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Rat Submandibular Gland during the Maturation Process: Changes in Enzyme Activities, Protein and Lectin-Binding Profiles

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Summary

The total protein glycosylation profile and specific activity of lysosomal enzymes were investigated in rat submandibular glands isolated from very young (1-month), young (1.5-months) and adult rats (3-months) rats. The specific activity of lysosomal hydrolases (i.e. acid phosphatase, arylsulfatases A and B, β-N-acetyl-D-glucosaminidase, β-galactosidase and β-glucuronidase) decreased in parallel to increasing age of the animals. Furthermore, the thermal stability of acid phosphatase and β-N-acetyl-D-glucosaminidase was influenced by the age of rats. Age-related changes in protein profile regarding the intensity of particular bands as well as the appearance of certain proteins limited to special age groups were also demonstrated as revealed by Coomassie and lectin staining. Moreover, the marked age-related increase in structures Man (α1-2, α1-3, α1-6) Man, Fuc (α1-6) GlcNAc as well as Gal (β1-3) GlcNAc was observed, whereas staining with terminal NeuAc and GlcNAc showed an inverse correlation. The reaction with (β1-6) branched N-glycans and Gal (β1-3) Gal structures was limited to 1-month-old rats. No significant changes in a specific reaction with NeuAc (α2-3) Gal were observed. We speculate that the observed differences with respect to protein and glycosylation profiles between 1-month-old rats and older ones could be caused by a modification of the diet composition as well as by the functional and morphological maturation of the rat submandibular gland.

Key words
Lectins • Lysosomes • Maturation • Oligosaccharides • Submandibular gland

Introduction

The submandibular gland is the major salivary gland secreting a considerable portion of glycoprotein and several functionally important low-molecular weight proteins as well as water and electrolytes into the whole saliva. Its postnatal development in rats has attracted considerable attention over many years, since this gland is incompletely developed and transitory at birth, and the fetal type secretory units are completely replaced by those of adult type over a short time (Cutler and Chaudhry 1975, Cutler 1980, Moreira *et al.* 1990). Apart from the morphogenic and cytodifferentiation changes observed during pre- and postnatal development of this gland, the appearance of the secretion process as well as the chemical composition of the secretory products have been studied in detail (Materazzi *et al.* 1973, Cutler *et al.* 1991). More recently, increasing interest has been

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attributed to glycosylation pattern of salivary mucins (Zhang *et al.* 1994, Accili *et al.* 1999) as a primary means of defense of teeth and oral mucosa against mechanical, chemical and microbal insults (Nieuw Amerongen *et al.* 1995, Tabak 1995). Accordingly, the present paper was designed to investigate development-related changes in the specific activity and thermal stability during the maturation process of the following rat submandibular gland lysosomal enzymes: β-galactosidase (EC 3.2.1.23), β-N-acetyl-D-glucosaminidase (EC 3.2.1.52), β-glucuronidase (EC 3.2.1.31), arylsulfatases A and B (EC 3.1.6.1), as well as acid phosphatase (EC 3.1.3.2). We also studied lectin-binding profiles of tissue proteins as a mixture of proteins that should represent a full spectrum of glycosylation processes, whereas a single protein may not provide a complete picture of development-related changes.

Methods

Materials

A Glycan Differentiation Kit containing the digoxigenin-labeled lectins (Table 1), PNGase-F and Nonidet P-40 were purchased from Roche Molecular Biochemicals. PVDF membranes were obtained from Millipore. N-acetyl-D-glucosamine, methyl-α-D-mannopyranoside, p-nitrocatechol sulphate, fucose, proteinase and phosphatase inhibitor coctail (cat. No. P. 2714), phenolphtalein-β-D-glucuronide, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl N-acetyl-β-D-glucosaminide, SDS, and High Molecular Weight Standards were from Sigma. N-acetylneuraminic acid, galactose and 4-nitrophenylphosphate Na_2-salt were from Serva. All remaining reagents were of analytical grade.

Table 1. Lectins used and their sugar binding specificity.

