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Enzymatic Analysis of Cyanogenic Glycosides. II.[1] A Simple Method by Using a Microdiffusional Apparatus

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A simple method for the determination of cyanogenic glycosides by using a Conway's microdiffusional apparatus was investigated. The hydrolysis of cyanogenic glycosides by enzymes and the isolation of cyanide liberated were simultaneously performed in the same apparatus. The liberated hydrogen cyanide was determined by Epstein's method using pyridine–pyrazolon reagent. By this method, the recoveries of linamarin and amygdalin were over 90% and the coefficients of variation were 0.2 to 3.6% for both glycosides in the range of 0.5—400 μg of cyanide. This method was applied to the determination of cyanogenic glycosides in pastes of butter beans and apricot kernels and the results were compared with those obtained by the steam distillation method.

Keywords——cyanogenic glycoside; amygdalin; linamarin; enzymatic hydrolysis; almond emulsin; linamarase; Conway's microdiffusional apparatus; Epstein's method

Some food crops contain cyanogenic glycosides,[2] which are considered to be enzymatically hydrolyzed to produce cyanide when they are ingested by living organisms. Therefore, the determination of cyanogenic glycosides in food is as important as that of free cyanide. However, procedures for the determination of cyanogenic glycosides so far used are not convenient as an assay method for food.

In this paper, we describe a simple method for the liberation and the isolation of cyanide from cyanogenic glycosides by using a Conway's microdiffusional apparatus, in which the enzymatical hydrolysis of cyanogenic glycosides and the isolation of free cyanide are simultaneously performed. We determined the cyanide contents of standard cyanogenic glycosides, linamarin and amygdalin, with this method, and then the procedure was applied to the measurement of cyanogenic glycosides in pastes of butter beans and apricot kernels.

Experimental

Materials——Almond emulsin (Sigma Chemical Co.) and linamarase were used for the enzyme preparation. Linamarase was purified as described previously[3] and the preparation obtained by CM-Sephadex column chromatography was used for the hydrolysis of linamarin. The enzymes were dissolved in 0.05 M sodium citrate buffer, pH 5.2, to give a β-glucosidase activity of 4 U/ml. Linamarin was prepared from butter beans as described previously,[4] and amygdalin was purchased from Nakarai Chemicals. A standard solution of cyanide was prepared with potassium cyanide (Kanto Chemicals Co.) using 0.01 M NaOH and was tested for concentration by titration with $AgNO_3$ according to Liebig–Denige's method. Apricot kernels were purchased from Tanuma Co., and butter beans were kindly supplied by Dr. M. Uchiyama, National Institute of Hygienic Sciences.

Enzymatic Hydrolysis of Cyanogenic Glycosides——The absorbing solution, 5.0 ml of 0.5 M NaOH, was poured into the outer compartment of the Conway's microdiffusional apparatus (Fig. 1, a semimicro type, Shibata Scientific Technology). High-vacuum silicone grease (Dow Corning Co.) had previously been spread over the ground parts of the dish and the cover. Five milliliters of the sample (dissolved or suspended in 0.05 M sodium citrate buffer, pH 5.2) was poured into the inner compartment. The dish was covered immediately after addition of the enzyme solution, linamarase (100 μl) or almond emulsin (200 μl), to the inner compartment. The dish was rolled gently, and incubated at 50 °C for 90 min. After the incubation, the dish was allowed to stand at room temperature for 3 h. An aliquot of the

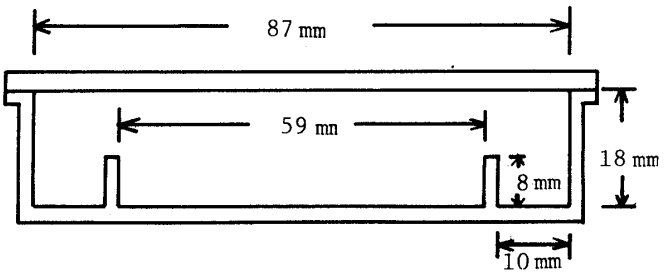

Fig. 1. A Conway's Microdiffusional Apparatus (Semimicro Type)

absorbing solution in the outer compartment (1—3 ml) was transferred quantitatively to a volumetric flask containing a drop of phenolphthalein reagent, and neutralized with 2 M acetic acid in an ice box. Water was added to the volumetric flask to make 5 or 10 ml, and 1—3 ml of this solution was used for the determination of cyanide.

