Immunohistochemical Localization of Carbonic Anhydrase Isozymes I, II and III in the Bovine Salivary Glands and Stomach

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Summary. The immunolocalization of carbonic anhydrase (CA) isozymes in bovine salivary glands and stomach were first investigated in order to discuss their biologic functions. In parotid glands, CA-II was located in serous acinar cells, whereas CA-III, in the duct segments. In contrast, a strong reaction was shown for both CA-II and CA-III in the duct segments of the submandibular gland, especially CA-III was selectively located in basal cells of the interlobular ductal epithelium; however, these glands essentially lacked CA-I. On the other hand, epithelial cells of the rumen, reticulum and omasum showed cytoplasmic reaction for CA-I, II and III in all layers of the epithelium, except the stratum corneum. The parietal cells in the abomasal epithelium were more intensely stained for CA-II, but not for CA-I and CA-III. Immunolocalization of CA isozymes in serous cells in the parotid gland indicates their primary function in secreting macromolecules, whereas localization of CA in striated and excretory ducts in the parotid and submandibular gland suggests their traditional function in fluid and electrolytic transport. The biologic function of CA isozymes in the ruminal, reticular and omasal mucosa are postulated to influence the absorption and excretion of volatile fatty acid and NH₃; the abomasal parietal cell is considered to be involved in ion transport.

Carbonic anhydrases (CA) catalyze the hydration of CO₂ and the dehydration of H₂CO₃ (CO₂ + H₂O ↔ HCO₃⁻ + H⁺), reactions which can occur at extremely high rates. Two principal carbonic anhydrase isozymes are found in mammalian erythrocytes; a high activity isozyme (CA-II) and a low activity isozyme (CA-I) (Funakoshi and Deutsch, 1963; Armstrong et al., 1966). These isozymes also have been shown, by immunostaining, to be distributed in mammalian tissue, (Kumpulainen and Korhonen, 1978; Kumpulainen, 1979; Spicer et al., 1979). Moreover, a third isozyme, CA-III has recently been discovered in the skeletal muscle of rabbits (Register et al., 1978), rats (King et al., 1974; Carter et al., 1981), horses (Nishita and Deutsch, 1981) and cows (Engberg et al., 1985). CA-III has poor CO₂ hydrase activity and low sensitivity to sulfonamide inhibitors (Carter et al., 1981). This very low activity enzyme (CA-III) was observed mainly in Type 1 muscle fiber from humans (Shima et al., 1983), rats (Jeffery et al., 1986), and horses (Nishita et al., 1987). Väänänen and Autio-Haromainen (1987) reported that CA-III was located in smooth muscle and myoepithelial cells of some human tissues.

The distribution of CA isozymes in salivary glands of a ruminant has been studied biochemically to determine its presence in the parotid submandibular glands of sheep (Fernley et al., 1979, 1988) and of sheep, goats and cows (Matsumoto et al., 1982). However, there are no published data on the localization of CA isozymes I, II and III in the main bovine salivary glands which are capable of producing large amounts of bicarbonate.

Evidence of the distribution of carbonic anhydrase in the bovine stomach, viz. the rumen, was first obtained by a standard histochemical approach (Schultz, 1962a, b, 1963). Later, Carter (1971) and Laurent et al. (1977) isolated a low activity isozyme (CA-I) which differed immunologically from bovine erythrocyte CA (CA-II) in a biochemical assay method. More recently, Galifi et al. (1982) has immunohistochemically demonstrated CA-I from ruminal epithelial cell. However, no published data is available on the localization of CA-I, II and III in the bovine entire stomach.
This study was conducted in order to determine the immunohistochemical localization of the CA isozymes in the bovine parotid and submandibular glands and the stomach, i.e., rumen, reticulum, omasum and abomasum, which have previously been neglected.

**MATERIALS AND METHODS**

Muscles weighing approximately 1 g and much ruminal epithelia of Holstein cows were homogenized with an equal volume of 0.01 M Tris-HCl, pH 7.5 and the homogenates centrifuged at 10,000 Xg for 30 min at 4°C. The supernatants of both tissues and hemo lysate of rats were used for double immunodiffusion and Western blotting.

**Purification of CA isozymes I, II and III**

CA-I was purified from erythrocytes of rats according to the method by ARMSTRONG et al. (1966) with some modifications. CA-II was further purified from CA-B (from bovine erythocytes) supplied by Sigma Chemical Co., U.S.A., using isoelectric focusing. CA-III was purified from equine muscle by the method previously reported with some modifications (NI-SHITA and DEUTSCH, 1981). Enzymatic activity was measured by the method of WILBUR and ANDERSON (1948).

