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Carbonic anhydrase II is secreted from bovine parotid glands

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Summary. Besides carbonic anhydrase VI (CA-VI), CA-II is suggested to be a second secreted isoenzyme in ruminant saliva. Therefore, the aim of the present study was to investigate the expression of salivary CA-II in bovine parotid glands at the protein level. Moreover, we intended to identify the cells which secrete the enzyme into the saliva. Two commercially available CA-II specific antibodies were tested for use in immunohistochemistry on frozen sections of bovine parotid tissue. Intense positive staining for CA-II was found in luminal duct cells and for the first time also inside the duct lumen, clearly demonstrating the expression and secretion of salivary CA-II in bovine parotid glands. The presence of CA-II protein was verified for parotid tissue and whole saliva using immunoblot analysis. Both salivary CA-II and CA-VI are highly active in supplying the alimentary tract with bicarbonate. It is suggested that a decrease in the expression of either one of these enzymes might severely disturb digestion and/or increase susceptibility to acidosis in ruminants.

Key words: Carbonic anhydrase II, Cattle, Immunohistochemistry, Parotid gland, Salivary secretions

Introduction

Saliva in ruminants is primarily supplied by parotid glands, which secrete rapidly and continuously (Piatkowski et al., 1990). This is highly important for the animals, because they need to relocate nitrogenous and phosphorous compounds, which are essential factors for microbial growth in the forestomach (Breves et al., 1987; Piatkowski et al., 1990). In contrast to humans, ruminants secrete large volumes of alkaline and well buffered saliva, mostly for lubricating and swallowing food particles, but also to provide a constant pH for ruminal microorganisms (Kay, 1960). In this context, salivary carbonic anhydrase (CA; carbonate dehydratase, EC 4.2.1.1) is of major interest, because it participates in various basal processes, such as local pH regulation of the oral cavity and the alimentary tract, in bicarbonate transport as well as in electrolyte balance (Parkkila and Parkkila, 1996). Since the initial discovery of the intracellular 29-kDa CA-II in bovine erythrocytes, different CA isoenzymes were found in various mammalian tissues (Sly and Hu, 1995). The only isoenzyme so far known to be secreted into saliva is CA-VI, which is exclusively characterized by an apparent molecular weight of 42 kDa (Sly and Hu, 1995). CA-VI was first described from sheep salivary secretions (Fernley et al., 1988, 1989). Likewise, CA-VI is present for example in saliva of cattle (Asari et al., 2000; Mau et al., 2006), humans (Kivelä et al., 1999), rats (Breiner-Feldstein and Silverman, 1984), pigs (Nishita et al., 2001) and goats (Lamy et al., 2008; Mau et al., 2009). Only recently, a second CA isoform, most likely CA-II, was found in saliva of ruminating mammals such as cattle, goats and Bactrian camels (Mau et al., 2009). Both CA-II and CA-VI were described earlier from human and bovine salivary pellicle (Leinonen et al., 1999; Li et al., 2004; Mau et al., 2006). Both enzymes are further present in human as well as in bovine parotid and submandibular glands (Asari et al., 1989, 2000; Parkkila et al., 1990; Ogawa et al., 1993).

However, knowledge on the exact site of CA-II secretion from bovine salivary glands is so far completely lacking. Therefore, the aim of the present study was i) to find evidence for CA-II secretion from bovine parotid glands and ii) to identify the CA-II secreting cells by using immunohistochemistry and immunoblotting. The expression of specific enzymes

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controlling the homeostasis of the digestive tract in ruminating animals could elucidate salivary adaptations to provide and/or maintain the specialized functions of the ruminant digestive system.

Materials and methods

Tissue and saliva samples

Left and right parotid glands of 2 cows (*Bos primigenius f. taurus*) were provided by the Research Institute for the Biology of Farm Animals in Dummerstorf, Germany. Right after slaughter parotid glands were excised and immediately blocks of 1x1 cm were taken from the inner parts of the organs and frozen with liquid nitrogen. Transport of samples to the working laboratory was done on dry ice without interruption of the cooling chain. For immunoblotting, 140 mg of parotid tissue were homogenized in 0.5 ml 10 mM TBS (pH 7.4) with added protease inhibitors. Protein concentrations were determined against BSA standard using the BCA method (Kit BCA-1 and B 9643, Sigma-Aldrich, Taufkirchen, Germany). Bovine whole saliva was obtained from 4 different cows by natural salivation. Larger food particles were removed by sedimentation at 300xg, 10 min, 4°C. Tissue samples and saliva were kept frozen at -80°C until use.

