Separation and identification of carp pituitary proteins and glycoproteins

H. Ryšlavá[1], M. Janatová[1], G. Čalounová[1], I. Selicharová[2], J. Barthová[1], T. Barth[2]

[1]Department of Biochemistry, Faculty of Science, Charles University of Prague, Prague, Czech Republic

[2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ABSTRACT: Carp pituitary proteins and glycoproteins were separated by the combination of immobilized metal affinity chromatography (IMAC) and affinity chromatography on Con A-Sepharose. The protein fractions were analysed by SDS-PAGE. Luteinizing hormone (in the $\alpha_1\beta$ and $\alpha_2\beta$ forms), growth hormone, free α_1 subunit and β subunit of thyroid-stimulating hormone were identified by N-terminal amino acid sequencing. N-linked oligosaccharide chains of thyroid-stimulating hormone (β TSH) were separated after fluorescent labelling on a GlycoSep N column and treated by exoglycosidases. Among the saccharide components, complex and hybrid structures terminating SO_4-GalNAc-GlcNAc-Man-, and high mannose structures, with 1 to 8 mannose units attached to the oligosaccharide core $(GlcNAc)_2(Man)_3$, were found.

Keywords: carp hormones; glycoproteins; immobilized metal ion affinity chromatography (IMAC); oligosaccharide chains

Pituitary gland cells synthesize, store and secrete many protein and glycoprotein hormones: luteinizing hormone (lutropin, LH), follicle-stimulating hormone (follitropin, FSH), thyroid-stimulating hormone (TSH), somatotropin, prolactin, corticotropin, etc. These hormones were isolated and characterized as far as their protein structure, biological function and receptors in target tissues are concerned, mainly in mammals (Gray, 1988; Apparaily et al., 1994; Bousfield et al., 1996; Hearn and Gomme, 2000; Lewis at al., 2000; Dias and Van Roey, 2001; Szkudlinski et al., 2002), and partly in birds (Ishii, 1993). These hormones were also isolated from the pituitaries of some fish species (Suzuki et al., 1988; Tanaka et al., 1993; Van Der Kraak et al., 1993; Okada et al., 1994; Santos et al., 2001). Although the primary structure of glycoprotein hormones in fish and mammals is conserved, their biological functions, regulation and protein expression differ (Santos et al., 2001).

Ionex chromatography was used for the separation of LH, FSH and TSH (Suzuki et al., 1988; Tanaka et al., 1993; Van Der Kraak et al., 1993; Okada et al., 1994). For further purification, affinity chromatography on immobilized Concanavalin A (Hulova et al., 1998), gel chromatography (Tanaka et al., 1993; Hulova et al., 1998), reversed phase HPLC (Okada et al., 1994; Hulova et al., 1998) or immunoaffinity chromatography were applied (Byamungu et al., 1991).

Immobilized metal affinity chromatography (IMAC) was used for the isolation of human prolactin and growth hormone (Maisano et al., 1989; Ueda et al., 1991) and for the separation of LH and

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FSH from rainbow trout pituitary (Govoroun et al., 1997).

In this paper we describe the separation of carp pituitary proteins and glycoproteins by IMAC, their identification by N-terminal sequencing and further characterization of the glycan component of the β chain of thyroid-stimulating hormone.

MATERIAL AND METHODS

Carp pituitaries were collected at the fishery in Vodňany and dried in acetone. Con A-Sepharose 4B and Sepharose 6B linked with iminodiacetic acid were from Pharmacia (Uppsala), GlycoSep N column was from Oxford GlycoSciences, UK, PNGase F from New England Biolab, other enzymes and chemicals were from Sigma (St. Louis).

Extraction. 0.2 g of acetone-dried carp pituitaries was homogenized in 10 ml of 0.05M Tris-HCl buffer pH 6.8 containing 1.5mM phenazine methosulphate (PMSF). The homogenate was centrifuged at 5 000 × g for 15 min and the supernatant was stored at 4°C.

