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ARYLAMIDASE OF HUMAN LIVER

FRANCIS J. BEHAL, GWYNNE H. LITTLE *AND ROBERT A. KLEIN

Departments of Biochemistry and Microbiology, School of Graduate Studies, Medical College of Georgia, Augusta, Ga. (U.S.A.)

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SUMMARY

1. Arylamidase from human liver, which catalyzes the hydrolysis of certain amino acid derivatives of β-naphthylamine, was purified 2120-fold by salt fractionation, gel filtration, ion-exchange chromatography, and adsorption chromatography.

2. Acrylamide gel electrophoresis and analytical ultracentrifugation of the final enzyme preparation indicated that it was a single protein with a $s_{20,w}$ value of 8.5.

3. Only α-amino acid β-naphthylamines of the L configuration were susceptible to arylamidase catalyzed hydrolysis; alanine-β-naphthylamine had the highest v_{max} value. Several other substrates in which the amino acid residue had a larger non-polar or basic R group such as methionine-β-naphthylamine or arginine-β-naphthylamine had the lower K_m values.

4. This enzyme had no dipeptidyl arylamidase activity, but rather cleaved amino acid residues from dipeptide-β-naphthylamine in a step-wise manner beginning with the N-terminal residue.

INTRODUCTION

Isozymic forms of arylamidase of human origin have been the subject of a number of reports in the literature recently[1–5]. Although similar in terms of enzymatic properties, these isozymes differ in chromatographic and electrophoretic behavior. It is now recognized that these enzymes are not leucineaminopeptidase, as they were earlier thought to be on the basis of their catalyzing the hydrolysis of L-leucine-β-naphthylamide. The term, arylamidase, has gained wide usage in the literature and we have utilized it in the same sense as other investigators have, pending a definitive elucidation of the role of this enzyme. Changes in plasma levels of these enzymes have been attributed to certain neoplastic diseases, particularly of the hepatobiliary tract[6,7]. Their biological function is unclear, although some authors suggest a role in regulating certain pressor substances such as angiotensin[8]. The arylamidases of human plasma have been separated in our laboratory and components were found which corresponded chromatographically and electrophoretically to isozymes from small intestine, liver, and pancreas[3]. That there is a group of human tissue specific

arylamidase isozymes is now clear and experiments in our laboratories are directed toward possible methods for utilizing selective arylamidase isozyme assays to detect early specific tissue damage.

Reports from our laboratory have described the properties of partially purified arylamidase from human liver and duodenum and contrasted these properties to those of crystalline swine leucineaminopeptidase[9,19]. We have now prepared the isozyme from liver in pure form. The purification, properties, and action of human liver arylamidase is the subject of this report.

EXPERIMENTAL PROCEDURE

Materials

The amino acid-β-naphthylamines, amino acid-p-nitroanilides, and dipeptide-β-naphthylamine were obtained from Mann Research Laboratories, and International Chemical and Nuclear Corporation. The specific rotations of the substrates used in this study were in agreement with literature values available. Human liver tissue was obtained less than 6 h post-mortem and was frozen at $-20°$ until used.

Methods

Arylamidase assay. The rate of liberation of β-naphthylamine from amino acid-β-naphthylamine or dipeptide-β-naphthylamine was determined fluorometrically at $37°$ in an incubation mixture containing 4.0 μmoles of substrate, 8.0 μmoles of Co^{2+}, and 40 μmoles of Tris–maleate buffer (pH 6.8), in a 4.0 ml reaction mixture. β-Naphthylamine is excited at 335 mμ and fluorescences at 410 mμ. Fluorescence was determined with a Beckman Model 77204 ratio fluorometer and a linear strip chart recorder. The unit of arylamidase activity was defined as that amount of enzyme that catalyzes the hydrolysis of 1 μmole of substrate per min under these conditions. Specific activity was defined as units of enzyme activity per mg of protein. A colorimetric β-naphthylamine assay method involving diazotization as modified by BEHAL, KLEIN AND DAWSON[9] was also utilized in some instances. Alanine-β-naphthylamine was the substrate used for the determination of arylamidase activity unless stated otherwise.

