

Inhibition of Alanine and Aspartate Aminotransferases by β -Nitropropionic Acid

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ABSTRACT: The inhibition of serum alanine and aspartate aminotransferases (SALT and SAST) by β nitropropionic acid (β NPA), toxic metabolite of some fungi higher plants) in vitro was studied. The results indicated that both SALT and SAST were competitively inhibited by β NPA and the enzymes recovered their original activity by dialysis, indicating that the inhibitory effect of β NPA is reversible. The inhibition of both SALT and SAST by β NPA was found to be slow and showed the characteristic of a first order reaction up to 30 minutes. The rate constants characterizing this inhibition, namely: the binding constant (K_B) (90 μ M and 225 μ M for SALT and SAST, respectively) and bimolecular velocity of inhibition k_i (666 and 714 (M min)⁻¹ for SALT and SAST, respectively) were determined. K_n (rate of nitrification of the enzymes) for SALT and SAST were 0.06/min and 0.18/min, respectively.

INTRODUCTION

It is well known that transamination provided important links between the metabolism of three amino acids: aspartate, glutamate, and alanine, and the analogous α -keto acid members of the citric acid cycle.⁽¹⁾ Serum alanine aminotransferase (SALT) reversibly transfers the amino group from alanine to α -ketoglutarate, forming pyruvate and glutamate. Aspartate aminotransferase (AST) transfers an aminogroup from aspartate to α -ketoglutarate forming oxalacetate in this

process. The liver is a rich source of aminotransferases (ALT & AST) and their measurement has been of value in the diagnosis of liver disorders. These enzymes are most abundant in the liver. They are not secreted into the blood, and therefore any elevation of their plasma levels is due to leakage from damaged cells. Transaminase levels are used for diagnosis of liver diseases. In viral hepatitis, plasma levels of both enzymes can be 20-100 times above upper limit of normal range. Enzyme elevations are

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proportional to extent of ongoing tissue damage, and they can be demonstrated before fever and jaundice develop. SALT is specific for liver diseases, but SAST is also increased in muscles diseases and acute myocardial infarction.⁽²⁾

Transamination is the major process for removing N_2 from aminoacids or transfer N_2 to α -keto acids. All aminoacids except lysine and threonine undergo trans-reactions. For most of these reactions, α -keto glutarate and glutamate serve as one of the α -ketoacid-aminoacid pairs. Pyridoxal phosphate is a necessary cofactor for these reactions.⁽³⁾

β NPA has been formed as a metabolite of higher plants and fungi, which is responsible for toxic symptoms produced in dairy cattle.⁽⁴⁾ It has been identified as a *Penicillium atrovenetum* product from streptomycetes species⁽⁵⁾ as well as some strains of.⁽⁶⁾ It is easily derived from β propiolactone (now considered to be a carcinogenic substance)⁽⁷⁾ by reacting with sodium

nitrite.⁽⁸⁾ Birch *et al.*,⁽⁹⁾ found that aspartic acid-4C¹⁴ was converted to β -NPA-1C¹⁴ by *Penicillium atrovenetum*. Marshall⁽¹⁰⁾ emphasized the possibility of β -alanine being an intermediate in fungal nitrification and considered aspartic acid to be its precursor.

Previous work^(11,12) indicated that β NPA propionate manifests inhibitory effects on whole brain acetyl cholinesterase (AChE) as well as monoamine oxidase extracted from rat brain.⁽⁷⁾ These findings gave an idea to study its effect on serum aminotransferases (SALT and SAST) as these two enzymes are of great value as liver function tests, especially that β NPA has structural similarity to their substrates alanine and aspartate. Kinetic measurements were carried out to evaluate its inhibitory power, such as bimolecular velocity of inhibition, affinity constant nitration rate as well as the type of inhibition of SALT and SAST by β NPA.

MATERIAL AND METHODS

β NPA used was prepared by the chemical reaction between β -propiolactone (BDH) and sodium nitrite.⁽¹³⁾ Samples also were purchased from Aldrich Chem. Co., England. Fresh human serum (obtained from Medical Research Institute Blood Bank) was used as source of the enzymes. The enzymes SALT and SAST activities were assayed by the method of Reitman and Frankel.⁽¹⁴⁾ In this method, the keto acid formed was measured colorimetrically after combination with 2,4-dinitrophenyl hydrazine, then the hydrazone formed was measured at 530 nm.