Immobilized metal affinity chromatography (IMAC). Sepharose 6B activated with iminodiacetic acid in a column (2 × 5 cm) was charged by washing with 50 ml of 0.5% $CuSO_4.5H_2O$. The column was then equilibrated with 50 ml of 20mM phosphate buffer pH 7.5 containing 0.5M NaCl (starting buffer). 10 ml of pituitary extract (concentration 5 mg/ml) was applied to the column. The column was then washed with 70 ml of starting buffer. The proteins adsorbed on the column were eluted with 0.1M acetate buffer containing 0.5M NaCl in two steps: the first step pH 4.8 (100 ml), the second step pH 4.0 (100 ml). The column was then washed with 50 ml of starting buffer and elution continued with 1-methylimidazole in starting buffer again in two steps: the first one 10mM (100 ml) and the second 50mM (50 ml). The concentration of proteins in the eluate was monitored by measuring the absorbance at 280 nm.

Affinity chromatography on Con A-Sepharose. Protein fractions from IMAC were applied to the Con A-Sepharose column and separation was done according to Hulová et al. (1998). The concentration of proteins was monitored by measuring the absorbance of the eluate at 280 nm.

SDS electrophoresis. The proteins and glycoproteins were analysed using discontinuous polyacrylamide gel electrophoresis in the presence of SDS according to Laemmli (Laemmli, 1970). The analysis was performed under reducing (5% sulphanylethanol (2-mercaptoethanol) in sample buffer) and non-reducing conditions. 3% stacking gel and 15% separating gel were used, the proteins were stained by Coomassie Brilliant Blue R-250. Lysosyme (m.w. 14 500) and its polymers (m.w. 29 000, 43 500, 58 000) were used as molecular weight markers.

N-terminal amino acid sequence. For N-terminal sequencing the proteins separated by SDS-PAGE were blotted onto PVDF membrane Millipore PSQ. The proteins visualized by Coomassie Brilliant Blue R-250 were subjected to N-terminal amino acid sequencing in Beckman LF 3 600 sequencer.

Deglycosylation. Glycoproteins were deglycosylated enzymatically by PNGase F (glycopeptide N-glycosidase EC 3.5.1.52) in 20mM bicarbonate buffer pH 8.6 containing 0.1% SDS and 1% NP-40 at 37°C for 24 h. The proteins were precipitated by three volumes of ice-cold ethanol and separated by centrifugation at 16 000 × g for 15 min.

Fluorescent labelling of oligosaccharides. Derivatization of oligosaccharides with 2-amidobenzamide (2AB) was done according to Bigge et al. (1995). The glycans liberated from 1 mg of glycoprotein were dissolved in 0.35M 2AB in 1M $NaBH_3CN$ and 30% acetic acid in dimethyl sulphoxide (DMSO), derivatization proceeded 2 h at 60°C and products were purified by paper chromatography.

Separation of 2 AB labelled oligosaccharides. The HPLC system LCP 4 000 (Ecom, Czech Republic) was fitted with spectrofluorometer LS 5B (Perkin Elmer, USA). Chromatographic separation of 2 AB labelled oligosaccharides was achieved using GlycoSep N column (4.6 × 250) (Oxford GlycoSciences, UK). The elution solution was composed of solvent A (acetonitrile) and solvent B (50mM ammonium formiate pH 4.4). Elution started with 35% B at a flow rate of 0.4 ml/min, followed by a linear gradient of 35–53% B over 72 min, followed by 53–100% over the next 3 min. The flow rate was then increased to 1 ml/min over the next 2 min and the column was washed by 100% B for 5 min before being re-equilibrated in 35% B for injection of the next sample. Column temperature was 30°C, λ_{ext} = 330 nm, λ_{emiss} = 420 nm (Guile et al., 1996).

