enzyme is still capable of binding isocitrate, α-ketoglutarate and $Mn^{2+}$. Since $TPN^+$ and TPNH, in contrast to isocitrate, do not protect against inactivation by iodoacetate, there is no indication that the coenzyme binding site is affected by this reagent. All the available information thus points to an involvement of methionine in catalysis at a step subsequent to formation of the enzyme–substrate complex.

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## Partial purification and some properties of bovine heart arylamidase

Arylamidases are found widely distributed in nature and are capable of hydrolyzing aminoacyl-β-naphthylamides[1–4]. Enzymes isolated from different sources appear to have differing requirements for catalysis. This communication reports on the partial purification of bovine heart arylamidase, its specificity and the effect of selected thiol reagents and alkylating agents on enzymic activity.

Bovine heart arylamidase is a soluble enzyme since essentially all the enzyme activity found in heart homogenates is recoverable in 14 600 × *g* supernatant fractions (Table I). The enzyme has been purified from homogenates to the extent of 1261-fold by $(NH_4)_2SO_4$ fractionation, column hydroxylapatite adsorption and DEAE-Sephadex column chromatography (Fig. 1; Table I). The DEAE-Sephadex pooled peaks of enzyme show one enzyme band by the zymogram technique[3] and eight bands of protein by disc electrophoresis[5] (Fig. 2). These data suggest that either several enzyme peaks are inactivated during electrophoresis or that different aggregates of enzymes or isozymes may exist and be separable by DEAE-Sephadex but are indistinguishable by disc gel electrophoresis. Impurities may also be present.

Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

TABLE I

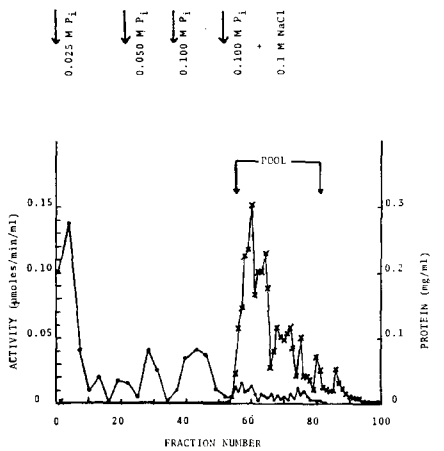
PURIFICATION OF BOVINE HEART ARYLAMIDASE

The enzyme was assayed with arginine β-naphthylamide as the substrate.

| *Preparation* | *Total units (μmoles β-naphthylamide hydrolyzed per min)* | *Specific activity (nmoles β-naphthylamide hydrolyzed per min per mg protein)* | *Purification (-fold)* | *Recovery over previous step (%)* | *Overall recovery (%)* |
|---|---|---|---|---|---|
| Homogenate | 473 | 0.011 | — | — | — |
| 14 600 × *g* supernatant fraction | 386.5 | 0.053 | 4.9 | 81.8 | 81.8 |
| 40–60% $(NH_4)_2SO_4$ sediment | 257 | 0.118 | 10.9 | 66.5 | 54.4 |
| Hydroxylapatite | 215 | 0.704* | 65.1 | 83.3 | 45.3 |
| 75% $(NH_4)_2SO_4$ sediment | 99.7 | 0.45 | 41.5 | 48.7 | 21.1 |
| DEAE-Sephadex | 64.5 | 3.47* | 321 | 65.3 | 13.8 |
| DEAE-Sephadex Fraction 62 | | 13.65 | 1261 | | |

* These specific activities are for the pooled peaks.

Assays were carried out at pH 7.0 with arginine-β-naphthylamide as described earlier[4]. Experiments were performed with the pooled DEAE-Sephadex peaks. The purified arylamidase is capable of hydrolyzing the β-naphthylamides of arginine, lysine, phenylalanine, leucine, glycine, tyrosine and proline at the following respective rates, relative to arginine (100%): 124, 119, 67.2, 47.3, 36.8, 30.3, and 23.9. The



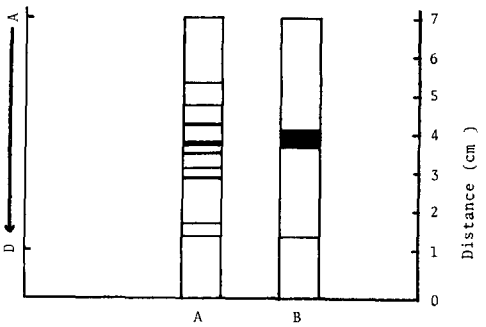




Fig. 1. Purification of arylamidase on DEAE-Sephadex. The enzyme, previously sedimented with $(NH_4)_2SO_4$, chromatographed on hydroxylapatite, and concentrated with $(NH_4)_2SO_4$, was adsorbed onto DEAE-Sephadex, and eluted sequentially with 0.025 M, 0.05 M, 0.1 M, and 0.1 M potassium phosphate (pH 7.5) containing 0.1 M NaCl. ×—×, enzyme; ●—●, protein.

Fig. 2. Polyacrylamide disc gel electrophoresis of purified arylamidase. The protein pattern was run according to DAVIS[5], and the zymogram was run according to MARKS *et al.*[3]. A, protein bands; B, zymogram.

