Bulletin of Okayama Prefectural Junior College Vol. 37 22~27 (1992)

A Sensitive Radial Diffusion Method for the Determination of Several Proteases

Hiroyuki SUMI, Chieko YATAGAI and Nobuyoshi NAKAJIMA

SUMMARY

A sensitive radial diffusion method for determing esterolytic activities of some proteases using a pH indicator and synthetic ester substrate was developed. When Bromothymol Blue and N^{α}-p-tosyl-L-arginine methyl ester are included in agar gel, this method permits detection of as little as 0.1 ng (4×10^{-15}mole) trypsin, 10 ng subtilisin BPN, 100 ng of nattokinase or subtilisin Carlsberg, and 10 to 100 ng pancreatic kallikrein per 25 μl each solution. Under the moderate conditions of screening of plant enzymes or routine assay of chromatographic fractions, this method was satisfactorily used without any interferences. The procedure is simple, sensitive and no special techniques and materials are required.

INTRODUCTION

A radial diffusion method is one of the convenient and sensitive methods for screening enzymes of plant, bacteria and animal origins.[1~8] It is also useful in routine assay of chromatographic fractions. For protease screening in plant extracts, Santarius and Ryan[8] have reported a sensitive radial diffusion method using protein substrates such as gelatin and casein. However, a synthetic substrate, N^{α}-benzoyl-DL-arginine-p-nitroanilide, in agar gel was found much inferior in its sensitivity compared to gelatin method.[8]

We have found that a radial diffnsion method utilizing synthetic ester substrates, especially arginine derivatives such as N^{α}-p-tosyl-L-arginine methyl ester (TAME) and N^{α}-benzoyl-L-arginine ethyl ester (BAEE) in the presence of a pH indicator, Bromothymol Blue (BTB) in agar gel, is especially sensitive and useful for the detection of some proteases. Its sensitivity is even higher than the gelatin method described above.

In this paper we report the assay procedure, the effects of acids, bases and buffer concentrations on this TAME-BTB radial diffusion method and the applicasion to isolate some plant euzymes.

MATERIALS AND METHODS

Materials

Bovine trypsin was purchased from Boehringer. α-Chymotrypsin, subtilisin BPN' (protease type VII), subtilisin Carlsberg (protease type VIII), papain, soybean trypsin inhibitor (STI) and chloramphenicol from Sigma Chemical Co., Trypticase soy agar from BBL, noble and bacto-agar from Difco. Gelatin, BAEE and TAME were obtained from Nakarai Chemicals, N^{α}-acetyl-L-arginine methyl ester hydrochloride (AAME) and N^{α}-p-tosyl-L-lysine methyl ester hydrochloride (TLME) from Protein Research Foundation, and N^{α}-acetyl-L-tyrosine ethyl ester (ATEE) from Tokyo Kasei. Nattokinase (Lot 002) was purified as described previously.[9] Porcine pancreatic kallikrein was a gift from Dr. H. Hiratani (Japan Chemical Research, Co.,

Abbreviations: TAME, N^{α}-p-tosyl-L-arginine methyl ester; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; BTB, Bromothymol Blue; STI, soybean trypsin inhibitor; AAME, N^{α}-p-tosyl-L-lysine methyl ester hydrochloride; ATEE, N^{α}-acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl) aminomethane; VAI, *Vicia angustifolia* proteinase inhibitor.

Ltd.). The other chemicals were of the reagent grade. Proteinase inhibitor from *Vicia angustifolia* L. var. *segetalis* Koch seeds (VAI) was prepared as described previously.[10] *V. angustifolia* leaves were collected in the field around Fukuoka. Young, mature, healthy green leaves were selected for enzyme purification.

Purity of the trypsin sample used was determined by titration with p-nitrophenyl-p'-guanidinobenzoate[11] and found to be 64% active. The other proteases in this paper were used without determination of their purities.

Preparation of Gel Plates

Gels containing gelatin as substrate were prepared as describec by Santarius and Ryan[8] and the plates were autoclaved at 1 kg /㎠ and 121℃ for 20 min and cooled.

Gels containing synthetic ester substrates were prepared as follows: TAME (37.9 mg, 1 mM) was dissolved in 45 ml dislilled water and to this was added 5 ml 0.2% aqueous BTB solution prepared in advance (20 mg BTB dissolved in 0.5 ml 0.1 M NaOH and diluted to 10 ml with distilled water) and then added 50 ml 1.5% Trypticase soy agar. The solution (about 10 ml per plate) was poured into 9 cm diameter petri dishes and autoclaved under the conditions described above.

