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Dual-times Detection System for On-line Assay of Enzymatic Activity Based on Valve-switching Technique Using Size Exclusion Chromatography

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A dual-times detection system in size exclusion chromatography was developed for carboxypeptidase A assay. In the system the enzymatic activity was detected 2 times by circulating the sample solution (mobile phase) in a reaction tube through a fluorescence detector.

Assay of the enzymes by size exclusion chromatography (SEC) is useful for characterizing the enzymes because it gives the information of molecular weights (MW) to help identification of the enzymes of interest. In such studies, enzymatic activity is preferably measured by a sensitive and selective detection method for the enzymes.

In the measurement of enzymatic activity by conventional liquid chromatography, a batch method is generally used and the activity is measured for each fraction by spectrophotometry or spectrofluorometry after incubation with the proper substrate. On the other hand, in high performance liquid chromatography (HPLC) such a batch method is not convenient because it is difficult to collect many fractions at every short time-intervals, followed by the detection of enzymatic activity for each fraction manually. Thus an on-line post-column detection of enzymatic activity has been usually employed in enzyme assay.[1-6] In the HPLC chromatograms obtained by the on-line post-column detection, however, many interfering peaks are usually observed, which sometimes makes it difficult to identify the peaks of the analyte enzymes. It is thus required to explore some additional methods for the confirmation whether the peaks in the chromatogram are arisen from the enzyme of interest or not. One of the ideas for such a confirmation of the chromatographic peaks due to the enzymes is that the enzymatic activity is detected at least 2-times at a reasonable time-interval during incubation with the substrate, and if any increase of the peak intensity is observed, it can be attributed to the enzyme. Fulton et al. reported a convenient assay

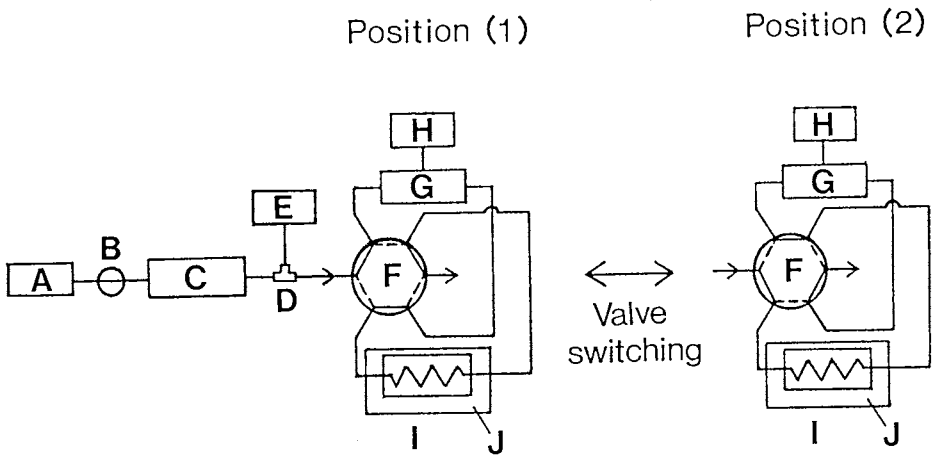

Fig. 1. Schematic diagram and manifold of the instrumental system for the detection of enzymatic activity in on-line SEC.
A: Pump (Eluent), B: Sample injector, C: Separation column, D: T-tube, E: Pump (Substrate solution), F: Six-port valve, G: Fluorescence detector, H: Recorder, I: Reaction tube, J: Thermostat.

system with two detectors and a computer, with which the enzymatic activity was automatically detected 2-times and observed as the increase of the peak intensity.[7] In the present work, we have developed a versatile system for the on-line postcolumn measurement of enzymatic activity based on the valve-switching technique.