Paper chromatography. Chromatography was carried out at $27°$ for 20 h on Whatman No. 1 filter paper using the ascending technique with a n-butanol–glacial acetic acid–water (4:1:5, by vol., upper phase) solvent. Free β-naphthylamine was visualized first by viewing the chromatograms under ultraviolet light. Then amino acids and dipeptides were revealed by spraying the chromatograms with 0.25% ninhydrin in acetone and heating them at $100°$ for 10 min. The amino acids and dipeptides selected for study and β-naphthylamine had distinctly different R_F values.

Protein determination. Protein determination was carried out according to the method of LOWRY *et al.*[10]. Protein concentration of individual fractions from column chromatography was estimated by measuring the absorbance at 280 mμ in a 1.0-cm cell; under these conditions most proteins at a concentration of 1.0 mg/ml have an absorbance near 1.0.

Acrylamide gel electrophoresis. Disc electrophoresis as described by DAVIS[11] was used to monitor the purity of enzyme preparations. The gel concentration was 7.0%.

RESULTS

Arylamidase purification

Livers obtained at post-mortem were frozen and stored at −20° until used. Arylamidase activity was purified as described below. All procedures were carried out at 4° unless specified otherwise.

Step 1. A 500-g sample of liver was homogenized in 2000 ml of 0.01 M phosphate buffer at pH 7.0. The homogenate was allowed to autolyze for 7 days to effect solubilization of the arylamidase activity. The autolysate was then centrifuged at 16 000 × *g* for 30 min to remove any solid material.

Step 2. The pH of the supernatant from Step 1 was brought to 8.0 with 35% ammonium hydroxide, and then 0.1 vol. of a solution, containing 0.4 mole of zinc acetate, 0.005 mole of sodium acetate and 0.01 mole of glycine per l of 90% methanol, was added and the pH of the solution was readjusted to 8.0. The mixture was allowed to stand overnight and then centrifuged at 16 000 × *g* for 30 min. The precipitate was discarded. The supernatant was brought to 80% saturation with solid ammonium sulfate and allowed to stand overnight. The material was then centrifuged as before and the supernatant discarded. The precipitate was suspended in 250 ml of 40% saturated ammonium sulfate at pH 8.0 and allowed to stand overnight. Undissolved material was removed by centrifugation at 27 000 × *g* for 15 min.

Step 3. The supernatant from Step 2 was brought to 70% saturation by adding a saturated ammonium sulfate solution; then the mixture was allowed to stand overnight. Then 100 g of Celite 545 was added and the resulting slurry was poured into a 2.5 cm × 60 cm column and allowed to settle. The column was gradient eluted with a solution at pH 8.0 having a decreasing ammonium sulfate concentration. The initial concentration was 70% saturation and the limiting concentration was 40% saturation. The total volume of eluant was 2000 ml. The eluate was collected in fractions containing approx. 20 ml each. The fractions containing arylamidase activity were combined and concentrated by pressure dialysis to a volume of 50 ml then dialyzed against 0.1 M sodium borate buffer at pH 7.5 containing 1.0 M NaCl per l.

Step 4. The material from Step 3 was applied to a Pharmacia K-50 column packed with Sephadex G-200 equilibrated with 0.1 M borate buffer at pH 7.5 containing 1.0 mole of NaCl per l. Elution from this column was carried out with 2000 ml of the same buffer. Fractions containing approx. 20 ml each were collected and those containing arylamidase activity were pooled. The combined fractions were desalted by application to a 4.0 cm × 80 cm column packed with Sephadex G-25 equilibrated with 0.005 M phosphate buffer at pH 8.6. Elution was carried out with 1000 ml of the same buffer. Fractions containing 20 ml each were collected. Active fractions were pooled and concentrated by pressure dialysis to a volume of about 25 ml.

Step 5. The material from Step 4 was applied to a 2.5 cm × 60 cm column, packed with DEAE-cellulose which had been equilibrated with 0.005 M phosphate buffer at pH 8.6. The column was gradient eluted by means of a solution having an increasing NaCl concentration at pH 7.0. The initial NaCl concentration was 0.01 M and the limiting concentration was 0.2 M. The total volume of eluant was 2000 ml. Fractions containing 20 ml each were collected. Active fractions were pooled, concentrated by pressure dialysis to a volume of 5.0 ml and dialyzed against 0.001 M phosphate buffer at pH 7.0.