When Trypticase soy agar was replaced by noble or bactoager, 0.1 M Tris-HCL buffer, p H 7.4, was used instead of distilled water. The other conditions were the same as above.

Assay and Detection Methods of Esterolytic Activities

Usually 4 wells of 4 mm in diameter were made in the substrate-agar gels with a thin glass tube and 25 μl enzyme solutions were introduced into the wells. The dishes were covered and left to stand at 37℃ for 16 hr.

Clearly detectable bluish zones were produced against blnish green background in the case of Trypticase soy agar and blue background slightly different from the color of the diffused zones in the case of noble or bacto-agar-Tris system after enzymatic hydrolysis of the substrate, TAME or BAEE. Enzymatic hydrolyses of TLME, AAME and ATEE (for chymotrypsin assay) produced diffused zones with yellowish orange against bluish bacrground and in these cases boundaries of the diffused zones were not so clearly detectable.

Recording of the experimental results were made easily by taking pictures using instant color films rather than black-and-white films.

Detection of Proteinase Inhibitors

Varying amounts of a proteinase inhibitor such as STI[12] or VAI[10,13,14] was premixed with constant amounts of trypsin in 0.1 M Tris-HCl buffer (pH 7.4) and 25 μl of the mixture was applied to the substrate gels.

Esterlytic Activities in Plant Extracts

Crude extracts from plant leaves were prepared according to the procedure described by Santarius and Ryan[8] except that several foldsof gauzes were used to obtain juice from the tissue homogenates.

Parlial Purification of Crude Enzyme from V. angustifolia Leaves

Crude enzyme from leaves of *V. angustifolia* was prepared[8] and further purified, after dialysis against distilled water and subsequent lyophilization, by gel filtration on Sephadex G-100.

RESULTS

Effects of Buffer Concentrations Used for Noble and Bacto-Agar Gel Preparations

For the preparation of gels with noble or bacto-agar, a buffer should be used to maintain pH of the gels which was weakly acidic (about pH 6.1) when prepared with distilled water. Various concentrations (1 mM, 10 mM, 0.1 M and 0.2 M) of Tris-HCl, pH 7.4, were examined and 0.1 M was found to be suitable for use and produced comparable results to those of the Trypticase soy agar prepared with distilled water. When 0.2 M buffer was used, diffused zones caused by the hydrolysis of TAME tend to be larger, however, the color difference between the zones

and the background become closer and more difficult to estimate. When lower concentrations of the buffer were used, the pH of the gel was poorly maintained at the desired pH and hence the sensitivity became lower.

Sensitivities of Various Synthetic Ester Substrates against Several Proteases

Of the 4 synthetic ester derivatives basic amino acids, two arginine esters (BAEE and TAME) exhibited the highest sensitivities against bovine trypsin (Fig.1 and Table I). In the case of TAME, we could detect as

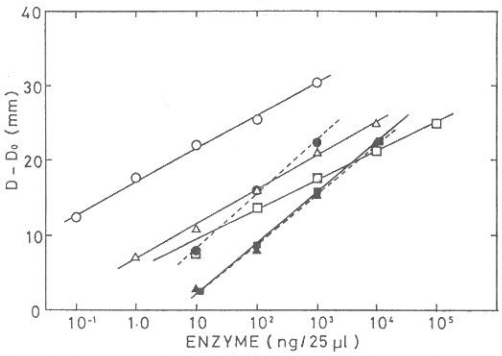

Fig.1. Comparison of the sensitivities of radial diffusion methods against several proteases. Trypsin was assayed by both TAME-BTB (○−○) and gelatin (△−△) methods. The other proteases, subtilisin BPN' (□−□), kallikrein (●···●), nattokinase (■−■) and subtilisin Carlsberg (▲···▲), were assayed by TAME-BTB method. Activities were expressed by the net diameter of the diffused zones. D, final radial diffusion diameter in mm ; D_0, well diameter in mm.

