Determination of Ultratrace Zinc by Enzymatic Activity of Carbonic Anhydrase

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A method for the determination of trace zinc has been investigated by using enzymatic activity of carbonic anhydrase. The recovery of enzymatic activity of apo-carbonic anhydrase was proportional to the amount of zinc in solution theoretically and experimentally, when it was monitored spectroscopically using the substrate, *p*-nitrophenyl acetate. The amount of zinc in the sample solutions was therefore determined from the curve, zinc concentration *vs.* the enzymatic activity. The limit of detection is 10 ng/ml or 2 ng. The method has been applied to the analysis of zinc in fruit juices and water samples.

Of many enzymes, the metalloenzymes have specific metal ions located at their active centers, and the metal ions play essential roles in determining the enzymatic activity and in the processes of enzymatic reactions. This allows the metalloenzymes to show their selectivity and specificity to substrates which are concerned in enzymatic reactions. Such characteristics of the metalloenzymes have been subjects of recent interest, particularly as regards their application to the determination of trace metals.[1,2]

The enzymatic activity of the metalloenzyme for substrates is determined by the amount of active metal ion in the enzyme. The assay of the metalloenzyme is performed by measuring the recovery of the enzymatic activity of the inactive metal-free apoenzyme, when the metal can be removed from the enzyme. The assay of the enzyme is very sensitive and selective to the amount and kind of metal ion added, being useful for the analysis of trace metal ions. Townshend and Vaughan reported the detection of zinc based on the re-activation of apo-alkaline phosphatase.[3] Lehky and Stein studied the determination of zinc in serum using aminopeptidase.[4] Several workers investigated the determination of zinc and copper by enzymatic activity of metalloenzymes.[5,6]

In the present paper, a theoretical consideration is given on the relationship between the recovery of enzymatic activity and the amount of zinc in the case of one active metal ion, taking into account the chemical equilibria between the apoenzyme and the metal ion. A general case, *i.e.*, one in which more than one metal ion is necessary, is given in Appendix II. The relationship is confirmed experimentally and applied to the determination of zinc in fruit juices and waters, where apo-carbonic anhydrase was used as one of the representative zinc-containing enzymes found widely in biological systems.[7]

Theoretical Consideration

The enzymatic reaction in the case of carbonic anhydrase can be described as follows (definitions of all the signs are summarized in Appendix I):

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P. \quad (1)$$

The reaction velocity v in this system is given by the Michaelis-Menten expression:

$$v = \frac{V_{max}[S]}{K_m + [S]}, \text{ where } K_m = \frac{k_{-1}+k_{+2}}{k_{+1}}, \quad (2)$$

It is assumed that the following equilibrium between the apoenzyme and metal ion (zinc in the present case) exists in solution:

$$E_a + M \rightleftharpoons E \quad (3)$$

$$K = [E]/[E_a][M]. \quad (4)$$

Thus, the concentration of metalloenzyme is given by

$$[E] = K([E_a]_0 - [E])([M]_0 - [E]). \quad (5)$$

Under the conditions $[S] \gg [E_a] \gg [M]$, the equation is written as follows:

$$[E] = \frac{K[E_a]_0[M]_0}{1 + K[E_a]_0}. \quad (6)$$

If the concentration of substrate is sufficiently high as assumed in Eq. 6, the concentration of the product in Eq. 1 is proportional to that of the enzyme. Thus,

$$[P] = k_0 t[E], \quad (7)$$

where k_0 is molecular activity of enzyme and t the reaction time. Since we can assume that $K[E_a]_0 \gg 1$, the following expression can be obtained from Eqs. 6 and 7.

$$[P] = k_0 t \cdot \frac{K[E_a]_0}{1 + K[E_a]_0} \cdot [M]_0 \simeq k_0 t[M]_0. \quad (8)$$

Differentiating, we have

$$\frac{d[P]}{dt} \simeq k_0[M]_0. \quad (9)$$

This shows that the formation rate of the product in Eq. 1 is proportional to the initial concentration of metal ion in solution. Thus the metal concentration in the sample solution can be determined by measuring enzymatic activity, if the formation rate of the product can be obtained, for example, by absorption measurement of the product.