β-naphthylamides of isoleucine, valine, benzoyl-DL-arginine, glutaryl-L-phenylalanine as well as the 4-methoxy-β-naphthylamide of leucine were not hydrolyzed. Whereas arginine- and phenylalanine-β-naphthylamides are rapidly cleaved, neither benzoyl-DL-arginine-β-naphthylamide nor glutaryl-L-phenylalinine-β-naphthylamide is hydrolyzed, indicating that a free α-amino group is essential for activity. Alteration of the naphthyl moiety by the introduction of a methoxy group in the 4-position results in total loss of activity, suggesting that the increased bulk of the 4-methoxy group makes the leucine-4-methoxy-β-naphthylamide unavailable to the enzyme which is capable of hydrolyzing leucine-β-naphthylamide. The purified enzyme will not hydrolyze such dipeptides as Ala–Gly, Gly–Gly, and Leu–Gly, nor will it split such tripeptides as Leu–Gly–Gly, Ala–Gly–Gly, Arg–Gly–Gly, Phe–Gly–Gly, and Gly–Gly–Gly. It does not act upon the oxidized B chain of insulin.

Thiol reducing groups such as cysteine, mercaptoethanol, and dithiothreitol activate the enzyme and maintain activity of refrigerated, stored enzyme. While activity is essentially constant over a 4–60 mM range of thiol for freshly purified enzyme, the activity drops markedly during 3 weeks' storage in the cold. Mercaptoethanol appears to be the most effective activating agent, preserving the greatest amount of enzyme activity for the longest period of time, over the widest concentration range. Purified enzyme is inactivated by freezing.

Arginine- and leucine-β-naphthylamides are optimally hydrolyzed at pH 6.5, whereas phenylalanine- and alanine-β-naphthylamides are optimally split at pH 7.0. Arylamidase is a heat-labile enzyme since all hydrolytic activity against the β-naphthylamides of arginine, leucine, phenylalanine, and alanine is lost upon heating

TABLE II

THE EFFECT OF TOSYL-L-PHENYLALANINE CHLOROMETHYLKETONE, TOSYL-L-LYSINE CHLOROMETHYL KETONE, PHENYLMETHYLSULFONYL FLUORIDE (PMSF) 2,4-DINITROFLUOROBENZENE AND DIPHENYL CARBAMYL CHLORIDE ON PURIFIED BOVINE HEART ARYLAMIDASE

The enzyme was assayed with L-arginine naphthylamide. Concentration of inhibitors, 1 mM. Concentration of dithiothreitol, 2 mM.

| *Order of addition to dialyzed enzyme* | | *β-Naphthylamide liberated (nmoles/min per mg protein)* | *Inhibition (%)* |
|---|---|---|---|
| — | Dithiothreitol | 793.0 | 0 |
| Tosyl-L-phenylalanine chloromethyl ketone | + Dithiothreitol | 167.0 | 79.0 |
| Tosyl-L-lysine chloromethyl ketone | + Dithiothreitol | 500.0 | 36.8 |
| PMSF | + Dithiothreitol | 418.0 | 41.3 |
| 2,4-Dinitrofluorobenzene | + Dithiothreitol | 125.0 | 84.2 |
| Diphenyl carbamyl chloride | + Dithiothreitol | 272.0 | 65.8 |
| Dithiothreitol | | 1000.0 | 0 |
| Dithiothreitol + Tosyl-L-phenylalanine chloromethyl ketone | | 209.0 | 79.0 |
| Dithiothreitol + Tosyl-L-lysine chloromethyl ketone | | 376.0 | 62.6 |
| Dithiothreitol + PMSF | | 962.0 | 4.2 |
| Dithiothreitol + 2,4-Dinitrofluorobenzene | | 209.0 | 79.0 |
| Dithiothreitol + Diphenyl carbamyl chloride | | 358.0 | 64.3 |

for 10 min at 60°. At a level of 1.6 mM, iodoacetate and *N*-ethylmaleimide inhibit arylamidase activity by 41.2 and 58.8%, respectively. *p*-Chloromercuribenzoate inhibits the enzyme by 21.4% at an 80 $\mu$M concentration.

Alkylating agents which react with thiol, amino, and imidazole groups will inhibit arylamidase activity (Table II). Tosyl-L-phenylalanine chloromethyl ketone, tosyl-L-lysine chloromethyl ketone, PMSF, 2,4-dinitrofluorobenzene, and diphenyl carbamoyl chloride inactivate the enzyme in the absence of dithiothreitol. In the presence of dithiothreitol, only PMSF fails to inhibit the enzyme, thereby suggesting that the other alkylating agents may react with groups on the enzyme other than thiols in order to effect an inhibition[6-9]. Anhydrides such as succinic anhydride also inhibit arylamidase.

The addition of EDTA to bovine heart arylamidase results in a 68.7% inhibition of the enzyme. The inhibition can be partially reversed by the subsequent addition of 3.3 mM $Ca^{2+}$ or $Mn^{2+}$, suggesting that metal ions may be involved in activity or in maintaining a stable configuration of the enzyme.

Sources of peptides and alkylating agents: Tosyl-L-phenylalanine chloromethyl ketone, 2,4-dinitrofluorobenzene, and PMSF were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Tosyl-L-lysine chloromethyl ketone, Phe–Gly–Gly, and Arg–Gly–Gly were purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. Diphenyl carbamyl chloride, Ala–Gly, Gly–Gly, Leu–Gly, Leu–Gly–Gly, Ala–Gly–Gly, Gly–Gly–Gly, and the oxidized B chain of insulin were purchased from Mann Research Laboratories, New York, N.Y., U.S.A.

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