Histological staining

Bovine parotid gland tissue was cut into 6 µm sections using a cryostat microtome (Leica, Nussloch, Germany). Tissue sections were mounted on glass slides (Superfrost Plus, 9161155, Thermo Scientific, distributed by Menzel, Braunschweig, Germany), and immediately fixed in ice-cold methanol for 30 min. After rehydration for 5 min in distilled water, tissue samples were stained by haematoxylin/eosin for 5 and 15 min, respectively and dehydrated using 2-propanol (70, 80, 90, 100%) and Rotihistol (100%; 6640.1, Carl Roth, Hamburg, Germany). For Masson-Goldner staining, tissue sections were pre-treated with haematoxylin for 4 min. Thereafter, sections were incubated with fuchsine-ponceau (1.35 mM ponceau xylidine, 0.6 mM acid fuchsine, 0.2% [vol/vol] acetic acid) for 15 min. Samples were rinsed with water and treated with a solution of 5% [wt/vol] phosphomolybdic acid – 2% [wt/vol] orange G for 7 min. Sections were counterstained with 0.2% [wt/vol] light green for 7 min and dehydrated with Rotihistol (100%; Carl Roth). All samples were air-dried and mounted in Roti-Histokitt (6638.2, Carl Roth). For the histological examination of the bovine parotid glands the image analysis system Leica DM LB (Leica Microsystems, Wetzlar, Germany) was used.

Immunohistochemistry

Parotid tissues were cut in serial sections of 6 µm as described above and immediately fixed in ice-cold methanol for 15 min. After washing with 0.01 M TBS (pH 7.4; 3x10 min), slides were incubated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity followed by subsequent rinsing in 0.01 M TBS (2x5 min). Prior to incubation with the primary antibody, the sections were blocked with normal goat serum for 30 min. Either a polyclonal rabbit anti-bovine CA antibody (1789-9950, AbD Serotec, Düsseldorf, Germany, dilution of 1:50 in TBS with 1% goat serum) or a polyclonal rabbit anti-human CA-II antibody (H-70, sc-25596, Santa Cruz, Heidelberg, Germany, dilution of 1:50 in TBS with 1% goat serum) were added. The primary antibodies were omitted in the negative controls. All sections were incubated overnight at 4°C with continuous shaking. Thereafter, sections were washed in TBS (5x2 min) and incubated with the secondary antibody, a goat anti-rabbit IgG linked with horseradish peroxidase (4030-05, SouthernBiotech, USA, distributed by Biozol, Eching, Germany, dilution of 1:500). After another washing with TBS (2x5 min), tissue sections were incubated for 7 min with AEC (AEC kit; BZL 00733 + BZL 00735, Biozol, Eching, Germany) as the chromogen staining substrate. After rinsing with TBS (2x1 min), all sections were counterstained with haematoxylin for 30 sec and mounted in glycerol jelly for evaluation under a light microscope (Leica DM LB; Leica Microsystems).

Gel electrophoresis and immunoblotting

Two-dimensional gel electrophoresis and subsequent immunoblotting were performed to visualize the carbonic anhydrase isoenzymes II and VI in bovine saliva using the identical CA-specific antibodies as used for immunohistochemistry. Whole saliva samples containing 100 µg of total protein were mixed with rehydration buffer (8 M urea, 4% CHAPS (3-[3-cholamidopropyl dimethylammonio]-1 propane-sulfonate), 0.4% DTT (dithiothreitol), 2% IPG buffer and 10 µl/ml bromophenol blue). Samples were subjected to isoelectric focussing (IEF) at 20°C on 11 cm IPG strips pH 4-7 for 1 h at 500 V, 1 h at 1,000 V and 1.5 h at 8,000 V. After that, proteins on the IPG strips were equilibrated using 6 M urea, 50 mM Tris-HCl pH 6.8, 0.1 mM EDTA, 30% glycerol, 2% SDS, 0.01% bromophenol blue supplemented with 1% DTT for 15 min, then alkylated for 15 min with the same buffer solution plus 65 mM iodacetamide instead of DTT. If not otherwise stated, chemicals were obtained from Sigma Aldrich, Taufkirchen, Germany and GE Healthcare, Munich, Germany. The equilibrated strips were applied on top of a 12% SDS-PAGE gel and proteins were separated for 2 h with a constant current of 30 mA according to Laemmli (1970).

To further verify antibody specificity for bovine CA-II, parotid tissue homogenates were supplemented with calculated volumes of sample-buffer (K929.1; Roth, Hamburg, Germany) to reach a concentration of 5 µg/µl

of total protein for each sample. Carbonic anhydrase II isolated from bovine erythrocytes (15882, Serva Electrophoresis, Heidelberg, Germany) served as positive control. Proteins were heated for 5 minutes at 95°C and 6 or 10 µl of samples were separated on 5.6% stacking and 12% resolving gels using one-dimensional SDS-PAGE. Fermentas prestained protein standard (SM0671, Fermentas, St. Leon-Rot, Germany) was used to determine protein molecular masses. Gels were constantly run at 125 V for 2 h.