Since the absorption of substrate is not negligible in the present case because of the overlapped spectra, Eq. 9 should be modified. The total absorption of the substrate and product at λ is given by

$$A_\lambda = (e_S^\lambda[S] + e_P^\lambda[P])l. \quad (10)$$

By differentiation we have

$$\frac{dA_\lambda}{dt} = \frac{d}{dt}(e_P^\lambda - e_S^\lambda)[P] \cdot l \simeq (e_P^\lambda - e_S^\lambda)k_0 l \cdot [M]_0. \quad (11)$$

We see that the time-dependence of the total absor-

bance at λ (dA_λ/dt) is also proportional to the initial concentration of metal. The amount of zinc in the solutions can be determined from a plot of (dA_λ/dt) *vs.* $[M]_0$.

Experimental

Chemicals. Bovine carbonic anhydrase C-7500 (EC 4.2.1.1, abbreviation BCA, Sigma Chem. Co.) was used. Substrate (*p*-nitrophenyl acetate) and buffer (tris(hydroxymethyl)methanamine=Tris) were purified by means of solvent extraction using a 0.001% dithizone–chloroform solution.[8] *p*-Nitrophenyl acetate was dissolved in 0.03 M Tris buffer solution (pH 7.8).[9] (1 M=1 mol dm^{-3}.) The distilled water was prepared by a Daiken Sekiei subboiling distiller.[10] Zinc chloride was used for the preparation of standard solution of zinc.

Instruments. A Shimadzu UV-210 spectrophotometer with a SPR-5 temperature controller was used for the measurements of enzymatic activity of carbonic anhydrase, the optical path of the absorption cell being 10 mm. A Hitachi 170-50 atomic absorption spectrophotometer with a Jarrell-Ash FLA-100 carbon rod atomizer was used to determine the zinc content in the sample solutions.

Preparation of the Apoenzyme. A zinc-free enzyme (apoenzyme) was prepared by a modified method of Lindskog and Malmstöm[11] as follows. BCA (10 mg) was dissolved in 1 ml of water and dialyzed with 0.01 M 1,10-phenanthroline and 0.1 M of sodium acetate buffer (pH 5.0) for 5—10 days. After further dialysis with 10^{-4} M EDTA and 0.06 M Tris (pH 7.8) for half a day, the chelating reagents were eliminated by dialysis with water. The zinc content in the enzyme was reduced to less than 3% of the initial content. The concentration of enzyme protein was determined by measuring the absorbances at 260 nm and 280 nm.[12]

Measurement of Enzymatic Activity. The enzymatic reaction of BCA for the substrate, *p*-nitrophenyl acetate, proceeds as follows:

$$O_2N\text{-}C_6H_4\text{-}OCOCH_3 + H_2O \xrightarrow{BCA} O_2N\text{-}C_6H_4\text{-}OH + CH_3COOH \quad (12)$$

The absorbance of the product, *p*-nitrophenol, was measured at 348 nm.[9] We see from the Michaelis-Menten plot (reaction velocity *vs.* concentration of substrate) that 1 mM of *p*-nitrophenyl acetate is sufficient to satisfy the conditions for Eq. 6.

The procedure for enzymatic activity measurement was as follows: 2 ml of the substrate solution and 50—200 μl of the zinc-containing sample were mixed in a cuvette. 100 μl of 10^{-4} M apo-BCA was then added to the mixed solution, the time at which addition was made being taken as $t=0$. The increase in absorbance at 348 nm, which corresponds to the increase of the product in Eq. 12, was measured subsequently. All the experiments were carried out at 25 °C.

Results and Discussion

Calibration Curve for Zinc. The relationship between the change in absorbance at 348 nm (reaction rate) and the concentrations of zinc in the solution is shown in Fig. 1. The change in absorbance corresponds to the enzymatic activity. The change of absorbance per minute was adopted for measurement of enzymatic activity. The enzymatic activity was calculated from the absorbance change during the period 1—5 min after addition of enzyme.