Lectin origin, acronym	Sugar specificity	Inhibitory sugar
Aleuria aurantia AAA	Fuc (α1-6) GlcNAc	L-Fuc
Datura stramonium DSA	Gal (β1-4) GlcNAc	D-Gal, D-GalNAc
Galanthus nivalis GNA	Man (α1-2, α1-3, α1-6) Man	methyl-α-D-mannopyranoside
Maackia amurensis MAA	NeuAc (α2-3) Gal	NeuAc
Sambucus nigra SNA	NeuAc (α2-6) Gal	NeuAc
Arachis hypogaea PNA	Gal (β1-3) GalNAc	D-Gal
Phaseolus vulgaris PHA-L	(β1-6) branched N-glycans	
Triticum vulgaris (wheat germ) WGA	terminal NeuAc and GlcNAc	NeuAc, GlcNAc

Fuc – L-fucose; Man – D-mannose; NeuAc – N-acetylneuraminic acid; Gal – D-galactose; GalNAc – D-N-acetylgalactosamine; GlcNAc – D-N-acetylglucosamine;

Animals

Three series of male Wistar rats, aged 1, 1.5 and 3 months were distributed into experimental groups of ten animals each. Rats housed in a controlled environment (12 h light - 12 h dark, temperature 20 °C, humidity 50 %) were fed *ad libitum* a conventional diet and had free access to food and drinking water. The rats of various age were killed by decapitation between 08:00 h and 09:00 h after a 24-hour fast. The submandibular glands were dissected, weighed and frozen at –70 °C until assayed.

Preparation of tissue homogenates

The glands were homogenized on ice in 5 ml of 20 mM Tris/HCl buffer (pH 7.0, containing 0.15 M NaCl and a proteinase and a phosphatase inhibitor coctail) per g of the tissue with a glass-glass homogenizer (10 000 rpm, 3 times for 15 s each) and then centrifuged at 30 000 x *g* for 1 h.

Determination of enzymatic activities

Acid phosphatase (AP) activity was measured by using 4-nitrophenylphosphate Na_2-salt by the modified methods of Igarashi and Hollander (1968). Arylsulfatases A and B (AS) activities were determined by using p-nitrocatechol sulphate according to Worwood *et al.* (1973) with some modification. β-N-acetyl-D-glucosaminidase (AM) activity and β-galactosidase (GAL) activity were measured by using p-nitrophenyl-N-acetyl-β-D-glucosamine and p-nitrophenyl-β-D-galacto-

pyranoside, respectively, both according to the modified method of Kaplan and Jamieson (1977). β-glucuronidase (GLU) activity was measured by using phenolphtalein-β-D-glucuronide according to the modified method of Barret and Heath (1977). The reaction mixture contained: 25 µl of the substrate solution, 25 µl of the appropriate buffer solution and aliquots of 100 µg protein in 25 µl of tissue homogenate. The reaction mixtures were incubated at 37 ºC for 10 min (AP and AM), for 30 min (GLU), for 45 min (GAL) or for 120 min (AS). The enzyme reaction was stopped by the addition of 500 µl of glycine buffer, pH 10.7 and monitored in a ELISA reader at 405 nm for GAL, AP and AM; at 570 nm for GLU, and at 490 nm for AS.

Determination of protein stability to heating

The mixture containing 25 µl of the appropriate buffer solution and aliquots of 100 µg protein in 25 µl of tissue homogenate was incubated in 56 ºC for 5 to 120 min. The preincubated samples were then analyzed for enzyme activities as described above. The rate of decrease of enzymatic activity establishes the rate of irreversible heat denaturation. The residual enzyme activities were then plotted against preincubation time. The samples without preincubation at 56 ºC were used as control (100 %).

Peptide N-glycosidase F (PNGase F) digestion

Five µg of protein from tissue homogenates was dissolved in 50 µl of 20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.1 % SDS, 5 % β-mercaptoethanol and boiled at 100 °C for 5 min. After cooling to room temperature, 10-fold excess of Nonidet P-40 compared to the concentration of SDS was added. PNGase F (EC 3.2.2.18) digestion was carried out for 19 h at 37 °C using one unit of the enzyme. The control sample was processed in the same way, with addition of the reaction buffer instead of PNGase F.

SDS-PAGE and blotting

Equal amounts of protein (50 µg) were separated on 12.6 % polyacrylamide gels in the presence of sodium dodecylsulphate in reducing condition according to Laemmli (1970) and then transferred onto a PVDF membrane using a wet blotter in the transfer buffer (25 mM Tris and 192 mM glycine in 20 % methanol, pH 8.4) for 90 min at a constant voltage of 100 V.

Lectin blotting studies

Specific lectin-blotting was performed according to Haselback *et al.* (1990) as described previously in detail (Przybyło and Lityńska 2000).

