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Development of Proteolytic Activity of Pepsinogen A Isozymes, Their Distribution in Abomasum and Duodenum of Sheep Foetuses

Rozwój aktywności proteolitycznej izoenzymów pepsynogenu A, ich rozmieszczenie w trawieńcu i dwunastnicy płodów owcy

In sheep stomach like in other animal species (6, 21, 22) two electrophoretically distinct groups of proteinases have been observed: fast moving group (FMG) and small moving group (SMG) (26). FMG in sheep shows main proteolytic activity and immunologically is related to cattle pepsinogen A (PG A) (3). FMG in lambs (Astrachan breed) contained four and in some animals (20%) five proteolytic fractions named Pg 1, Pg 2, Pg 3, Pg 4 and Pg 5 according to their decreasing electrophoretical mobilities towards the anode with Pg 1 having the highest mobility (13). SMG contains probably pepsinogen C (PG C) known also as progastricsin, prochymosin and small moving protease (SMP) (13) corresponding to that observed in humans (19), which is not distinguishable from cathepsin E (15). Both groups are present in fundic and pyloric parts of abomasum and in proximal duodenum but the number of proteolytic bands and their activity in these parts differ (26). FMG of lamb abomasum in first days after birth contains all proteolytic fractions (except Pg 1 in some animals) but their relative proteolytic activity differs from that of adults (27). Developmental qualitative changes in the production of aspartic proteases in different animal species show characteristic pattern (8). Some information is available concerning developmental change of pepsinogens in rats (10), mice (29), hens (30), pigs (24, 25) and humans (6) but no information from ovine foetuses has been reported.

The aim of this study was to investigate the beginning of production and distribution of proteolytic fractions in fundic, pyloric parts of abomasum and proximal duodenum in development of sheep foetuses.

MATERIALS AND METHODS

15 ovine foetuses obtained under general anesthesia from primiparous pregnant ewes at 70 to 135 days of gestation (Polish Merinos Breed) and 4 lambs (1 to 3 hours after birth before suckling), 1 milk-fed lamb 5 days old. Lambs were anesthesized by vetbutal and then blood-drained. The abomasa and proximal duodenum (about 10 cm) were removed immediately and then washed with cold water and 0.1 mol/l phosphate buffer at pH 7.5. Lamb and sheep abomasa after removal were washed in cold water, immersed for 10 min in solution of 2% $NaHCO_3$ and after that in 0.1 mol/l

natrium phosphate buffer at pH 7.5. Each stomach was divided in 2/3 anterior fundic part and 1/3 (before pyloric posterior sphincter) as pyloric part. Each part was extracted separately. The same day the mucosa was scraped off with a scalpel and disintegrated for 3 min in a Potter-Elvehem homogenizer using 5 ml of 0.01 mol/l natrium phosphate buffer for 1 g of tissue. Suspended tissue was separated by centrifugation at 5000 f for 15 min at 4C and homogenized for the second time with the same volume of the buffer. Supernatants of both homogenizations were pooled and stored over a night at 4C. The next day before electrophoresis clotting activity was determined in unacidified extracts to exclude the possibility of errors in sample identification due the artifacts caused by active enzymes. The recovery of milk clotting activity from fundic mucosa extracts was more than 96% of total activity.

Clotting activity was measured according to procedure of Berridge (4) except that the volume of milk substrate was reduced to 2 ml and 0.2 ml of examined extract. Milk-clotting activity was determined in unacidified and acidified extracts to pH 2.0 by addition of 0.1 mol/l HCl. Acidified extracts after 30 min kept in 19–20°C were brought to pH 6.3 by addition of 0.2 mol/l Na_2HPO_4 while the unacidified extracts were adjusted pH 6.3 by addition of 0.2 mol/l NaH_2PO_4. 12 g of low-fat milk powder (SM Gostyń, Poland) was solubilized in 100 ml of 0.01 mol/l $CaCl_2$, pH of milk solution was 6.3. Extracts were diluted with distilled water which could blott the milk between 5 to 6 min at 30°C. The activities were expressed in coagulant units (CU). One CU was the amount of enzymes that clotts 10 ml of substrate in 100 s. All samples were analysed twice using milk powder from the same lot.

