employ the peak height procedure both for peptides and proteins. The use of peak height provides a simpler approach to the determination of concentration of components of such chromatographic effluents without loss of precision.

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Colorimetric detection of peptides with *tert.*-butyl hypochlorite and potassium iodide

The detection of peptides on paper chromatograms by exposure to chlorine gas to form the labile N-chlorinated derivatives of peptide bonds has been proposed by RYDON AND SMITH[1]. Recently, MAZUR, ELLIS AND CAMMERATA have used a solution of *tert.*-butyl hypochlorite in cyclohexane instead of chlorine gas[2]. The application of starch–iodide or *o*-tolidine–iodide to chlorinated peptides on a paper chromatogram reveals the peptides as blue black spots[1–5]. This is due to the liberation of iodine from the potassium iodide and the reaction of the starch or *o*-tolidine with iodine. The spectrophotometric measurement of the triiodide ion is known as a sensitive method for the determination of iodine[6].

In this paper, the chlorination of peptides with *tert.*-butyl hypochlorite, followed by the colorimetric measurement of the triiodide ion which is formed from the potassium iodide solution, is adopted for the monitoring of peptides eluted from a chromatographic column with a volatile buffer system.

Materials

Egg white lysozyme (6 × recrystallized) and *tert.*-butyl hypochlorite were standard commercial products (Seikagaku Kogyo Co., Tokyo). All chemicals were reagent grade and used without further purification. A tryptic digest of carboxymethylated lysozyme was prepared according to the method of CANFIELD AND ANFINSEN[7]. The peptides were a gift from Dr. N. IZUMIYA.

Colorimetric measurement

The peptide solution, in glacial acetic acid or in 50% acetic acid, and the aliquots of the effluent fractions obtained from the column were completely evaporated in a vacuum desiccator. 0.1 ml of 1% *tert.*-butyl hypochlorite in acetic acid–dichloroethane (1:9, v/v) was added to the dried sample, and the reaction mixture was evaporated immediately. A mixture of 0.5 ml of glacial acetic acid and 3.0 ml of 0.05 *M* KI was added to the chlorinated peptides in a test tube. After standing for 30 min, the optical density of the reaction mixture at 355 mμ was measured against the blank, a mixture of 0.5 ml of glacial acetic acid and 3.0 ml of 0.05 *M* KI.

Results and discussion

Two typical peptide maps of a tryptic digest of carboxymethylated lysozyme were obtained by both the ninhydrin reaction and the peptide reagent method, as shown in Fig. 1. It was apparent from Fig. 1 that the map by the latter method was