Other methods

The protein concentration was determined according to the routinely used procedure (Bradford 1976) with bovine serum albumin as a standard. Densitometric tracing of the lectin-stained blots was performed using GelScan 4 Program (Kucharczyk TE, Poland). The levels of significance for the differences between mean values were computed using Duncan's new multiple range test. $P<0.05$ values were considered significant.

Results

Lysosomal enzyme activities

To determine the influence of postnatal development on lysosomal enzymes in rat submandibular gland, GAL, AM, GLU, AS, and AP activities were measured in rats aged 1, 1.5 and 3 months. We observed that a decrease of the specific activity tended to run parallel to increasing age for all tested enzymes (Fig. 1) and it appeared to take place in two distinct phases. In the first phase, we found a decrease in specific activity of all enzymes between age 1 and 1.5 months. In the second phase, only specific activities of GAL, AP, and GLU were further reduced in 3-month-old rats but at a slower rate. Moreover, the specific activities of the tested enzymes exhibited large differences (Fig. 1). Among them, AM showed the highest specific activity in comparison to other enzymes in each age group, and activity values followed a decreasing order: AM >>> AP >> GAL > GLU > AS.

Thermal stability of enzymes

To determine whether the age-related decrease in enzyme specific activities was due to kinetic properties of the enzymes, we measured thermal stability of AM, AP and GLU. Each of enzymes tested reacted differently to heat denaturation. The decay of enzyme specific activities was observed to be from 60 % to 95 % in 2 h of preincubation in 56 °C with different rate of decay (Fig. 2). The activity of GLU was not affected in glands from 1.5- and 3-month-old rats, whereas in 1-month-old rats the activity dropped to 60 % after 2 hours. On the contrary, the activity of AP significantly declined in all

groups after 5 min of incubation, reaching only 6 % of activity after 2 h. The most sensitive to heat denaturation was AP from 1-month-old rats. The activity of AM was also significantly reduced after 2 h of incubation. The decrease of specific activity tended to run parallel to the time of preincubation for all age groups up to 30 min, and then the activity of the enzyme from adult rats declined significantly faster than did in the others. In case of AM the enzyme from adult animals was the most thermolabile. This suggested that the thermal stability of AP and AM was influenced by the age of rats. We also measured the thermal stability of AM activity after its deglycosylation. The enzyme deprived of N-glycans was much more sensitive to thermal denaturation showing only 5 % of specific activity after 30 min as compared to 65 % in the whole glycoprotein (data not shown).

