Atrioactivase, a Specific Peptidase in Bovine Atria for the Processing of Pro-atrial Natriuretic Factor

PURIFICATION AND CHARACTERIZATION

Atrioactivase, a Specific Peptidase in Bovine Atria for the Processing of Pro-atrial Natriuretic Factor

Aeryl protease which catalyzes conversion of pro-atrial natriuretic factor (ANF) to the active circulating form, ANF(99-126), was purified from a particulate fraction of bovine atria. The enzyme was solubilized with 1.6 M KCl. The molecular mass of the purified enzyme was 580 kDa on gel filtration, whereas by sodium dodecyl sulfate-polyacrylamide gel electrophoresis a cluster of six bands with molecular masses around 30 kDa was observed. The purified enzyme produced ANF(99-126), from partially purified bovine pro-ANF by the selective cleavage of the arginyl peptide bond in the-Pro97-Arg98-Ser99- sequence in pro-ANF. The enzyme was localized mainly in the microsomal fraction rather than the granule fraction. It is likely that the enzyme selectively cleaves the Arg98-Ser99 peptide bond in pro-ANF during the process of secretion.

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Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was performed using 10% acrylamide gel (11). Proteins were stained by the silver staining method according to Wray et al. (12). A molecular weight standard kit from Sigma was used for calibration.

For fluorography of [3H]DFP-labeled enzyme, the gel was soaked in an Enhancing (Du Pont-New England Nuclear) solution for 30 min before drying and exposed to Kodak XAR-5 film for 3 days at -70°C.

Purification of the Enzyme

Enzyme Extraction—Ten bovine atria (360 g) freshly obtained from a local slaughterhouse were minced and homogenized in a Polytron homogenizer for 30 s in 2 liters of 1 mM HEPES, pH 7.0, containing 0.25 M sucrose and 1 mM EDTA, followed by centrifugation for 1 h at 10,000 × g. The sediment was homogenized again and centrifuged under the same conditions in order to remove cytosolic proteins. The resulting sediment was homogenized in a Polytron homogenizer for 30 s in 2 liters of a high ionic concentration buffer, 10 mM HEPES, pH 7.0, containing 0.25 M sucrose, 1 mM EDTA, and 1.6 M KCl to release the enzyme from the membrane fraction. The homogenate was centrifuged for 1 h at 10,000 × g, and the supernatant was dialyzed against 10 mM HEPES, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA (buffer A).

Chromatography on Heparin-Agarose—The dialysate was applied to a heparin-agarose column (2.7 × 9 cm) equilibrated with buffer A and subsequently washed with the buffer. The enzyme was eluted with a linear gradient from 0.1 to 1.5 M NaCl in buffer A. Fractions containing activity were collected and dialyzed against buffer A containing heparin at 10 μg/ml (buffer B).

Chromatography on Arginine-Agarose—The eluate from heparin-agarose was applied to an arginine-agarose column (1.3 × 4 cm) equilibrated with buffer B, and the column was eluted by a linear gradient of NaCl from 0.1 to 1.0 M. Fractions containing the enzyme (total 10 ml) were directly concentrated in a Centricon-30 cartridge (Amicon) to 1 ml. The recovery was nearly 90%.

Gel Filtration on Sephacryl S-300—The concentrate was applied to a Sephacryl S-300 column (1.8 × 100 cm) equilibrated with buffer B. The active fraction eluted just after the void fraction.

Chromatography on Aprotinin-Agarose—The eluate from Sephacryl S-300 was applied to an aprotinin-agarose column (1.3 × 4 cm) equilibrated with buffer B and eluted with a linear gradient of NaCl from 0.1 to 1.0 M.

Rechromatography on Arginine-Agarose—This step was performed essentially by the same method as the previous step except that elution was with a gradient of NaCl from 0.1 M to 0.5 M.

Determination of pH Optimum

The pH optimum for the enzyme was determined by measuring the activity in buffers of different pH. The buffers were all 50 mM in concentration and were citrate-phosphate for pH 5.0-7.0, Tris for pH 7.0-9.0, and carbonate for above pH 9.0.