The assay mixture for SALT contains: 1 ml phosphate buffer (0.1 M, pH 7.4) containing 0.2 M DL-alanine, 2mM α -oxoglutarate and 0.2 ml serum (containing an amount of enzyme equivalent to 25 U/l), then incubated at 37°C for 30 minutes. For SAST, the assay mixture contains: 1ml phosphate buffer (0.1M, pH 7.4) containing 0.1 M L-aspartate, 2mM α -oxoglutarate and 0.2 ml serum (containing an amount of enzyme

equivalent to 25 U/l), then incubated at 37°C for 60 minutes.

For the determination of the type of inhibition and the enzyme-inhibitor dissociation constant (K_i) the substrate concentration was varied: 75, 100, 150, and 200 mM DL-alanine for SALT, as well as 25, 50, 75, and 100 mM L-aspartate for SAST. β NPA was kept at a constant concentration for each experiment: 4.25, 8.50, or 17 mM in case of SALT and 17, 34, or 68 mM in case of SAST. The inhibitor and substrate were added simultaneously to above mentioned incubation mixtures.

For the determination of the binding constant (K_B) and the bimolecular velocity of inhibition (k_i) of SALT and SAST by β NPA; the experimental technique developed by Main⁽¹⁵⁾ was used. According to this technique, β NPA was added to the above mentioned assay mixtures in the following concentrations: 1.7, 3.4, 6.8, 13.6, and 17.0 $\times 10^{-5}$ M in case of SALT, and 8.5, 17.0, 25.5, 34.0, and 42.5 $\times 10^{-5}$ M in case of SAST. The mixture was assayed

after incubation of the enzyme and β NPA at 37°C for several time intervals: 5, 10, 15, 20, 25, and 30 minutes, then the substrate was added to measure the remaining activity.

Dialysis: the enzyme (0.2 ml for each of SALT and SAST) with β -NPA (17 mM) were dialyzed overnight against phosphate buffer at 4°C, with occasional change of buffer. Control of enzyme without inhibitor dialyzed and undialyzed were also taken.

RESULTS

The activities of SALT and SAST were measured at substrate concentration 0.2 M alanine and 0.1 M aspartate, respectively. Normal values obtained were 25 and 20 U/L for SALT and SAST, respectively. Using a relatively low concentration of β NPA (4.25 mM in case of SALT and 17 mM in case of SAST) the activities of the two enzymes were decreased to 75% of control. It has been found that the concentrations of β NPA which caused 50% inhibition of the activities of SALT

and SAST were 8.5 mM and 34 mM, respectively.

Figure 1a (taking SALT curves as example) shows that the double reciprocal curves of reaction velocity (v) plotted against substrate concentration (s), when keeping the inhibitor at constant concentration in each experiment and changing the substrate concentrations were in accordance to that mentioned by Dixon and Webb⁽¹⁶⁾ for the competitive type of inhibition. These were confirmed by replot of the slopes obtained from figure 1a against inhibitor concentration [I]. A linear line was obtained (figure 1b) indicating that the inhibition of each of SALT and SAST by β NPA is of the linear competitive type (after Cleland nomenclature).⁽¹⁷⁾ The values obtained for K_m (Michaelis constant) and K_i (enzyme-inhibitor dissociation constant) are listed in table 1. Graphical representation of the inhibition curves of SALT as well as SAST at different concentration of β NPA obtained by plotting log velocity (v) of SALT as well as SAST remaining uninhibited against

time (t) show straight lines (figure 2a taking SAST as an example). This indicated that the inhibition of each of SALT and SAST by β NPA follows first order reaction kinetics.⁽¹⁸⁾ The rate constant characterizing the inhibition was calculated using the following equation:⁽¹⁸⁾

$$k_i \cdot [I] \cdot \Delta t = 2.303 \log \frac{v_0}{v_0 - v_i}$$

Where k_i is the biomolecular velocity of inhibition (M min)⁻¹, v_0 and v_i are the velocity of uninhibited and inhibited reactions respectively. The slopes of the lines were used to construct the relationship between [I] against $\Delta t/2.303 \Delta$

$\log v$ as described by Main and Hastings.⁽¹⁸⁾ The nitration rate k_n is obtained from 1/slope of the straight line figure 2b while the binding constant K_B is obtained from the intercept of the straight line on [I] axis, while the intercept on the vertical line gave $1/k_i$ ($k_i = k_n/K_B$). The values of K_B , k_n , and k_i are given in table (2).

The inhibitory effect of β NPA on SALT as well as SAST could be abolished by dialysis. The dialyzed enzymes recovered 85% of their original strength as compared with the control subjected to the same conditions.

