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MEASUREMENT OF PEPTIDASE ACTIVITY OF BOVINE PANCREAS CARBOXYPEPTIDASE A BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

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The authors have established a method for the determination of peptidase activity of bovine pancreas carboxypeptidase A (CPDase A) by means of indirect atomic absorption spectrophotometry. The method is based on the determination of L-phenylalanine cleaved from the substrate hippuryl-L-phenylalanine(HPA) after converting the amino acid first to a Schiff base by salicylaldehyde and then to the copper-Schiff base chelate, which is extractable with methyl isobutyl ketone (MIBK) as the ternary complex in the presence of bathophenanthroline. Atomic absorption method of CPDase activity gives accurate values than the conventional UV method.

Peptidase activity of carboxypeptidase A(CPDase A)[EC 3.4.2.1] is usually assayed by measuring the rate of hydrolysis of the substrate by the UV method[1]; the assay is performed at 25°C in 0.05 M Tris—buffer(pH 7.5) containing 0.5 M sodium chloride. Apparent proteolytic coefficient is calculated from the linear portion of the first order reaction plots observed under the conditions, when hydrolysis does not exceed 15%, measuring the increase in absorbance at 254 nm of hippuryl-L-phenylalanine(HPA) as the substrate. Esterase activity[2] of CPDase A is determined by means of pH titration of the substrate hippuryl-dl-β-phenyllactic acid.

The present authors have extended a series of indirect atomic absorption spectrophotometry(AAS) to the measurement of enzyme activity. When either substrates or products are measurable, indirect AAS will be applicable[3]. The authors have established the determination of the peptidase activity of bovine pancreas CPDase A which exhibits specificity for the cleavage of carboxyl terminal L-amino acids from proteins. The peptidase activity of CPDase A is determined by measuring the amino acid[4] cleaved from the substrate following the reaction with salicylaldehyde to form a Schiff base whose copper complex is extractable into methyl isobutyl ketone(MIBK) in the presence of bathophenanthroline by forming the ternary complex.

HPA is incubated with CPDase A at 25°C in N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid(HEPES) buffer (pH 7.5) containing 0.5 M sodium chloride, and the L-phenylalanine cleaved is determined by AAS quantitatively. The authors have established the determination conditions of the peptidase activity of bovine pancreas CPDase A, using HPA, and compared this AAS method with the conventional UV method. This paper describes the new applicability of the indirect AAS method to the measurement of enzyme activity.

EXPERIMENTAL

Reagents

HPA(0.0168 g) was dissolved in 50 ml of 0.025 M HEPES buffer(pH 7.5) containing 0.5 M sodium chloride to make a 1.0×10^{-3} M solution.

CPDase A(bovine pancreas, Boehringer Mannheim(West Germany)) was dissolved in 10% lithium chloride solution at a concentration of 5.0-19 mg/ml.

Reagents used for the quantitative measurement

1.0×10^{-3} M glycine solution: 0.07507 g of glycine was dissolved in 1000 ml of deionized water.

2.0×10^{-2} M salicylaldehyde: 2.4426 g of salicylaldehyde was dissolved in 1000 ml of deionized water.

1.0×10^{-3} M copper acetate: 0.01996 g of copper acetate was dissolved in 1000 ml of deionized water.

2.0×10^{-3} M bathophenanthroline: 0.3324 g of bathophenanthroline was dissolved in 500 ml of MIBK.

Apparatus

Atomic absorption spectrophotometer: Shimadzu AA/Flame emission AA-630-12 with a Cu hollow cathode lamp; air-acetylene was used for flame AAS. AAS measurement conditions were as follows: wavelength, 324.7 nm; Cu; lamp current, 10 mA; slit width, 1.9 Å; air flow rate, 13 l/min; acetylene flow rate, 2.0 ml/min; rate of aspiration, 2.2-2.8 ml/min.

pH meter: Orion Research Model 801/digital pH meter was used.

