A Method for the Detection of β-Nitropropionic Acid in Crude Biological Extracts

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ABSTRACT

Methods now available for the detection of β-nitropropionic acid (BNP) in crude biological extracts are either nonspecific for BNP, require extensively purified samples, require large quantities of the unknown, or are too laborious to be utilized as a general screening method. These problems are especially acute where quantities of BNP are low and contaminants are in large excess. BNP is detected in crude biological systems using the technique of thin-layer chromatography. The sample is extracted with ether at pH 2.0 to 2.5 and evaporated to dryness. The extract is dissolved in 1.0 ml. of acetone and spotted on silica gel plates for either one- or two-dimensional thin-layer chromatography. The method can detect as little as 30 γ of BNP, which makes it feasible for the determination of small quantities of BNP in a large excess of contaminating materials. The method is flexible in that several solvent systems can be used.

β-Nitropropionic acid (BNP) is a metabolite of a number of fungi and plants (1). Reports indicate that it is present in the culture filtrates of such fungi as *Aspergillus flavus* (2), *A. oryzae* (3), and *Penicillium atrovenetum* (4). Concentrations as high as 10 mg. of BNP per liter of culture medium have been reported for *A. flavus* while *P. atrovenetum* is reported to produce up to 1.0 g. per liter of culture medium after 8 days of growth (4). BNP has been shown to be toxic when fed to chicks (5) or cattle (6) and a preliminary report indicates that the material may also be carcinogenic (7).

Since a large number of enzymes presently in use in the food industry are derived from *A. flavus* and *A. oryzae*, a method for detecting the presence of BNP in these preparations would be a valuable tool.

Several methods are available for the detection of BNP, most of which are not specific for this compound. Extracts of fermentation media or crude enzyme preparations usually contain colored impurities and other substances that interfere with color determinations and spectrophotometric assays. The Victor-Meyer test for nitroparaffins (8) or the determination of BNP at 250 nm. in 0.1N NaOH (9) are not specific for BNP and are subject to interference by other compounds present in the extract. Determination of the melting point depression of crystalline BNP with unknown (4) requires large amounts of extensively purified extract and is not feasible for routine analysis.

The method described by Matsumoto et al. (10) also suffers from a lack of specificity. BNP is hydrolyzed to produce nitrite ions which are then detected using the sulfanilic acid, 1-naphthylamine reagent (SANA). The sensitivity of the method is a function of the temperature of hydrolysis. Hydrolysis at 100°C. is less than half as sensitive as hydrolysis at autoclave temperatures of 120°C. Interfering substances

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are removed by dilution, making the detection of small quantities of BNP in a large excess of interfering substances difficult.

Shaw (11) has described a method for the determination of the methyl ester of BNP by gas chromatography. However, it is too laborious to serve as a general screening procedure.

Several chromatographic methods are available for the specific detection of BNP. Hatcher and Schmidt (12) describe a single-solvent system for the paper chromatography of BNP, the detecting reagent being SANA; whereas Marshall and Alexander (13) first reduced BNP with tin in HCl and subjected the resulting β-alanine to paper chromatography. Shaw and McCloskey (14) extracted BNP from culture filtrates and chromatographed them on silica gel thin-layer plates in a single solvent system, but only after extensive purification of the compounds. Harwig and Scott (15) have detected BNP in crude extracts by thin-layer chromatography on silica gel plates utilizing toluene: ethyl acetate:90% formic acid(6:3:1) as the solvent system. They also mention the solvent system benzene:methanol:acetic acid (24:2:1) but do not indicate that BNP was run in this solvent.

The chromatographic methods described above have generally made use of only a single solvent system. For some extracts, this may not sufficiently separate BNP from interfering substances and, therefore, cannot be used as a general method. The use of SANA as the detecting reagent is specific for nitrite ions and, therefore, requires the prior treatment of the chromatogram with base at elevated temperatures to hydrolyze BNP. This is then sprayed with the SANA reagent followed by HCl (14). We have found that, on standing, the SANA reagent darkens with time and must be prepared fresh at frequent intervals.