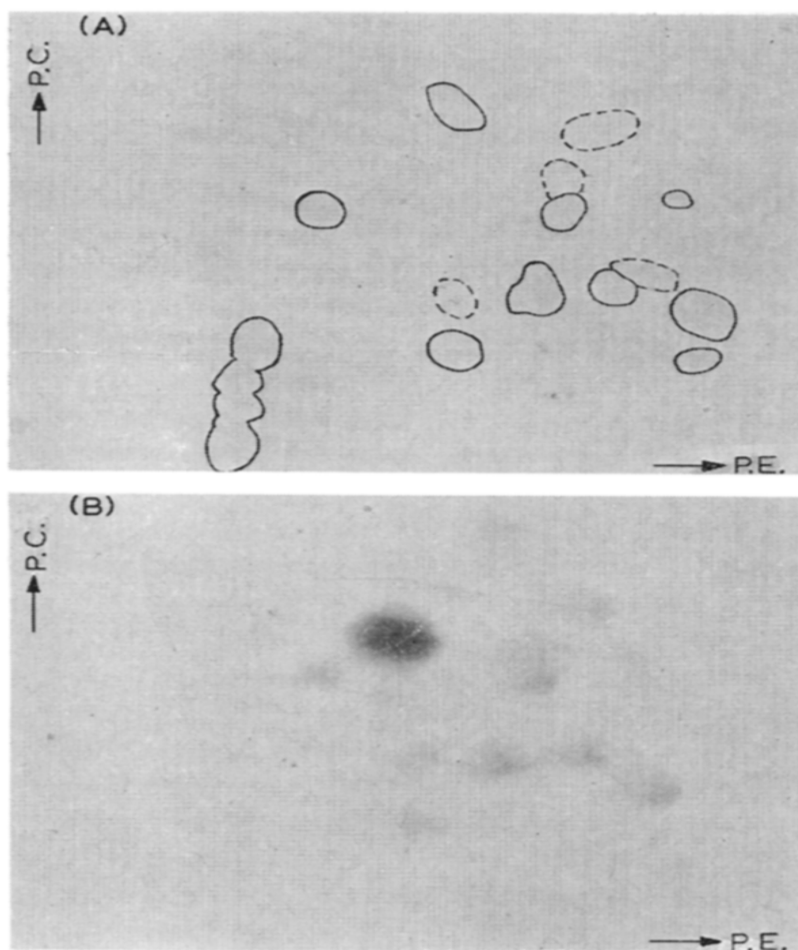

Fig. 1. Peptide maps of a tryptic digest of carboxymethylated lysozyme. Sample: 0.025 μmoles of carboxymethylated lysozyme. Solvent for paper chromatography: *n*-butanol–pyridine–H_2O (1:1:1). Solvent and conditions for paper electrophoresis: 0.04 *M* ammonium carbonate, 2,000 V and 60 min. Map (A): color was developed by spraying with 0.1% ninhydrin in water saturated *n*-butanol, followed by aeration at 50°. Map (B): color was developed by spraying with 1% *tert.*-butyl hypochlorite in acetic acid–dichloroethane (1:9) followed by aeration for 30 min at 50° and then by dipping the paper in the mixture of *o*-tolidine saturated in 2% acetic acid and 0.05 *M* KI (equal volume).

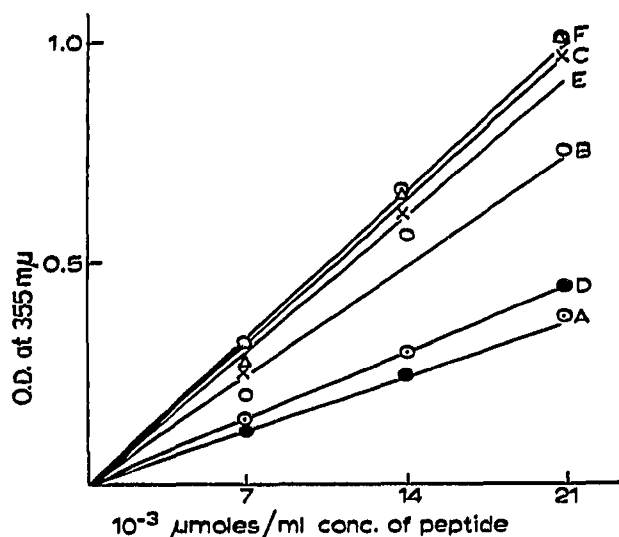

Fig. 2. Colorimetric determination of peptides by chlorination and liberation of the triiodide ion. Peptides: (A) = Gly–Gly; (B) = Gly–Gly–Gly; (C) = Gly–Gly–Gly–Gly; (D) = L-Tyr–Gly–Gly; (E) = Gly–L-Tyr–Gly–Gly; (F) = Gly–L-Tyr–L-Tyr–Gly. For assays: see text.