All 1D and 2D gels were subsequently blotted onto PVDF membranes (Hybond-P, RPN303F, GE Healthcare, Munich, Germany) with a constant current of 1 mA/cm^2 for 50 minutes using a semi-dry electro blotter (Starlab, Ahrensburg, Germany) and a continuous buffer according to Bjerrum and Schafer-Nielsen (1986). PVDF membranes were blocked for 1.5 h with a synthetic blocking solution (RotiBlock, A151.2, Roth) diluted 1:10 in TBS-Tween20 (200 mmol/l Tris, 1.37 mol/l NaCl, 0.05% Tween20; Roth) and then incubated with the primary antibody overnight at 4°C with continuous shaking (1:400; rabbit anti-bovine CA, AbD Serotec or 1:400; rabbit anti-human CA-II, Santa Cruz). After washing with TBS-Tween20 (3x10 min) membranes were incubated with the secondary antibody (1:40,000; SouthernBiotech) for 1.5 h at room temperature. In addition to three washing steps with TBS-Tween20 (3x10 min), membranes were rinsed with TBS (200 mmol/l Tris, 1.37 mol/l NaCl; 3x10 min). Immediately after, 1 ml of SuperSignal West Pico

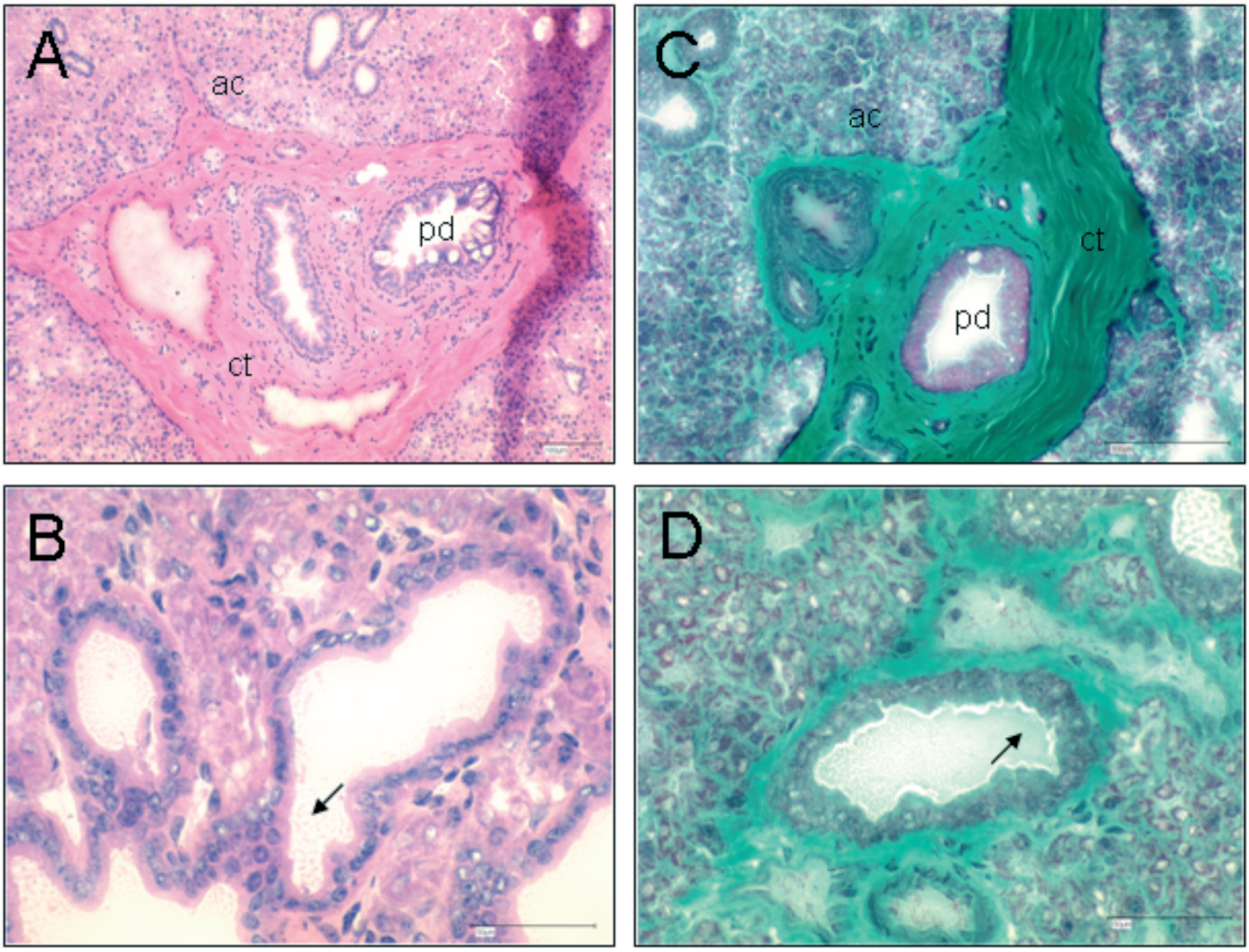

Fig. 1. Histological staining of bovine parotid gland using haematoxylin/eosin (HE; **A, B**) and Masson-Goldner trichrome stain (MG; **C, D**). **A.** Morphology of bovine parotid tissue showing secretory acini (ac), connective tissue (ct) and large parotid ducts (pd). HE. **B.** Detailed view of a parotid duct, which might contain saliva secretions indicated by reddish staining inside the duct lumen (black arrow). HE. **C.** MG staining of bovine parotid tissue to show secretory acini (ac), large parotid ducts (pd) and connective tissue (ct), characterized by green colour. MG. **D.** Detailed view of a parotid duct, which contains mucus (black arrow) most likely representing saliva residues. MG. Scale bars: A, C, 100 µm; B, D, 50 µm

Chemiluminescent substrate (1:1; 34080, Pierce, Rockford, USA) was added in the dark and incubated there for 5 minutes. Antibody reactivity was visualized using X-ray films (34090, Pierce).

