Emzyme-Linked Immunosorbent Assay for Detection of Canine Chromogranin A by Use of Immunological Cross-Reactivity of Rabbit Anti-Bovine Chromogranin A Antibody

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ABSTRACT. Bovine and canine chromogranin A were extracted and purified from each specie’s adrenal glands. Isolated bovine 70 kDa protein showed 100% identity to bovine CgA reported previously, whereas isolated canine 68 kDa protein showed 83.3% identity to bovine CgA by the NH2-terminal amino acid sequence analysis. Rabbit antibody to purified bovine protein (CgA) was found to immunologically cross-reacted with purified canine protein (CgA). In sandwich ELISA with anti-bovine CgA, concentration-dependent curves were obtained ranging from 0.3 to 20 µg/ml for canine CgA. From these findings, sandwich ELISA with anti-bovine CgA is found to be useful to determine the concentration of canine CgA.

KEY WORDS: canine, chromogranin A, ELISA.

Chromogranin A (CgA) belongs to a family of highly acidic proteins, chromogranins. CgA is co-released with epinephrine or norepinephrine from cromaffin granules of adrenal medulla [1, 3]. CgA is well known to be stored in the secretory granules in wide variety of neuroendocrine tissues and neuroendocrine tumors [4, 8, 14]. CgA of different animal species share considerable homology, and mammalian CgA has been reported to immunologically cross-react with CgA of reptiles, amphibians, fish, and Drosophila tissues [18]. Although the functions of CgA are still poorly understood, CgA is associated with hormone packaging, stabilization of the granule against osmotic pressure, and excretion of intracellular calcium. Extracellular peptidases cleave CgA into biologically active peptides, which act as regulators of hormone secretion [5]. Most of the CgA-derived peptides have been reported to decrease hormone secretion.

The assessment of cortisol and catecholamine levels is well recognized to be a valuable way to investigate stress since these hormones reflect the activity of two important stress responsive axes: hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medullary (SAM) axis. Human CgA is used not only as a marker of pheochromocytoma but also as an index of stressed conditions [15]. For amino acid sequencing, the purified canine 68 kDa protein and bovine 70 kDa protein separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Atto Co., Ltd., Tokyo, Japan). The PVDF membrane was stained with Coomassie brilliant blue R-250 (CBB), and the transferred 68 kDa or 70 kDa band was excised and subjected to NH2-terminal amino acid sequence analysis (HP G1005A Protein Sequencing System; Hewrett-Packard Co., Palo Alto, CA).

Rabbit antisera to bovine CgA were produced by hyperimmunization of albino male rabbits. For immunization, 1 mg of purified bovine CgA was mixed with Freund’s complete adjuvant (DIFCO LABORATORIES, Detroit, U. S. A.)*
were washed with PBS. Fifty BSA in PBS for 1 hr at room temperature. Then, the wells
night. Uncoated sites in the wells were blocked with 0.2% BSA. Fifty
BSA in PBS for 1 hr at room temperature. Then, the wells
were washed with PBST. Fifty
BSA in PBS for 1 hr at room temperature. Then, the wells
adsorbed to each well of a 96-well ELISA plate at 4°C. After washing
the nitrocellulose membrane 5 times in 0.15 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.05% Tween 20 and 0.1% bovine serum albumin (BSA) (PBSTB)). After washing, nitrocellulose membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted to 1:5,000 in PBSTB, Sigma Chemical Co.). After washing the nitrocellulose membrane 5 times in 0.15 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.05% Tween 20 (PBST), the specific antigen was detected by use of 3,3′-diaminobenzidine (Sigma Chemical Co.).

Direct enzyme-linked immunosorbent assay (ELISA) was performed using microtiter ELISA plate (Iwaki., Tokyo, Japan). Fifty µl of bovine and canine CgA (1 or 10 µg/ml) in 0.1 M sodium carbonate buffer, pH 9.5, was adsorbed to each well of a 96-well ELISA plate at 4°C overnight. Uncoated sites in the wells were blocked with 0.2% BSA in PBS for 1 hr at room temperature. Then, the wells were washed with PBST. Fifty µl of diluted purified either bovine CgA or canine CgA diluted in PBS containing 0.1% BSA (PBSTB) was added to each well and incubated for 1 hr at 37°C. After the plates were similarly washed, 50 µl of anti-bovine CgA (IgG) (diluted to 1:500 in PBSTB) was added to each well and incubated at 37°C for 1 hr. After washing, 50 µl of 500-fold diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) was added to each well. After washing, 100 µl of the substrate solution containing 0.2 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) and 0.04% H2O2 in 0.05 M citrate buffer, pH 4.2 was added to each well; the plates were held at room temperature for 30 min for color development. To stop the reaction, 50 µl of 1N HCl was added to each well. After washing, 50 µl of 500-fold diluted horseradish peroxidase-conjugated streptavidin (Sigma Chemical Co.) was added to each well. Then, color development, stopping the enzyme-substrate reaction and determination of absorbance at 405 nm were performed as mentioned above.

The concentrations (µg/ml) of bovine CgA and canine CgA was determined by the Lowry’s method[12] using BSA as the standard.