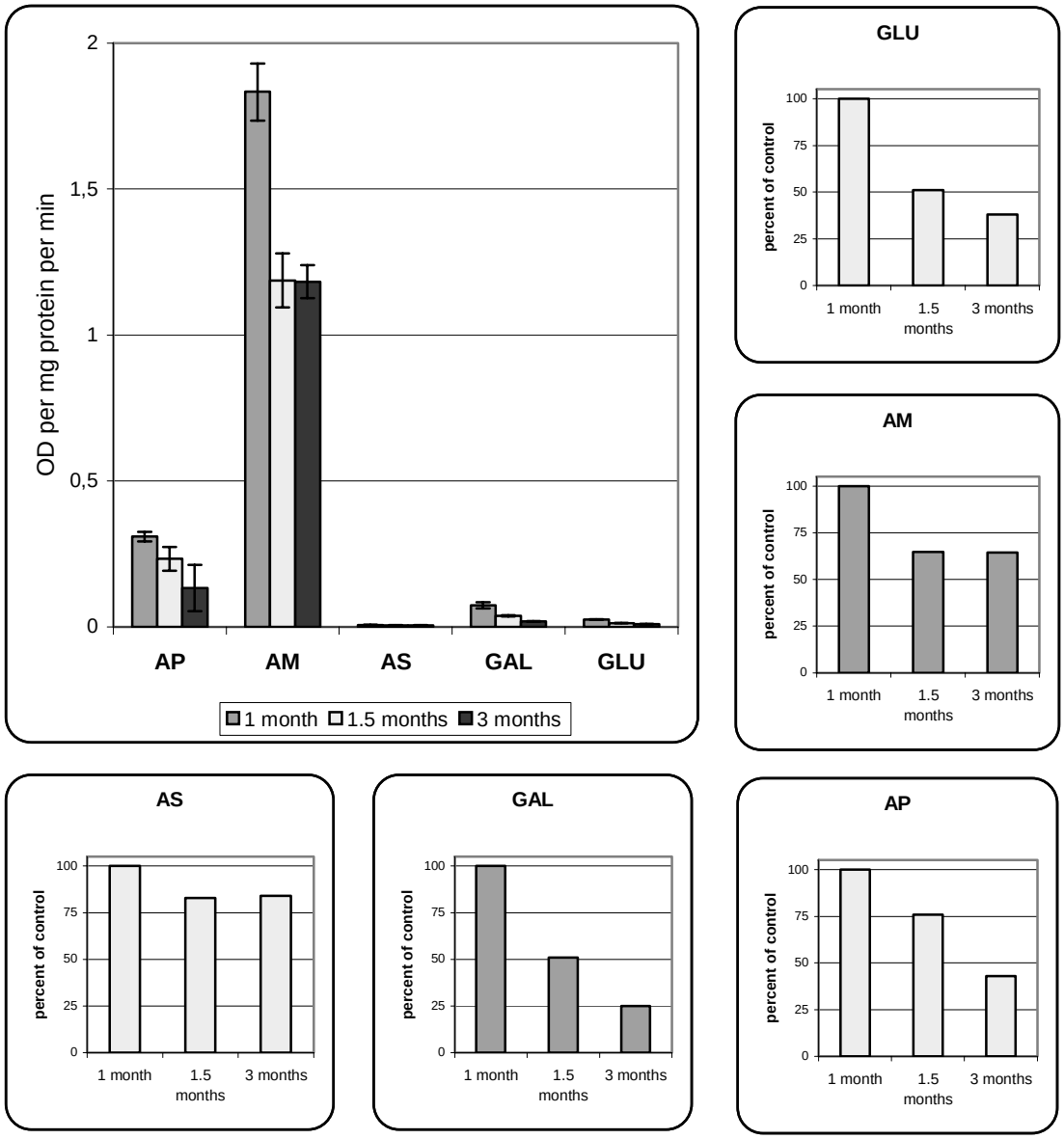

Fig. 1. Age-dependent changes in the specific activities of five rat submandibular gland lysosomal enzymes. Specific activity is expressed either as the optical density (OD)/(mg protein x min) (large figure) or as percentage of activity of the sample from 1-month-old rats (control) (small insets). Values are presented either as mean ± SD of two separate assays performed in triplicate (large figure) or as the mean of two separate assays each in triplicate (small insets).

Protein profile

The pattern of rat submandibular gland proteins aged 1, 1.5 and 3 months, stained with Coomassie Brilliant Blue (CBB) is shown in Figure 3. In SDS-PAGE proteins were separated between less than 25 kDa and up to 170 kDa and marked changes in the stainable materials were present in particular age groups. In general, proteins from 1.5- and 3-month-old rats showed a similar pattern,

which was quite different from protein profile obtained from 1-month-old rats in several bands. The bands corresponding to 90 kDa and 66 kDa appeared to be broader in 1-month-old rats, whereas the bands corresponding to 37 kDa and 31 kDa stained with less intensity than did the corresponding band in the 1.5- and 3-month-old rats. We also observed that the appearance of special proteins was limited to particular age groups.