A technique has been developed using thin-layer chromatography on silica gel plates for the detection of BNP in crude extracts of commercial enzymes and fermentation liquors. The system utilizes three different solvent systems and is designed to be used as either a one- or two-dimensional system depending on the relative migration of BNP.

Although the method is designed primarily for fermentation products, it is applicable to any liquid or solid sample of biological material. The method can detect as little as 30 γ of BNP.

MATERIALS

Silica gel thin-layer plates, 250 μ thick were purchased from Analtech, Inc., Newark, Del. Plates were activated at 110°C. for 2 hr. and stored in a desiccator cabinet for up to 3 days. Plates were reactivated, if necessar, for 1 hr. at 110°C.

BNP was purchased from Aldrich Chemical Co., Inc., Cedar Knolls, N. J. and was recrystallized twice from chloroform (2).

Bromcresol green spray reagent was prepared by dissolving 0.3 g. of bromcresol green in 100 ml. of 80% methanol (v./v.) to which 8 drops of 30% NaOH was added.

Speedex was purchased from the Great Lakes Carbon Corp. New York, N. Y.

The standard BNP was dissolved in 95% ethanol at a concentration of 10 mg. per ml.

β-NITROPROPIONIC ACID

TABLE I. R_f VALUES OF BNP[a]

Solvent System[b]	R_f of BNP
A	0.80 (0.71)[c]
B	0.49
C	0.29

[a]$R_f = \dfrac{\text{distance of migration of BNP (cm.)}}{\text{distance of migration of solvent (cm.)}}$

[b]Solvent A consisted of 1-butanol:acetic acid:1% ammonium hydroxide (4:1:1)(13); solvent B, benzene:methanol:glacial acetic acid (45:8:4)(16); solvent C, benzene:ethanol:ammonium hydroxide (10:20:0.5)(16).

[c]R_f of BNP in solvent 1 to 2 days old.

METHODS

Extraction of the Sample

Enzyme samples and fermentation liquors are utilized directly. Tissue samples may be treated by grinding or other means prior to extraction.

For solid preparations, dissolve 30 g. in 200 ml. of water. For liquid preparations, start with 200 ml. of sample. The solution is adjusted to pH 2.0 to 2.5 with $4.5NH_3PO_4$ and any precipitate formed is filtered off in the presence of a filter aid, e.g., 5% Speedex. The filtrate is transferred to a 1-liter separatory funnel and extracted three times with 1 volume of ether (4).

In some cases a gummy substance is extracted and remains suspended in the ether layer. When this occurs, the ether layer is separated and a sufficient amount of Speedex is added to occlude the gum. The suspension is filtered and the cake washed with 2 volumes of ether (400 ml.). Any water trapped by the gum is removed by decanting the ether and recombined for further extraction.

The ether extracts are combined and evaporated to about 200 ml. over steam. The ether is dried by the addition of 10 g. of anhydrous $MgSO_4$ (5). After stirring for 5 min., the material is filtered and the cake washed with 100 ml. of ether. The combined ether fractions are transferred to a 600-ml. beaker and evaporated over steam to 25 to 30 ml. and finally to dryness at 40°C. in a vacuum desiccator. Care must be taken not to allow the sample to go dry over steam as destruction of the sample may occur at temperatures above the melting point (68° to 69°C.) of BNP (2).

The extract is quantitatively transferred, in ether, to a 5-dram vial and evaporated to dryness at 40°C. under a stream of nitrogen and stored at 2°C. prior to use.

Representative enzyme samples derived from *A. flavus* and *A. oryzae* were assayed for the presence of BNP. A sample of enzyme prepared from *A. oryzae* grown under submerged and semisolid conditions was tested as well as samples from different strains of *A. flavus-oryzae*. Enzymes prepared from *A. flavus-oryzae* grown in different media as well as a sample of fermentation liquor were also tested.

