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Distribution and biological activity of β-thymosins

Review Article

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Summary. β-Thymosins, a group of highly homologous peptides consisting of about 40 amino acid residues, were found to be distributed from mammals up to echinoderms. Althogh they have first been isolated from mammalian thymus tissue preparations, their occurrance is not organ-specific and they are present even in different types of cells. For thymosin β_4 several biological activities have been reported, stating that this peptide acts as a thymus peptide hormone and is also involved in the neuroendocrine and immune system. However, it was recently demonstrated that thymosin β_4 has actin-sequestering properties and therefore might play an important role in the regulation of the microfilament system. This fact gives a new outlook on the real biological function of β-thymosins.

Keywords: Amino acids – β-Thymosins – Phylogenetic distribution – Actin sequestration – Immunoassays – Immunohistochemistry

The term β-thymosins is restricted to a group of highly homologous peptides with an average molecular weight of 5 kDa. Because they were originally isolated from thymus tissue preparations, they were termed "thymosins" and postulated to possess immunomodulating or hormonal functions. However, the phylogenetic distribution of β-thymosins is ranging from mammals down up to echinoderms, i.e. they are also found in species containing no thymus gland. Usually, two highly homologous β-thymosins are present in one species. In mammals, the main β-thymosin is thymosin β_4 (Low et al., 1981), which is accompanied by thymosin β_{10} (human, horse, cat, rabbit, rat and mouse), thymosin β_9 (bovine), thymosin β_9^{Met} (pig) or thymosin β_{13} (whale) (Erickson-Viitanen et al., 1983a, b; Hannappel et al., 1989; Horecker and Morgan, 1984; Hörger, 1993; Voelter, 1993). In rabbit and frog tissue two peptides with slight deviations from the thymosin

β_4 ($T\beta_4$) structure were found. $T\beta_4^{Ala}$ in rabbits differs only in the first amino acid (Ala instead of Ser) (Erickson-Viitanen et al., 1983a). From oocytes of Xenopus laevis a β_4-like peptide was isolated, namely $T\beta_4^{Xen}$, whose primary structure is different in three amino acids from $T\beta_4$ (Hannappel et al., 1988). No β-thymosins could be detected in invertebrates like gypsy moth larvae, the earthworm (Erickson-Viitanen et al., 1983a), the meal worm as well as in funghi like yeast (Hörger, 1993).

Thymosin β_4 ($T\beta_4$), a peptide consisting of 43 amino acids and rich in glutamate and lysine residues (Fig. 1), is the most investigated β-thymosin up to

	5	10	15
Tß4	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-		
Tß4^{Ala}	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-		
Tß4^{Xen}	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-*Ala*-		
Tß9	Ac-*Ala*-Asp-Lys-Pro-Asp-*Leu*-*Gly*-Glu-Ile-*Asn*-*Ser*-Phe-Asp-Lys-*Ala*-		
Tß9^{Met}	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Asn*-*Ser*-Phe-Asp-Lys-*Ala*-		
Tß10	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Ala*-*Ser*-Phe-Asp-Lys-*Ala*-		
Tß11	Ac-Ser-Asp-Lys-Pro-*Asn*-*Leu*-*Glu*-Glu-*Val*-*Ala*-*Ser*-Phe-Asp-Lys-*Thr*-		
Tß12	Ac-Ser-Asp-Lys-Pro-Asp-*Leu*-Ala-Glu-*Val*-*Ser*-*Asn*-Phe-Asp-Lys-*Thr*-		
Tß12^{perch}	Ac-Ser-Asp-Lys-Pro-Asp-*Ile*-*Ser*-Glu-*Val*-*Thr*-*Ser*-Phe-Asp-Lys-*Thr*-		
Tß13	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Ala*-*Ser*-Phe-Asp-Lys-*Ala*-		
Tß14	Ac-*Ser*-ASp-Lys-Pro-Asp-*Ile*-*Ser*-Glu-*Val*-*Ser*-*Ser*-Phe-Asp-Lys-*Thr*-		

	20	25	30
Tß4	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
Tß4^{Ala}	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
Tß4^{Xen}	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
Tß9	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
Tß9^{Met}	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
Tß10	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
Tß11	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-*Thr*-		
Tß12	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-*Thr*-		
Tß12^{perch}	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
Tß13	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
Tß14	Lys-Leu-Lys-Lys-Thr-Glu-Thr-*Ala*-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		