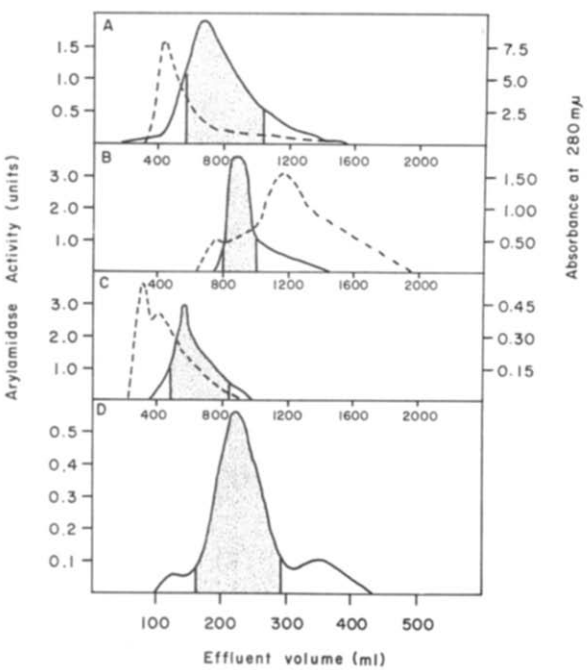
TABLE I

PURIFICATION OF ARYLAMIDASE

Step	*Vol. (ml)*	*Units*	*Protein (mg)*	*Specific activity*	*Purification*
1. Homogenate	2000	80	52 200	0.00153	1.0
2. $(NH_4)_2SO_4$ ppt., 40–80% satd.	250	35	1 250	0.028	18.3
3. $(NH_4)_2SO_4$–celite elution	665	38	930	0.041	26.8
4. Gel filtration	260	29	110	0.26	170.0
5. Ion-exchange chromatography	520	29	79	0.36	235.0
6. $Ca_3(PO_4)_2$ chromatography	90	11	3.4	3.24	2120.0

Step 6. The sample was then applied to a column of 1.0 cm × 50 cm containing 8.0 g of $Ca_3(PO_4)_2$ (Hypatite, Clarkson Chemical Co.) equilibrated with 0.001 M phosphate buffer (pH 7.0). Gradient elution was carried out with 500 ml of phosphate buffer (pH 7.0), the initial and limiting molarities were 0.01 and 0.05, respectively. The active fractions were pooled and concentrated by pressure dialysis.

Table I summarizes the results of a typical purification procedure. Fig. 1 shows



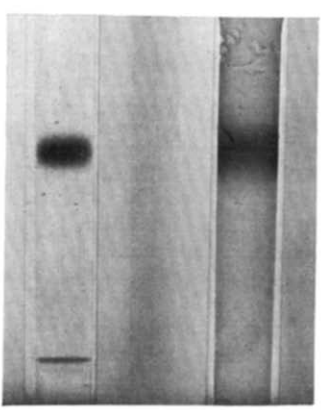


Fig. 1. Elution of arylamidase from (A) column packed with $(NH_4)_2SO_4$ precipitated enzyme–celite slurry; (B) Sephadex G-200 column; (C) DEAE-cellulose column; and (D) hydroxylapatite column. ———, arylamidase; — — —, $A_{280\ m\mu}$. Shaded areas indicate fractions pooled for subsequent steps.

Fig. 2. Polyacrylamide gel electrophoresis of purified liver arylamidase at pH 8.9. Gel on the left is stained for protein; gel on the right is stained for enzyme activity.

the chromatographic elution profiles from $(NH_4)_2SO_4$ slurry, gel filtration, ion exchange, and hydroxylapatite chromatography. The final recovery was 13% and the final purification was 2120-fold.

Acrylamide gel electrophoresis at pH 8.9 of the final preparations from the above procedure produced a single protein band when stained with Amido Black; duplicate gels stained for arylamidase with the histochemical method produced a single band at the same distance from the tracker dye. These results are shown on Fig. 2. Sufficient enzyme protein was not present in the final preparation to permit measurement of the sedimentation coefficient dependence on concentration. However, a Schlieren pattern with a single symmetrical peak was obtained for each of two separate preparations resulting from the procedure described above. The samples were centrifuged at pH 6.8 in 0.01 M Tris–maleate buffer at 172 296 $\times$ *g* in a Spinco SW 50-L rotor. The protein concentrations were 0.7 and 0.8 mg/ml and the $s_{20,w}$ values were 8.6 and 8.5, respectively.