Initially, for the purposes of this investigation, two portions of each sample were extracted with ether. BNP (10 mg.) was added to one portion prior to extraction in order to monitor the compound throughout the procedure.
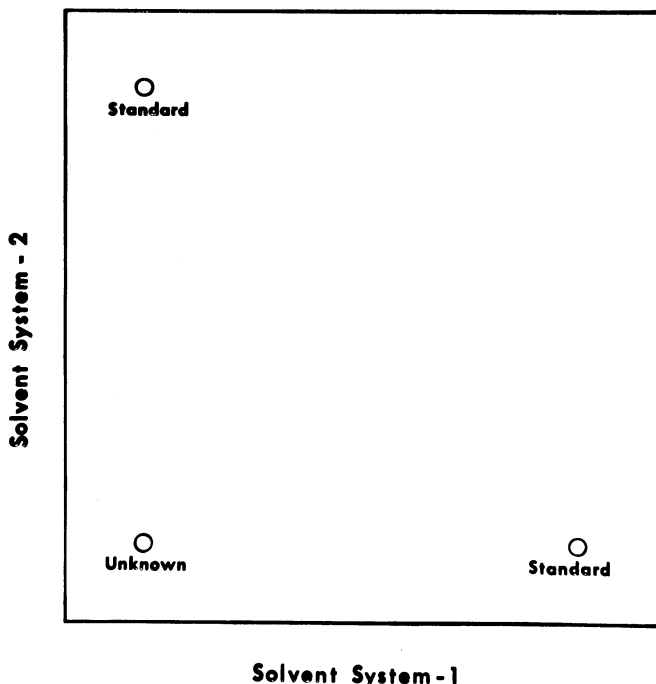
**Fig. 1.** Procedure for spotting and developing the two-dimensional chromatogram.

Chromatography

The dried sample is dissolved in 1.0 ml. of acetone and 15 μl. spotted on silica gel plates. Table I indicates that the relative rate of migration (R_f) of BNP varied with each of the three solvent systems: A, 1-butanol:acetic acid:1% ammonium hydroxide (4:1:1)(13); B, benzene:methanol:acetic acid (45:8:4); and C, benzene:ethanol:ammonium hydroxide (10:20:0.5)(16).

An R_f of 0.80 was obtained in freshly prepared 1-butanol:acetic acid:1% ammonium hydroxide (4:1:1); however, as the solvent system aged, the relative rate of migration of BNP decreased.

The unknown samples are first subjected to one-dimensional chromatography in each of the three solvent systems. It is useful to compare the unknown to a similar sample containing BNP. For investigative purposes we have extracted two portions of the unknown, one of which contains 10 mg. of added BNP standard, In practice, however, it is not necessary to extract two separate portions of each sample. A single extraction is sufficient provided that an additional spot of the unknown is included on the plate, to which 50 γ of BNP has been added directly to the spot prior to chromatography.

The plate is developed until the extent of migration of the solvent system is about 2 in. from the top. The extent of solvent migration is marked and the plate dried at 120°C. for 10 min. in a draft oven. After cooling to room temperature, the
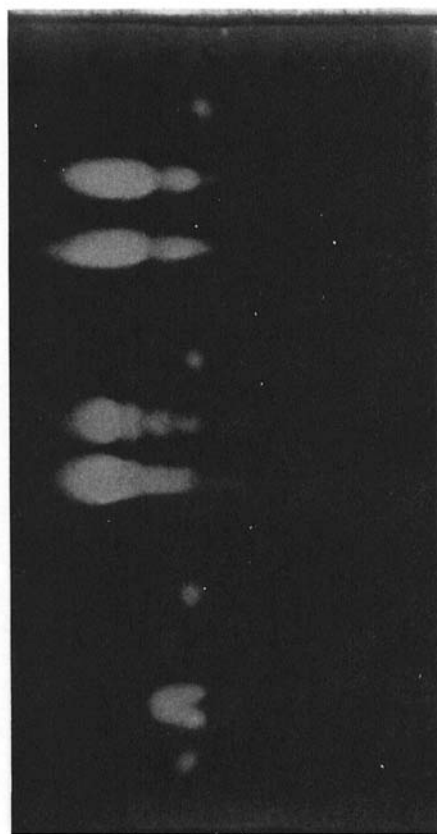
1. BNP