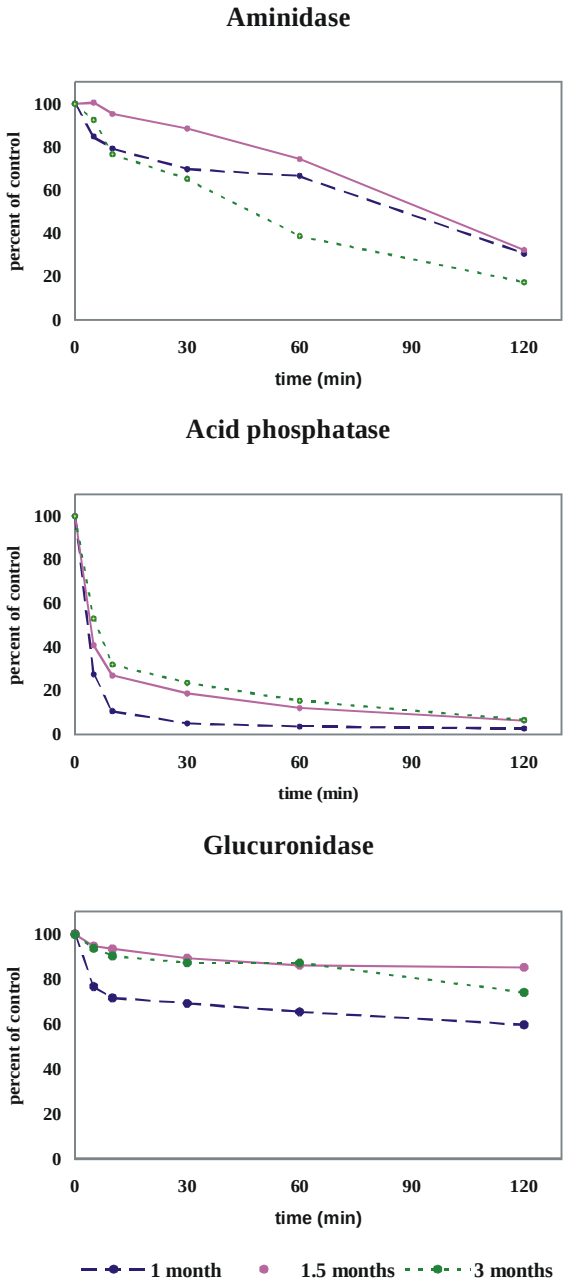

Fig. 2. The effect of age on thermal stability of rat lysosomal hydrolase. The residual specific activity after preincubation at 56 °C was measured up to 120 min as described in Materials and Methods and expressed as percent of activity of the sample without preincubation at 56 °C (control). Values represent the average of two separate assays performed in triplicate of each age group.

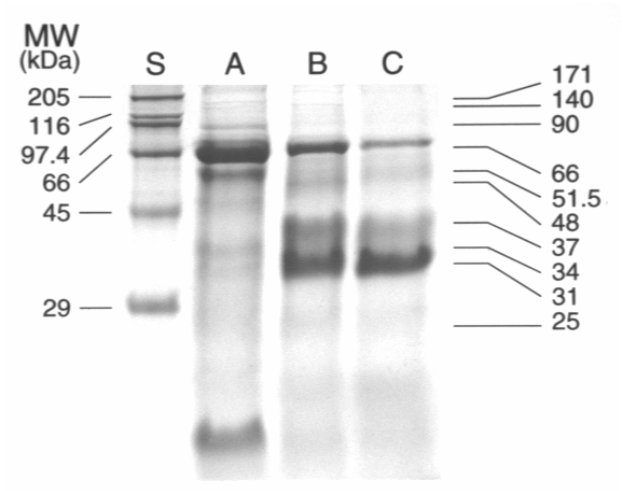

Fig. 3. Protein composition of submandibular gland protein from rats aged 1 month, 1.5 months and 3 months following SDS-PAGE on 12.6 % acrylamide slab gel. Fifty microgram proteins were applied per lane. The gel was stained with Coomassie Blue. Lane S – molecular weight standards, lane A – 1-month-old rats, lane B – 1.5-month-old rats, lane C – 3-month-old rats

Lectin-binding studies on tissue proteins

Lectin binding showed stronger staining of many bands that occurred with CBB staining under similar loading concentrations. It was shown that a large number of proteins distributed between an apparent molecular weight of 10 kDa to 205 kDa, possessed high mannose types (reaction with GNA) as well as sialylated α2-6 Gal complex type glycans, whereas fewer proteins in the range of 10 to 78 kDa had fucosylated (reaction with AAA) oligosaccharides (Fig. 4). On the contrary, only limited number of proteins in particular age groups had glycans with β1-6 GlcNAc branchig, sialic acid α2-3 Gal or O-linked Gal-GalNAc moieties (Fig. 4). Moreover, there was a striking difference in the staining patterns given by SNA and MAA, lectins that can bind to sialic acids, which is likely to reflect a difference in the position of terminal sialic acid linkage. The most remarkable observations were: (1) the presence of large diffused zone between 33 and 45 kDa that showed strong staining with AAA, WGA, SNA and DSA; (2) the presence of proteins containing α-mannosyl termini of high mannose glycans as shown by staining with GNA with an increasing number of bands in older animals; (3) the presence of two additional bands lower than 29 kDa in 1.5- and 3-month-old rats stained with AAA, lectin specific for fucose; (4) MAA was found to react only with glycoprotein of molecular mass larger than 205 kDa with the same intensity in all age groups; (5) PHA-L was found to bind only to high molecular weight glycoprotein larger than 205 kDa in 1-month-old rats; (6) the reaction with PNA

was only limited to 1-month-old rats; (7) the marked age-related increase in GNA, AAA and DSA staining was observed, whereas staining with agglutinin WGA showed inverse correlation.