Radioimmunoassay

Radioimmunoassay was performed by the method for human ANF described previously (13). Since bovine ANF (1-42) has the same sequence as human ANF (1-41), human ANF (1-41) was used as standard. The amount of ANF was expressed as equivalents of human ANF (1-41). For determination of the amount of bovine pro-ANF, a factor of 0.4 was used for immunoreactivity.

Subfractionation of Atrial Homogenate

Subcellular fractions of bovine atrial tissues were isolated essentially by the method of DeBold and Bencosme (15). Briefly, 60 g of bovine atrial tissue was minced and homogenized using a Polytron homogenizer for 5 s in 10 mM HEPES, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. The suspension was further homogenized by 5 strokes of a Teflon homogenizer. The resultant homogenate was subfractionated by centrifugation, and the fractions collected were: nuclear fraction (1,900 × g for 10 min), mitochondrial fraction (second centrifugation at 1,900 × g for 10 min), crude granule fraction (32,000 × g for 10 min), and microsomal fraction (145,000 × g for 90 min). Each fraction was resuspended in the buffer used for homogenization.

The radioimmunoactive ANF was measured by radioimmunoassay after boiling the samples in 1 N acetic acid. Alkaline phosphatase activity was measured by the method of Ray (16). For measurement of general protease activities, fractions were mixed with 5 volumes of 2.0 M KCl, 10 mM HEPES, pH 7.4, containing 1 mM EDTA. After incubation for 15 min at 22 °C the mixtures were centrifuged at 145,000 × g for 90 min. The resultant supernatants were used for the measurements.

Cleavage of Bovine Pro-ANF by the Purified Enzyme

To a mixture of 0.4 ml of 50 mM Tris buffer, pH 8.0, containing 0.1% bovine serum albumin and 0.1 ml of pro-ANF solution (1.7 nmol eq of immunoreactive ANF 1-98 (11)) was added 0.1 ml of the enzyme solution (1 μg/ml) or buffer B (control) followed by incubation for 1 h at 37 °C. After adding 0.4 ml of 1 N acetic acid, the mixture was boiled for 15 min to stop the reaction and centrifuged to obtain a clear supernatant, which was lyophilized and reconstituted in 0.5 ml of 0.1% trifluoroacetic acid in preparation for HPLC.

Characterization of ANF Generated by the Atrial Enzyme

High performance gel filtration was performed on a Bio-Rad TSK 125 column (30 × 7.5 mm) using a solvent system consisting of 30% acetonitrile, 0.1% trifluoroacetic acid, and 0.2 M NaCl (17). Fifty μl of the atrial enzyme-treated pro-ANF or control pro-ANF were applied. A flow rate of 0.5 ml/min was used, and the eluate was collected in 0.25-ml fractions.

Reverse-phase HPLC was run on a Vydac C18 column (0.46 × 25 cm, Alltech) eluted isocratically in 22% acetonitrile and 0.1% trifluoroacetic acid. Fifty μl of sample or control pro-ANF were applied, and the eluate was collected in 0.25-ml fractions. The fractions from both runs were evaporated in a Speed Vac concentrator (Savant) and redissolved in the radioimmunoassay buffer (13).

Purification of ANF Generated from Pro-ANF by Enzyme Treatment

Immunoreactive ANF (1-96) fractions obtained by reaction of the purified enzyme with partially purified pro-ANF by reverse-phase HPLC (on Vydac C18) were collected and further purified using a combination of a Zorbax CN column (0.75 × 30 cm, Du Pont-New England Nuclear) and the Vydac C8 column by the method described previously (18).

Amino Acid Sequence Analysis

Amino acid sequence analysis was performed on an automated gas-phase instrument (model 470A, Applied Biosystems) by the method described by the manufacturer.

Preparation of Bovine Pro-ANF

Partially purified bovine pro-ANF was prepared by the method of Trippodo et al. (19). Bovine atrium was boiled in 1 N acetic acid for 15 min, followed by homogenization for 10 s using a Polytron homogenizer. After centrifugation to obtain a clear supernatant, the crude extract was applied to a Sephadex G-100 column (1.0 × 90 cm). The pro-ANF peak fractions identified by radioimmunoassay and elution position were pooled. The concentration of immunoreactive pro-ANF was estimated by radioimmunoassay.