The enzymatically active fraction was pooled and the purity of CA isozymes was analysed by the method of SDS-polyacrylamid gel electrophoresis (SDS-PAGE). Single bands of rat CA-I, bovine CA-II and equine CA-III were observed by the SDS-PAGE, respectively.

**Antisera**

The antibody against purified CA-I, II and III were raised in rabbits, respectively, by the method of

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**Fig. 1.** The specificity of the anti-rat CA-I serum.  
A. Double immunodiffusion test. With the anti-rat CA-I serum (5), the rat CA-I (2) and the extracts of rumen (3) from the cow, spurs were observed. Antiserum did not react with bovine CA-II (1) and equine CA-III (4).  
B. Electrophoretic blotting after SDS-PAGE. A single band of CA-I from rat CA-I (2) and bovine rumen (3) are observed after staining by the immunohistochemical method using anti-rat CA-I serum.

**Fig. 2.** The specificity of the anti-bovine CA-II serum.  
A. Double immunodiffusion test. The antibody raised to bovine CA-II (5) produces a single precipitin line against bovine CA-II (2) and bovine rumen (3). Antiserum does not react with rat CA-I (1) and equine CA-III (4).  
B. Electrophoretic blotting after SDS-PAGE. A single band of CA-II from bovine CA-II (2) and bovine rumen (3) is observed after staining by the immunohistochemical method using anti-bovine CA-II serum.
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FUNAKOSHI and DEUTSCH (1970) with some modifications. The specificity of the antiserum of CA-I, II and III was examined by double immunodiffusion and Western blotting techniques. The cross-reactivity among anti-rat CA-I for bovine CA-I and anti-equine CA-III for bovine CA-III was also tested.

**Immunohistochemical methods**

Immunodiffusion tests were performed in 1% agarose gels prepared in 0.15 M sodium chloride. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously (NISHITA and MATSUSHITA, 1988).

**Tissue**

The parotid and submandibular glands and stomach, i.e., rumen, reticulum, omasum and abomasum, from two healthy Holstein cows were used. Tissue species were fixed rapidly in Bouin’s solution for 16 h, then dehydrated in ethanol and embedded in Paraplast (Monject, St, Louis, MO, U.S.A.)

**Immunohistochemical staining**

The antibodies to CA-I, II and III were separated from rabbit antisera by precipitation with 33% saturated ammonium sulphate solution. Each precipitate was dissolved in phosphate-buffered saline (PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and the solutions were dialyzed against this buffer. The concentration of each immunoglobulin G (IgG) fraction was adjusted to 1.8 mg/ml. Normal rabbit IgG (1.8 mg/ml), prepared by the same method as described above, was used as a control in place of the antiserum against CA isozymes. Deparaffinized and rehydrated sections 5 μm thick were cut from each tissue sample. Sections were pretreated with 0.3% H₂O₂-methanol for blocking endogenous peroxidase activity and with normal goat serum (2% in PBS) for 20 min to block fragment-crystallizable (FC) receptors. For the primary reaction, monospecific antisera against CA isozymes, diluted at 1:3000, were used to localize the respective isozymes. After incubation in antiserum for 1 h, binding of the antibody was visualized using a Vectastain Elite ABC-POD reagent Kit (Vector; Burlingame, CA, U.S.A). The staining procedure was performed according to the method recommended by the manufacturer. Finally, the preparations were incubated with 0.02% H₂O₂ and 0.1% diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl (pH 7.6) for 5 min. Sections were stained with hematoxylin for the nucleus, then dehydrated through graded concentrations of alcohol and coverslips applied using HSR solution. Normal rabbit IgG (1.8 mg/ml) was used for the control in place of the antiserum against the CA isozymes.

**RESULTS**

The antiserum to rat CA-I formed a single precipitin line with rat CA-I and with a crude extract of bovine rumen (with spur), and did not react with equine CA-III and bovine CA-II (Fig. 1A). The antiserum to bovine CA-II and with a crude extract of bovine rumen, and all of the precipitin line fused completely (Fig. 2A). Anti-bovine CA-II did not react with rat CA-I and equine CA-III. The antiserum to equine CA-III formed a single precipitin line with equine CA-III and with crude extract of bovine muscle (with spur), and did not react with bovine CA-II and rat.
CA-I (Fig. 3A). The specificity of the antisera against CA isozymes was also evaluated by Western blotting. A single band of CA isozymes can be observed in each column on nitrocellulose sheets, respectively (Figs. 1B, 2B, 3B).