Exoglycosidase digestion of 2-AB labelled oligosaccharides. Digestion by enzymes was performed at 37°C for 1 to 4 h in 50mM formiate buffer pH 5.0. 1 U of α-neuraminidase, β-galactosidase,

β-N-acetylhexosaminidase and α-mannosidase was used in 50 µl reaction mixtures.

RESULTS

The proteins extracted from carp pituitaries were separated by immobilized metal ion affinity chromatography (IMAC). By this procedure proteins were separated into 5 fractions (Figure 1a) which were analysed by SDS-PAGE (Figure 1b) and the N-terminal amino acid sequences of some proteins were determined (Table 1). In the unabsorbed fraction were proteins of various molecular masses, some proteins of this fraction bound to Con A-Sepharose, thus proving to be glycoproteins.

In the fraction which was eluted from the IMAC column under acidic conditions (pH 4.8, fraction

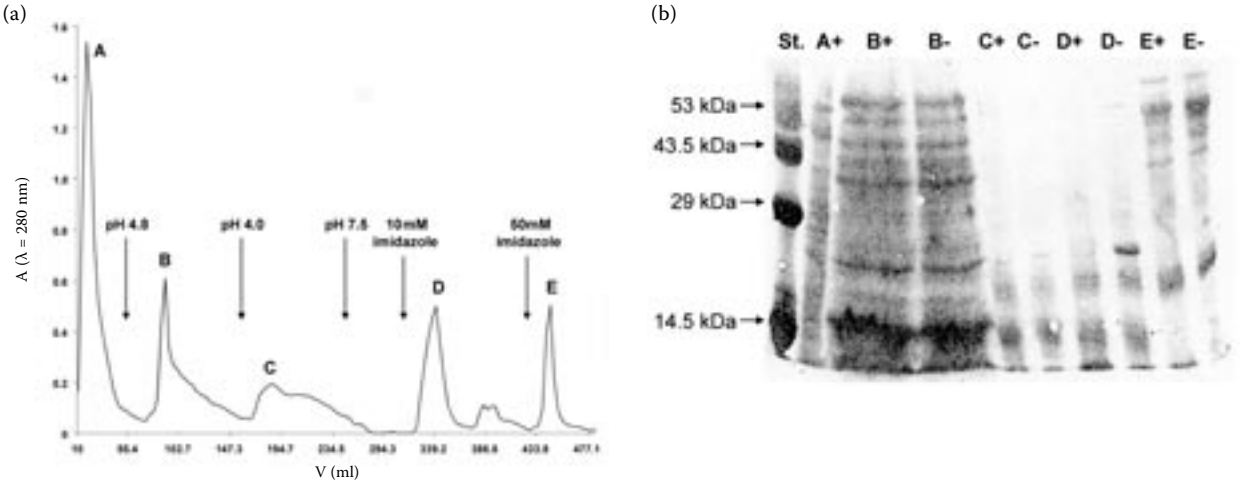

Figure 1. Separation of carp pituitary proteins by immobilized metal ion affinity chromatography

(a) IMAC separation of carp pituitary proteins: A – proteins not interacting with immobilized Cu^{2+}, B – proteins eluted with 0.1M acetate buffer pH 4.8, C – proteins eluted with 0.1M acetate buffer pH 4.0, D – proteins eluted with 10mM 1-methylimidazole, E – proteins eluted with 50mM 1-methylimidazole

(b) SDS-PAGE of protein fractions obtained by IMAC in reducing (–) and non-reducing (+) conditions, St – standards of molecular mass