2. Submerged Medium of A. flavus-oryzae

3. Same as 2 plus BNP

4. Carbohydrase derived from 2

5. Same as 4 plus BNP

6. Carbohydrase from semi-solid grown A. oryzae

7. Same as 6 plus BNP

8. Protease from submerged A. oryzae

9. Same as 8 plus BNP

10. Lipase from submerged A. flavus-oryzae

11. Same as 10 plus BNP

12. BNP

Fig. 2.   One-dimensional chromatography of the enzyme extracts. The solvent system was benzene:methanol:acetic acid (45:8:4) (16). Where indicated, either 50 γ of BNP was added to the extract as described in the text or 10 mg. of BNP was added to the enzyme solution prior to extraction.
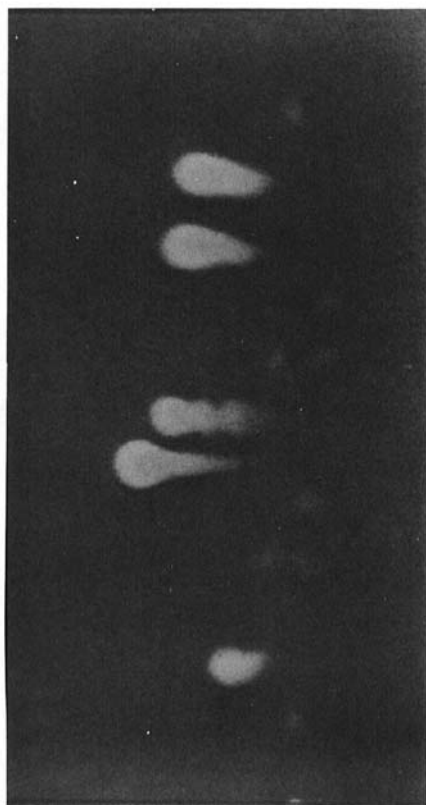
1. BNP

2. Submerged Medium of A. flavus-oryzae

3. Same as 2 plus BNP

4. Carbohdrase derived from 2

5. Same as 4 plus BNP

6. Carbohdrase from semi-solid grown A. oryzae

7. Same as 6 plus BNP

8. Protease from submerged A. oryzae

9. Same as 8 plus BNP

10. Lipase from submerged A. flavus-oryzae

11. Same as 10 plus BNP

12. BNP

Fig. 3.   One-dimensional chromatography of the enzyme extracts. The solvent system was 1-butanol:acetic acid:1% NH_4OH (4:1:1)(13). Where indicated either 50 γ of BNP was added to the extract as described in the text or 10 mg. of BNP was added to the enzyme solution prior to extraction.
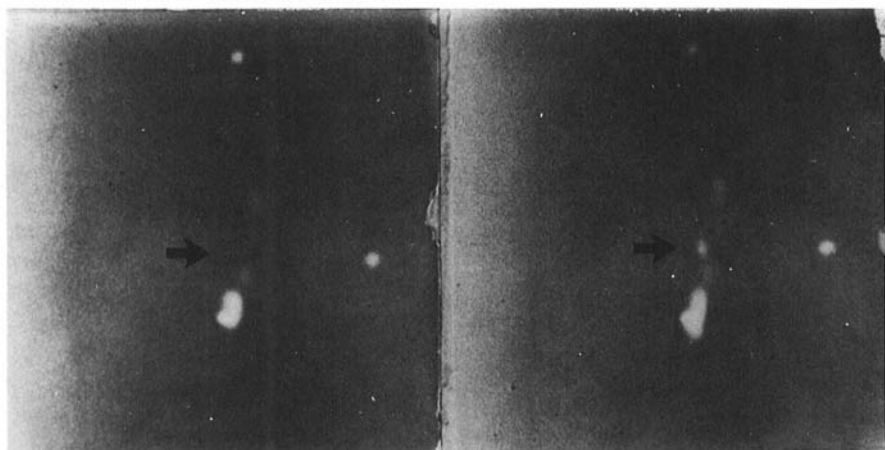
TWO DIMENSIONAL CHROMATOGRAPHY   TWO DIMENSIONAL CHROMATOGRAPHY OF SUBMERGED
OF SUBMERGED MEDIUM OF A. FLAVUS-ORYZAE   MEDIUM OF A. FLAVUS-ORYZAE WITH BNP ADDED.

