THE ISOLATION OF ANILINOTHIAMINE FORMED FROM THIAMINE AND ANILINE BY BACILLUS ANEURINOLYTICUS KIMURA ET Aoyama

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The thiaminase of Bacillus aneurinolyticus Kimura et Aoyama (BKA) was demonstrated by Fujita et al. (1) to decompose thiamine into 2 methyl 4 amino 5 hydroxymethylpyrimidine (Pm·CH₂OH) and 4 methyl 5 β hydroxyethylthiazole (Th), entirely differing from the mode of thiamine decomposition by Bacillus thiaminolyticus Matsukawa et Misawa, which was proved to be a base exchange reaction (3). However, anilinothiamine, i.e. N (2 methyl 4 amino pyrimidyl 5 methyl) aniline (AT) was proved by paper partition chromatography (PPC) (2, 4) to be formed after incubating bacterial suspension together with thiamines and aniline.

On the other hand, the thiaminase activity of the bacterial cells was inhibited, rather than activated, by aniline. It is also inhibited by both pyridine and quinoline without producing any base exchange products (2).

In order to test the real production of AT exceptionally from aniline alone, the authors attempted to isolated the supposed AT from the decomposition products and the crystals formed by the enzymatic action were in fact identified as AT.

EXPERIMENTAL

1. Preliminary Experiment.

Various isolation procedures were attempted using both synthetic AT and the enzymatic product, but fractionation with organic solvents, e.g., chloroform, ether, benzene, did not lead to crystallization, possibly being prevented by an existing brown oily substance. Various precipitation reactions using picric acid, phosphotungstic acid or weakly alkaline silver nitrate were combined, but it failed to isolate the crystals. Various chromatographies were then attempted and some cation exchange resins i.e., Amberlite IR 120 and IRC 50 were found the most effective.

Both resins were ground in a mortar after drying. Amberlite IR 120
was used in 80–100 mesh range, IRC – 50 over 100 mesh range. They were washed with water, then with 5 per cent sulfuric acid, followed by thorough washing with water. AT is adsorbed on both H and Na-forms of Amberlite IR - 120 from its acid solution. It is hardly eluted either by 2N HCl or by 25 per cent KCl in 0.01 N HCl, but readily by 1 N NH₄OH-ethanol (1 : 1). Being however a displacement chromatography as shown in Fig. 1, the separation of AT was not sufficient and on applying the enzymatic materials, the eluate was not free from oily substances and the crystallization was inhibited. But the adsorbability of the resin being very high, hardly affected either by acids or by salts and Pm·CH₂OH being completely removed by eluting with 100ml 2N HCl, the sample was first adsorbed on the resin.

AT is adsorbed on either the H or buffered form (pH 4.2) of Amberlite IRC – 50, but fails on the Na-form of the resin. Since the elution by 0.1 N HCl takes a form of displacement chromatography, the separation of AT was insufficient. For obtaining an elution chromatography, AT was allowed to be adsorbed on the resin² column, buffered at pH 4.2, followed by elution with 1 M acetate buffer, pH 4.2.

In this case the elution took a form of elution chromatography, but the volume of the effluent was too large, the subsequent procedure being difficult. 1 M NaCl in 0.01 N HCl was then used as an eluting solution and the clear-cut separation was effected as shown in Fig. 2. The crystallization of AT was thus attained.

² The resin was converted to a Na-form from H-form, followed by washing with water. 1 M acetate buffer, pH 4.2, was then passed through until the effluent became pH 4.2, followed by thorough washing with water.
2. *Isolation and Identification of AT formed by BKA.*

BKA was cultured aerobically in an ordinary broth at 37° for 3 days. It was centrifuged and the bacterial cells were washed with a saline solution, and suspended in water. It was used as an intracellular enzyme.

100 mg Thiamine and 600 mg aniline hydrochloride were dissolved in 40 ml of M/15 phosphate buffer, pH 6.5 and the pH was corrected to 6.5. The suspension containing the bacteria grown in 4l media was then added and the total volume was made up to 400 ml with distilled water. The whole was incubated at 38° for 72 hours. Thereafter, 3 volumes of ethanol was added and the mixture was allowed to stand for 30 minutes. The supernatant after centrifugation was concentrated to about 40 ml *in vacuo* at below 60°. An aliquot subjected to PPC revealed the presence of AT, Th and Pm-CH₂OH together with a little undecomposed thiamine. The remaining deproteinized solution was adjusted to pH 4.5 with 1 N HCl and extracted five times with 20 ml each of ether, whereby both Th and a considerable amount of oily substance were removed.