Table 1. Proteins identified in carp pituitary

Fraction from IMAC	Molecular mass	N-terminal amino acid sequence	Interaction with	Identified protein
A		ADVTXLXENV	ConA+	protein homologous to growth differentiation factor
		KQVTMKYEDVI	ConA+	–
B	36 300	XPSDETDPVYDNRQY		–
	22 400	YPRNDMNFGXEEX		α_1 subunit
	15 300	GQDGEVDXGXDFLKSAYXDI		–
C	22 900	SYLDPXEPVXETVAV		β LH
	15 100	YPRNDMNNFGXEEX		α_1 subunit
D	22 900	SYLDPXEPVXETVAV	ConA+	β LH
		SDNQRLFNAVIRVQHLHQLA	ConA–	growth hormone
	15 100	YPRNDMNNFGXEEX	ConA+	α_1 subunit
		YPRNYMNNFGXEEX	ConA+	α_2 subunit
		NDMNNFGXEEX	ConA+	α_1 subunit*
E	22 000	MXAPTEYTIYIES	ConA+	β TSH

α_1 – subunit lacking the first three amino acids at the N-terminal; X – Cys or glycosylated Asn

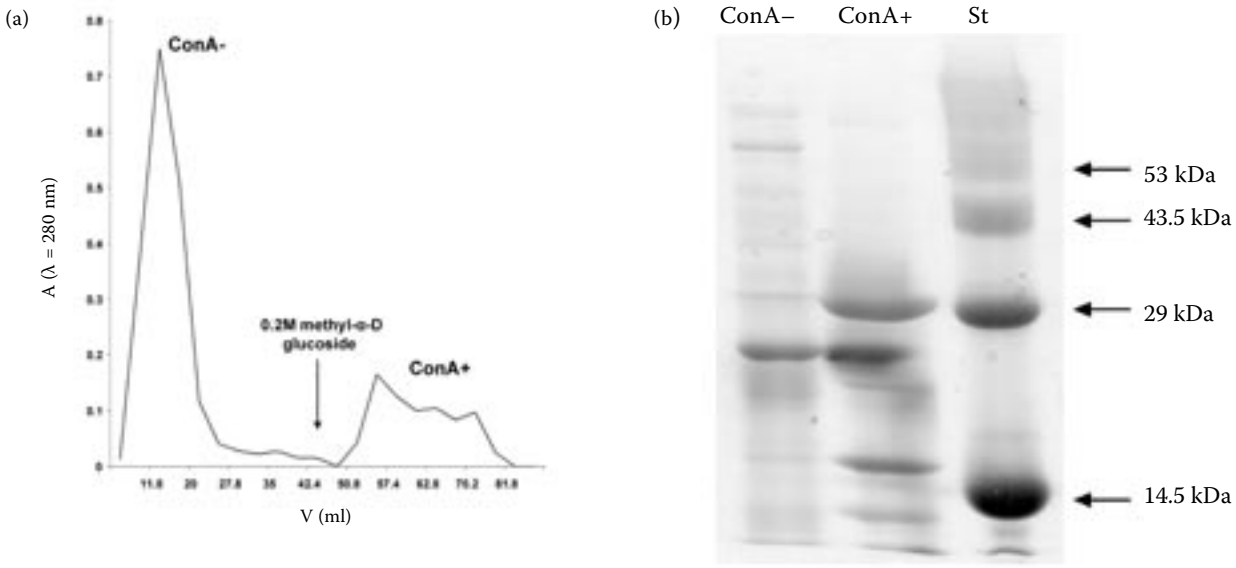

Figure 2. Affinity chromatography of fraction D (Figure 1) of carp pituitary proteins on Con A-Sepharose

(a) Affinity chromatography on Con A-Sepharose: *Con A–*: proteins not interacting with immobilized Con A, *Con A+*: glycoproteins eluted with 0.2M methyl α-glucopyranoside
(b) SDS-polyacrylamide gel electrophoresis of fractions obtained by affinity chromatography: St – standards of molecular mass