Agar gel electrophoresis. It was carried out using 1% solution of agar (Agar, Noble, Difco, Detroit, Michigan USA) containing 1 mg of casein (Serva) in 1 ml (w/v) of agar gel in 0.05 mol/l natrium phosphate buffer at pH 7.5 and poured over 20 by 20 cm glass plates to uniform thickness of 1.5 mm as described earlier (13) using abomasal extracts of 5 days old lambs as fraction reference. Relative activity of the separated fractions was visually estimated considering the wideness and intensity of the clear areas on the background of undigested and staining caseine, as follows: the highest activity was marked by circling the number of the fraction; e. g. 2, 3, 4 means the lowest activity of Pg 2, the highest activity of Pg 3 and high activity of Pg 4.

RESULTS

Milk-clotting activity in the fundic part of abomasum at a 70-days old foetus was small and increased gradually as foetus developed. The highest value was observed during the first days after birth (Table 1). Proteolytic activity in pyloric part appeared at 85th day, increased with development. In proximal duodenum at 120 days was observed a small value of proteolytic activity which slightly increased at birth. The high value of activity was in fundic part, low – in the pyloric part of abomasum and the lowest in proximal duodenum.

Electrophoretic analysis demonstrated in fundic part of abomasum of 5-day-old lambs the existence of Pg 1 of small, Pg 2 of high, Pg 3 and Pg 4 of approximative equal activity. Slow moving group contained in lamb – four and in foetus – three fractions of which prochymosin was main. In fundic part of a 70-day-old foetus already occurred Pg 3 and Pg 4 of equal activity, following development of foetus Pg 3 which demonstrated slightly higher proteolytic activity than Pg 4.

In 85-day-old foetuses Pg 3 and Pg 4 of equal activity appeared in pyloric parts and on the following days Pg 3 demonstrated higher activity than Pg 4.

Tab. 1. Developmental changes in milk clotting activity in abomasal mucosa of ovine foetuses and lambs. Values are expressed in chymosin units per 1 g of wet mucosa
Zmiany rozwojowe w aktywności ścinania mleka w błonie śluzowej płodów owiec i jagniąt. Wartości wyrażone w jednostkach chemozyny na 1 g mokrej błony

Age of foetuses in days	Abomasum		Proximal duodenum
	fundic part	pyloric part	
70 (1)*	9.7	0.0	0.0
85 (2)	12.1 (11.0– 13.2)**	4.5 (2.4– 6.7)	0.0
90 (4)	21.0 (11.4– 26.4)	9.1 (4.8–17.0)	0.0
105 (2)	36.8 (32.7– 41.0)	14.3 (11.8–16.8)	0.0
120 (2)	128.8 (125.7–132.0)	16.8 (14.5–19.1)	2.4
135 (4)	150.0 (87.3–279.3)	19.8 (7.7–28.8)	2.9
birth (4) 0 days	239.3 (148.5–305.2)	22.3 (14.5–24.0)	2.8
5 (1)	336.0	23.3	3.0

* Number of animals examined in each age group; ** Scattering of values in each age group.
* Liczba badanych zwierząt w każdej grupie wiekowej; ** Rozrzut wartości w każdej grupie wiekowej.

Tab. 2. Developmental changes of pepsinogen A isozymes in fundic and pyloric parts of abomasum and proximal duodenum of ovine foetus and lambs
Zmiany rozwojowe izoenzymów PGA w części dennej i odźwiernikowej żołądka właściwego i dwunastnicy płodów owiec i jagniąt

Gestation in days	Fundus	Pylorus	Proximal duodenum
70	3 , 4	traces	0.0
85	③, 4	3 , 4	0.0
90	③, 4	③, 4	traces
105	③, 4	③, 4	traces
120	②, ③, 4	②, ③, 4	3 , ④
135	②, ③, 4	②, ③, 4	3 , ④
Lambs 0 day	2 , ③, 4	②, ③, 4	3 , 4
Lambs 5 days	①, ②, ③, 4	②, ③, 4	3 , 4

Proximal duodenum contained Pg 4 and was slightly better expressed than Pg 3 in foetus whereas in lambs both isozymes were equal. At a 120-day-old foetus Pg 2 appeared for the first time as a small proteolytic band which was the same at 135 days either in fundic and pyloric parts of abomasum. Pg 2 increased in course of time and displayed as a main proteolytic fraction in fundic part of a 5-day-old lamb.

Schematic presentation of proteolytic fractions and their relative intensities in abomasum fundic part of foetuses in development shows Fig. 1.