The aqueous phase was then adjusted to pH 10 with a strong ammonia and extracted five times with 20 ml each of chloroform, whereby both AT and Pm-CH₂OH were transferred into a chloroform phase. The chloroform phases were collected, washed with water, dehydrated with sodium sulfate, and the solvent was evaporated. The residue was extracted by warming three times with 10 ml each of benzene. The benzene-insoluble residue was negative for the Dragendorff reaction. The benzene solution was shaken with 15 ml of 0.1 N HCl, whereby the substance was transferred into the aqueous phase. The benzene solution was further washed four times with 10 ml each of water. The aqueous phase and the washings were combined and it is passed through a column of Amberlite IR-120, 4.0 g, 0.5 × 15 cm, at a rate of 0.7 ml/min, followed by washing with 71 ml of water, 0.8 ml/min. The column was developed with 95 ml of 2 N HCl, 0.8 ml/min, whereby Pm-CH₂OH was removed. After washing with 30 ml of water, 1.0 ml/min, it is eluted with a solvent-system, 1 N NH₄OH-ethanol³ (1 : 1) at a rate of 0.6 ml/min.

The fraction showing an intensive absorption at 245 mµ as shown in Fig. 1 was collected and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of 0.05 N HCl, to which 5 ml of 1 M acetate buffer, pH 4.2, was added and the whole was made up to 50 ml with distilled water. It was then adsorbed on a column of Amberlite IRC-50⁴, 4.0 g, buffered at pH 4.2, 1.0 × 9.0 cm, 0.7 ml/min. After washing with 32 ml of water, 0.8 ml/min, it was eluted by 1 M NaCl in 0.01 N HCl, 0.6 ml/min. The fraction showing an intensive absorption at 245 mµ was collected and adjusted to pH 10 with a strong ammonia. The mixture was extracted five times with 20 ml each of

³ As AT is scarcely eluted both in acid and neutral reactions, it was discharged with ammonia. Ethanol was added up to 50 per cent, since AT is hardly soluble in water.

⁴ It is a weakly acidic cation exchanger and adsorbs AT well. As it has an abnormally intensive affinity for hydrogen ion, it was buffered with acetate at pH 4.2 for increasing the exchange capacity, thus avoiding the direct adsorption on IRC-50 of AT which had been transferred from benzene into HCl.
chloroform and the chloroform phases were collected, followed by washing with water. After dehydrating with sodium sulfate, the solvent was evaporated. The residue was almost colorless, partially crystallized.

By recrystallization from 50 per cent ethanol, 8.5 mg of crystals were obtained. Further five recrystallizations gave about 2 mg of colorless prisms, mp 169-170° (uncorrected).

By mixed melting point, elementary analysis and absorption spectra, the crystals were identified with the synthetic compound.

Melting point: Enzymatic product, mp (macro) 169 170° (uncorrected)
Synthetic compound, mp (macro) 169 171° (  )
mixed mp 168 170° (  )

Elementary analysis:
Found, sample 1.775 mg : N₂, 0.3861 ml (17°/762 mm)
N, 25.67 %
Calculated for C₁₂H₁₄N₄ : N, 26.15 %

Absorption maxima:
Enzymatic product (in 99.5 per cent ethanol)
240, 274 mμ
'' '' (in 0.1 N HCl)
243 mμ
Synthetic compound (in 99.5 per cent ethanol)
239, 274 mμ
'' '' (in 0.1 N HCl)
244 mμ

SUMMARY

The production of anilinothiamine by *Bacillus aneurinolyticus* Kimura et Aoyama in the presence of both thiamine and aniline was confirmed by isolating the crystals and by comparing the elementary analysis and physical constants with those of the synthetic compound. The yield of the isolated anilinothiamine was not clear, because the pyrimidine moiety which is produced simultaneously absorbs remarkably at 245 mμ, thus making the separate determination of anilinothiamine impossible. For isolating the crystals the application of ion exchange resin column chromatography was proved to be efficient.

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REFERENCES