Fig. 4.   Two-dimensional chromatography of the submerged medium of *A. flavus-oryzae* extract. The solvent for the first dimension consisted of benzene:methanol:acetic acid (45:8:4) (16). The solvent for the second dimension consisted of 1-butanol:acetic acid:1% NH_4 OH (4:1:1)(13). BNP (50 γ) has been added to the sample on the right prior to chromatography.

plate is sprayed with bromcresol green to develop the chromatogram. The BNP as well as other acidic spots appears as bright yellow against a sky blue background. The clear distinction of BNP in two of the three solvent systems compared with the known indicates a positive test.

For some highly impure samples, the one-dimensional system may not sufficiently separate the BNP spot from contaminating substances. In this case, the sample is subjected to two-dimensional chromatography; the combinations of solvent systems employed will depend on the relative rate of migration of BNP compared to the contaminating material. If possible, at least one of the solvent systems employed should separate BNP from the bulk of the contaminants. Figure 1 indicates the procedure for spotting and running the two-dimensional chromatogram.

The standard (50 γ of BNP in 5 μl. of 95% ethanol) is spotted in the lower right-hand corner about 1 in. from the side and bottom edges of the plate. The unknown (15 μl.) is spotted equidistant in the lower left-hand corner. In the two-dimensional system, it is also useful to compare a positive plate with the unknown. In this case, an amount of standard equal to at least 50 γ of BNP may be spotted on top of the unknown on a separate plate. The plate is developed in solvent 1 to a point about 2 in. from the top. The extent of migration of the solvent is marked, and the plate dried in the hood for 1 hr. or in a 40°C. draft oven for 10 min. The plate is then rotated 90° to the left and another 50 γ sample of BNP spotted 1 in. from each edge in the lower left-hand corner. The plate is then
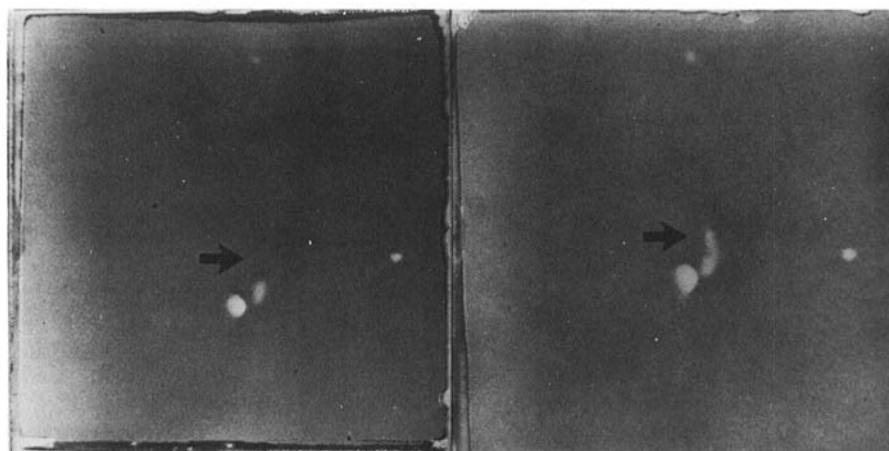
CARBOHYDRASE FROM SEMI-SOLID GROWN A. ORYZAE   CARBOHYDRASE FROM SEMI-SOLID GROWN
                                                                    A. ORYZAE WITH BNP ADDED