Results

Histological staining

Morphology of secretory acini (ac), connective tissue (ct) and large parotid ducts (pd) was visualized in bovine parotid glands by haematoxylin/eosin (HE; Fig. 1A) and Masson-Goldner trichrome staining (MG; Fig. 1C). Using HE stain parotid ducts revealed a reddish material present inside the lumen that most likely was remaining saliva (Fig. 1B). Using MG stain connective tissue and remaining salivary mucous were identified within bovine parotid ducts by green colour (Fig. 1D).

Immunohistochemistry

Protein expression of bovine CA-II was studied in the parotid glands of two different cows (Figs. 2, 3). In the first animal sample, the anti-bovine CA antibody (AbD Serotec) caused weak reddish staining mostly at the luminal cell layer of the parotid ducts (Fig. 2A,B, right part). Likewise, the anti-human CA-II antibody H-70 (Santa Cruz) showed strong reddish staining at the

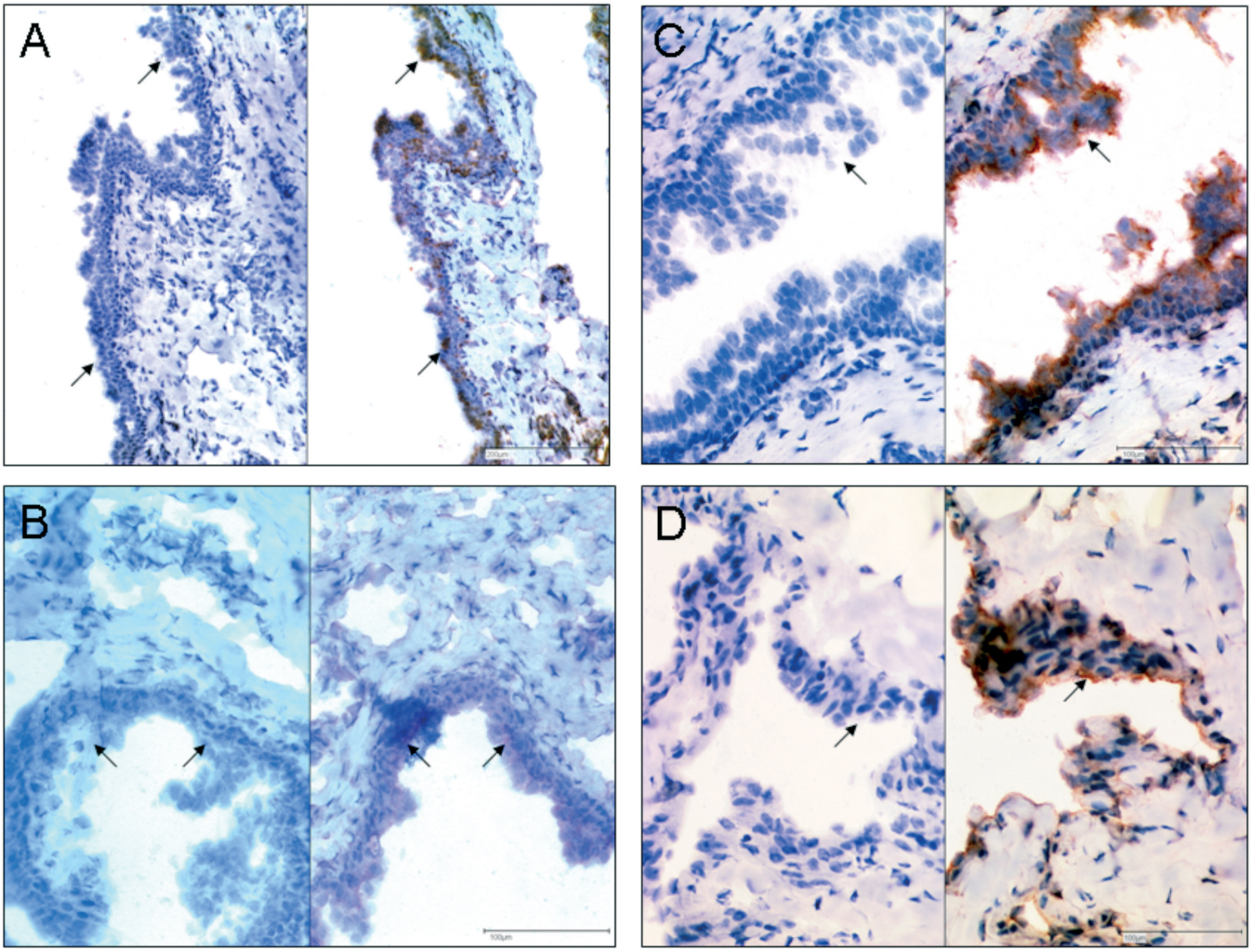

Fig. 2. Immunohistochemical localization of CA-II in the bovine parotid gland. The left part of each figure shows the negative control, in which primary antibodies were omitted. All negative controls only stained blue with haematoxylin and were free of non-specific staining by the secondary antibody. The right part shows identical positions in the tissue sections treated with primary and secondary antibodies. Expression of carbonic anhydrase is visualized by red or brownish staining. **A, B.**The anti-bovine CA antibody (AbD Serotec) caused weak staining mostly at the luminal cell layer of the parotid ducts (arrows). **C, D.** The anti-human CA-II antibody (Santa Cruz) showed strong positive reactions also at the luminal cell layer (arrows). Scale bars: 100 µm.

luminal cell layer, clearly indicating the presence of bovine CA-II in parotid duct cells (Fig. 2C,D, right part). At identical positions, negative controls were only stained by haematoxylin and showed no staining caused by the secondary antibody (Fig. 2A,B, left part; Fig. 2C,D, left part).

In the second animal sample, the anti-bovine CA antibody (AbD Serotec) caused strong reddish staining at the luminal cell layer of the large parotid ducts (Fig. 3A,B, right part) as did the anti-human CA-II antibody H-70 (Santa Cruz; Fig. 3C,D, right part). Both negative controls were again free of non-specific binding by the secondary antibody (Fig. 3A,B, left part; Fig. 3C,D, left part). In addition, positive staining for CA-II was observed as clear reddish brinks in the cell free lumen of bovine parotid ducts using both primary antibodies (Fig. 3B,D, right part). In one case, a red staining for CA-II could also be observed within the parotid gland acini (Fig. 4).

Immunoblot analysis

To provide further evidence that both antibodies used are highly specific for immunohistochemical staining of bovine parotid CA-II, immunoblots were performed on bovine whole saliva (Fig. 5) and on extracts from bovine parotid tissue (Fig. 6). Although bovine saliva contains numerous proteins (Fig. 5A) both