Table 2. Suggested oligosaccharide structures present in β TSH of carp

Oligosaccharide structure	GU_{exp}	GU_{theor}	GU_{exp} mann. treat.	GU_{theor} mann. treat.	GU_{exp} desulph. hexos. treat.	GU_{theor} desulph. hexos. treat.
C	4.15	4.30	4.15	4.30	4.15	4.30
C.Man	5.23	5.20	4.15	4.30	4.15	4.30
$C.Man_2$	5.97	6.10	4.15	4.30	4.15	4.30
$C.Man_3$	7.03	7.00	4.15	4.30	4.15	4.30
$C.Man_4$	8.19	7.90	4.15	4.30	4.15	4.30
$C.Man_5$	8.92	8.80	4.15	4.30	4.15	4.30
$C.Man_6$	9.61	9.70	4.15	4.30	4.15	4.30
$C.Man_7$	10.45	10.60	4.15	4.30	4.15	4.30
$C.Man_8$	11.18	11.50	4.15	4.30	4.15	4.30
$C(Man_2)(HexNAc.HexNAcSO_4)$	7.03	7.13	5.28	5.33	6.00	6.10
$C(HexNAc.HexNAcSO_4)_2$	6.21	6.36	6.21	6.36	4.10	4.30

C – the oligosaccharide core of N-linked saccharides in glycoproteins, C.Man–$C.Man_8$ – high mannose structures
$C(Man)_2(HexNAc.HexNAcSO_4)$ – hybrid structure, $C(HexNAc.HexNAcSO_4)_2$ – bianternary complex structure
$HexNAc.HexNAcSO_4$ = probably $GlcNAc.GalNAcSO_4$ (according to analogy with other glycoprotein hormones)
GU_{exp} – number of glucose units determined from the positions of a standard dextran hydrolyzate (Figure 3)
GU_{theor} – theoretically calculated number of glucose units of suggested oligosaccharide structure using different increments of different monosaccharides (Guile et al., 1996)
mann. treat. – oligosaccharide chains after α-mannosidase treatment
desulph. hexos. treat. – oligosaccharide chains after desulphatation and β-hexosaminidase treatment

"B") the N-terminal sequence responding to the α_1 subunit of carp glycoprotein hormones was identified (Table 1). The carp luteinizing hormone was found in two fractions: in the "C" fraction eluted with 0.1M acetate buffer pH 4.0 containing 0.5M NaCl and in fraction "D" eluted with 10mM 1-methylimidazole. The "D" fraction contained a higher amount of LH, but there was a difference between LH molecules in these two fractions. LH from the "C" fraction contained only the $\alpha_1\beta$ structure. In fraction "D" the hormone was found both in the $\alpha_1\beta$ and $\alpha_2\beta$ forms (Table 1). The "D" fraction also contained the growth hormone. Its molecular weight is very similar to the β subunit of LH, the separation of these two hormones was done on a Con A-Sepharose column (Figure 2). In the fraction which was eluted from the IMAC column by 50mM 1-methylimidazole, the β chain of thyroid-stimulating hormone was found (Table 2). This hormone was specifically bound to Con A-Sepharose, which showed that it had the character of a glycoprotein and its saccharide component was further studied. The β subunit of TSH was enzymatically deglycosylated by PNGase F and after fluorescent labelling, the oligosaccharide chains were separated by normal phase chromatography on a GlycoSep N column (Figure 3). The positions of the individual oligosaccharide chains were expressed as the number of glucose units (GU) by comparison with glucose oligomer standards (Figure 3a). The chromatogram (Figure 3b) documents the oligosaccharide profile of the β subunit of carp TSH. There are many oligosaccharide structures with various numbers of GU from 4.3 to 11.18 (Table 2).