Table (1): Enzyme-inhibitor dissociation constant (K_i) for SALT and SAST in the presence of β NPA. K_m is the Michaelis-Menten constant.

| Constant | Enzyme | |
|----------------------|---------|----------|
| | SALT | SAST |
| K_i (slope replot) | 5.60 mM | 17.00 mM |
| K_m | 0.14 mM | 0.12 mM |

Table (2): Inhibition of SALT and SAST by β NPA. Calculations of the binding constant (K_B) and the rate of nitration (k_n) from measurements of $2.303 \Delta \log v/\Delta t \pm$ S.D., at different concentrations of β NPA [I]. k_i is the bimolecular velocity of inhibition of the enzyme by β NPA.

| [I] 10^{-5} M | SALT $2.303 \Delta \log v/\Delta t$ | [I] 10^{-5} M | SAST $2.303 \log v/\Delta t$ |
|---|--|---|---------------------------------|
| 1.7 | 6.9 \pm 0.50 | 8.5 | 4.6 \pm 0.20 |
| 3.4 | 16.1 \pm 0.90 | 17.0 | 5.7 \pm 0.30 |
| 6.8 | 25.3 \pm 1.25 | 25.5 | 9.2 \pm 0.82 |
| 13.6 | 43.7 \pm 2.35 | 34.0 | 12.4 \pm 0.93 |
| 17.0 | 48.3 \pm 3.16 | 42.5 | 25.3 \pm 1.50 |
| $K_B = 90 \mu\text{M}$ | | $K_B = 255 \mu\text{M}$ | |
| $k_n = 0.06 \text{ min}^{-1}$ | | $K_n = 0.18 \text{ min}^{-1}$ | |
| $k_i = 666 \text{ M}^{-1} \text{ min}^{-1}$ | | $k_i = 714 \text{ M}^{-1} \text{ min}^{-1}$ | |

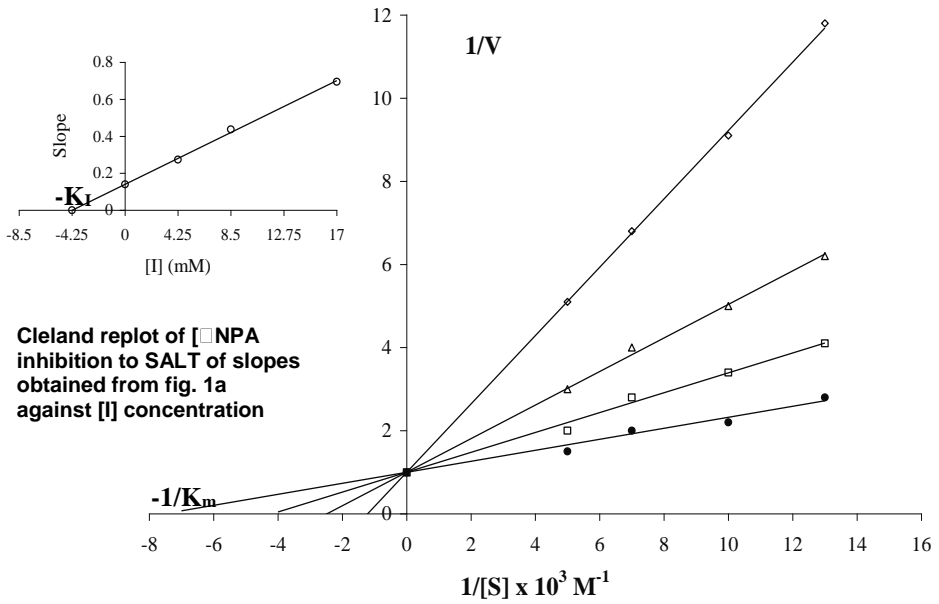


Figure (1b): Cleland replot of \square NPA inhibition to SALT of slopes obtained from fig. 1a against $[I]$ concentration

Figure (1a): Lineweaver-Burk plot of the inhibition of SALT by \square NPA, the concentrations used were (\blacklozenge) 0, (\square) 4.25, (Δ) 8.5 and (\diamond) 17mM

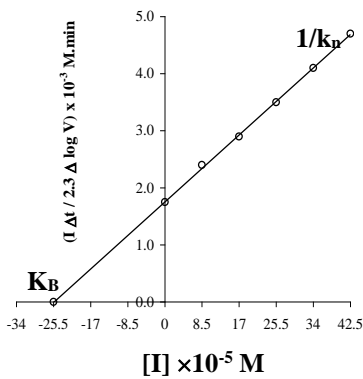


Figure (2b): Main plot after the data of inhibition obtained by plotting $[I]/\text{slopes}$ of the curves in fig.(2a) against \square NPA concentrations.