Separation of Cyanide by Steam Distillation——The sample pastes of butter beans and apricot kernels were prepared by grinding beans and kernels which had been boiled for 30 min. The sample paste (2 g) containing cyanogenic glycosides in 50 ml of 0.05 M sodium citrate buffer, pH 5.2, was incubated with the enzyme at 37 °C for 15 h in a distillation flask. Then steam was allowed to pass into the vessel and to a trap containing 10 ml of 1 M KOH solution. The distillate (about 70 ml) was neutralized with 2 M acetic acid and the total volume of the solution was adjusted to 100 ml with water. One milliliter of the solution was used for the determination of cyanide.

Analytical Methods——Free cyanide was determined by Epstein's method using pyridine–pyrazolon reagent.[5] β-Glucosidase activity was measured by the procedure described previously[6] using *p*-nitrophenyl (PNP-)β-glucoside (Sigma Chemical Co.). The assay mixtures contained 2 mM PNP-β-glucoside and the enzyme in a total volume of 0.2 ml of 0.05 M sodium citrate buffer, pH 6.0. After incubation for an appropriate time at 37 °C, the reaction was terminated by the addition of 1.4 ml of 0.2 M sodium borate buffer (pH 9.8). The quantity of *p*-nitrophenol liberated was measured at 400 nm. One unit of β-glucosidase was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per min under the conditions mentioned above.

Results and Discussion

Enzymatic Hydrolysis of Cyanogenic Glycosides

It has been reported that almond emulsin hydrolyzed amygdalin but had little effect on linamarin, and the enzyme from the butter beans, that is linamarase,[3] hydrolyzed linamarin but not amygdalin.[1] Therefore, linamarase was used for the hydrolysis of linamarin and almond emulsin was used for amygdalin. The pH optima of linamarase toward linamarin and of almond emulsin toward amygdalin were 5.0—5.2 and 4.5—5.5, respectively. The hydrolysis of the cyanogenic glycosides by the two enzymes were performed in pH 5.2 medium.

The effects of temperature on the hydrolysis by linamarase and almond emulsin were studied (Fig. 2). Both enzymes were most active at 55°C. However, the hydrolysis of cyanogenic glycosides by the two enzymes was carried out at 50 °C so as to avoid inactivation of the enzymes during prolonged incubation.

Recovery of Free Cyanide by Using a Microdiffusional Apparatus

We developed a method for the liberation and isolation of cyanide from cyanogenic glycosides using a microdiffusional apparatus as described in the experimental section. In the sealed apparatus, cyanogenic glycosides are hydrolyzed by the added enzyme, and cyanide liberated is simultaneously absorbed in the sodium hydroxide solution during incubation at 50 °C for 90 min followed by standing at room temperature for 3 h. To check whether free cyanide is absorbed quantitatively in the sodium hydroxide solution by this method, the recovery of cyanide in the standard solution was investigated. Five milliliters of the standard cyanide solution containing 0.1—500 μg of cyanide was put into the inner compartment of the apparatus, and the dish was covered immediately after 0.5 ml of 0.5 M citrate buffer, pH 5.2, was added. The dish was incubated and allowed to stand as described in the experimental section, and then the amount of cyanide in the absorbing solution was determined. As shown

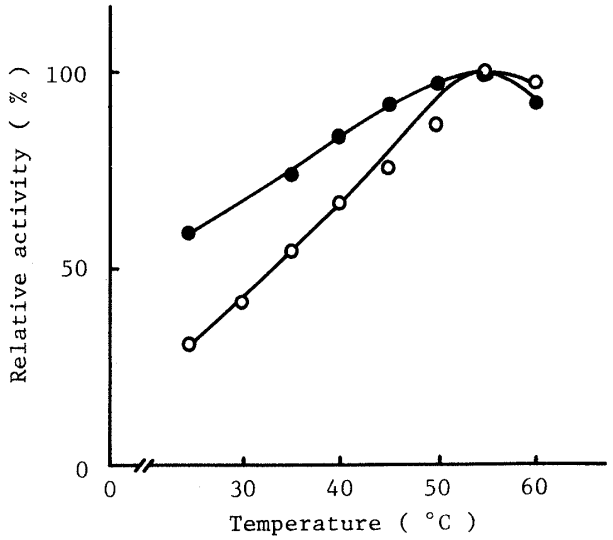

Fig. 2. Effects of Temperature on the Hydrolysis by Linamarase (○) and Almond Emulsion (●)

Enzyme activities were assayed with PNP-β-glucoside as the substrate during incubation for 10 min at various temperatures in the range of 25—60 °C.