TABLE I. SENSITIVITY OF TAME-BTB RADIAL DIFFUSION METHOD AGAINST SEVERAL PROTEASES

Enzyme	Substrate	Sensitivity[a] (ng/25 μl)
Trypsin	TAME	0.1
	BAEE	1
α-Chymotrypsin	ATEE	10-100
Subtilisin BPN'	TAME	10
Nattokinase	TAMD	100
Subtilisin Carlsberg	TAME	100
Kallikrein	TAME	10-100

[a] Minimum enzyme amounts which produce clearly detectable diffused zones.

little as 0.1 ng trypsin as a clear detectable zone in either Trypticase soy agar or bacto-agar. On the other hand, using TLME or AAME as the substrate, we could detect 100 ng bovine trypsin. The other major differences between the former two and the latter are the color of the diffused zones, that is, the former bluish and the latter yelkowish orange.

TAME was also applicable for the detection of other proteases, such as subtilisins (BPN' and Carlsberg), nattokinase, kallikrein and papain. Minimum detectable amounts for these enzymes are 10 ng for subtilisin BPN', 100 ng for nattokinase or Carlsberg, and 10 to 100 ng for kallikrein per 25 μl sample solution (Table I). Papain was also detectable in our preliminary experiments, however, we did not determine the precise detectable amount.

ATEE was used for the detection of α-chymotrypsin, however, the sensitivity was not so high as TAME for trypsin. We could detect 10 ng chymotrypsin and the color produced by the enzymatic hydrolysis of the substrate was yellowish orange, and thus similar to TLME and AAME.

Effects of Acids, Bases, Salts and Buffers

When Trypticase soy agar and 10 ng trypsin were used, bases such as ammonia and sodium hydroxide contained in the sample solution produced diffused zones similar to that produced by the hydrolysis of substrates (TAME and BAEE). These bases were usable without serious effects at lower concentrations than 0.05 M and the acids such as acetic and hydrochloric acids at lower than 0.1 M. Neutral salts (KCl and NaCl) were usable without any effects under the conditions used. However, $CaCl_2$ slightly supressed TAME-hydrolytic activity at higher conceutration (1.8 M) possibly by the acidic property of the salt. Buffers such as Tris-HCl (pH 7.2) and K-phosphate (pH 7.2) were able to be used up to about 0.1 M safely. At concentrations higher than 0.25 M, diffused zones produced by these buffers overcome those produced by enzymatic hydrolysis of TAME. Ammonium bicarbonate (pH 8.5) was usable without any effects up

to 0.2 M and ammonium acetate (pH 7) up to 4.5 M, although the latter at high concentration produced orange zones and slightly depressed enzyme activity.

Application to the Detection of Proteinase Inhibitors

Correlation between the amounts of STI and residual tryptic activities are shown in Fig.2.

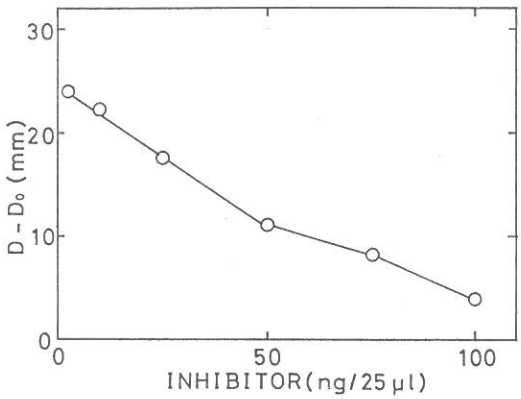

Fig.2. Detection of soybean trypsin inhibitor by TAME-BTB radial diffusion method. Aliquots (25 μl each) containing 100 ng trypsin and varying amounts (2.5-100 ng) STI in 0.1 M Tris-HCl (pH 7.4) were assayed by the TAME-BTB method as described in the text. Residual activities are expressed as in Fig.1.

This suggests that the TAME-BTB method could be used at least for qualitative detection of the inhibitors such as STI. However, under the same condition no significant inhibition was observed even with 100 ng VAI, which was found to be a kind of temporary type proteinase inhibitor for trypsin and chymotrypsin.[14]

Esterolytic Activities of the Extracts from Various Plant Leaves

Extracts from leaves of 10 plants all exhibited esterolytic activities by the TAME-BTB radial diffusion method (Fig.3 and Table II). Gelatinolytic activities were also detected in all of them although the diffused zones were smaller than those produced by the TAME-BTB method. By both the methods leguminous plants displayed the highest activities in all the plants examined.