The schematic diagram of the instrumental system is shown in Fig. 1. An HPLC system (FPLC from Pharmacia-LKB, Bromma, Sweden) was used, which was assembled from a LCC-500 plus controller (Pharmacia-LKB), an 821-FP spectrofluorometer (JASCO, Tokyo) and a VL-611 six-way valve (JASCO). A SEC column of TSK G3000SW (Toso, Tokyo) with the permeation limit in the range of 300000 - 1000 MW was employed for size exclusion separation. An MH-20B pH meter (TOA, Tokyo) was used with a GST-155C pH electrode (TOA). In the enzymatic activity measurement, 0.01 mM (M = mol/dm^3) dansylglycyl-L-tryptophan (DNS-Gly-Trp) dissolved in a Tris buffer solution (0.05 M Tris/1 M NaCl/HCl buffer solution of pH 7.6) was used as the substrate. Carboxypeptidase (CPase) A obtained from bovine pancrease (ICN Biochemicals, Cleveland, OH, USA, Cat. No. 100403), which was dissolved in a Tris buffer solution, was examined for enzyme assay. Other chemicals and solvents were of analytical or chromatographic grade, if not stated.

Purified water was prepared by a Milli Q deionization system (Millipore Kogyo, Tokyo).

The dual-times detection of enzymatic activity in SEC was performed by using the instrumental system shown in Fig. 1. The sample was separated by the SEC column at a flow rate of 0.3 ml/min. The substrate (DNS-Gly-Trp) solution was mixed into the column effluent through a T-tube connected at the end of the column. The flow rates of the eluent and substrate solutions were both 0.3 ml/min (thus 0.6 ml/min in a reaction tube). Just after the column outlet the effluent was once monitored by a fluorescence detector (Ex 283 nm, Em 367 nm)[8] before the enzymatic reaction proceeded appreciably. Then the eluate entered into a PTFE reaction tube of 0.5 mm i.d. x ca. 70 m length, which had the volume so as enough that all the eluate eluted in the time-interval of the exclusion and permeation limit of the SEC column could be stored. By switching the six-port valve from position(1) to position(2) in Fig. 1 at adequate time, the flow of the eluate was again introducted into the fluorescence detector without changing the flow direction. In this subsequent measurement, the chromatogram after the reaction time of 19-26 min was

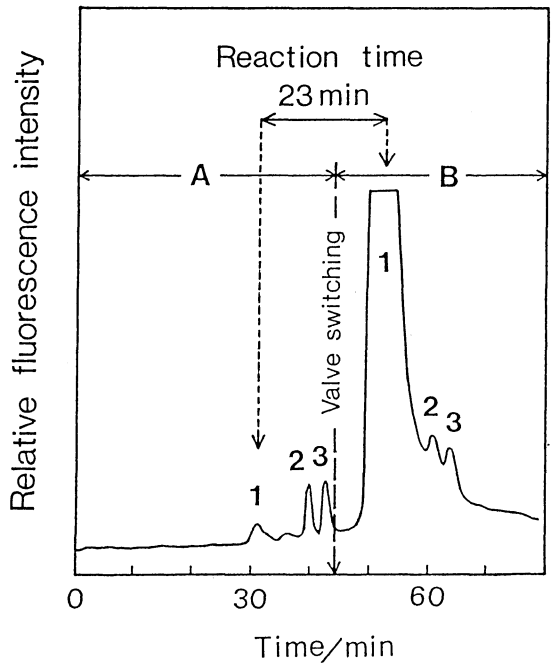

Fig. 2. SEC chromatogram of carboxypeptidase A measured by the dual-times detection system.
A: Detection range before incubation,
B: Detection range after on-line incubation.
1: Carboxypeptidase A, 2,3: Impurities.

obtained, where the fluorescence of triptophan was markedly detected at the above excitation and emission wavelengths.

As an example, the enzymatic activity of the CPase A was analyzed by the system developed in the present work. The chromatogram observed is shown in Fig. 2. By comparing the chromatograms in the regions A and B in Fig. 2, the intensity of Peak 1 increased significantly after the time interval of 23 min, while no appreciable difference was observed for Peaks 2 and 3. It is concluded from these experimental results that Peak 1 can be attributed to CPase and Peaks 2 and 3 to the impurities. In order to confirm the present conclusion, further experiment was performed as follows. The sample was incubated at 85 ℃ for 20 min, and it was injected into the on-line SEC system shown in Fig. 1. In consequence, Peak 1 disappeared due to denaturation of enzyme protein, and no change occurred for Peaks 2 and 3 in the chromatogram. This result indicates that Peak 1 shown in Fig. 2 can be assigned to CPase. As described above, it is noted that the dual-times detection system based on the valve-switching technique developed in the present work is useful for rapid and versatile enzyme assay.

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