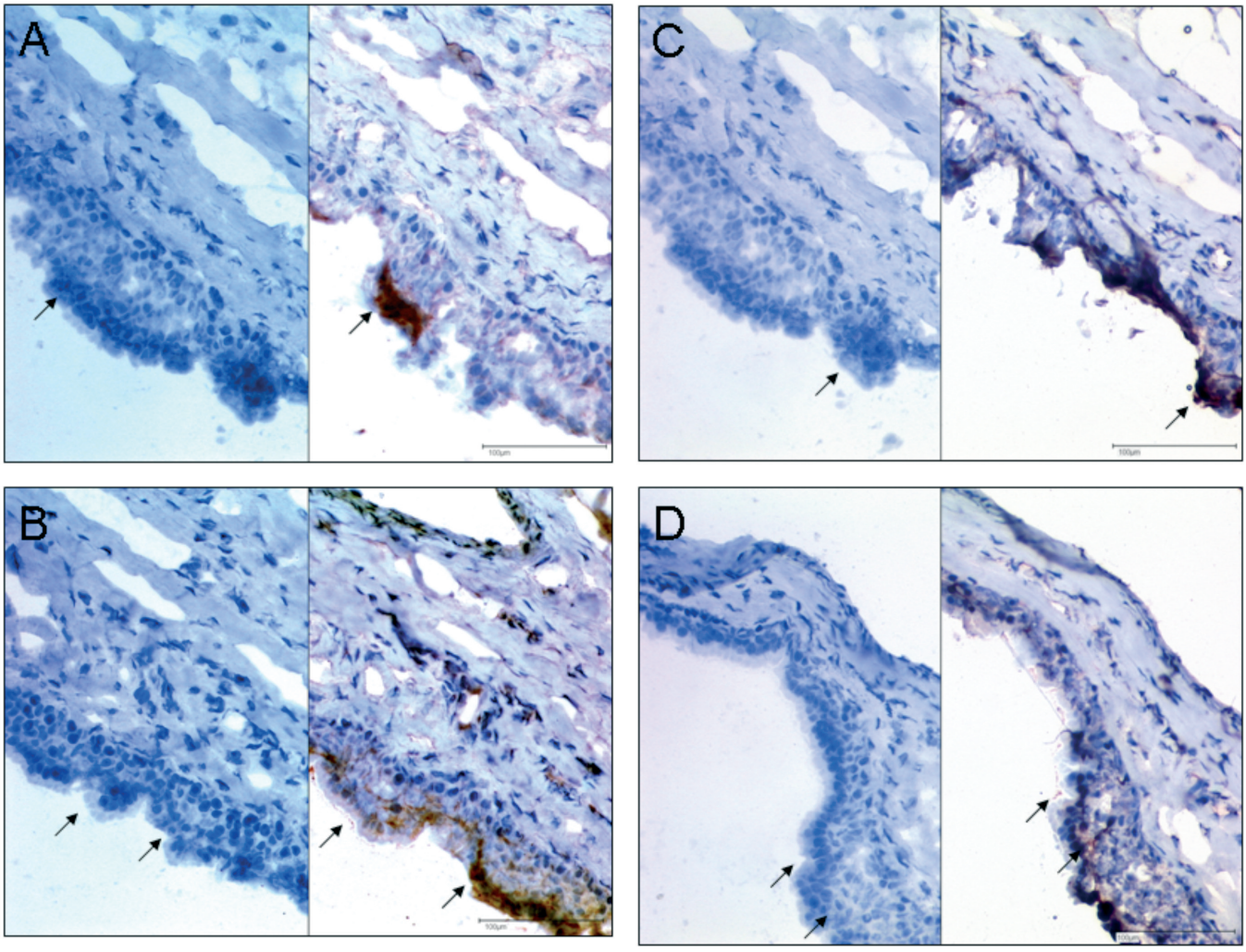

Fig. 3. Immunohistochemical localization of CA-II in the parotid gland of a second cow. The left part of each figure shows the negative control, which is characterized by blue counterstaining with haematoxylin only. The right part shows the identical position in the tissue sections treated with primary and secondary antibodies. Expression of carbonic anhydrase is visualized by red or brownish staining. **A, B.** The anti-bovine CA antibody (AbD Serotec) caused strong staining at the luminal cell layer of parotid ducts (arrows). **C, D.** The anti-human CA-II antibody (Santa Cruz) also reacted positively at the luminal cell layer (arrows). **B, D.** In addition, positive staining for CA-II was observed as clear reddish brinks in the cell free lumen of bovine parotid ducts, clearly demonstrating the secretion of CA-II into bovine parotid saliva. Scale bars: 100 µm.