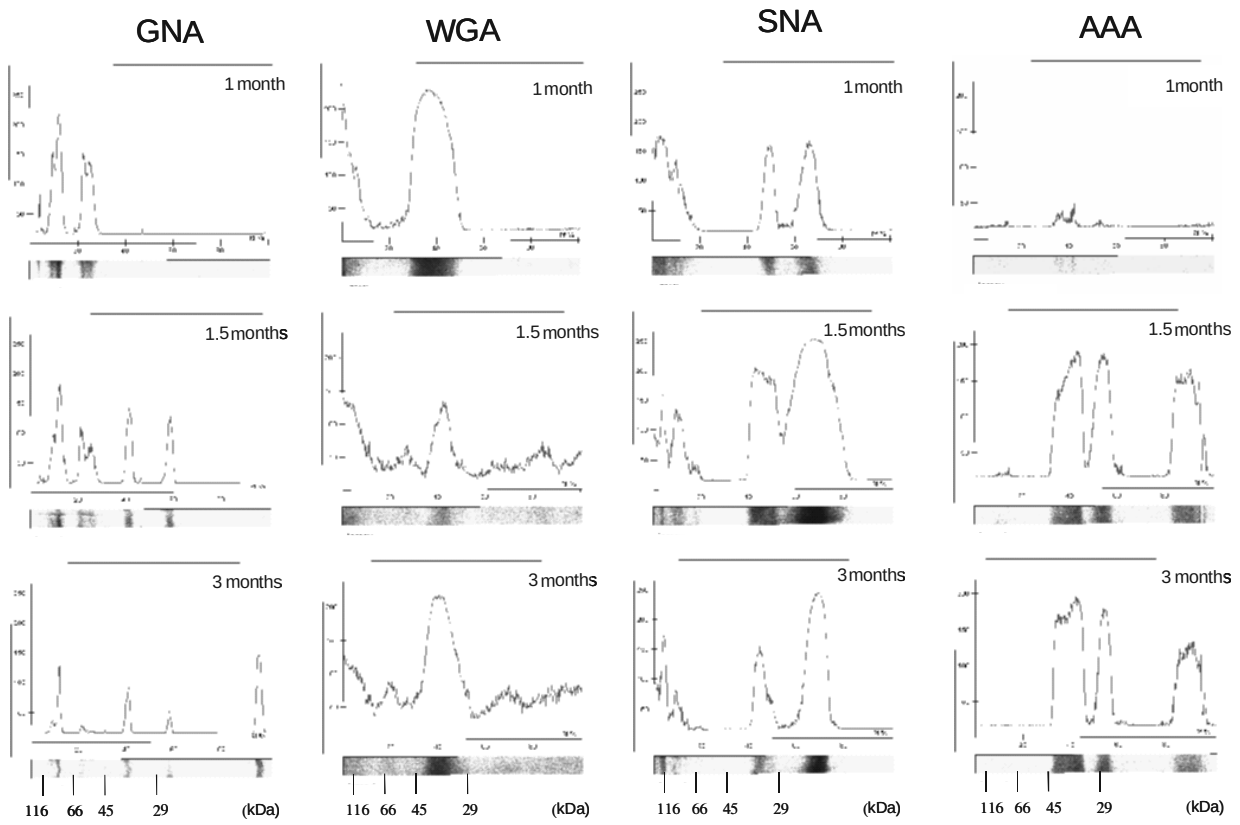

Fig. 4. Densitometric measurement of the intensity of lectin binding of rat submandibular gland proteins from different age groups. GNA, WGA, SNA, MAA acronyms of lectins, see Table 1.

Discussion

The present study provides evidence that the lysosomal system in the rat submandibular gland is clearly altered with the maturation process as we demonstrated an age-related decrease in specific activities of tested enzymes. The biochemical basis of the age-related changes is largely unknown. Their changes may reflect alterations in the regulation of synthesis and secretion pathways, or may be related to extracellular modifications of the biosynthetic products generated during the course of normal development. Age-dependent changes in the content of particular proteins as well as in their functions are well documented in the literature. A change in protein function requires a change in its shape or conformation, which can be induced through a variety of mechanisms, including conformational changes and covalent modifications. Nevertheless, it is difficult to define the nature of that change in detail. In rat and mouse the submandibular glands are not fully developed at birth and granular convoluted tubule (GCT) begins to develop by the 20th day of age. The course of postnatal development of several GCT cell products is correlated with the cytodifferentiation of these cells (Gresik 1980). The development and adult status of amylase activity parallels that of GCT (Gresik 1975), whereas the membrane-bound adenylate cyclase is fully developed already at the time of birth (Grand *et al.* 1975).

