Fluorometric Determination of Homopolymeric Peptides with 5-(*N*,*N*-Dimethylamino)naphthalene-1-sulfinic Acid after *N*-Chlorination

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A new fluorometric method of determination for oligopeptides based on the reaction of peptide bonds has been developed. The N-H groups in peptide bonds were chlorinated with *tert*-butyl hypochlorite, and the resulting N-Cl peptides were reacted with 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfinic acid. The product, 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfonyl chloride, which was formed quantitatively from N-Cl peptides, was extracted with chloroform, and the extract was hydrolyzed to give a highly fluorescent 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfonic acid. In the proposed method, the fluorescence intensity obtained from several glycine oligomers (di-, tri-, tetra-, penta- and hexa-glycine) increased in proportion to the concentration of peptides and thus in proportion to the number of peptide bonds. Similar results were obtained with alanine oligomers (di- to hexa-alanine).

Keywords peptide; peptide bond; *N*-chlorination; *tert*-butyl hypochlorite; 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfinic acid; 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfonic acid; fluorometric determination; glycine oligomer; alanine oligomer

For the quantitative estimation of peptides and proteins, relative values with respect to a certain standard protein have often been employed instead of measuring each protein directly. In this case, the result may be subject to unavoidable variation if the methods of determination[1-5] are based on the chemical reaction of specific amino acid residues or free amino and/or carboxyl groups because of the variation of the contents of these functional groups in various peptides and proteins.

It is a purpose of this paper to describe a new fluorometric method of determination of oligopeptides based on the reaction of a common chemical entity of proteins, *i.e.*, peptide bonds, using *tert*-butyl hypochlorite (TBH) and 5-(*N*,*N*-dimethylamino)naphthalene-1-sulfinic acid (DNS-H).

Experimental

Materials 5-(*N*,*N*-Dimethylamino)naphthalene-1-sulfonyl chloride (DNS-Cl) and TBH were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo. DNS-H was synthesized from DNS-Cl according to the method of Scully *et al.*[6] Di-, tri- and tetra-glycine were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Penta- and hexa-glycine and other peptides were from Sigma Chemicals Co. Other chemicals were of the highest grade available commercially.

Instrument Fluorescence intensity was measured with a Hitachi F-3000 fluorescence spectrophotometer.

Procedure One-tenth milliliter of a sample solution containing 0.02—0.5 μmol of peptide in 50% acetic acid in a small test tube was mixed with 0.05 ml of 10% TBH reagent in acetic acid–dichloroethane (7:3, v/v). The reaction mixture was evaporated to dryness after 30 min at 40 °C and 0.1 ml of 1 mM DNS-H reagent in 5 mM $NaHCO_3$ was added to the residue. The resulting DNS-Cl was dissolved in 0.5 ml of chloroform and the solution was poured onto an Extrelut (Merck) column (7 mm i. d. × 30 mm) which had been moistened with 0.2 ml of 0.5% $NaHCO_3$ prior to sample application. The test tube was washed twice with 0.5 ml of chloroform, and the washings and 2 ml of chloroform were poured onto the column to elute the DNS-Cl thoroughly. The eluate was evaporated and the DNS-Cl was hydrolyzed to DNS-OH with 5 ml of 0.01 M NaOH solution in a boiling water bath for 10 min. After cooling, the fluorescence intensity (FI) was measured at excitation and emission wavelengths of 320 and 502 nm, respectively. Background was measured by the same procedure using 0.1 ml of 50% acetic acid instead of a sample solution.

Results and Discussion

Scully *et al.*[6] used DNS-H for the determination of *N*-chloramines. Application of this method for the determination of oligopeptides has been attempted by chlorinating peptides with active chlorine, followed by the reaction of the resulting N-Cl peptides with DNS-H to give DNS-peptides (Chart 1). However, it was found that DNS-peptide was not formed from di-glycine after chlorination of the peptide followed by treatment of the resulting N-Cl di-glycine with DNS-H, but DNS-Cl was formed quantitatively instead. Therefore, the DNS-Cl thus formed was extracted with chloroform, and the extract was hydrolyzed to DNS-OH. It was shown that the FI of DNS-OH increased in proportion to the concentration of di-glycine, which indicates that the method could be applicable to the determination of peptides and proteins as depicted in Chart 1.

One percent TBH reagent, used by Kimura *et al.*[7] for the chlorination of peptides, was found to be insufficient to chlorinate peptide bonds of various glycine oligomers (from di- to hexa-glycine). Thus, the reaction conditions were reexamined employing hexa-glycine, which was the least reactive among the glycine oligomers. The TBH concentration of 10% was chosen from the viewpoints of the efficiency of chlorination and the time required to remove excess TBH. As for the reaction temperature for chlorination with 10% TBH, it was found that, the fluctuation of FI developed from hexa-glycine at temperatures higher than 40 °C tended to become too large for quantitative determination although the higher temperature gave a higher rate of reaction. The time course was studied at 40 °C with 10% TBH reagent. As FI reached its maximum