antibodies detected either CA-VI and/or CA-II isoenzymes with high specificity. Using the anti-bovine CA antibody (AbD Serotec) both the CA-VI (42 kDa; red circle) and CA-II (29 kDa; green circle) were clearly detected in bovine whole saliva (Fig. 5B). The anti-human CA-II antibody (Santa Cruz) showed a positive reaction only with the CA-II isoenzyme secreted into bovine saliva (Fig. 5C; green circle). In parotid homogenates both antibodies detected the same protein exactly comigrating with the CA-II positive control at 29 kDa (Fig. 6A,B). Non-specific staining by the secondary antibody used was excluded in additional experiments.

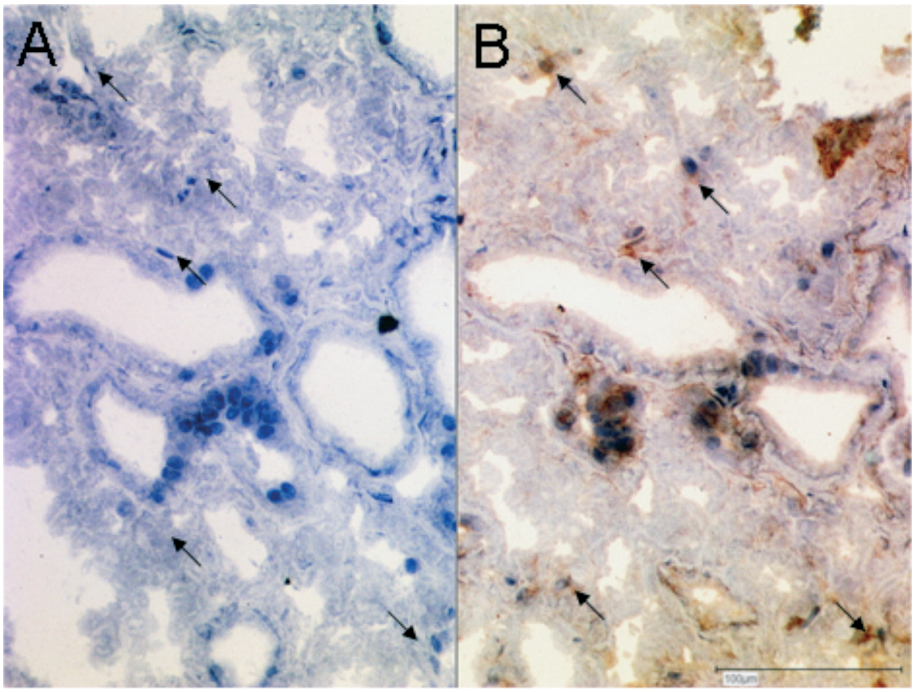

Fig. 4. Immunohistochemical localization of CA-II in bovine parotid acinar cells. **A.** Negative control without primary antibody. **B.** The anti-bovine CA antibody (AbD Serotec) caused reddish staining of CA-II in parotid acinar cells (arrows). Scale bars: 100 µm.