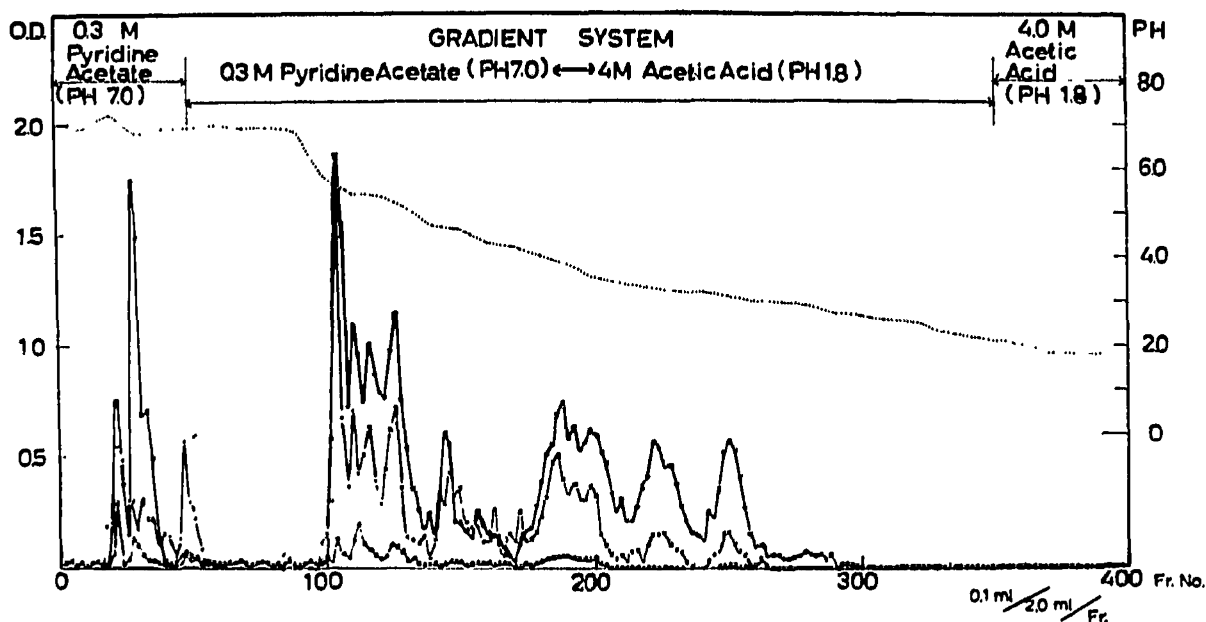

Fig. 3. Elution profile of a tryptic digest of carboxymethylated lysozyme. Sample (3.6 μmoles as protein) was chromatographed on Dowex-1 (2X, 200–400 mesh). The column (0.9 × 150 cm) was run with a gradient of 4*N* acetic acid running into a mixing chamber containing 300 ml of 0.3 *M* pyridine–acetate buffer, pH 7.0. The elution was carried out at a flow rate of 40 ml per h at room temperature and each 2.0 ml fraction was collected into a test tube. Aliquots of the effluent fractions were monitored by the direct ninhydrin method (0.5 ml) (▲-▲-▲); the ninhydrin method after alkaline hydrolysis (0.1 ml) (×-×-×); and the peptide reagent method (0.1 ml) (●-●-●).

more clear-cut for the detection of peptide spots than that of the former. Some spots were hardly detectable by the peptide reagent, but they were distinctly seen when the color was developed by the ninhydrin reaction. These spots corresponded to free amino acids, such as lysine, arginine and leucine, which could be theoretically liberated by the tryptic digestion of carboxymethylated lysozyme.

The triiodide ion formed from the chlorinated peptide in 0.05 *M* KI solution gave an absorption spectrum with maxima near 290 mμ and 355 mμ (*cf.* ref. 6). It was evident from Fig. 2 that the absorbance at 355 mμ *versus* the concentration of peptide followed Beer's law. The absorbances obtained from di-, tri- and tetra-glycine bore a certain linear relationship to the number of peptide bonds. However, it was difficult to calculate exactly the number of peptide bonds by colorimetric measurements with the peptide reagent, because the chlorination of peptide bonds and the liberation of triiodide ion from the chlorinated peptide might be effected by the kind of amino acid residues which composed the peptide bonds. Furthermore, some amino acids, such as tyrosine, histidine and proline, partially react with *tert.*-butyl hypochlorite.

Chromatographic elution patterns of a tryptic digest of carboxymethylated lysozyme obtained by the direct ninhydrin method[8], by the ninhydrin method after alkaline hydrolysis[9] and by the peptide reagent method, are illustrated in Fig. 3. These results showed that the the colorimetric measurements with the peptide reagent were useful for the monitoring of peptides and proteins eluted from a chromatographic column with a volatile buffer system.

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