The protein from canine adrenal glands showed a protein band at 68 kDa by SDS-PAGE, whereas that from bovine adrenal glands at 70 kDa as shown in Fig. 1. Their molecular weights are quite similar to that of bovine CgA reported previously [9]. To confirm whether or not the bovine 70 kDa and canine 68 kDa proteins are CgA, their NH2-terminal amino acid sequence was determined by Microplate reader (Bio-Rad Model 550, Japan) at 405 nm. Sandwich ELISA carried out in triplicate with microtiter ELISA plate. Fifty µl of rabbit anti-bovine CgA (IgG) (10 µg/ml) in 0.1 M sodium carbonate buffer, pH 9.5, was adsorbed to each well of the plate at 4°C overnight. Uncoated sites in the wells were blocked with 0.2% BSA in PBS for 1 hr at room temperature. Then, the wells were washed with PBS. Fifty µl of purified either bovine CgA or canine CgA diluted in PBSTB was added to each well and incubated for 1 hr at 37°C. After the plates were similarly washed, 50 µl of biotin-labeled anti-bovine CgA (IgG) (diluted to 1:500 in PBSTB) was added to each well and incubated at 37°C for 1 hr. After washing, 50 µl of 500-fold diluted horseradish peroxidase-conjugated streptavidin (Sigma Chemical Co.) was added to each well. Then, color development, stopping the enzyme-substrate reaction and determination of absorbance at 405 nm were performed as mentioned above.

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The protein from canine adrenal glands showed a protein band at 68 kDa by SDS-PAGE, whereas that from bovine adrenal glands at 70 kDa as shown in Fig. 1. Their molecular weights are quite similar to that of bovine CgA reported previously [9]. To confirm whether or not the bovine 70 kDa and canine 68 kDa proteins are CgA, the NH2-terminal amino acid sequence of the purified proteins was determined. As shown in Table 1, NH2-terminal amino acid sequence of the purified bovine 70 kDa protein showed 100% (7/7) homology to those of bovine CgA as reported previously [9], indicating that purified bovine 70 kDa pro-
This study investigated the immunological cross-reactivity between bovine and canine chromogranin A (CgA). Western blotting was carried out to study the immunological cross-reactivity between bovine and canine CgA. In Western blots with antibody against bovine CgA, bovine CgA showed a sharp band at 70 kDa, whereas canine CgA showed a band at 68 kDa (Fig. 1), indicating that there are immunological cross-reactivities between bovine and canine CgA. In Western blots with antibody against bovine CgA, bovine CgA showed a sharp band at 70 kDa, whereas canine CgA showed a band at 68 kDa (Fig. 1), indicating that there are immunological cross-reactivities between bovine and canine CgA. 

The purified bovine 70 kDa protein was found to be available to determine the concentration of canine CgA. Thus, the purified bovine 70 kDa protein was used as an immunogen for production of rabbit anti-bovine CgA antibody. On the other hand, the NH2-terminal 12 amino acids sequence of the purified bovine CgA as reported previously [9], suggesting that the purified bovine 68 kDa protein may be bovine CgA. Since immunological cross-reactivities have been reported among CgA from different animal species [18], Western blotting was carried out to study immunological cross-reactivity between bovine and canine CgA. In Western blots with antibody against bovine CgA, bovine CgA showed a sharp band at 70 kDa, whereas canine CgA showed a band at 68 kDa (Fig. 1), indicating that there are immunological cross-reactivity between bovine and canine CgA in this study.

In ELISA with CgA at 10 µg/ml, bovine CgA showed 2-fold higher absorbance than canine CgA (Table 2). Similar findings were also obtained with CgA at 1 µg/ml (Table 2). These findings indicate that the heterologous antigen-antibody reaction was lower than the homologous reaction.

To determine the concentration of canine CgA, sandwich ELISA was carried out with anti-bovine antibody as both coating and probing antibodies. In the ELISA with anti-bovine CgA antibody (IgG) (10 µg/ml) as the coating antibody, concentration-dependent curves were obtained ranging from 0.04 to 10 µg/ml for bovine CgA and from 0.3 to 20 µg/ml for canine CgA, respectively (Fig. 2). In the ELISA with purified canine CgA as an antigen, on the other hand, coefficients of variance of the same day and different day determinations were <16% and <17%, respectively (Table 3).

From the present findings, sandwich ELISA with anti-bovine CgA, was found to be available to determine the concentration of canine CgA.

**REFERENCES**


**Table 2. Immunological cross-reactivity of canine CgA with anti-bovine CgA antibody determined by direct ELISA**

<table>
<thead>
<tr>
<th>CgA concentration (µg/ml)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine CgA (n=5)</td>
<td>Canine CgA (n=5)</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>0.985 ± 0.015</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>0.613 ± 0.010</td>
</tr>
</tbody>
</table>

| a) Concentrations of bovine and canine CgA used for ELISA. Absorbance at 405 nm indicates absorbance at 405 nm–absorbance of blank at 405 nm. Data were expressed as mean ± SD. 

**Table 3. Precision assays of sandwich ELISA for determination of canine CgA concentrations**

<table>
<thead>
<tr>
<th>CgA concentration (µg/ml)</th>
<th>Absorbance at 405 nm (α)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Mean (µg/ml)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.23</td>
<td>0.015</td>
<td>6.56</td>
<td>1.34</td>
<td>0.21</td>
<td>15.66</td>
</tr>
<tr>
<td>0.3</td>
<td>0.04</td>
<td>0.014</td>
<td>11.76</td>
<td>0.35</td>
<td>0.05</td>
<td>14.29</td>
</tr>
</tbody>
</table>