General properties

The enzyme was very sensitive to low concentrations of EDTA. Activity was restored upon removal of the EDTA and addition of any one of several divalent cations. The most potent activator was Co^{2+} at a concentration of 1.2 μmoles/ml; other activators and their effectiveness relative to Co^{2+} were Mn^{2+} (90%), Mg^{2+} (87%), and Ca^{2+} (70%). The enzyme had a sharp pH optimum value of 6.8, and its activity was not enhanced by thiol compounds.

Substrate specificity

The rates of hydrolysis of 27 amino acid-β-naphthylamines and amino acid-*p*-

TABLE II

ARYLAMIDASE SUBSTRATE SPECIFICITY

The value, 100, was assigned to that substrate hydrolyzed at the highest rate. The substrate concentration was 1.0 μmole/ml in each case. A relative rate of hydrolysis of zero was obtained for the following derivatives of β-naphthylamine: L-α-aspartic acid, L-histidine, L-proline, L-hydroxyproline, D-alanine, β-alanine, D-leucine, acetyl-L-alanine, formic acid and propionic acid. A relative rate of hydrolysis of zero was also obtained with D-alanine-*p*-nitroanilide and D-leucine-*p*-nitroanilide.

Substrate	*Rate of hydrolysis*
L-Alanine-β-naphthylamine	100
L-Phenylalanine-β-naphthylamine	63
L-Methionine-β-naphthylamine	58
L-Leucine-β-naphthylamine	36
L-Arginine-β-naphthylamine	28
L-Tryptophan-β-naphthylamine	19
Glycine-β-naphthylamine	14
L-Lysine-β-naphthylamine	13
L-Serine-β-naphthylamine	6
L-Threonine-β-naphthylamine	6
L-α-Glutamic acid-β-naphthylamine	4
L-Valine-β-naphthylamine	3
L-Isoleucine-β-naphthylamine	3
L-Alanine-*p*-nitroanilide	10
L-Leucine-*p*-nitroanilide	4

nitroanilides by liver arylamidases were determined and are presented in Table II. An unsubstituted α-amino acid residue of the L configuration was required for susceptibility to arylamidase catalyzed hydrolysis.

Effect of substrate concentration on reaction velocity

The effect of substrate concentration on the rate of arylamidase catalyzed hydrolysis was determined for 10 amino acid-β-naphthylamines. The results are shown in Table III. Alanine-β-naphthylamine had the highest v_{max} value. Values for v_{max}

TABLE III

EFFECT OF SUBSTRATE CONCENTRATION ON REACTION VELOCITY

Substrate	$K_m \times 10^4$ *(M)*	v_{max} *(moles/l per min)* $\times 10^6$
L-Alanine-β-naphthylamine	6.4	56
L-Methionine-β-naphthylamine	1.7	32
L-Leucine-β-naphthylamine	3.2	25
L-Arginine-β-naphthylamine	3.5	24
L-Lysine-β-naphthylamine	1.5	3
L-Phenylalanine-β-naphthylamine	2.5	15
L-Isoleucine-β-naphthylamine	3.3	4
L-Valine-β-naphthylamine	3.2	2
L-Serine-β-naphthylamine	17	6
Glycine-β-naphthylamine	13	7

were higher for substrates with amino acid residues having straight chain or γ-branched R groups. Low v_{max} values were associated with β-branched R groups. The lower K_m values were associated with those substrates having amino acid residues with non-polar or basic R groups, regardless of the branch point.

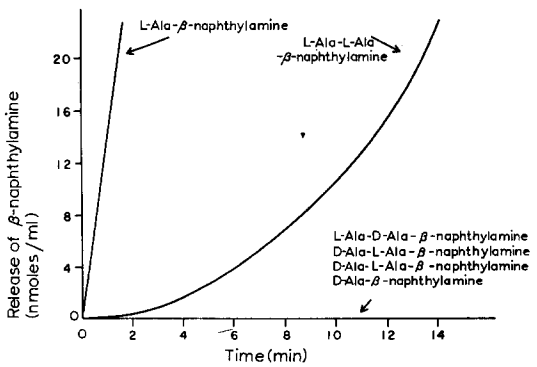


Fig. 3. The rates of arylamidase catalyzed release of β-naphthylamine from optical isomers of alanyl-alanine-β-naphthylamine. Enzyme concentration was the same in each case; rate of appearance of β-naphthylamine was determined fluorometrically.