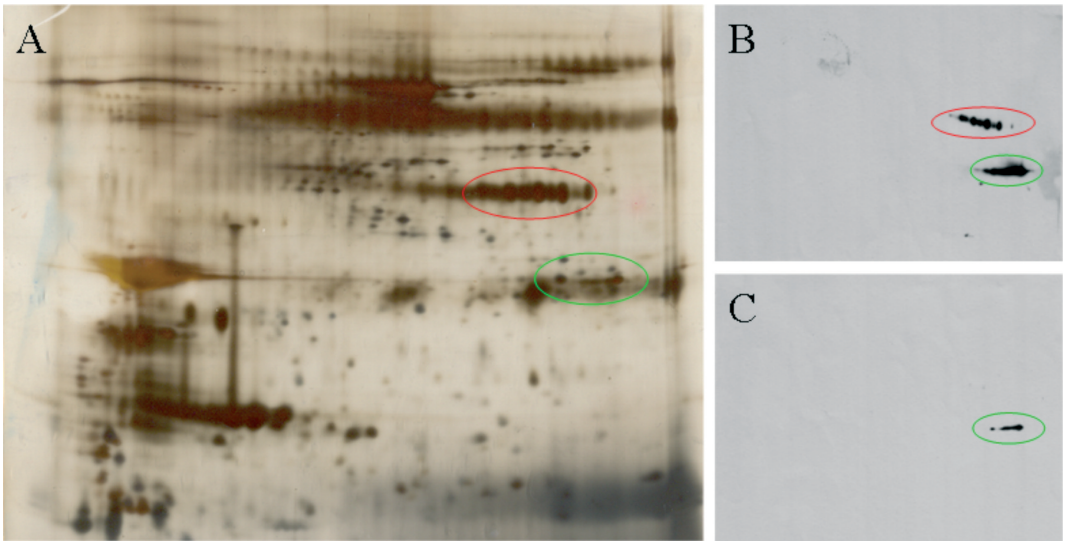

Fig. 5 Two dimensional gel electrophoresis of bovine whole saliva **(A)** and immunoblot analysis for the expression of carbonic anhydrases (CA) using an anti-bovine CA specific antibody **(B)** and an anti-human CA-II specific antibody **(C). A.** Bovine saliva was subjected with 100 µg of total protein to isoelectric focussing and proteins were resolved in a 12% SDS-PAGE. Beside CA-VI (red circle), a second carbonic anhydrase, CA-II (green circle), was present in bovine saliva (Mau et al., 2009). **B.** Using the anti-bovine CA specific antibody (AbD Serotec) both CA-VI (red circle) and CA-II (green circle) reacted positively, with molecular weights of 42 kDa (CA-VI) and 29 kDa (CA-II). **C.** Using the anti-human CA-II specific antibody (Santa Cruz) only the secreted CA-II isoform at 29 kDa showed positive reaction

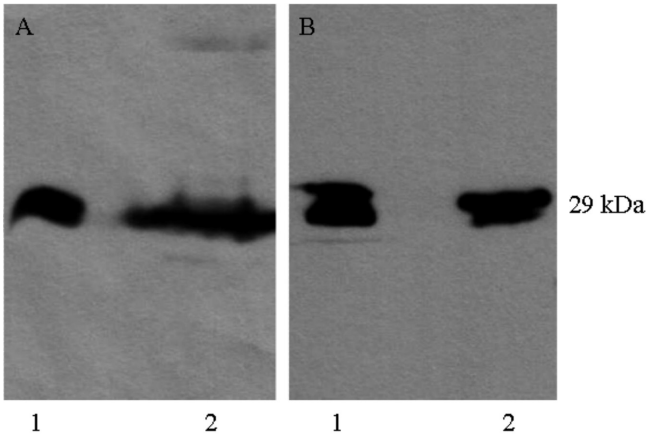

Fig. 6. Immunoblot analysis of bovine parotid CA-II to test for antibody specificity. The membrane was divided for incubation with **(A)** anti-bovine CA antibody (AbD Serotec) or **(B)** anti-human CA-II antibody (Santa Cruz). **A.** In bovine parotid gland homogenate (2) the antibody detected a strong, single band at 29 kDa, which comigrated exactly with the CA-II from bovine erythrocytes used as positive control (1). **B.** The human CA-II specific antibody produced exactly the same signals at 29 kDa, clearly demonstrating the presence of CA-II in the parotid gland extracts (2). *29 kDa* molecular weight of CA-II; 1, bovine CA-II from erythrocytes used as positive control; 2, bovine parotid gland homogenate.

Discussion

The present study provides first evidence for the secretion of CA-II from bovine parotid glands into saliva. The presence of CA-II was demonstrated in parotid tissue by immunohistochemistry and immunoblotting. Two commercially available antibodies were tested. The polyclonal anti-bovine CA antibody (AbD Serotec) was raised against CA-II from bovine erythrocytes and, although not tested for immunohistochemistry before, worked well with both methods. Interestingly, in immunoblotting the antibody cross-reacted with salivary CA-VI. A cross-reaction of CA-II- and CA-VI-specific antibodies was also observed by Murakami and Sly (1987). Their CA-VI and CA-II antisera recognized an amino acid sequence that is homologous between the two isoenzymes. The polyclonal anti-human CA-II antibody H-70 (Santa Cruz) was raised against amino acids 191-260 of CA-II isolated from human erythrocytes. Since the bovine CA-II shares approximately 85% sequence identity with human CA-II in these amino acids, the antibody showed a high cross reactivity with bovine CA-II.