The first and small proteolytic band of SMG localized behind Pg 4 represented probably progastricsin which was divided by stained strip of protein that

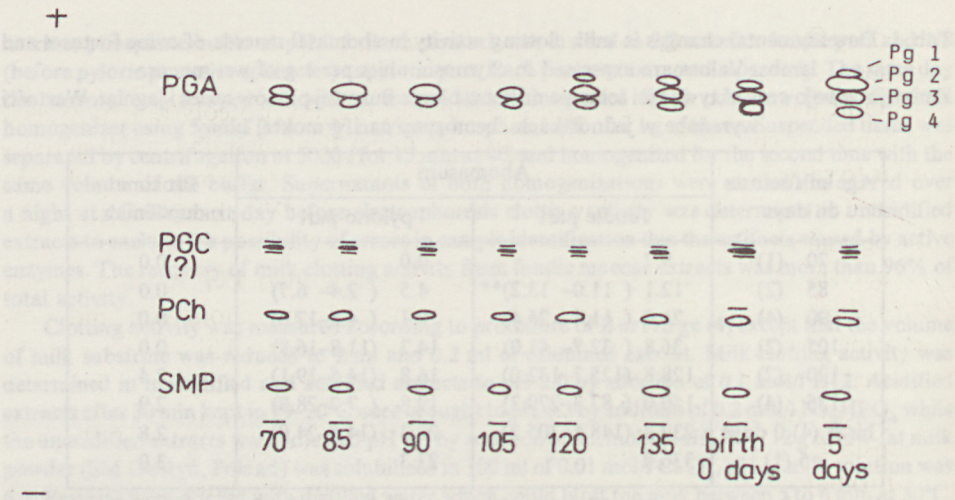

Fig. 1. Electrophoregrams of fundic abomasum extracts showing protease fractions and their relative intensities of ovine foetuses in development

Elektroforegramy wycinków z dna żołądka wykazujące frakcje proteazy oraz intensywność względna płodów owcy w trakcie rozwoju

electrophoretical migration corresponded to PG C in cattle. The principal proteolytic fraction in abomasum belonging to SMG was prochymosin. The highest proteolytic activity of the SMG was observed in fundic part, less pronounced in pyloric region and small in duodenum. The last small fraction was situated near the line of start on agar gel and corresponded to SMP in a man (19).

Crude extract from foetal stomach exhibited about 1.3% and that from lambs varied from 2% to 4% of total proteolytic activity.

DISCUSSION

The study of development of abomasal glands by histological methods demonstrated that peptic cells contained some pepsinogen granules at 85-day-old foetuses and number of these cells increased in 150-day-old ovine foetuses (12). In this investigation the production of gastric proteases was started before 70-day-old foetus and occurred earlier than previously reported. This difference may be explicated by different method and animal breed used. In the calf foetus prochymosin has been produced in 10 weeks while pepsinogen was not produced in the 20th week at first in fundic part and later in pyloric part of abomasum (1).

Proteolytic activity in fundic, pyloric part of abomasum and duodenum appeared in different time in ovine foetus analogically as was found in calf foetus (1, 28).

Individual differences in proteolytic activities were observed. This may be

caused by different expression of protease gens during development process of foetuses.

Fundic part of abomasum demonstrated the highest proteolytic activity as it was found in sheep (26, 27), while pyloric tissue demonstrated small activity similar to rats (10), lambs (27), sheep (26) and calves (1, 28). Proximal duodenum of ovine foetus displayed the lowest activity as in lambs (27), sheep (26), man (20, 23) dog and cat (16, 17).

Analysis of zymograms demonstrated fast migrated PG A isozymogens comprising initially Pg 3 and Pg 4 in abomasum and duodenum and slow migrating group of 3 fractions in foetuses or 4 in the lambs. This protease group consisted probably of prograstricsin, chymosin (in lamb only), prochymosin which demonstrated main activity in fundic part of abomasum and SMP or cathepsin E.

Chromatographically isolated lamb prochymosin contained the main and two small components (3). Zymograms of mucosa extracts of foetuses revealed only one prochymosin component like previously observed in lambs (27).

Among pepsinogen A isozymogens – Pg 3 and Pg 4 were first in fundus and as foetus developed successively appeared Pg 2 which was more intensive at birth and finally was major fraction in 5-day-old lambs. Pepsinogen 1 was isozyme that appeared in one-day-old lambs and this is in accordance with previous results (27).

