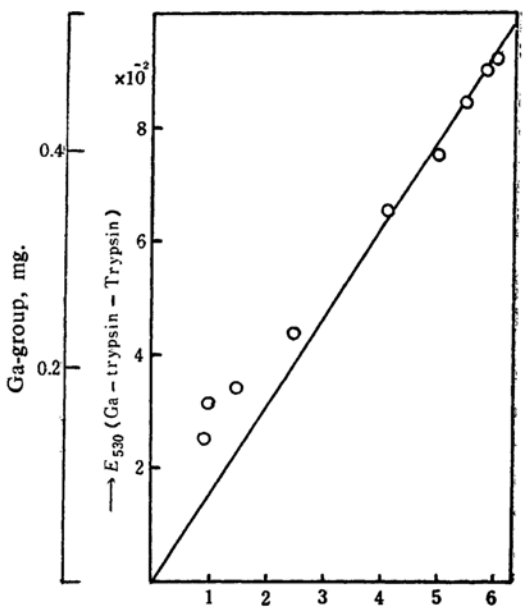

Fig. 1. Estimation of the Ga-group combined with enzyme.

4) C. H. Chervenka and P. E. Wilcox, *J. Biol. Chem.*, **222**, 621, 635 (1956).
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combined glucosylamidyl residue was determined by the modified Brückner's method[21] as follows: Namely, 1 ml. of water and 1 ml. of 4% aqueous solution of orcinol were added to the resulting precipitate of enzyme-derivative. Then, 8 ml. of 31.2 N sulfuric acid, cooled with cold water, was added to it. After mixing them completely, it was heated in a boiling water bath exactly for 5 min., then cooled with tap water. The absorbance of the solution was measured at 530 mμ, using a whole blank solution which contains the same concentration of untreated enzyme and which has been treated similarly in the reference cell. The value of the combined glucosylamidyl residue was calculated by multiplying the molecular ratio for the value obtained from the estimation of glucosylamidyl glycine.

Measurement of the Activity.—One half milliliter of the reaction mixture was transferred into 10 ml. of 1/15 M phosphate buffer, pH 7.1 at certain time intervals. By using 1 ml. of this diluted solution, the enzymatic activity was measured according to the modified Anson's method by using casein as substrate[22].

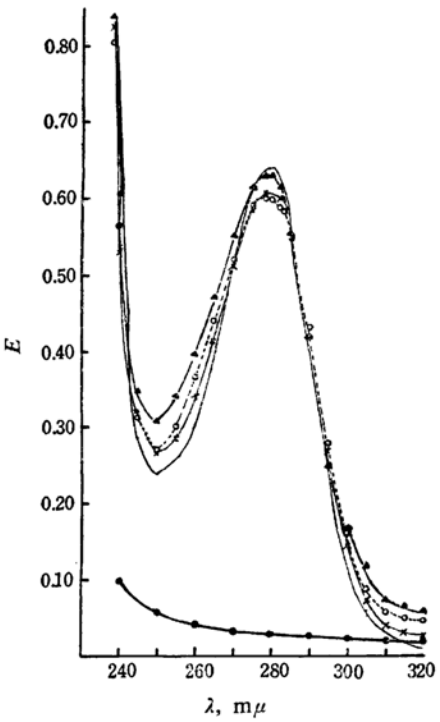

Fig. 2. UV-absorption spectrum of Ga-trypsin.
——— Trypsin
×—× Ga-trypsin
○···○ Ga-trypsin heated for 10 min. at 100°C
▲—▲ Ga-trypsin heated for 60 min. at 100°C
●—● Ga-glycine

Determination of the Guanidino-group and Amino Nitrogen.—The guanidino-group was determined by Sakaguchi's reaction. The sample was treated in the same manner as described in the determination of glucosylamidyl residue, because the Sakaguchi's reaction was inhibited by *S*-methylisothiourea and *S*-methylglucosylisothiourea. Namely, 0.5 ml. from the reaction mixture described above was pipetted out and the enzyme was precipitated by adding TCA solution and washed several times with TCA solution, then further washed with absolute alcohol. The precipitate was dissolved in 0.1 N sodium hydroxide and the guanidino-group was determined by Weber's method[24].

The amino group of the precipitate which was prepared by the same method as in the determination of glucosylamidyl residue was determined by ninhydrin reaction according to the method of Troll and Cannan[23].

Absorption Spectrum and Optical Rotation.—The sample for measurement of absorption spectrum was prepared by exhaustive dialysis against water of pH 3 (with hydrochloric acid). The optical rotation was determined by using the sample prepared as follows. A large amount of the reaction mixture was brought to pH 3.4 with hydrochloric acid, and precipitated at 70 per cent saturation of ammonium sulfate and washed several times with 70% ammonium sulfate. The precipitate was dissolved in water.

$$[\alpha]_D^{25}=\frac{+0.16\times 6000}{35\times 0.5\times 1.058}=+51.8^\circ$$

Table I. Reaction of *S*-methylglucosylisothiourea and trypsin

Reaction time	Combined sugar component, mg. / mol. protein	Relative activity %	NH_2-group mol. / mol. protein
24 hr.	3.3	89	7.86
48 〃	4.6	78	
72 〃	5.7	71	
96 〃	6.0	65	6.76
6 days	6.3	58	
14 〃	6.5	53	6.07
20 〃	6.5	53	

Table II. Reaction of *S*-methylisothiourea and trypsin

Reaction time	Increase of amidine group %	Relative activity %	Relative activity (with 0.01 M $CaCl_2$)	NH_2-group mol. / mol. protein
24 hr.	115	95	78	7.86
48 〃		94	68	
72 〃		94	57	
96 〃	190	93	49	6.92
193 〃	220	93	43	
15 days		92	38	

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Table III. Enzymatic hydrolysis of glucosylamidyl casein by glucosylamidyl trypsin or amidyl trypsin*

Substrate	Enzyme		
	Trypsin (with 0.01 M) $CaCl_2$, %	Glucosylamidyl trypsin	Amidyl trypsin
Casein	82	93	100
Glucosylamidyl casein	70	79	93

* Enzyme preparation of trypsin and the two trypsin derivatives were taken from the incubating mixture in 24 hr. from starting.

Results and Discussion

The results are shown in Tables I to III. Under the condition presented, trypsin (mol. wt. 23800)[15,16] was combined with 6~7 mol. of glucosylamidyl residue in 4 days. As the result, the color intensity of ninhydrin reaction decreased about 14 per cent. The Sakaguchi's reaction has no change by being combined with glucosylamidyl residue. By the reaction of trypsin and *S*-methylisothiourea, the guanidino group of enzyme derivative clearly increases.

By glucosylamidination or amidination of trypsin, the enzyme does not suffer from the loss of the enzymatic activity, but becomes rather stable against the autodigestion. It is well known that peptide linkage which involves α-amino group of lysine becomes less easily digested by trypsin, by blocking the ε-amino group of the substrate with acetyl group and others[25,26]. From the result reported here, it is proved that glucosylamidyl casein and amidyl casein are resistive to the tryptic digestion, in accordance with a report of Weil et al.[26]

On the other hand, amidyl trypsin and glucosylamidyl trypsin have enzymatic activity, but it seems likely that homoarginine or glucosylhomoarginine linkage which exists in the modified enzyme molecule is not digested by trypsin and the modified trypsins.

It is probably deduced that ε-amino group of lysine and other groups which are to be glucosylamidinated or amidinated have nothing to do with the tryptic activity.

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