Measurement of peptidase activity of CPDase A using HPA

a)Measurement of the peptidase activity by AAS

Fifteen milliliters of 1.0×10^{-3} M HPA solution was taken into a 50 ml test tube. Fifty microliters of enzyme solution(0.1-0.5 mg/ml) was added to substrate solution, and it was mixed well and incubated for 2,4,6, and 10 min, respectively. The measurement was carried out at the end of each incubation. Two ml of the enzyme solution was manipulated for the enzyme activity.

b)Manipulation of amino acid determination

The determination was performed according to the method of Uno et al.[4] To a 50 ml centrifuge tube were taken 10.0 ml of 0.04 M borate buffer(pH 10.0), 5 ml of 2.0×10^{-2} M salicylaldehyde solution and 1.0×10^{-3} M copper acetate solution, and to this mixture was added 2 ml of incubated HPA solution with and without the enzyme. After being kept for about 10 min, it was then extracted with 10 ml of chloroform for 10 min by mechanical shaking, followed by centrifugation for 5 min at 3000 rpm. Five milliliters of the aqueous layer was taken exactly into a 50 ml centrifuge tube. To this were added 2.0 ml of 2.0×10^{-3} M bathophenanthroline and 5.0 ml of MIBK, and the mixture was shaken well for 5 min. Copper content in the MIBK extract was determined by AAS. The amino acid amount extracted with MIBK was calculated from the working curve in which glycine was used as a standard.

RESULTS AND DISCUSSION

Selection of buffer solution

Optimum pH of CPDase A was reported to be 7.5[1]. Various buffer solutions, such as HEPES (0.025 M, pH 7.5), borate-HCl(0.025 M, pH 7.5), Tris-HCl(0.025 M, pH 7.5), and phosphate (0.025 M, pH 7.5) were prepared to determine the buffer effect upon the CPDase A activity (Fig. 1). As Tris possesses the amino group which reacts with salicylaldehyde, the value of blank prepared with the Tris-buffer was higher. Phosphate buffer is considered to be an inhibitor of CPDase, and CPDase activity have not been observed in the buffer. Borate and HEPES buffers showed nearly the same AAS values. HEPES buffer showed much better linearity up to 9 min incubation. Then, the authors employed HEPES buffer in the measurements. HEPES is not considered to react with any metals.

Examination of enzyme concentration and incubation time

Effect of CPDase A concentration on the incubation time was studied (Fig. 2). The enzyme in various concentrations (0.51-1.78 μg/ml) was incubated with a constant conentration (1.0×10^{-3} M) of HPA. At the time of 6 min of incubation, the difference between the scale reading of sample and that of blank is correlated linearly with the enzyme concentration (inset of Figure 2). Therefore, with the conditions, the enzyme specific activity can be determined by AAS method.

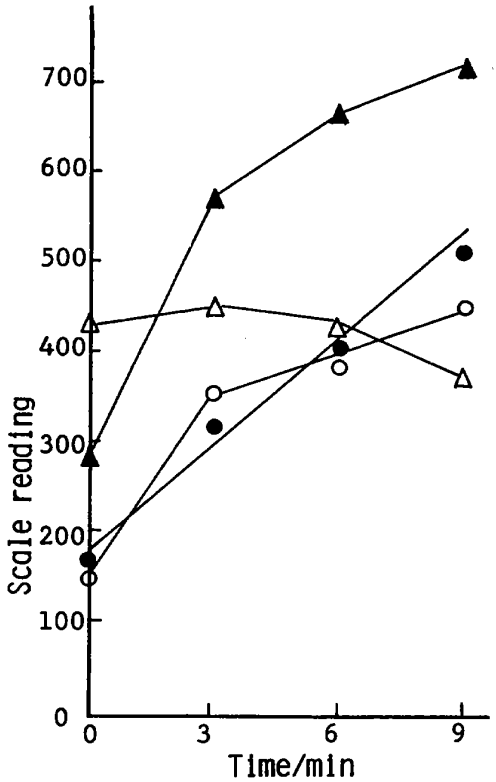

Fig.1 Effect of various buffers on CPDase A activity
△:0.025 M Phosphate buffer(pH 7.5);▲:0.025 M Tris-HCl buffer (pH 7.5);○:0.025 M Borate buffer (pH 7.5);●:0.025 M HEPES buffer (pH 7.5)