The results obtained are summarized in Table 1.

In the parotid glands, immunostaining localized CA-II throughout the cytoplasm of serous acinar cells but the duct segments appeared devoid of this isozyme. These glands essentially lacked CA-I. The duct segments, such as short segments of the intercalated duct, the striated duct, interlobular and excretory duct, showed reactivity with CA-III antiserum (Fig. 4a–c). The submandibular glands also essentially lacked CA-I. The duct segments revealed strong staining and slight staining in the serous demilane for CA-II, especially in the striated ducts. An increase in CA-III reactivity occurred in the basal cells of the

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**Fig. 4.** Photomicrographs showing immunoreactivities for CA (brown) in the bovine parotid gland. Counterstaining with hematoxylin. a. CA-I. No immunoreactivity in serous acinar cells and duct segments. b. CA-II. Uniform immunostaining of serous acinar cells in contrast with the lack of reactivity in the duct segments (arrows). c. CA-III. Uniform staining of intercalated and striated ducts in contrast with the lack of reactivity in serous acinar cells. a–c: ×180

**Fig. 5.** Photomicrographs showing CA-I, II and III-immunoreactivities (brown) counterstained with hematoxylin in the bovine submandibular gland. a. CA-I. No staining of acinar cells, serous demilane and duct segments. b. CA-II. Uniform staining of striated ducts and slight staining of serous demilane, in contrast with the lack of reactivity in mucous acinar cells. c. CA-III. Strong staining of the basal cells of the interlobular duct in contrast with the lack of reactivity in acinar cells. a–c: ×180
interlobular and excretory ducts rather than in the striated ducts. The distribution of the two isozymes in the striated and excretory ducts was demonstrated as heterogeneous in staining some cells of the parotid and submandibular glands (Figs. 4, 5).

Epithelial cells of the rumen, reticulum and omasum showed cytoplasmic reactions for CA-I, II and III in str. granulosum, str. spinosum and str. basale, but no specific reaction occurred in str. coroneum and other mucous membrane and muscle layers (Fig. 6a–c). On the other hand, the parietal cells in the abomasal epithelium were strongly stained for CA-II, but not for CA-I and CA-III (Fig. 7a–c).

Control sections exposed to normal rabbit serum did not stain to any significant extent in the areas described as immunoreactive sites.

**DISCUSSION**

The cross-reactivity among antisera for rat CA-I, II and III was examined by radioimmunoassay by Jeffery et al. (1986), which reported a maximum cross-reactivity of 0.01%. We reported on the cross-reactivity among anti-equine CA-III for equine CA-I and CA-II, examined by enzyme immunoassay.
The maximum cross-reactivity obtained was 0.035%. These data, combined with the results of this study, indicate that monospecific polyclonal antisera formed against carbonic anhydrase isozymes are sufficient to indicate selectivity to the cytochemical system.

In the present study, only CA-II (high activity isozyme) was located in serous acinar cells while CA-III (very low activity isozyme) was strongly observed in the duct segments of the parotid gland. By contrast, both CA-II and CA-III were recognizable only in the duct segments of the submandibular glands; however, these glands essentially lacked CA-I. With regard to the localization of CA-I and II in the salivary glands, a detailed description for the submandibular and parotid glands of mice and rats was presented by HENNINGER et al. (1983). In that report, they also studied differences in immunoreactivity among various fixative fluids, suggesting that fixations with Bouin's and Carnoy's fluids provided the best retention of affinity for antibodies as well as being the most consistent for immunostaining.

The main points are as follows: In mice parotid glands immunostaining localized throughout the cytoplasm of the serous acinar cells upon using the Bouin fixation. On the other hand, in submandibular glands, CA-I was localized in the serous acinar cells whereas CA-II was not demonstratable even after both fixations. As for the ductal segments in mice parotid, in the striated and excretory ducts, CA-I and II were not demonstratable, although in the submandibular glands CA-I and II were localized with Bouin's fluids. In rat parotid glands, immunostaining localized CA-I and II in the serous acinar cells with both fixations, and in the submandibular glands, CA-I and II were demonstrated with Carnoy's fluids. For ductal segments in rat parotid, in the striated and excretory ducts, CA-I was not demonstratable with either fixation but CA-II was localized in Bouin's fluids. On the other hand, using Bouin's fluids, CA-I and II were localized in these ducts of the submandibular glands.