Arylamidase catalyzed hydrolysis of dipeptide-β-naphthylamines

The rate of arylamidase catalyzed release of β-naphthylamine from the four optical isomers of alanyl-alanine-β-naphthylamine was determined fluorometrically. L-Alanyl-L-alanine-β-naphthylamine was the only one of these substrates yielding β-naphthylamine. There was a lag in the rate of appearance of β-naphthylamine and then the rate increased to approach that of L-alanine-β-naphthylamine hydrolysis. These results are shown on Fig. 3.

Aliquots of the reaction mixture withdrawn at intervals during the course of the reaction were chromatographed to establish the nature of the reaction products. Only alanine and β-naphthylamine appeared as reaction products. Samples taken as early as 3 min showed no trace of dipeptide on the paper chromatograms although the amount of liberated alanine increased in 1 h. These results suggested that the hydrolysis of L-alanyl-L-alanine-β-naphthylamine proceeds stepwise from the N-terminal residue thus explaining the lag in the rate of β-naphthylamine liberation.

To further characterize the enzyme, the products of a reaction mixture containing L-alanyl-D-alanine-β-naphthylamine were also studied by paper chromatography. Analysis of the reaction products showed that even after 16 h incubation neither alanyl-alanine nor β-naphthylamine was liberated; alanine and alanine-β-naphthylamine were formed. D-Alanyl-L-alanine-β-naphthylamine and D-alanyl-D-

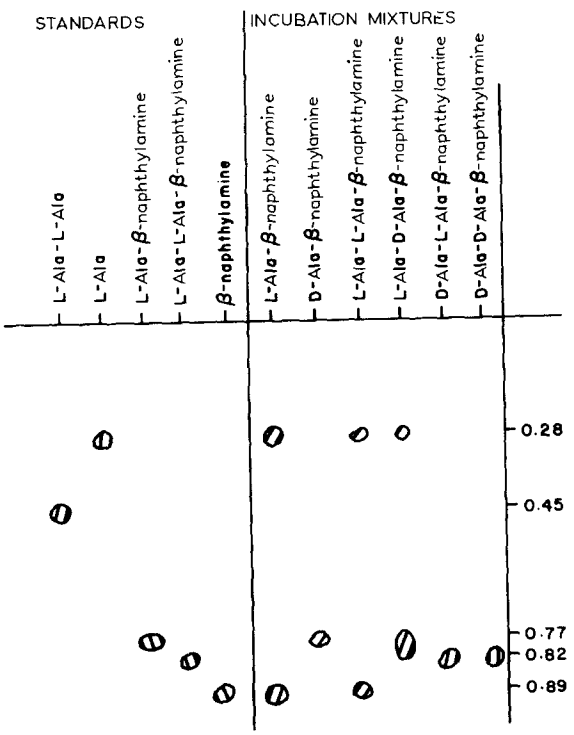


Fig. 4. Composite paper chromatogram showing R_F values for standards (on left) and products of of arylamidase catalyzed hydrolysis of optical isomers of alanine-β-naphthylamine (on right). In no case did alanylalanine appear as a reaction product.

alanine-β-naphthylamine remained completely unreacted after 16 h incubation with the enzyme. These results are shown on a composite chromatogram on Fig. 4.

DISCUSSION

When substrates such as L-leucine-β-naphthylamine were first utilized, it was generally believed that the enzyme, leucineaminopeptidase, was chiefly responsible for their hydrolysis[7]. Subsequently a group of enzymes, having no leucineaminopeptidase activity, but which hydrolyze a variety of amino acid-β-naphthylamine have been reported[3,9]. These enzymes have been classified as arylamidases. A similar enzyme has been reported that hydrolyzes dipeptide-β-naphthylamine to yield dipeptide and β-naphthylamine[12–14]. Some investigators have postulated that the role of arylamidase is to inactivate regulatory peptides such as angiotensin II amide. A previous report from our laboratory described the predominance of arylamidase in certain gram negative bacteria among a representative group of gram positive and gram negative bacteria[15]; this enzyme lacked the divalent cation requirement characteristic of arylamidase from animal sources. The isozymic nature of arylamidase of human origin has been reported from our laboratory[3] and subsequently reported by other investigators[1,2,4,6].