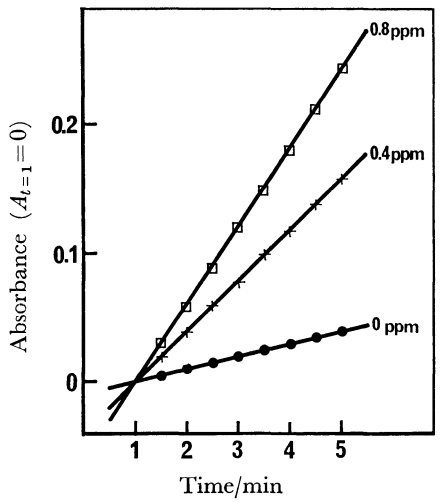

Fig. 1. Time dependence of absorbance change at several zinc concentrations. Absorbance at $t=1$ min ($A_{t=1}$) after the addition of apoenzyme was assumed zero.
Zinc solution: 100 μl, 1 mM substrate solution: 2 ml, 0.1 mM BCA solution: 100 μl. ●—●: [Zn]=0 μg/ml, ×—×: [Zn]=0.4 μg/ml □—□: [Zn]=0.8 μg/ml.

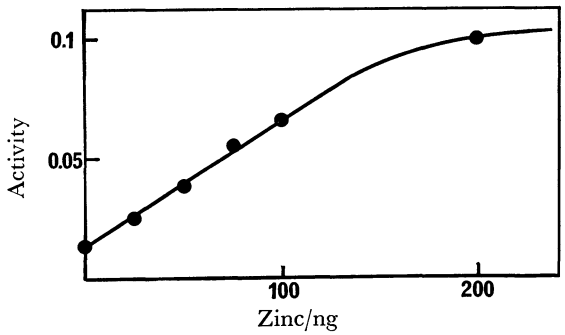

Fig. 2. Calibration curve for zinc measured by enzymatic activity of BCA. The enzymatic activity was measured as the change of absorbance (dA/dt) at 348 nm. 1 mM substrate solution: 2 ml, 0.1 mM BCA solution: 100 μl.

As shown in Fig. 2, the calibration curve is linear in the range 10—100 ng of zinc. For the amount of zinc over 100 ng, the condition $[E_a] \gg [Zn]_0$ is no longer valid and the calibration curve starts to bend. However, the dynamic range of the calibration curve can be extended by varying the amount of sample added to the buffer solution. The actual dynamic range, therefore, may be extended from 10 ng/ml to 1 μg/ml. A higher concentration of zinc ion might be determined if $[E_a]$ can be increased. However, increase of background absorption and difficulty of mixing the enzyme and sample homogeneously at the start of reaction result in poor accuracy. Thus 10 nmol enzyme is the maximum working amount for the determination of zinc under the present conditions.

The integral absorbance (A) can also be applied to the determination of zinc instead of the change in absorbance (dA/dt), as can be seen from Eq. 10.

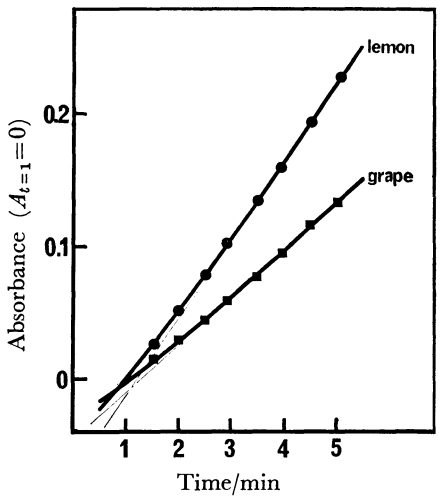

Fig. 3. Time dependence of absorbance change in grape and lemon juice samples. 1 mM substrate solution: 2 ml, 0.1 mM BCA solution: 100 μl, sample solution: 100 μl. ■—■: grape juice, ●—●: lemon juice.