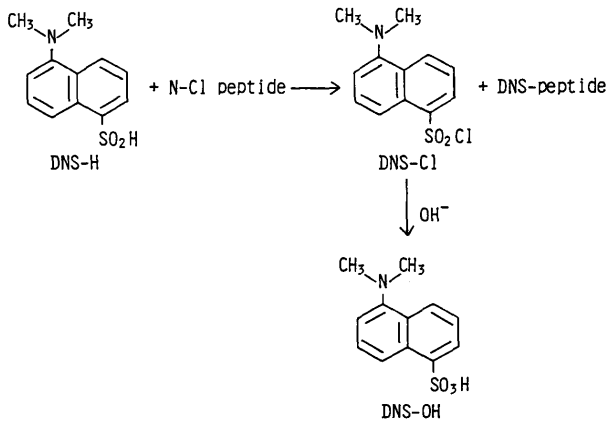

Chart 1

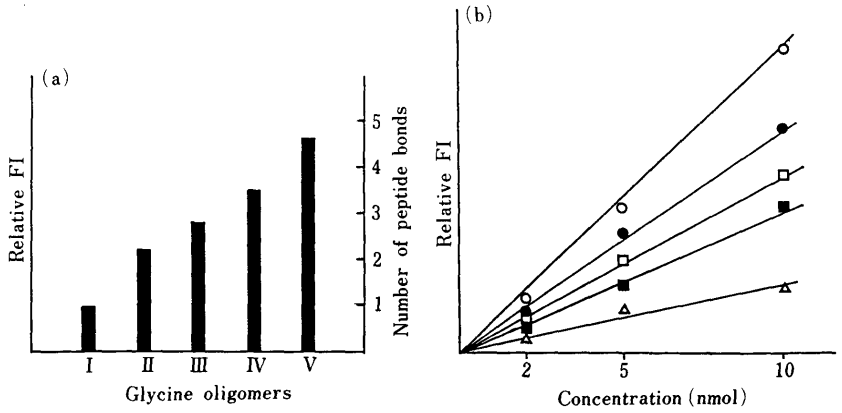

Fig. 1. Relative FI (a) and FI (b) Developed from Glycine Oligomers

(a) I, Gly-Gly; II, Gly-Gly-Gly; III, Gly-Gly-Gly-Gly; IV, Gly-Gly-Gly-Gly-Gly; V, Gly-Gly-Gly-Gly-Gly-Gly. (b) △, Gly-Gly; ■, Gly-Gly-Gly; □, Gly-Gly-Gly-Gly; ●, Gly-Gly-Gly-Gly-Gly; ○, Gly-Gly-Gly-Gly-Gly-Gly.

after 25 min, 40 °C and 30 min were chosen for the chlorination of peptides in the standard assay procedure.

As N-Cl peptides reacted readily with DNS-H to form DNS-Cl, the DNS-Cl was extracted immediately by using an Extrelut column. Recoveries of DNS-Cl from an Extrelut column and conditions for hydrolysis of the extracts were examined with authentic DNS-Cl (50 nmol). A volume of 3 ml of chloroform was used as an eluent in the standard procedure as the average recovery of DNS-Cl from the Extrelut column was 97.3% when 1.5 to 4 ml of chloroform was used as the eluent. Hydrolysis of the extracts was performed in 5 ml of 0.01 M NaOH in a boiling water bath for 10 min as the FI reached a plateau in 10 min when hydrolysis was examined with authentic DNS-Cl.

When FI's were measured for glycine oligomers (2—10 nmol), they increased in proportion to the concentration of the oligomers, and so to the number of peptide bonds (Fig. 1a, b). In addition, FI obtained from di-glycine was linearly related to concentration in the range of 2—50 nmol, and the coefficient of variation at 20 nmol ($n=5$) was 1.68%.

As for other homooligopeptides, it was found that there were some variations in reactivity under the same conditions as those for glycine oligomers. There was a tendency that peptide bonds involving amino acids with bulky side chains required more vigorous conditions. For example, when alanine oligomers were chlorinated under the conditions of the standard assay procedure except for a reaction time of 3 h instead of 30 min, FI's of alanine oligomers increased linearly in proportion to the number of peptide bonds as in the case of glycine oligomers (Fig. 2). Furthermore, the FI of even the least reactive peptide, penta-phenylalanine, increased gradually on prolonged reaction with TBH for chlorination (data not shown).

Kinoshita *et al.*[8] reported a fluorometric determination of proteins based on a similar principle, using thiamine after *N*-chlorination of peptide bonds (thiamine method).

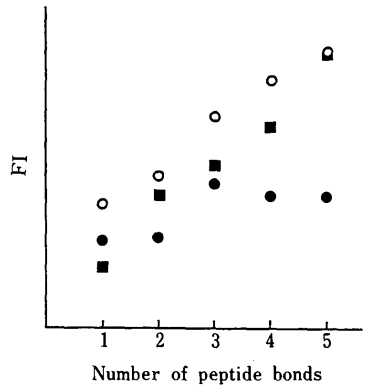

Fig. 2. FI Developed from Alanine and Glycine Oligomers

○, alanine oligomers, 40 °C, 3 h, 10% TBH reagent; ●, alanine oligomers, 40 °C, 30 min, 10% TBH reagent; □, glycine oligomers, 40 °C, 30 min, 10% TBH reagent.

As 0.5 μg of protein can be determined by the thiamine method and 0.26 μg of di-glycine by the proposed method, it appears that the sensitivities of both methods are in the same range.

References and Notes

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