A highly purified liver arylamidase having been prepared, it was then possible to study (a) the substrate specificity of the enzyme; (b) the relationship between substrate structure and susceptibility to arylamidase catalyzed hydrolysis; and (c) the mode of action of this enzyme on dipeptide-β-naphthylamine.

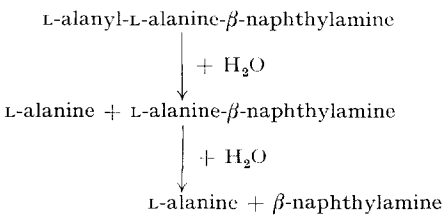
The substrate specificity study summarized in Table II established that the amino acid residue of the substrate must have an unsubstituted α-amino group of the L-configuration, and that amino acid -*p*-nitroanilide as well as amino acid-β-naphthylamine were susceptible to arylamidase catalyzed hydrolysis. The results also showed that substrates having amino acid residues with straight chain or γ-branched R groups were much more susceptible to hydrolysis than those substrates with β-branched or acidic R groups on the amino acid residue.

The relative resistance of β-branched N-terminal residues to hydrolysis has been noted even in the case of non-enzymatic (acid or alkaline) hydrolysis of valyl-glycine and isoleucyl-glycine[16,17]. This factor is probably reflected in the enzymic rate of hydrolysis of these amides as was pointed out by SMITH, SPACKMAN AND POLGLASE[18]. However, it is probably not the only one involved, since it has also been reported by SMITH, SPACKMAN AND POLGLASE that neither isoleucinamide or valinamide inhibits leucineaminopeptidase catalyzed hydrolysis of L-leucinamide when present in equal concentrations. These findings were interpreted to indicate that the β-branched amino acid derivatives do not interact with the enzyme as strongly as do the γ-branched derivatives, *i.e.*, leucinamide or leucylglycine[18].

Although the highest v_{max} value was obtained with alanine-β-naphthylamine, lower K_m values were associated with substrates having amino acid residues with larger R groups. This pattern was observed when the R group was either non-polar or basic. The low v_{max} values observed with valine-β-naphthylamine or isoleucine-β-naphthylamine indicated the marked effect that β-branching of the R group has on the susceptibility of these substrates to arylamidase catalyzed hydrolysis. This same pattern has also been observed in the case of hydrolysis of leucyl-glycine,

norleucyl-glycine, norvalyl-glycine, methionyl-glycine, valyl-glycine, and isoleucyl-glycine by leucineaminopeptidase from human liver[3,9]; the latter two dipeptides with β-branched R groups were hydrolyzed much slower than were the first four dipeptides.

The enzyme also catalyzed the hydrolysis of alanyl-alanine-β-naphthylamine. The question then arose whether or not the enzyme acted upon alanyl-alanine-β-naphthylamine to yield (a) alanyl-alanine and β-naphthylamine or (b) alanine and alanine-β-naphthylamine as initial hydrolytic products. D-Alanyl-D-alanine-β-naphthylamine and D-alanyl-L-alanine-β-naphthylamine were completely resistant to hydrolysis; L-alanyl-D-alanine-β-naphthylamine yielded L-alanine and D-alanine-β-naphthylamine as the only reaction products, even after prolonged incubation. Therefore, hydrolysis of the N-terminal amino acid residue is dependent on the residue being of the L-configuration whereas the penultimate residue may be of either configuration. The results indicated that the enzyme acted to cleave amino acids residues sequentially, beginning with the N-terminal residue, rather than releasing dipeptide from β-naphthylamine as the initial step. This mechanism was confirmed in studies of the hydrolysis of L-alanyl-L-alanine-β-naphthylamine. If the N-terminal alanine residue were hydrolyzed first, a lag in the appearance of β-naphthylamine would be expected and no free alanyl-alanine should be detected, even early in the reaction. The data in Figs. 3 and 4 for the hydrolysis of L-alanyl-L-alanine-β-naphthylamine was consistent with this hypothesis. The action of this arylamidase is therefore as follows:



This mechanism of action is the same that we have previously reported for an arylamidase from human duodenum[19] and an arylamidase from *Neisseria catarrhalis*[20], but is not the same as that reported for arylamidases of the pituitary by ELLIS and coll.[12–14]; these enzymes catalyzed the hydrolysis of various dipeptide-β-naphthylamine to yield dipeptide and β-naphthylamine as reaction products.

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