However, zinc determination due to the integral absorbance causes large error in the analytical results. A typical example is shown in Fig. 3, where the absorbance changes at 345 nm are shown for grape and lemon juices. The rate of increase of absorbance is low at the initial stage of the reaction, and the amount of zinc obtained from the apparent absorbance might be less than the actual value. The slow increase of absorbance at the initial stage of the reaction might be due to the redistribution of zinc in the enzyme and sample. Measurement of the enzymatic activity should therefore be started after a few minutes (at least 1 min).

The sensitivity of the present method is *ca.* 10 ng/ml or 2 ng. As can be seen from Eq. 11, the sensitivity depends on the molecular activity (k_0) and absorption coefficient of the product (e_p). It may be improved by selection of a more appropriate substrate, although *p*-nitrophenyl acetate was used as a substrate for the sake of convenience.

In order to use the metalloenzyme method for metal determination, the following conditions should be satisfied: 1) A metal-free enzyme; 2) an enzyme stable in the metal-free state; 3) an enzyme easily obtained and convenient to work with; 4) little interference of other metal ions; and 5) linearity between the metal concentration and the rate of product formation. So far, alkaline phosphatase and aminopeptidase have been applied to zinc determination.[3,4] These enzymes can be easily obtained and are readily manipulated. The analytical sensitivity of the methods using aminopeptidase and alkaline phosphatase is comparable with that of the present method. However, more than one zinc atom is required for reactivating these enzymes from the zinc free state. As can be seen in Eqs. 2′ and 3′ in Appendix II, the relationship between zinc content and recovery of activity is complicated, not being linear. This indicates that the standard addition method may not be available, even when the interference of the matrices is large. For this reason, the use of carbonic anhydrase is preferable to these enzymes.

Table 1. Effect of anions on enzymatic activity of carbonic anhydrase[a)]

Anions	Amount/μmol	Relative activity[b)]/%
Cl^-	1	88±18
NO_3^-	1	82±16
HSO_4^-	1	85±19
ClO_4^-	1	74±15
CN^-	0.2	12±2
CN^-	1	5±3

a) The amounts of zinc and apo-carbonic anhydrase were 1.5 nmol and 10 nmol, respectively. b) The ratio (%) of the enzymatic activities measured with and without the anions.

Table 2. Effect of metal ions on enzymatic activity of carbonic anhydrase[a)]

Zinc amount/ng	Amount/ng	Relative activity[b)]/%
0	Co(II) 0	10±3
0	100	55±12
100	20	95±18
100	100	145±22
0	Hg(II) 0	25±2
0	100	12±3
100	20	74±5
100	100	59±7
0	Cu(II) 0	6±1
0	100	7±1
100	20	87±9
100	100	88±8

a) The amounts of zinc and apo-carbonic anhydrase were 1.5 nmol and 10 nmol, respectively. b) Enzymatic activities based on the activity when only 100 ng zinc is added.

Interference of Coexistent Ions. A few studies have been made on the effect of the coexisting cations[13] and anions[14] on the activity of carbonic anhydrase. The interference of the concomitants in the present system is summarized in Tables 1 and 2. Excess amounts of cyanide and perchlorate ions interfere severely with the analytical results.

In the case of cations, Fe^{2+}, Mn^{2+}, Ca^{2+}, Na^+, and Mg^{2+} do not interfere as long as their amounts are less than twice the amount of zinc ion. Hg^{2+} reduces the enzymatic activity by 50%, and Co^{2+} at a concentration equal to that of zinc ion increases the enzymatic activity by 50%. However, the level of mercury or cobalt in real samples is generally much lower than that of zinc (in the environmental and biological samples).[15,16] Thus, the selectivity of this method is maintained at such low levels in many cases. If the interference of the concomitants can not be avoided, the standard addition method should be employed. It would improve the accuracy of the procedure.