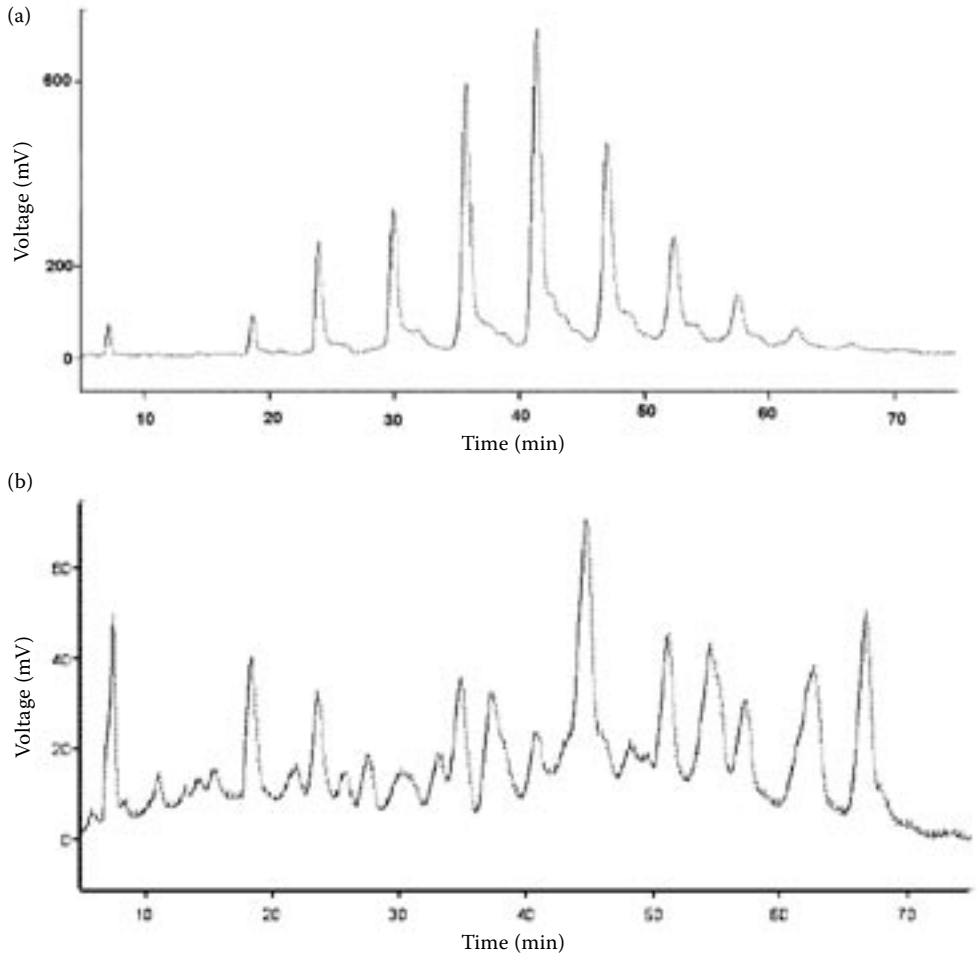

Figure 3. Chromatographic separation of 2AB labelled oligosaccharides of β TSH of carp on GlycoSep A column (fluorescence detection λ_{ext} = 330 nm, λ_{emiss} = 420 nm)
(a) Glucose oligomer standards G3–G11
(b) Oligosaccharide profile of β TSH after PNGase treatment

Labelled oligosaccharides were subjected to digestion by exoglycosidases: α-neuraminidase, β-galactosidase, β-N-acetylhexosaminidase and α-mannosidase. Some oligosaccharides were susceptible to α-mannosidase treatment (Table 2), some oligosaccharides could be hydrolyzed only after treatment with HCl in methanol. By this procedure the terminal sulpho groups were liberated (Table 2).

DISCUSSION

Immobilized metal affinity chromatography takes advantage of protein affinity to metal ions (Okada et al., 1994). It is used especially for the purification of recombinant proteins with oligohistidine tags. However, naturally occurring proteins with accessible histidyl, partially cysteinyl, glutamyl, aspartyl residues on the protein surface could also contribute to binding to IMAC support (Ueda, 2003). Prolactin and growth hormone which have a zinc binding domain were purified by this technique (Maisano et al., 1989; Ueda et al., 2001).