Mechanism of developmental PG A changes is not known. It is suggested that these changes are controlled by hormones. Studies demonstrated that precocious development of gastric proteolytic activity and precocious change of pepsinogen fraction in young rats (2–9 days old) resembling that of an adult patterns were obtained after treatment with glucocorticoids (10). Also young mice undergo similar changes (14). An increase in the synthesis or proteases after treatment of pigs with ACTH was obtained (24, 25).

An increase of activity of Pg 2 was observed at birth and this may be caused by corticosteroids whose high concentration in ovine foetus blood is involved in the initiation of partituration (2). This period corresponds well with the time when production of proteolytic enzymes increased. It is possible that the mechanisms of the digestive development in ovine foetus in terminal period of intrauterine life and first days after birth of lambs are similar to those after ACTH treatment of rats (10) and pigs (24).

The occurrence of PG A in gastric mucosa of ovine foetus is similar to that of the calf foetus and very different from that of pig (7, 25) and human (6) where the pepsinogen is produced just now in postnatal period. The pyloric part of abomasum in the foetus and lamb demonstrated the existence of pepsinogen A as well as in the cat and dog (16, 17), cattle (1, 28) and opposite to man (6) and pig (24), that contain progastricsin only. The main pepsinogen in fundic part of new-born lambs in this and other sutdy was Pg 3 (27) while in 3–5-day-old lambs

and sheep was Pg 2 (26). The main pepsinogen of sheep pyloric region appeared Pg 3 whereas in the foetus and lambs Pg 3 and Pg 4 were of equal intensity. Foetal and lamb duodenum contained also both Pg 3 and Pg 4 of similar activity.

Ovine pepsinogen/pepsin studied by chromatographic method was homogeneous (3, 9) but fast protein liquid chromatography revealed 3 and 4 fractions (3), 3 fractions (18). Agar gel electrophoresis in this and other study (5, 13, 26) demonstrated 4–5 proteolytic fractions. These discrepancies may be explicated by using different methods and animals.

CONCLUSIONS

1. Milk-clotting activity in abomasum was present in a 70-day-old foetus, increased gradually as foetus developed, the greatest value was observed in the first day of lambs' life. In pyloric part and duodenum proteolytic activity was observed in 85- and 120-day-old ovine foetus, respectively.

2. Prochymosin and pepsinogen A isozymes – Pg 3 and Pg 4 in fundic part were found in a 70-day-old foetus; in pyloric part – Pg 3 and Pg 4 of equal intensities was found in a 85-day-old foetus and the same isozymes in duodenum – in a 120-day-old foetus.

3. The main proteolytic fraction of pepsinogen A was Pg 3 whose relative activity decreased whereas Pg 2 increased in fundic part of lambs after birth. Pyloric region of foetal abomasum initially contained Pg 3 and Pg 4 of equal activity and in 1-day-old lambs contained small band of Pg 2 with Pg 3 and Pg 4 of high and equal activity.

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STRESZCZENIE

Badania przeprowadzono na wyciągach z błony śluzowej od 15 płodów od 70 do 135 dni po zapłodnieniu oraz 5 jagniąt 1- i 5-dniowych. Celem badań było określenie początku syntezy proteinaz żołądkowych i ich względna aktywność w rozwoju płodów owcy. Aktywność proteolityczną oznaczano testem ścinania mleka, liczbę frakcji proteolitycznych i ich względną aktywność określano przy pomocy elektroforezy w żelu agarowym. Część denna żołądka płodów 70-dniowych wykazywała aktywność proteolityczną, która zwiększała się, uzyskując najwyższą aktywność u jagniąt po urodzeniu. W części odźwiernikowej mała aktywność pojawiała się w 85 dniu, a w dwunastnicy w 120 dniu, zwiększając się wraz z wiekiem płodu. W badaniach elektroforetycznych stwierdzono trzy do czterech izoenzymy PGA nazwane zgodnie z malejącą migracją od Pg 1 do Pg 4 oraz trzy frakcje wolnomigrujące, wśród których główna była prochymozyna. Zmiany rozwojowe izoenzymów PGA w części dennej i odźwiernikowej żołądka polegały na obecności Pg 3 i Pg 4 początkowo o równej aktywności, a następnie aktywność Pg 3 przewyższała Pg 4 i w miarę rozwoju płodu pojawiała się Pg 2 oraz u jagniąt Pg 1 w części dennej żołądka.