	35	40	
Tß4	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH		
Tß4^{Ala}	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH		2%
Tß4^{Xen}	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-*Thr*-*Ser*-Glu-Ser-OH		7%
Tß9	Lys-Glu-thr-Ile-Glu-gln-Glu-Lys-Gln-Ala-*Lys*-OH		22%
Tß9^{Met}	Lys-Glu-thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Lys*-OH		20%
Tß10	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Arg*-*Ser*-*Glu*-*Ile*-Ser-OH		26%
Tß11	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Ser*-OH		22%
Tß12	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Thr*-*Ala*-OH		19%
Tß12^{perch}	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Ala*-Ala-*Ala*-*Thr*-*Ser*-OH		21%
Tß13	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Lys*-OH		20%
Tß14	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Thr*-*Ala*-OH		29%

Fig. 1. Amino acid sequences of β-thymosins and their deviation (in %) from $T\beta_4$ primary structure

now. It was first isolated in 1981 by Low et al. from calf thymosin fraction 5. Because $T\beta_4$ induces the activity of TdT (terminal deoxynucleotidyl transferase) in vitro and in vivo, it was thought to be a member of peptide hormones, which are produced and secreted by the thymus gland and influence T cell differentation. It was also reported in the literature that $T\beta_4$ expresses phenotypic changes on a human T cell line (Kokkinopoulos et al., 1985), induces the secretion of luteinizing hormone-releasing factor (LHRH) from rat hypothalamus (Rebar and Miyake, 1981), inhibits the migration of guinea pig macrophages (Thurman et al., 1981, 1984) and enhances the capacity for antigen presentation on macrophages (Tzehoval et al., 1989). $T\beta_4[1-39]$, which has been isolated from bovine hypothalamus, acts in vitro as a stimulator in activating calmodulin-dependent enzymes (Galoyan et al., 1992). Later on it was shown that $T\beta_4$ is not a thymus-specific peptide but is also present in different rat tissues even in high concentrations (Hannappel 1987). It was reported by Gomez-Marquez et al. (1989) that several rat tissues express the $T\beta_4$ gene. High amounts of $T\beta_4$ were detected in human white blood cells, especially in neutrophils and in macrophages (Hannappel and van Kampen, 1987). Different mammalian cell lines, not derived from the reticuloendothelial system, are capable of synthesizing $T\beta_4$ (Goodall et al., 1983a). However, no secretion of this peptide was observed (Hannappel and Leibold, 1985; Schöbitz et al., 1991). These facts are contrary to the assumption for a hormonal or secretory function of this peptide, also no signal peptide was found after $T\beta_4$ biosynthesis (Wodnar-Filipowicz et al., 1984; Gondo et al., 1987). There is no evidence for the existence of a large polypeptide precursor of $T\beta_4$ although this peptide has been isolated from different sources. $T\beta_4$ is the primary translation product, as was demonstrated by in vitro translation assays with rat spleen mRNA (Wodnar-Filipowicz and Horecker, 1983).

Lenfant et al. (1989) reported that the tetrapeptide AcSDKP shows inhibitory activity on the proliferation of hematopoietic pluripotent stem cells. As the sequence of this small peptide is the same as $T\beta_4[1-4]$, it was stated that it might be generated in vivo from $T\beta_4$ (which in this case acts as the precursor molecule) through enzymatic digestion. In a bovine bone marrow stem cell preparation, AcSDKP is cleaved from $[^3H]T\beta_4$, probably by the enzyme Asp-N-endoproteinase (Grillon et al., 1990). ^1H and ^{13}C NMR studies demonstrated that AcSDKP exists in a random coil conformation which is attached to a helical part of $T\beta_4$. Therefore, the enzymatic cleavage at this site could be conformationally facilliated (Freund et al., 1992). AcSDKP was also found in human white blood cells but not in plasma (Pradelles et al., 1990) as well as in different murine tissues (Pradelles et al., 1991). Additionally, it was reported for AcSDKP to be able to inhibit partially the formation of E-rosettes between human lymphoma T-cells and sheep erythrocytes (Thierry et al., 1990).

Actually, $T\beta_4$ has raised a lot of scientific interest since Safer et al. (1991) observed that it forms a 1 : 1 complex with muscle G-actin under physiological ionic conditions and thus prevents its polymerization to F-actin. Therefore, $T\beta_4$ seems to be involved in the microfilament system, acting as an actin-sequestering peptide like gelsolin or profilin. The k_d-values are 2–3 μM for skeletal muscle actin or 0.4–0.7 μM for platelet actin (Nachmias et al., 1991; Weber et al., 1992). $T\beta_4$ binds not to F-actin or to filament ends (Weber et al., 1992). Microinjection