Published reports that compare purified proteins from young and old animals have demonstrated differences in their specific activity or catalytical efficiency (Stadtman 1988) as well as in their susceptibility to denaturation by heat (Rothstein 1984). These observations led to a general conclusion that the specific activity is lower for the enzyme from older animals, and that the decrease in the enzyme activity by heat denaturation is much faster in the old animal (Levine and Stadtman 1995). Our results regarding the decline of specific activity for all the tested enzymes with age are in line with these observations.

There are numerous age-associated changes in lysosomal hydrolase activities, but the alterations seem to vary depending on the hydrolase assayed and the tissue used (Cuervo and Dice 1998a,b). Nevertheless, the altered hydrolase activities do not cause abnormal lysosomal function in any of these cases. On the other hand, age-related changes in the lysosomal system might affect some cellular processes as lysosomal hydrolases participate in many fundamental cell processes including continuous turnover of proteins inside cells, elimination of abnormally synthesized or incorrectly modified proteins, cell differentiation, cell cycle progression, antigen presentation, and intracellular traffic of proteins (Goldberg and Dice 1974, Doherthy and Mayer 1992).

Our results concerning the decline in specific activities of tested submandibular gland enzymes in developing rats are partly supported by data obtained by Sanchez-Martin and Cabezas (1997). These authors evaluating the activities of seven lysosomal hydrolases in sera of rats aged 1, 2, 3, 6, and 12 months, only found decrease in the enzyme activity of AM. Nevertheless, they observed marked differences in specific activities the hydrolases assayed, which correspond to the pattern obtained by us. The difference in the specific activity of enzymes belonging to hydrolases family could be influenced by several factors influencing the activities of lysosomal enzymes in biological fluids (Cabezas 1985, Sanchez-Martin *et al.* 1996). Some of these factors, such as modifications in the cell, might be altered depending on the age and aging processes.

Krahling *et al.* (1979) studying postnatal development of peroxisomal and mitochondrial enzymes in rat liver, found that the enzyme activities increased from very low level at birth to maximum activities in rats aged one to three weeks, and thereafter decline to a lower steady state level at six weeks of age. They suggested that diet and weaning might influence these changes in activity. Since the rats were weaned at three weeks of age and had begun to eat some dry food prior to this time, the peak of activities prior to three weeks of age could be attributed to the change in food intake from milk to the laboratory chow.

It is also well known that the rat submandibular gland develops during both the prenatal and postnatal period (Ogawa *et al.* 1998) and the morphological changes that are under hormonal control may influence the level and type of produced proteins. However, from studies of Tanaka *et al.* (1990) and Nilina *et al.* (1990) concerning protein secretion under β_1- and α_1-adrenoceptor stimulation, it has been concluded that the functional maturation precedes morphological maturation of the rat submandibular gland.

Proteins from young and old animals may differ in their susceptibility to denaturation by heat. Sometimes the pattern observed in the protein from the older animals is biphasic, implying a molecular heterogeneity. In our study, no general pattern of specific activity changes in thermal stability was observed. Among enzymes tested, AP was the most sensitive while GLU showed the greatest resistance against heat. A possible explanation for these results is the greater resistance to quaternary structure disruption at high temperature in case of GLU (Flores and Ellington 2002). On the other hand, at least in the case of AM, the presence of N-glycans exerted a protective action, since heat denaturation was significantly higher after its deglycosylation. In rat tissues, there are two major forms of AM, A and B, which are composed of two subunits (αβ and ββ, respectively). The consequence of AM subunit structure is the differential thermostability of its isoforms. In our studies, we did not estimate proportion between thermolabile izoenzyme A and thermostabile izoenzyme B in relation to age. However, it is possible that age-related changes in their relative proportion could influence the different AM reactivity to heat denaturation in the individual age groups.

Cellular glycoconjugates are known to be modified with the development, differentiation and maturation of cells. The present study has elucidated that glycoconjugates change with the transition from the young-type to adult-type of the rat submandibular gland. The main changes occurring around the 4th week of life widely alter the glycoproteins of submandibular gland; in particular modifications of AAA, SNA, DSA and GNA binding pattern and intensity of staining indicate relevant fluctuations of glycoconjugates during postnatal development. Our results are in accordance with the previous studies on submandibular gland (Kim and Allen 1994, Ito *et al.* 1995, Accili *et al.* 1999).