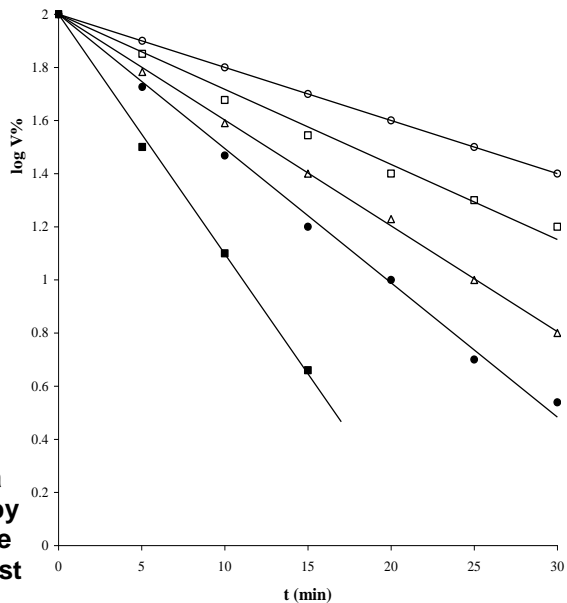


Figure (2a): Log (activity %) of SAST plotted against time (t) at various concentrations of \square NPA $[I]$. (\circ) 0.85, (\square) 1.7, (Δ) 2.55, (\bullet) 3.4, (\blacksquare) 4.25×10^{-4} M

Discussion

From the kinetic studies of transamination of the amino acids alanine and aspartate to their corresponding keto acids pyruvate and oxaloacetate catalyzed by the enzymes SALT and SAST respectively in the absence or presence of β NPA, it was possible to evaluate their type of inhibition and the enzyme-inhibitor dissociation constants (K_i).

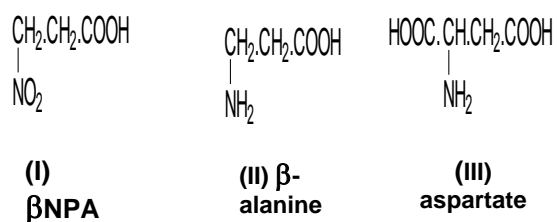
It was possible to determine the type of inhibition of both SALT and SAST by β NPA which was of the competitive type. A similar observation was noticed when β NPA was tested as an inhibitor for each of AChE⁽¹¹⁾ and MAO.⁽¹⁹⁾

It could be formulated that β NPA nitrates SALT and SAST (with a nitration rate $k_n = 0.06/\text{min}$), as organophosphates phosphorylate,⁽¹⁵⁾ carbamates carbamylate⁽²⁰⁾ and dimethyl sulfoxide sulfonylates AChE.⁽²¹⁾

Moreover, the bimolecular velocity of inhibition (k_i) indicated that the inhibition of the two enzymes is of the same order and

magnitude (666/M min for SALT and 714/M min for SAST). It is considered to be the most reliable criterion to measure the inhibitory power of an inhibitor to the enzyme.⁽¹⁵⁾

From the present results, it seems that the inhibition of SALT and SAST by β NPA takes place through the competition with their amino acid substrates, as β NPA shows structural similarity to them:



The competition will be thus easier with alanine (3C) than that with the aspartic acid (4C).

The concentrations of β NPA which caused 50% inhibition of the enzymes SALT and SAST were 8.5 mM and 34 mM, respectively, indicating that the amount of β NPA needed to inhibit SALT was lower than

that needed to inhibit SAST, this is due to the similarity between β NPA and the substrate alanine (both containing 3C atoms). This was also confirmed with the values of K_i which was for SALT about 1/3 that of SAST. Also the values of K_B , K_n and k_i in the case of SALT were lesser than those of SAST with β NPA.

The inhibition of SALT and SAST results in the decrease of the transformation process of alanine and aspartate to their keto acids. Therefore, a portion of these amino acids will proceed via deamination to join tricarboxylic acid cycle and hence be oxidized to CO_2 and H_2O or converted into glucose or glycogen (gluconeogenesis). The fate of the major part of aspartate will be through the urea-ornithine cycle, resulting in more urea formation, which may be one of the causative toxic symptoms produced in dairy cattles as a result of eating plants contaminated with β NPA produced by certain fungi.^(5,6)

In conclusion: the present work provides β NPA as an amino transferase

inhibitor in addition to its inhibitory action on cholinesterase⁽¹¹⁾ and monoamine-oxidase⁽¹²⁾

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