Estimation of the Molecular Mass of the Enzyme

The purified enzyme was applied to a TSK G 3000 SW column (0.75 × 60 cm) pre-equilibrated and eluted in a solution of 50 mM phosphate buffer, pH 6.8, containing 0.1 M NaCl, 1 mM EDTA. The column was eluted at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The enzyme activity of each was measured with the synthetic substrate. The protein concentration was determined by the method of Lowry et al. with Peterson's modifications (20). The column was calibrated with the following standards: blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), γ-globulin (150 kDa).

Deglycosylation of the Enzyme by N-Glycosidase F (N-Glycanase™)

The purified enzyme (100 ng) was treated with N-glycosidase F (N-Glycanase™) according to the instructions of the manufacturer.

RESULTS

Purification of the Pro-ANF Converting Enzyme Atrial Activi-
ty—Table I summarizes the overall purification of the enzyme. Activity in the crude homogenate was readily lost in a high ionic concentration solution. The low recovery from
the solubilization step and heparin-agarose chromatography may be due to instability of the enzyme. We found that heparin stabilizes the activity in a low ionic concentration buffer. Therefore, we added heparin to all the buffer systems after the heparin-agarose step. Without heparin activity was lost during purification.

**Estimation of Molecular Mass**—Fig. 1 shows the SDS-polyacrylamide gel electrophoresis pattern of the purified enzyme. As shown in lane A the final product consisted of three doublet bands with approximate masses of 30 kDa. The apparent masses of these pairs were estimated as 31.5 and 31.0 kDa, 29.0 and 28.5 kDa, and 26.5 and 26.0 kDa.

**Enzymatic Properties**—The enzyme has a single pH optimum between 8.0 and 8.5. Activity was inhibited by several serine-protease-specific inhibitors including DFP, aprotinin, leupeptin and benzamidine. Chelating or alkylating reagents had no effect (Table II).

**Effect of N-Glycosidase F (N-Glycanase™) Treatment of the Enzyme**—To determine whether the multiplicity of molecular forms of the enzyme is due to differences in glycosylation, the purified enzyme was treated with N-glycosidase F (Fig. 3).

The cluster of bands (Fig. 3, lane A) collapsed to two distinct bands (a major band at 28.0 kDa and a minor band at 30.0 kDa) as shown in Fig. 3 (lane B).

**Effect on Pro-ANF**—Partially purified pro-ANF was treated with the enzyme, and the resultant product was fractionated by gel filtration. Fig. 4a shows the elution profile. Partially purified bovine pro-ANF was eluted in fraction 35 in this system. After enzyme treatment the elution position of immunoreactive ANF was shifted to the elution position of the circulating form, ANF(99-126). The recovery was 76%.

Further examination of the product from the enzyme treatment by reverse-phase HPLC revealed that the elution position was shifted from that of pro-ANF to that of ANF(99-126). This position was distinguishable from those of ANF(105-121), ANF(102-126), ANF(101-126), or ANF(96-126), as shown in Fig. 4b. In order to obtain unequivocal identification of the ANF produced by the enzyme, the peak fractions (shown by a bar in Fig. 4b) were collected, purified by HPLC, and subjected to sequence analysis. The following amino-terminal sequence was determined: Ser-Leu-Arg-Arg-Ser-Ser-. In addition the amino acid composition of the peptide was compatible with that of ANF(99-126).

**Subcellular Distribution of the Enzyme**—The subcellular distribution of the enzyme was determined with fractions obtained from a homogenate of bovine atria (15). In addition to the enzyme activity detected by AGPR-MCA, we also measured nonspecific protease activity with Z-Phe-Arg-MCA (ZFR-MCA), immunoreactive ANF, and alkaline phosphatase.

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**TABLE I**

| Step                | Protein | Total activity  | Specific activity | Purity | Yield |
|---------------------|---------|----------------|------------------|--------|-------|
|                     | mg      | units          | units/mg         | %      | %     |
| 1.6 M KCl extract   | 6000    | 20,8           | 0.0035           | 1.0    | 100   |
| Heparin-agarose     | 40      | 11.7           | 0.293            | 83.7   | 56    |
| Arginine-agarose    | 2.31    | 4.58           | 1.98             | 565.7  | 22    |
| Sephacryl S-300     | 2.0     | 5.26           | 2.63             | 751.4  | 25    |
| Aprotinin-agarose   | 0.258   | 4.08           | 15.8             | 4514.3 | 19    |
| Arginine-agarose    | 0.097   | 3.09           | 31.9             | 9114.3 | 15    |

*One unit is defined as the enzyme activity which hydrolyzes 1 pmol of Boc-Ala-Gly-Pro-Arg-MCA/min.