Application to the Column Chromatography of Plant Enzymes

Sephadex G-100 gel filtration was chosen to illustrate the potential utility of the TAME-BTB method. Two distinct active peaks were

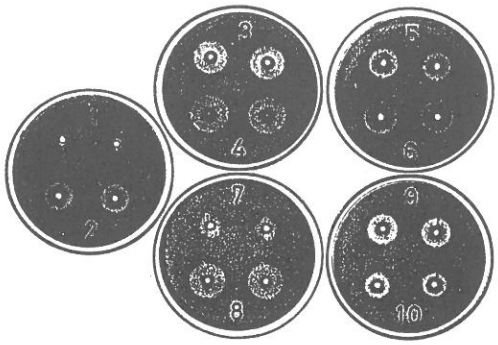

Fig.3. Photographic presentation of the TAME-BTB radial diffusion assay for plant enzyme screening. Duplicate aliquots (25 μl each) were assayed for each plant extract by the TAME-BTB method using bacto-agar gels prepared with 0.1 M Tris-HCl buffer (pH 7.4). Details are described in the text. The plant names numbered in the photography are given in Table II.

TABLE II
TAME-HYDROLYTIC ACTIVITY IN CRUDE EXTRACTS FROM PLANT LEAVES

Plant (Common mame)	Activity D-D_0(mm)		No.in Fig.1
Leguminosae			
Pisum sativum (Pea)	16.1	+++[a]	3
Vicia angustifolia (Vetch)	19.0	+++	4
Vicia faba (Broad bean)	16.2	+++	6
Compositae			
Artelisima vulagaris (Mugwort)	*13.7*	++	*2*
Calendula officinaeis	10.4	++	10
Lactuca sativa (Lettuce)	7.7	+	7
Iridaceae			
Iris sp. (Iris)	17.7	+++	8
Umbelliferae			
Petroselinum crhspum (Parsley)	14.0	++	9
Brassicaceae			
Brassica juncea (Brown mustard)	14.2	++	5
Polygonaceae			
Rumex crispus (Sorrel)	6.3	+	1

[a]Net diameter (D-D_0) of the radial diffusion zone was classified as weak (+, <10 mm), medium (++, 10-15 mm) and strong (+++, >15 mm).

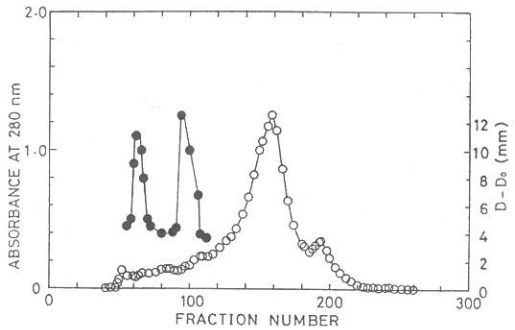

Fig.4. Gel filtration of the crude *V. angustifolia* leaf extract on a column (2.6 × 149 cm) of Sephadex G-100. Lyophilized crude extract (167 mg) was dissolved in 7 ml 0.01 M ammonium acetate, pH 7, and after clarification of the insoluble material by centrifugation, the supernatant was applied to the colum. Equilibration and elution were performed with 0.01 M ammonium acetate, pH 7.4 at a flow rate of 44 ml per h and fractions of 5 ml were collected. ○, Absorbance at 280 nm, ●, TAME-hydrolytic activity expressed by the net diameters of the diffused zones ($D-D_0$) as in Fig.1.

observed in the Sephadex fractions as shown in Fig.4. The same fractions, when assayed by the gelatin method, exhibited no detectable amount of gelatinolytic activity.

DISCUSSION

The method described in this paper based essentially on the slight change of gel pH resulted from the acid liberation by enzymatic hydrolysis of a synthetic ester substrate. The pH change was made visible by slight color changes between the diffused zones and the gel background by the aid of a pH indicator. Gel pH was initially adjusted to 7.3-7.4 and after enzymatic hydrolysis of TAME or BAEE, the pH of the produced zones declined and kept stable between 7.0, which is pK_a of BTB, and the initial gel pH, because of the homogeneous distributhon of the substrate in the gel matrix. Although the color change in these cases was maintained in alkaline side (blue), identification of the diffused zones was much easier than that of the acid color (orange).

The other substrates tested (TLME, AAME and ATEE) produced yellowish orange zones against bluish backgrounds. In these cases, boundaries of the diffused zones were ambiguous and not easy to estimate.

Of the several common pH indicators tested (Bromophenol Blue, Methyl Orange, Phenol Red and BTB) BTB was found to be most suitable for the detection of esterolytic activities of trypsin when TAME or BAEE was used as a ester substrate.