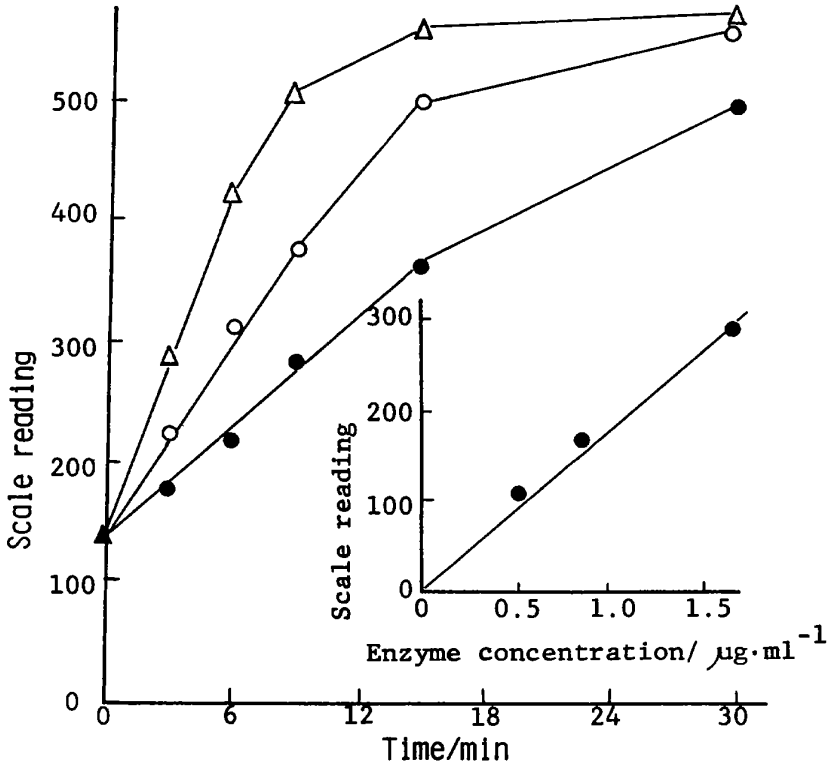

Fig.2 Relationship between enzyme concentration and incubation time
Enzyme concentration:●, 5.2×10^{-4} mg/ml;○, 9.3×10^{-4} mg/ml;△, 17.2×10^{-4} mg/ml(pH 7.5, 0.025 M HEPES buffer). Fifty microliters of the enzyme solution was used. ▲ Blank

Comparison of the specific activity determinations of CPDase by AAS using HPA substrate with UV method.

The conventional UV method was carried out as the reference[1]. The measurement conditions were the same as those of the AAS method.

In the UV method, 3 ml of HPA(1.0×10^{-3} M) was taken into the quartz cell exactly, and it was incubated with 10 μl of enzyme solution(0.28 mg/ml) at 25°C with agitation. The absorbance was then measured at 254 nm. Table 1 shows the comparison of the specific activity. Average specific activity of CPDase A by the AAS method was 43.1 unit/mg, while that by the UV method was 48.8 unit/mg with relative standard deviation being 7.4 and 10.2%, respectively. The ratio of the specific activity obtained by the AAS method to that by the AAS method was almost constant.

Table 1 Comparison of specific activity of CPDase A determined by the AAS method and the UV method.

No	AAS, Unit/mg	UV, Unit/mg	AAS/UV
1	42.0	45.3	0.927
2	37.8	41.0	0.922
3	44.3	52.9	0.838
4	41.8	51.8	0.806
5	44.3	41.9	1.057
6	45.8	49.1	0.933
7	43.6	51.0	0.855
8	47.1	46.0	1.024
9	46.7	52.7	0.886
10	37.5	56.0	0.670
$\bar{x}$	43.1	48.7	0.892
Standard deviation	3.19	5.01	
Relative standard deviation	7.4%	10.2%	

In the determination of CPDase A activity, usually the UV method detemines the peptidase activity while the titration method determines the esterase activity. Although the substrate specificity of CPDase A is comparatively broad, it reacts selectively with the C-terminal aromatic amino acids. This AAS method is to determine the cleaved amino acid, which is extracted into MIBK as the ternary Cu-Schiff base chelate in the presence of bathophenanthroline. As can be seen in Table 1, the standard deviation of the AAS method is smaller than that of the UV method, indicating that the AAS method is more precise. The present AAS method measures the net amino acid cleaved from the substrate. The authors recommend this AAS method as one of the useful methods in the measurement of CPDase A activity.

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Keyword phrases

indirect atomic absorption spectrophotometry of bovine pancreas carboxypeptidase A activity; amino acid Schiff base Cu chelate; peptidase activity of metalloenzyme; hippuryl-L-phenylalanine substrate.

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