In our study, a reddish staining of parotid acinar cells for CA-II was only present in one of the two animals. Positive staining for CA-II was mainly observed in excretory duct cells and within the lumen of bovine parotid excretory ducts. This supports other studies that showed earlier the presence of intracellular CA-II in parotid and/or submandibular glands of rats, humans and cattle (Asari et al., 1989; Parkkila et al., 1990; Ogawa et al., 1992, 1993). Similar to our study, in the rat submandibular gland CA-II staining was observed in the cytosol of epithelial cells of granular, striated and excretory ducts (Ogawa et al., 1992). Furthermore, in human parotid and submandibular glands CA-II was expressed primarily in the granules of serous acinar cells, as well as in duct epithelial cells, indicating its role in macromolecular and bicarbonate secretion (Parkkila et al., 1990; Ogawa et al., 1993). In the bovine submandibular gland CA-II was expressed primarily in the duct segments (Asari et al., 1989). However, in the bovine parotid gland a uniform staining for CA-II was observed in serous acinar cells only, and was completely lacking in duct segments (Asari et al., 1989). The latter is quite contradictory to our results. However, it is important to keep in mind that differences in the glandular sites of CA-II expression are often related to the fixation methods used. For example, Asari et al. (1989) used deparaffinized and rehydrated sections to study CA-II expression in bovine salivary glands. During fixation, embedding and rehydration the remaining saliva, together with secreted CA-II, most likely were dissolved away from the duct lumen and thus Asari and co-workers could not find CA-II in the lumen of parotid ducts. Likewise, Parkkila et al. (1990) used paraffin-embedded tissue to localize CA-II in human salivary glands and also failed to find the secreted enzyme. In contrast to these studies, we demonstrated that parotid ducts contained salivary remains, including secreted CA-II, when the tissue was immediately frozen right after slaughter without additional fixation. On the other hand, fixation and embedding are often necessary to unmask hidden antigen structures inside cells. Therefore, although we preserved the salivary remains due to the use of frozen sections, we might have weakened our chance to detect CA-II inside the parotid acinar cells.

Secretion of cytoplasmic CA-II via the apocrine export mode was first shown in rat coagulation glands (Wilhelm et al., 1998). Apocrine proteins like CA-II are synthesized in the cytoplasm and are directly translocated into aposomes without passing the endoplasmic reticulum or the Golgi apparatus (Wilhelm et al., 1998; Aumüller et al., 1999). Henningar et al. (1983) first postulated an export of CA-II from rodent salivary glands and later an apocrine-like type of secretion was also suggested for ruminant parotid glands (Stolte and Ito, 1996). Moreover, CA-II was demonstrated to be a part of extracellular in vivo salivary pellicle in humans (Li et al., 2004). Accordingly, a secreted CA isoenzyme of 29 kDa, most likely CA-II, was identified only recently in ruminant saliva (Mau et al., 2009).

The simultaneous secretion of CA-II and CA-VI into bovine saliva suggests that both enzymes may form a complementary system to regulate pH on epithelial surfaces of the bovine digestive tract. Although the cytoplasmic CA-II in salivary glands mediates HCO_3^-

secretion into saliva as part of the ion exchange process that is observed e.g. in parotid ducts, the secreted CA-VI and CA-II probably regulate salivary pH using the secreted HCO_3^- (Kaplan and Baum, 1993; Sly and Hu, 1995). The enzymes might thereby provide a suitable environment for rumen microbes which are necessary to maintain the digestion of fibre-rich grass diets. Because the isoenzymes are highly active in bicarbonate production, a decrease in the expression of either CA-II or CA-VI might lead to severe disturbances of digestion and/or to increased susceptibility to acidosis in ruminants. This assumption correlates well with results showing that the parotis has the highest CA activity of all ruminant salivary glands (Matsumoto et al., 1982). Since the saliva is well buffered at a constant pH of 8 and flows continuously into the rumen, salivary CA is considered to play an important role in maintaining a constant oral and ruminal milieu (Asari et al., 1989). This gets further support from studies on human alimentary tract showing a wide distribution of intracellular CA-II in epithelia of oesophagus, stomach, duodenum and colon (Parkkila et al., 1994). In bovine rumen and abomasum CA-II is also expressed in high quantities (Asari et al., 1989). Interestingly, a new 29-kDa colonic mucus CA, possibly contributing to maintain the intestinal pH microclimate, was described in humans and other mammals (Kleinke et al., 2005). Furthermore, CA-II was shown to be essential for the secretion of bicarbonate in the duodenum (Muallem et al., 1994), as well as for the production of regular amounts of saliva (Goto et al., 2008). It is also known that CA-II deficiency is the primary defect in the human syndrome of renal tubular acidosis and cerebral calcification. This might demonstrate the enzyme's importance in preventing the development of acidosis (Roth et al., 1992). Conclusively, the role of secreted salivary CA-II in avoiding acidosis and providing a suitable environment for microorganisms in the rumen of cattle and other ruminating animals should be studied further.

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