Although the TAME-BTB method is affected by acids and bases present in the sample solution, it is still a useful and sensitive method for screening proteases and for routine assay of chromatographic fractions. For these assays it is not necessary to use drastic conditions such as strong acids, bases or even buffers at high concentrations. Under moderate conditions they make no serious effects on this method.

The minimum detectable trypsin amount (0.1 ng) by the TAME-BTB method appeares essentially to be the same as that described by Santarius and Ryan for the gelatin method,[8] however, we have found this method actually more sensitive (larger in the diameters of the diffused zones) when both the methods were performed at the same time. Since activity and sensitivity of this method depend critically on the pH of the gels, Tris-HCl buffer to be used for the preparation of noble or bacto-agar gel should be prepared freshly each time or its pH should be checked just before use. Otherwise the pH of the buffer tends to rise slightly on standing and causes troubles.

We examined if the autoclaving process make any effects on the result of this method. For this purpose, chloramphenicol was included in the gel without autoclaving and the results were compared with that of the autoclaved gels. However, no essential difference was observed on the size of the diffused zones. On the other hand, in some cases heterogeneity of gel matrix was observed on non-autoclaved ones.

In addition to the protease assay this method

is also applicable to the assay of proteinase inhibitors. However, labile inhibitors such as VAI[14] was not clearly detectable by this method because of the long assay time. This may be a common feature of the radial diffusion methods.

Although two distinct peaks possessing TAME-hydrolyzing activities were detected in the Sephadex fractions, gelatinolytic activity was not detectable. The result was the same when assayed after lyophilization of these peaks. However, we could not confirm if the results were caused by the sensitivity difference between the two methods or by the own properties of the enzymes. Further studies need to be undertaken on the properties of these enzymes.

Limitation of the described method for the screening of proteases resides in the utilization of the small synthetic substrate, TAME or BAEE, which is applicable to detect trypsin-like activities only. On the contrary, gelatin is more general substrate for the detection of proteolytic activity. However, this method with its higher sensitivity, when used along with the gelatin method, might be useful to detect some kind of plant peptidases[15,16] which possess esterase activities also but no proteolytic ones.

Acknowledgemdnt. We appreciate the generous gift of purified kallikrein from Dr. H. Hiratani. This study was supported in part by a grant (17601) from the ministry of Education, Japan.

REFERENCES

1. J.Mestecky, F.W.Kraus, D.C.Hurst and S.A.Voight, *Anal. Biochem.*, 3,190 (1969)
2. W.-B.Schill and G.F.B.Schumacher, *ibid.*, 46,502 (1972)
3. G.F.B.Schumacher and W.-B.Schill, *ibid.*, 48,9 (1972)
4. M.Poberai und G.Savay, *Acta Histochem.*, 50,105 (1974)
5. H.W.Denker, *Histochemistry, 38, 331 (1974)*
6. A.Taufel, R.Friese and H.Ruttloff, *J.Chromatogr.*, 93,487 (1974)
7. H.Lowenstein and A.Ingild, *Anal.Biochem.*, 71,204 (1976)
8. K.Santarius and C.Ryan, *ibid.*, 77,1 (1977)
9. H. Sumi, H. Hamada, H. Tsuhima, H. Hiratani and H. Muraki, Experientia, 43, 1110-1111 (1986)
10. O.Abe, J.Ohata, Y.Utsumi and K.Kurolizu, *J.Biochem.*, 83,1737 (1978)
11. T.Chase and E.Shaw, "Methods in Enzymology", Vol.19, ed. by G.E.Perlman and L.Lorand, Academic Press Inc., New York, N.Y., 1970, p.20
12. M.Kunitz, *J.Gen.Physiol., 29,149 (1946)*
13. O.Abe, Y.Shimokawa, T.Araki and K.Kuromizu, *J.Biochem.*, 83, 1749 (1978)
14. O.Abe, Y.Shimokawa, J.Ohata and K.Kuromizu, *Biochim.Biophys.Acta*, 568,71 (1979)
15. W.C.Burger, N.Prentice, J.Kastenschmidt and M.Moeller, *Phytochemistry*, 7,1261 (1968)
16. E.C.Cameron and M.Mazelis, *Plant Physiol.*, 48,278 (1971)

Received Febrary 18, 1992
Accepted March 23, 1992