TABLE I. Isolation and Estimation of Cyanide by the Method Using the Microdiffusional Apparatus

Added (μg)	Found[a] (μg)	Recovery[a] (%)	C.V. (%)	n
0.10	0.121±0.012	121.0±12.0	9.9	4
0.50	0.490±0.002	98.0± 4.0	4.1	4
1.0	1.00 ±0.04	100.0± 4.0	4.0	4
10	10.0 ±0.3	100.0± 3.0	3.0	4
50	49.4 ±2.8	98.8± 5.6	5.7	4
100	96.4 ±1.3	96.4± 1.3	1.3	4
500	476.0 ±4.5	95.2± 0.9	0.9	4

a) Mean ± standard deviation (S.D.).

in Table I, cyanide in the standard solution was almost completely recovered in the range of 0.5—500 μg.

Liberation and Estimation of Cyanide from Cyanogenic Glycosides

The liberation and the estimation of cyanide from linamarin and amygdalin were investigated with the microdiffusional apparatus according to the proposed method. As shown in Table II, the recoveries of cyanide contained in linamarin and amygdalin were 96—101% and 90—97%, and the coefficients of variation (C.V.) were 0.5—2.0% and 0.2—3.6%, respectively, in the range of 0.5—400 μg of cyanide. This result indicates that cyanide contained in the cyanogenic glycosides is quantitatively estimated by this method over the wide range of 0.5—400 μg.

Determination of Cyanogenic Glycosides in the Sample Pastes

The amounts of cyanogenic glycosides in the pastes of butter beans and apricot kernels were determined by the present method using the microdiffusional apparatus. The endogenous enzymes which hydrolyze the cyanogenic glycosides in the native samples were previously inactivated by the boiling process used for preparing the sample pastes. The samples in 5 ml of 0.05 M sodium citrate buffer, pH 5.2, were put into the inner compartment of the microdiffusional apparatus, and then the enzyme solutions, linamarase and almond emulsin, were added to the inner compartment.

TABLE II. Recoveries of Linamarin and Amygdalin by the Present Method

Cyanogenic glycoside	CN in glycoside (μg)	Found[a] (μg)	Recovery[a] (%)	C.V. (%)	n
Linamarin	0.10	0.125±0.019	125.0±19.0	15.2	4
	0.50	0.482±0.008	96.4± 1.6	1.7	5
	5.0	5.00 ±0.05	100.0± 1.0	1.0	5
	10	9.9 ±0.2	99.0± 2.0	2.0	5
	100	100.7 ±0.5	100.7± 0.5	0.5	5
	400	405.6 ±4.4	101.4± 1.1	1.1	5
Amygdalin	0.10	0.110±0.005	110.0± 5.0	4.5	3
	0.50	0.450±0.005	90.0± 1.0	1.1	5
	5.0	4.69 ±0.02	93.8± 0.4	0.4	4
	10	9.5 ±0.1	95.0± 1.0	1.0	4
	100	97.0 ±3.5	97.0± 3.5	3.6	4
	400	376.4 ±0.8	94.1± 0.2	0.2	3

a) Mean±S.D.

TABLE III. Determination of Cyanogenic Glycosides in the Sample Pastes

Sample paste	Enzyme added	CN by steam distillation (μg/g)	CN by microdiffusion method (μg/g)
Butter beans	None	2.5	0
	Linamarase	105	101
Apricot kernels	None	2.1	0
	Almond emulsin	459	438

After the incubation, the cyanide content in the outer compartment was determined (Table III). The cyanide contents in the pastes of butter beans and apricot kernels were 101 and 438 μg/g, respectively. No cyanide was detected when the samples were incubated without linamarase or almond emulsin. This indicates that the values obtained are attributable to the hydrolysis of cyanogenic glycosides but not to free cyanide in the two samples. Free cyanide in the native samples may be volatilized during the boiling process used for preparing the sample pastes. Using the same sample, the isolation of cyanide by steam distillation was also carried out, and the amounts of cyanide estimated were compared with the results obtained by the microdiffusion method (Table III). The cyanide contents in the sample pastes obtained by the two methods were almost the same. The method using the microdiffusional apparatus seems to be very useful for liberation and isolation of cyanide from cyanogenic glycosides. The standard type of Conway's microdiffusional apparatus was also available for this analytical method as well as the semimicro type.

The microdiffusion method described here is considered to be advantageous for the analysis of cyanogenic glycosides in food for the following reasons; 1) the errors caused by loss of cyanide are decreased because the liberation and isolation of cyanide from cyanogenic glycosides were performed in the same sealed apparatus, 2) the period required for the liberation and isolation of cyanide is shortened as compared with that in the steam distillation method, 3) numerous samples can be treated simultaneously, 4) the sample weights for the determination can be decreased as compared with the steam distillation method. However, we recognize the possibility that some components contained in processed food may affect the enzyme activity. It is necessary to check this problem before application of the method to food

analysis. A method for the analysis of cyanogenic glycosides in processed food will be presented in detail elsewhere.

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