Accili *et al.* (1999) have shown changes in the expression of glycoconjugates in rat submandibular gland. The main changes of secretory component were noted around gestation day 18. *Dolichos biflorus* agglutinin (lectin specific for GalNAc α1-3 Gal and GalNAc α 1-3 GalNAc) and WGA seemed to act as markers of pre- and postnatal development in contrast to Con A lectin whose changeable binding was regulated during postnatal development. In our study the main

changes in lectin staining were observed between 4 and 6 weeks of age indicating the second transition point in rat submandibular gland glycosylation pattern. The age-dependent increase of high-mannose type glycans recognized by GNA is in accordance with the above observation. On the other hand, the reaction with WGA, a lectin, which can interact with di-N-acetylchitobiosyl sequences, repeated N-acetyl lactosamine sequences and some sialyl residues, was most intensive in 1-month-old rats but only mild in the older ones.

Ito *et al.* (1995) also revealed changes in glycoconjugates in hamster submandibular gland during the postnatal period. On the day of birth the fetal-type secretory cells were PNA, *Ulex europaeus* agglutinin (UEA I) (lectin specific for Fuc α1-2 Gal) and WGA positive and they were replaced by adult secretory cells that did not show PNA or UEA I staining, but were positive for WGA (similarly to our results). Hence, modulation of glycoconjugates expression takes place in submandibular gland and is due to replacement of fetal-type by the adult-type gland. The biological importance could be related to changes in the oral environment from the suckling period to the weaning period. In accordance with Ito *et al.* (1995) we observed the occurrence of PNA positive bands revealing the presence of the glycan Gal β1-3 GalNAc in 1-month-old rats only. However, in contrast to his results we observed the increasing with age reaction with AAA specific for fucose that is in agreement with Biol *et al.* (1992), suggesting nutritional and developmental regulation of glycosylation processes in rats digestive organs. The different processes in the rat intestine are dye-sensitive. The existence of a developmentally-controlled shift from terminal sialyl to fucosyl substitution in rat small intestine could be caused by modification of the diet composition at weaning since diet is rich in fat (milk) for suckling pups and becomes carbohydrate-rich (adult diet) after weaning (Biol *et al.* 1992). We did not find significant age-related changes in SNA reaction, whereas progressive fucosylation was observed.

Furthermore, the asparagin-linked oligosaccharides from mucin of adult female mouse submandibular gland were analyzed. It has been shown that these oligosaccharides lack fucose and they were of high mannose and hybrid types and all of the NeuAc were attached to Gal by the α2-3 linkages (Denny *et al.* 1995). In our study the reaction with MAA, a lectin specific for α2-3 NeuAc linkage, was observed only in species with Mr larger than 205 kDa with no age-related changes. The contribution of N-linked oligosaccharides to the properties of salivary mucin can only be speculative at this time. In general, the variety of function attributed to N-linked glycans is so broad that one is unable to identify specific role based on one class alone. One of the possibilities proposed by Denny *et al.* (1995) might be the formation of heterocomplexes with other salivary proteins and selective binding to oral bacteria and viruses. In our study, the major component that reacted with SNA, DSA, and AAA produced a diffused band between 45 and 29 kDa. This is in accordance with previous findings, revealing the presence of large diffused zone in sympathetic saliva of rat (el-Thaher *et al.* 1990) and cat (Windston *et al.* 1992) attributed to kallikrein. The diffuse appearance of kallikrein may be ascribed to the rich carbohydrate content comprising mono-, di-, tri- and tetrasialylated complex type oligosacharides (Watson *et al.* 1994). Our study also showed the reaction with AAA in this zone and age-related changes in the intensity of staining in 1.5- and 3-month-old animals suggesting that this proteins is one of those undergoing developmental changes. However, the observed changes in lectin binding intensity could be due to the increased amount of this protein as it was shown in protein staining with Coomassie Brilliant Blue.

In conclusion, rat submandibular gland undergoes functional changes during the maturation process and we speculate that the observed differences in protein and glycosylation profiles between different age groups could be caused by modification of the diet composition as well as by cytodifferentiation of GCT cells which is under synergistic control of androgens and thyroid hormones.

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