of $T\beta_4$ into cultured epithelial cells and fibroblasts leads to a depolymerization of F-actin (Sanders et al., 1992). In cells which possess a high rate of filament turnover like human neutrophils, a high concentration of $T\beta_4$ (160–200 μM) was found (Cassimeris et al., 1992), a fact that might indicate a possible role of $T\beta_4$ for the maintenance of G-actin. When PMN are stimulated by a chemoattractant, the amount of the $T\beta_4$-actin complex decreases (Cassimeris et al., 1992). Also, a stimulation of human platelets with thrombin leads to a decrease in the $T\beta_4$-actin complex, while the rate of F-actin increases (Nachmias et al., 1991). In contrast to profilin, another actin sequestering peptide, $T\beta_4$ acts as a potent inhibitor of actin nucleotide binding and exchange (ADP for ATP). It seems that both peptides could be involved together in the regulation of the filament turnover by controlling the ratio of ATP-actin (which poymerizes more rapidly) to ADP-actin (Goldschmidt-Clermont et al., 1992). $T\beta_4$ competes with the same actin binding site as profilin and actobindin. Like in actobindin, the NH_2-terminus of $T\beta_4$ is important for the binding on the actin molecule, especially the part 17–22 with the sequence LKKTET. This hexamotif is directly involved in actin binding, while the sequence 1–16 exerts a secondary effect which is necessary for the inhibitory activity of $T\beta_4$ (Vancompernolle et al., 1992). However, the COOH terminus is not effective in the inhibition of F-actin formation. Using the DNase I inhibition assay, Hannappel et al., (1993) demonstrated that the sequences 24–43 and 13–43 of $T\beta_4$ show no effect in the maintainance of G-actin. CD and NMR studies with $T\beta_4$ and $T\beta_9$ revealed a high probability for an α-helix formation in the NH_2-terminal (residues 4–16 for $T\beta_4$ and 5–25 for $T\beta_9$) and COOH-terminal (residues 30–40 for $T\beta_4$ and 30–41 for $T\beta_9$) part (Zarbock et al., 1990; Gallert et al., 1993). Similar results were obtained by calculation of the local backbone conformation of $T\beta_4$ (Sippl et al., 1992).

Schöbitz et al. (1990) reported that a stimulation of resting thymocytes with ConA results in a rapid increase of $T\beta_4$ in the early G1-phase of the cell cycle. However, the level of $T\beta_4$ mRNA increases in the S-phase of the cell cycle and this level is maintained during the G2 and M phase (Schöbitz et al., 1991; Otero et al., 1993). Thus, $T\beta_4$ could be also actively involved in actin turnover during the cytokinesis.

As shown in Table 1 and Fig. 1, another β-thymosin, highly homologous to $T\beta_4$, is present in the mammalian tissue. In bovine tissues, $T\beta_9$ occurs besides $T\beta_4$, but in lower concentrations (Hannappel et al., 1982). On $T\beta_9$, a peptide of 41 amino acids, less studies were done concerning its physiological relevance. Previously it has been reported that $T\beta_9$ as well as some fragments of it are active in the E-rosette assay (Kalbacher et al., 1990; Abiko and Sekino, 1982). Now, there is evidence that $T\beta_9$ might also play a role in actin sequestration like $T\beta_4$. It binds to muscle G-actin with a K_d of 0.8 μM by forming a 1 : 1 complex (Heintz et al., 1993). $T\beta_9^{Met}$ is the corresponding peptide in porcine tissues which differs from $T\beta_9$ in one amino acid, i.e. leucine in position 6 is replaced by methionine (Hannappel et al., 1989, Fig. 1). Low et al. (1990) reported for $T\beta_9^{Met}$ to enhance production of interleukin-2, γ-interferon and to stimulate the secretion of tumor necrosis factor. $T\beta_9^{Met}$ shows also activity in the E-rosette assay (Abiko and Sekino, 1990). In human, equine, cat, rabbit, rat and murine tissues, $T\beta_{10}$ is the accompanying β-thymosin to $T\beta_4$ (Erickson-Viitanen et al., 1983b, Haritos

Table 1. Phylogenetic distribution of β-thymosins

Species	1st β-thymosin	2nd β-thymosin
Human, horse, rat, mouse, cat	$T\beta_4$	$T\beta_{10}$
Calf	$T\beta_4$	$T\beta_9$
Pig, Sheep	$T\beta_4$	$T\beta_9^{Met}$
Rabbit	$T\beta_4^{Ala}$	$T\beta_{10}$
Guinea pig	$T\beta_4$	
Whale	$T\beta_4$	$T\beta_{13}$
Chicken, gecko	$T\beta_4$	
Frog	$T\beta_4^{Xen}$	
Rainbow trout	$T\beta_{11}$	$T\beta_{12}$
Perch	$T\beta_{12}$ (perch)	
Sea urchin	$T\beta_{14}$	