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**FIG. 2.** Estimation of molecular mass of the enzyme by gel filtration. The purified enzyme was applied to a TSK-G4000 SW column (0.75 × 60 cm) pre-equilibrated and eluted with 50 mM phosphate buffer, pH 6.8, containing 0.1 M NaCl and 1 mM EDTA. The column was eluted at a flow rate of 0.5 ml/min, and 1-min fractions were collected. AGPR-MCA hydrolyzing activity and protein are shown by O and ◦, respectively. The elution positions of standards are shown by arrows. Vo, void volume as determined with blue dextran; A, thyroglobulin (669 kDa); B, ferritin (440 kDa); C, catalase (232 kDa); D, γ-globulin (150 kDa).

**TABLE II**

| Inhibitors                  | Concentration | Inhibition % |
|-----------------------------|---------------|--------------|
| Soybean trypsin inhibitor   | 100 mg/ml     | 0            |
| Lima bean trypsin inhibitor | 100 mg/ml     | 0            |
| Aprotinin                   | 100 mg/ml     | 58           |
| p-Aminobenzamidine          | 1 mM          | 100          |
| DFP                         | 1 mM          | 100          |
| Pepstatin                   | 100 mg/ml     | 0            |
| Leupeptin                   | 100 mg/ml     | 100          |
| N-Ethylmaleimide            | 5 mg/ml       | 0            |
| Iodoacetate                 | 5 mg/ml       | 7            |
| Phosphoramidon              | 200 mg/ml     | 7            |
| EDTA                        | 6 mM          | 0            |
activity (Table III). The AGPR-MCA activity appeared mainly in the microsomal fraction, whereas ZFR-MCA hydrolysing activity was predominantly localized in the cytosolic fraction (high speed supernatant). Immunoactive ANF was found mainly in the granular fraction, as expected from previous studies (15).

The present study represents the identification and purification of a protease in bovine atrial membrane fractions capable of catalyzing the specific processing of pro-ANF to the circulating form of ANF. Although the amino acid sequence of active bovine ANF was shown to be the same as that of human ANF (14), the entire sequence of bovine pro-ANF has not yet been determined. However, we found the substrate used in the rat study served as a substrate for the bovine enzyme.

The purified enzyme has the following characteristics: 1) it has a high affinity to heparin-agarose and is stabilized by heparin; 2) the mass is 580 kDa by gel filtration, whereas on SDS-polyacrylamide gel electrophoresis a cluster of bands is seen around 30 kDa. We could not exclude the possibility of nonspecific aggregation of the enzyme, but a sharp single peak of the activity in Fig. 2 suggests that the enzyme exists in a 580-kDa form.

Recently several high molecular weight seryl proteases have been obtained from various tissues (22-26). The enzyme purified by us has characteristics similar to these, but does not seem identical. Tryptases isolated from human lung (23, 24) and human pituitary (22) were shown to have high affinity to

**DISCUSSION**

In the proteolytic conversion of rat pro-ANF to the circulating form ANF(99-126), specific cleavage of the Arg98-Ser99 peptide bond occurs in the following sequence (2, 3): -Ala95-Gly96-Pro97-Ser98-Leu99-Arg101-Arg102-Ser103. In the previous paper (10) we identified the presence of such a pro-ANF processing activity in rat atria using the synthetic fluorogenic substrate AGPR-MCA.

The enzyme was purified in the presence of N-ethylmaleimide. This cysteiny1 protease inhibitor, which has been shown not to inhibit the present enzyme (Table II), was added to all the buffers at 1 mM during purification to prevent proteolytic cleavage by cathepsin-like cysteiny1 proteases which are often present in the cytosol. The resultant product gave an identical pattern on SDS-gel electrophoresis.

In the third experiment, we treated the enzyme with an N-glycosidase. This treatment reduced the mass and the number of the bands, indicating that the enzyme exists in a 580-kDa form.