In this study the Bouin fixation was used, since, according to the results of HENNINGER et al. (1983), there were few differences between Bouin and Carnoy fixations with regard to the detection of the CA isozymes in the parotid serous acinar cells, and Bouin provided better immunostaining for the ductal segments. The fact that CA-II was localized in the bovine parotid serous acinar cells but not in submandibular, as seen as in this study, may be due to the different fixations. Thus, the wide variety of cell types in which each CA isozyme occurs indicates diverse functional roles for the enzyme.

The CA activity presumably serves different biologic activities of different cell types. For example, the enzyme mediates proton release to the lumen apparently in exchange for Na⁺ by gastric parietal cells (JACOBSON, 1978) and for K⁺ by intercalated cells of the renal collecting and distal convoluted tubule (SATO and SPICER, 1982). Pancreatic duct cells also carry out oppositely polarized transport in secreting HCO₃⁻ into the main lumen of the duct (JACOBSON, 1978; KUMPULAINEN and JOLAVAARA, 1981b).

In this study, we demonstrated the presence of CA-II and CA-III in the striated and excretory ducts. This finding provides evidence for an ion transport activity at these sites. Likewise, it is well-known that CA isozymes in the duct segments of the salivary glands have a similar function to CA in the pancreatic duct and kidney, providing H⁺ and HCO₃⁻ in exchange for other ions in a transcellular transport activity (HENNIGAR et al., 1983). On the other hand,
serous acinar cells in the parotid glands also showed CA-II reactivity which indicates their primary function in secreting macromolecules. Thus, it is apparent that the intracellular distribution of CA in exocrine cells corresponds with that of the secretory granules that largely fill the apical cytoplasm in the cell. The extracellular function of the enzyme at these sites possibly concerns the following activities: pH regulation; maintenance of ion balance in the fluid; and stabilization of macromolecules in the secretion process (Henningar et al., 1983).

The above findings correlate well with those results which showed that ruminant saliva is mainly secreted from the parotid glands, where the highest CA activity in the ruminant salivary glands was observed (Matsumoto et al., 1982), that it is composed of a well-buffered alkaline solution (pH 8), and flows continuously into the rumen (Phillis, 1976). Saliva in ruminants is known to be an essential factor for maintaining a constant milieu in the rumen.

Biological functions of the CA isozymes in the ruminal, reticulum and omasal mucosa are thought to influence the absorption and excretion of VFA (volatile fatty acid) and NH₄⁺ (Aafjes, 1967; Carter, 1971), and may involve some activity beyond simply catalyzing reactions between CO₂, HCO₃⁻ and water. Moreover, compared with the reticulum and omasum, CA in the rumen is considered to play an important role, together with saliva, in maintaining a constant milieu.

All three CA isozymes—I, II and III—were demonstrated for the first time in rumino-recticular and omasal epithelium. In particular, CA-II and III have not previously been isolated from these regions. The distribution of these three isozymes in the forestomach of the ruminant links them with physiological characteristics of this species. Previous reports by traditional biochemical assay have demonstrated only low activity isozymes (CA-I) in the rumen; the high activity type (CA-II), however, which occurs in the blood of non-ruminants, has not been observed in the rumen (Carter, 1971; Laurent et al., 1977; Galfi et al., 1982). In non-ruminants, the gastric mucosa of the mouse and guinea pig, the stomach contains only the high activity type (CA-II) (Carter and Parsons, 1970; Spicer et al., 1979). Carter (1971) has been able to isolate only the low activity isozyme from bovine ruminal epithelium, and not the high activity isozyme. He attributes the subsequent failure to isolate the latter to the fresh ruminal epithelial tissue which may contain both high and low activity isozymes (CA-I and II), because the selective loss of the CA-II through denaturation during the purification process could account for the inability to recover CA-II. Thus, a substantial loss of CA-II is likely to occur during preparation of the homogenate and at the subsequent purification stage. Consequently, there is an apparent lack of correspondence between the immunohistochemical and biochemical assays so far used, which indicates the failure of the latter method to detect reacting antibodies with greater sensitivity. The present finding that the selective parietal cells in the abomasal gland were strongly stained for CA-II indicates that the gastric parietal cell is considered a cell with high CA content and ion transport activity, as has previously been thought for some animals (Spicer et al., 1984).

It is known that the enzyme activity of CA-III is much lower than that of CA-I which has a lower activity than CA-II (Deutsch, 1987). Therefore, the significance of the presence of CA-III itself is uncertain. However, the fact that CA-I and II are inhibited by sulfonamides, such as acetazolamide, whereas CA-III is weakly inhibited by this reagent (Carter et al., 1981) suggests that CA-III has another important role for ion transport in duct cells of the salivary glands and in the epithelium of the forestomach.

REFERENCES