We separated LH, growth hormone, β subunit of TSH and free α_1 subunit from the carp pituitary homogenate by IMAC using elution solutions of decreasing pH value and increasing concentration of imidazole. Govoroun et al. (1997) successfully separated LH and FSH from the rainbow trout pituitary by this method. IMAC could be an alternative method to the separation of fish hormones by ion exchange chromatography, which has been used for the isolation of LH and FSH of mammals and birds. Ionex chromatography makes use of differences in the values of isoelectric points (pI). The pI of the hormone specific β subunit of LH and FSH of tuna is 4.91 and 4.76 resp., that of chum salmon 4.60 and 4.75 resp., that of carp 4.70 and 4.60 resp., however that of humans 8.30 and 5.91 resp., bovines 8.30 and 5.33 resp., porcines 8.30 and 5.12 resp., whereas the pI of α subunits in mammals and fish are very similar (www.expasy.org/cgi-bin/sprot-search-Ful). In our experiment FSH was not identified in any fraction eluted from the IMAC column with immobilized Cu^{2+} ions. Although there are structural similarities with mammalian hormones, the biological function of fish FSH is not fully understood, it is supposed to play a role in mediating puberty (Santos et al., 2001). It is possible that the protein of FSH is expressed especially in carps that are not fully mature yet.

The carp growth hormone seems to be a protein without saccharide in its molecule, because it did not bind to Con A-Sepharose and could be separated via this technique from LH which was glycosylated (Figure 2). In some fish species there are two types of α subunits (Huang et al., 1991) with a high degree of homology. The heterodimer $\alpha_2\beta$ was present only in fraction D, $\alpha_1\beta$ was in both fractions C and D. It is not clear if replacing the amino acids Asp, Phe, Gln, Ile by Tyr, Val, Arg, Val (which are the only differences between these two molecules) can influence the binding of such similar proteins to IMAC support. In the carp pituitary extract the free α_1 subunit was present, however it was not clear if it had dissociated from glycoprotein hormone molecules during isolation or whether it was the natural hormone form with hormonal activity like in mammals (Thotakura and Blithe, 1995).

The oligosaccharide component of the β chain TSH was studied in more detail. It is the first information concerning the saccharide moiety of TSH in fish species. The oligosaccharide chains are very heterogeneous (Figure 3), there are high mannose structures composed of 1 to 8 mannose monosaccharides attached to the oligosaccharide core $(GlcNAc)_2(Man)_3$ (Table 2). This type of oligosaccharide chains were identified neither in mammal pituitary glycoprotein hormones (Green and Baenziger, 1988a,b) nor in salmon LH (Manzella et al., 1995), however we have found high mannose structures in carp LH (data not shown). Mammalian LH and TSH hormones are characterized by a saccharide structure terminating SO_4-GalNAc-GlcNAc-Man- (Green and Baenziger, 1988a,b). In carp β TSH this structure was present in the form of a complex bianternary chain and a hybrid chain (Table 2). The oligosaccharides containing sialic acid, which is typical of mammalian FSH hormone (Green and Baenziger, 1988a,b), were not present in carp β TSH. Manzela et al. (1998) demonstrated the presence of this saccharide structures in LH of all vertebrate species (fish were represented by salmon). This oligosaccharide structure seems to be present in the glycoprotein hormones LH and TSH in all vertebrates, nevertheless the presence of a large amount of high mannose structures in carp TSH is interesting. It is not clear if these oligosaccharide structures are involved in receptor recognition or if they participate in the modulation of hormonal activity.

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Corresponding Author

RNDr. Helena Ryšlavá, Department of Biochemistry, Faculty of Science, Charles University of Prague, Albertov 2030, 128 40 Prague 2, Czech Republic
Tel. +420 221 951 281, e-mail: rysl@natur.cuni.cz

INSTITUTE OF AGRICULTURAL AND FOOD INFORMATION
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Tel.: + 420 227 010 111, Fax: + 420 227 010 116, E-mail: redakce@uzpi.cz

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