Three doublets observed on SDS-polyacrylamide gel electrophoresis around 30 kDa suggest that the enzyme is an aggregate of smaller subunits. To examine the possibility that these multiple bands arise from proteolytic cleavage of the native subunits or are due to heterogeneous glycosylation, three experiments were performed. First, the enzyme was purified in the presence of N-ethylmaleimide. This cysteiny1 protease inhibitor, which has been shown not to inhibit the present enzyme (Table II), was added to all the buffers at 1 mM during purification to prevent proteolytic cleavage by cathepsin-like cysteiny1 proteases which are often present in the cytosol. The resultant product gave an identical pattern on SDS-gel electrophoresis.

Second, we labeled the crude extract with [3H]DFP and subjected it to SDS-gel electrophoresis. The pattern of radioactive bands obtained from the crude extract was identical to that (Fig. 1) obtained from the purified and [3H]DFP-treated enzyme (data not shown). In the third experiment, we treated the enzyme with an N-glycosidase. This treatment reduced the mass and the number of the bands, indicating that the multiplicity of the bands is mainly due to heterogeneity in glycosylation, similar to the pattern observed with trypstase isolated from the pituitary gland (22).

Recently several high molecular weight seryl proteases have been obtained from various tissues (22-26). The enzyme purified by us has characteristics similar to these, but does not seem identical. Tryptases isolated from human lung (23, 24) and human pituitary (22) were shown to have high affinity to
heparin and to be stabilized by heparin. However, their masses are 120–130 kDa, which are much smaller than that of the atrial enzyme. While high ionic concentrations stabilize tryptases, the atrial enzyme rapidly lost activity under the same conditions.

An enzyme isolated from rat liver has been shown to have a mass of 600 kDa (25), which is close to that of the atrial enzyme. However, the liver enzyme exists only in the cytosol, and the substrate specificity is completely different from the atrial enzyme.

The enzyme we identified seems to be different from that reported by Baxter et al. (27). The present enzyme is a serine protease whereas their enzyme is a cysteine protease.

The observation that the present enzyme exists mainly in the microsomal fraction distinguishes it from the protease activity detected by ZFR-MCA which is localized in the cytosol. Twenty percent of the AGPR-MCA hydrolyzing activity has not been found in plasma, they have been reported in rat atrial granules. Twenty percent of the AGPR-MCA hydrolyzing activity has not been found in plasma, they have been reported in rat atrial granules containing pro-ANF, in agreement with the fact that ANF exists as pro-ANF in the granules rather than being processed in the granules. Recently Page et al. (28) suggested the involvement of the endoplasmic reticulum and Golgi in the processing of pro-ANF just before secretion as active ANF. This agrees with our observation of high activity in the microsomal fraction. However, the microsomal fraction obtained by the present method also contains plasma membranes, as indicated by the presence of alkaline phosphatase activity, a plasma membrane marker (as shown in Table III). Further investigation of the distribution of the enzyme may disclose the path of processing ANF.

HPLC and sequence analysis of the peptide produced from pro-ANF by this enzyme demonstrates that ANF(99-126) is the major or exclusive product. Since the amino acid sequence of bovine pro-ANF is not known and since antibodies to the amino-terminal segment of bovine pro-ANF are not available, it is not feasible to investigate whether this enzyme hydrolyzes other arginyl peptide bonds in the amino-terminal segment of pro-ANF. However, recently Michener et al. (8) made an interesting approach to this problem by utilizing an antibody to the NH2-terminal fragment of rat pro-ANF. They detected high activity in the microsomal fraction. However, the present enzyme is a seryl protease whereas their enzyme is a cysteine protease.

In view of the specific functional features of the enzyme we propose to call it "atrioactivase." One of the unique features of this enzyme is the specific cleavage of the arginyl peptide bond in -Pro95-Arg96-Ser97- instead of bonds involving a double basic residue such as -Arg101-Arg102-Ser103-. Cleavage of the latter peptide bonds gives rise to physiologically active peptides with 24 or 25 residues. Although such ANFs have not been found in plasma, they have been reported in rat brain (29, 30). In the brain the processing of pro-ANF may be catalyzed by proteases specific for double basic residues (31, 32), sequences which are commonly found in the processing of precursors of many peptide hormones such as preopiomelanocortin.

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