Short Communication

New Enzymatic Colorimetric Analyses to Evaluate the Activities of Carboxypeptidase A and B

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We have already reported the new enzymatic colorimetric reactions of benzoic acid derivatives,^{1,2)} which exploited the oxidative coupling of benzoic acid derivatives with a coupling reagent, 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) or 4-aminoantipyrine, in the presence of laccase. We also applied the laccase-catalyzed colorimetric reaction to a quantitative analysis of the activity of an angiotensin I converting enzyme,^{3,4)} and to the screenig of carboxypeptidase,⁵⁾ under the conditions of coexistence with *N*^α-benzyloxycarbonyl amino acid urethane hydrolase I (urethane hydrolase I).

In this paper, we present new enzymatic colorimetric analyses to evaluate the activities of carboxypeptidase A and B. These analytical methods each consist of three reaction steps:

(4-OH)Bz-Gly-Phe

(4-OH)Bz-Gly

(4-OH)BzOH

(4-OH)Bz-Gly-Arg

(4-OH)Bz-Gly

(4-OH)BzOH

The enzymes acted on the substrates to yield (4-OH)Bz-Gly and Phe or Arg. The former was then hydrolyzed in the presence of urethane hydrolase I to produce (4-OH)BzOH and Gly. Finally, oxidative coupling of (4-OH)BzOH with 4-aminoantipyrine was catalyzed by laccase, producing a red-colored compound, the concentration of which was measured at its absorbance maximum at 505 nm to evaluate the activity of carboxypeptidase A or B.

(4-OH)Bz-Gly-Phe and (4-OH)Bz-Gly-Arg were synthesized and used as substrates. Urethane hydrolase I from *streptococcus faecalis* RATCC 8043 was purified by the method described in the previous paper.⁶⁾ Laccase of *Polyporus versicolor* IFO 9791 was purified according to the method of Fåhræus and Reinhammer.⁷⁾ Carboxypeptidase A (from bovine pancreas, 60 units/mg of protein) and carboxypeptidase B (from porcine pancreas, 130 units/mg of protein) were obtained from Sigma. The activity of carboxypeptidase A was

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Abbreviations: (4-OH)Bz-, 4-hydroxybenzoyl; (4-OH)BzOH, 4-hydroxybenzoic acid. The abbreviated designations of amino acid derivatives, peptides and their derivatives obey the rules of the IUPAC-IUB Commission on Biochemical Nomenclature. The substrates used here contained amino acids of L-configuration.

estimated in 2.5 ml of 0.1 M sodium phosphate buffer at pH 6.0, containing $10\text{ }\mu\text{mol}$ of (4-OH)Bz-Gly-Phe, 5×10^{-8} units of urethane hydrolase I, and $2.5\text{ }\mu\text{mol}$ of CoCl_2 . The reaction was initiated by adding $100\text{ }\mu\text{l}$ of the carboxypeptidase A sample solution, and incubation was carried out at 37°C . After 20 min of incubation, the reaction was terminated by adding $100\text{ }\mu\text{l}$ of 0.25% 4-aminoantipyrine, $100\text{ }\mu\text{l}$ of laccase solution ($22\text{ }\mu\text{g/ml}$) being subsequently added, and the mixture incubated at 37°C . After 40 min, the absorbance was mea-

sured at 505 nm. The activity of carboxypeptidase B was measured by the same method as that for carboxypeptidase A, except that 0.1 M sodium phosphate buffer at pH 6.2, (4-OH)Bz-Gly-Arg, and the carboxypeptidase B sample solution were used.

As a result, the absorbance at 505 nm was linear with the sample concentration, and passed through the origin. As shown in Fig. 1, the present method gave a linear calibration curve for carboxypeptidase A up to $0.6\text{ }\mu\text{g}$ per ml, with a sensitivity of 8.9 ng of carboxypeptidase A per ml per 0.01 absorbance unit at 505 nm and with a 1 cm light path. The calibration curve for carboxypeptidase B gave linearity up to $0.4\text{ }\mu\text{g}$ per ml as shown in Fig. 2, and a sensitivity of 12.5 ng of carboxypeptidase B per ml per 0.01 absorbance unit at 505 nm. The developed methods were applied to the determination of carboxypeptidase A or B in a culture medium and in a human serum, the experimental results being in good agreement with the values of carboxypeptidase A or B added. The average analytical recovery of carboxypeptidase A or B added to a bouillon medium and human serum was $100 \pm 2\%$, being linear between 0.04 and $0.6\text{ }\mu\text{g}$ of added carboxypeptidase A or B per ml.

In order to evaluate the activity of carboxypeptidase A or B, several analytical methods have previously been developed such as the quantitative ninhydrin method and spectrophotometric methods. However, these methods suffered from the disadvantage that they were influenced by contaminants. The new assay methods presented in this paper could be applied to contaminated samples such as culture media and sera. More detailed descriptions of the new assay methods will be presented in papers to follow.

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FIG. 1. Calibration Curve for Carboxypeptidase A.

FIG. 2. Calibration Curve for Carboxypeptidase B.

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