Fig. 5.   Two-dimensional chromatography of the carbohydrase derived from semi-solid grown
A. oryzae. The solvent systems were similar to those described in Fig. 4. BNP (50 γ) has been
added to the sample on the right prior to chromatography.

developed in solvent system 2 until the extent of migration of the solvent system is
about 2 in. from the top. The extent of solvent migration is marked and the plate
dried and developed as described for the one-dimensional system.

RESULTS

Samples to which BNP had been added prior to extraction gave exactly the same
results as samples to which BNP had been spotted directly on the thin-layer plate.
The results in Figs. 2–5 indicate that samples that contain BNP can be readily
distinguished from samples that do not contain BNP. In some cases, the area in
which BNP migrates was obscured by contaminating substances in the
one-dimensional system. When these samples were assayed in the two-dimensional
system, the sample to which BNP had been added was readily distinguished. The
plates were similar to those shown in Figs. 4 and 5.

Generally,   if   there   are   no   interfering   substances,   one-dimensional
chromatography of the sample in two of the three solvent systems is sufficient for
the determination of BNP. As indicated in Figs. 2 and 3, one-dimensional
chromatography in two solvent systems was sufficient to demonstrate the absence
of BNP in the carbohydrase derived from a submerged fermentation of *A.
flavus-oryzae*, the protease from a submerged fermentation of *A. oryzae*, and the
lipase from a submerged fermentation of *A. flavus-oryzae*. The region where BNP
migrates was obscured by contaminating substances in both the submerged medium
of *A. flavus-oryzae* and the carbohydrase derived from semisolid-grown *A. oryzae*.
These extracts were therefore subjected to two-dimensional chromatography. Two

plates were run for each sample. On one plate, 50 γ of BNP was spotted directly on top of the sample prior to chromatography.

As indicated in Figs. 4 and 5, no BNP could be detected in either the submerged medium of *A. flavus-oryzae* or in the carbohydrase derived from a semisolid fermentation of *A. oryzae*. The plates to which BNP had been added to the sample prior to chromatography clearly indicated that BNP was readily detectable in this system.

DISCUSSION

Methods now available for the detection of BNP in crude extracts of biological materials are either lacking in specificity for the compound or cannot be readily utilized with a wide variety of different samples. These problems are especially acute if the concentration of BNP is low in relation to the contaminating materials.

A method has been devised which utilizes the technique of thin-layer chromatography for the specific detection of BNP in crude extracts of biological materials. The method is rapid and specific for BNP. BNP can be readily separated from contaminating substances by the technique of two-dimensional chromatography. The system is flexible in that various solvent systems may be used in conjunction with those mentioned here. The acid base reagent bromcresol green is employed as the detecting reagent, because this requires no harsh pretreatment of the plate prior to detection, and it is stable over many months at room temperature making it more convenient for large-scale screening of samples.

Studies using various quantities of BNP added to the sample indicated that the method can detect as little as 30 γ of BNP which corresponds to 70 p.p.m. for solid materials and 10 p.p.m. for liquid samples. The method is at least as sensitive and, in most cases, more sensitive than those previously reported (5,10,14). One report indicates that BNP can be detected at a level of 6 γ using a modification of the Matsumoto method (17). However, the method is not specific for BNP and is subject to interference by contaminating compounds.

The method, although designed for fermentation products, is applicable to any biological material including tissue previously extracted by grinding or other suitable means.

A number of representative samples of enzyme preparations produced by the *A. flavus-oryzae* group as well as a sample of the fermentation medium have been assayed for BNP. BNP was not found in any of the samples tested. This indicates that within the detection limits of the above test, BNP does not appear to be produced under the conditions employed for the industrial production of enzymes.

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