Application to Zinc Determination. The analytical results (the standard addition method) for the

Table 3. Determination of zinc in real sample

Sample	Zn content/(ng/ml)	
	present method[a)]	atomic absorption[b)]
Grape juice	340±50	370±20
Pear juice	180±50	250±20
Lemon juice	700±50	540±30
City water	2200±100	1800±100
Drain water	260±90	190±20

a) The amount of apo-carbonic anhydrase was 10 nmol at pH 7.8. b) Determined by atomic absorption spectrometry.

fruit juices and natural waters are given in Table 3, together with the amount of zinc in the same samples determined by atomic absorption spectrometry. The analytical values obtained by the present method are in line with those obtained by atomic absorption spectrometry. This indicates that the present method can be applied to the determination of zinc in biological and environmental samples.

However, when the "metalloenzyme method" is applied to metal determination in biological samples, the same enzyme intrinsically existing in the sample interferes with the results. For example, the determination of zinc in serum would not be possible by the present method because of the high activity of carbonic anhydrase in serum. The same situation occurs in the case of other esterases, when the samples related to digestive organ are analyzed. The appropriate selection of enzyme should be made with regard to the kind of sample. Pretreatment of sample for eliminating or inactivating the intrinsic enzyme is necessary.

Conclusion

The present method shows promise for determining trace zinc. In general, biochemical amplification and specificity to the metal ion analysed are important characteristics of metal determination by the activity of metalloenzymes. In the present system, one zinc ion caught in the enzyme protein can react with many specific substrates and produce many specific products if the reaction time can be prolonged. The catalytic reaction of metalloenzyme may enhance the sensitivity of the method. This is a great advantage of the "metalloenzyme method" as compared to the usual colorimetric method. In practice, however, the present method has some limitations due to the natural hydrolysis of the substrate and incompleteness of zinc-elimination from the enzyme. Nevertheless, it is superior in selectivity and comparable in sensitivity to convenient colorimetric methods such as the dithizone method.[17] The preparation of enzyme electrodes for trace metal analysis is an interesting application of the metalloenzyme method.

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Appendix I

E: holoenzyme
S: substrate
P: product
ES: complex of substrate and holoenzyme
v: initial reaction rate
V_{max}: maximum reaction rate
K_m: Michaelis constant
$k_{\pm 1,+2}$: rate constant
k_0: molecular activity of enzyme
t: reaction time
E_a: apoenzyme (inactive)
M: metal ion
K: stability constant between enzyme protein and zinc
e_S^λ: absorption coefficient of substrate at λ
e_P^λ: absorption coefficient of product at λ
A^λ: absorbance at λ
l: light path length in the cuvette
$[\ ]_0$: concentration at the condition of initial stage
λ: wavelength

Appendix II

In a system where more than one zinc ion is necessary to attain recovery of enzymatic activity, Eq. 5 should be modified as follows:

i) In the case where enzymatic activity appears for n bindings of zinc ions such as in aminopeptidase:

$$[E] = K_1K_2\cdots K_n[Zn]^n[E_a] \simeq \frac{K_1K_2\cdots K_n[Zn]^n}{1+K_1[Zn]+\cdots+(K_1K_2\cdots K_n[Zn]^n)}\cdot[E_a]_0, \quad (1')$$

$$[P] \approx \frac{K_1K_2\cdots K_n[Zn]^n}{1+K_1[Zn]+\cdots+(K_1K_2\cdots K_n[Zn]^n)}[E_a]_0k_0t. \quad (2')$$

ii) In the case where the molecular activity (k_0) of the enzyme changes in terms of the binding number of zinc ions to the enzyme, such as found in alkaline phosphatase, Eq. 8 should be replaced by

$$[P] \approx \frac{K_1K_2\cdots K_n[Zn]^n}{1+K_1[Zn]+\cdots+(K_1K_2\cdots K_n[Zn]^n)}\{(K_{01}K_1[Zn]) + \cdots + (k_{0n}K_1\cdots K_n[Zn]^n)\}t[E_a]_0. \quad (3')$$

k_{0i}: molecular activity of enzyme when the binding number of zinc ions to enzyme is "i".

K_i: step-wise stability constant, $K_i = \dfrac{[E_aZn_i]}{[E_aZn_{i-1}][Zn]}$. (4')

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