et al., 1985; Hörger, 1993). For $T\beta_{10}$ also no precursor molecule was found (Goodall and Horecker 1987). Comparison studies with human and rat $T\beta_{10}$ cDNA have shown a complete identity for the primary structure (McCreary et al., 1988). The nucleotide sequences are 95% identical. In adult rat testis an additional mRNA besides the common mRNA for $T\beta_{10}$ was found, which may be active only in later stages of spermiogenesis (Lin and Morrison-Bogorad, 1991). Therefore it was postulated that $T\beta_{10}$ may be involved in the regulation of the motility in mature sperm. However, $T\beta_{10}$ seems to play an imortant role in developing tissues, especially the brain, and high levels of $T\beta_{10}$ were found in human fetal brain and in several neuroblastomas (Hall et al., 1990). In human brain, the $T\beta_{10}$-content declines rapidly after birth. Investigations concerning the $T\beta_{10}$ mRNA revealed that this gene is present in embrionic brain and absent in the adult brain (Hall, 1991). Recombinant $T\beta_{10}$ was shown to have also an inhibitory activity on F-actin formation through binding to G-actin with a K_d of 0.7–1 μM. Like in the case of $T\beta_4$, the binding of $T\beta_{10}$ is not inhibited by polyphosphoinositides or regulated by calcium (Yu et al., 1993). In whale tissue, $T\beta_{13}$ was identified besides $T\beta_4$ (Voelter, 1993a, b), but up to now there is no evidence concerning its biological role.

Not only in mammals, but also in lower vertebrate species two β-thymosins were found. From trout spleen, $T\beta_{11}$ and $T\beta_{12}$ were isolated and their primary structure determined, which differs from that of $T\beta_4$ in their NH_2- and COOH-terminal region (Yialouris et al., 1992, Fig. 1). Both show a higher homology in their primary structure than the β-thymosin pairs in mammals, known up to now. However, another $T\beta_{12}$, different from that found in trout spleen, was isolated from perch liver (Low et al., 1992). Currently, there is no report about a coexisting β-thymosin in perch. In oocytes from the frog Xenopus laevis only one β-thymosin was identified, i.e. $T\beta_4^{Xen}$, which may replace the mammalian $T\beta_4$ (Hannappel et al., 1988). Also, in chicken and gecko, only $T\beta_4$ without an accompanying β-thymosin was found. More recently, another member of the β-thymosin family, namely $T\beta_{14}$, was isolated from sea-urchin and its primary structure determined (Hörger, 1993).

It is likely to assume that all identified β-thymosins possess a very similar biological relevance since they all show the LKKTET sequence in position 17–22 which is very important for the interaction with actin. $T\beta_9^{Met}$, $T\beta_4^{Xen}$ and $T\beta_{11}$ have shown in the DNase I inhibition assay to be able to inhibit actin-polymerization (Hannappel and Wartenberg, 1993).

Some members of the β-thymosin family as well as their fragments were chemically synthesized using different approaches. $T\beta_4$ was prepared by a conventional solution method (Abiko and Sekino 1990; Voelter and Kapurniotu, 1991; Kapurniotu et al., 1991, 1992, 1993) or solid phase synthesis using Boc-amino acids (Wang et al., 1981; Low et al., 1983). Synthesis of deacetyl-$T\beta_4^{Xen}$ was done classically by preparation of six fragments (Abiko and Sekino, 1989). A solution synthesis was also reported for $T\beta_9$ (Chandramouli et al., 1986) and for its C-terminal fragment 33–41, which is active in the E-rosette assay (Kalbacher et al., 1990). $T\beta_4^{Met}$ was also synthesized by a solid phase method (Abiko and Sekino, 1990) as well as trout $T\beta_{11}$ (Voelter et al., 1991) and $T\beta_{12}$ (Echner et al., 1993). A synthesis for AcSDKP, an inhibitory peptide on the hematopoietic stem cell proliferation which may be generated from $T\beta_4$ through enzymatic digestion, was described as well as for $T\beta_9$[1–4] (Freund et al., 1992).

In order to detect β-thymosins specifically, several immunoassays were established. First, a radioimmunoassay (RIA) was developed for the measurement of $T\beta_4$ in human serum (Naylor et al., 1984) or in different rat tissues (Goodall et al., 1983a). Increased levels of $T\beta_4$ in human newborn cord serum and in patients with AIDS have been measured (Naylor et al., 1986). Later on, Weller et al. (1987 and 1992) detected quantitatively $T\beta_4$ in human serum by an immunoassay. They found mean levels of 12.6 ng $T\beta_4$/ml serum with a slight enhancement with increasing age. However, all these assays were performed with antibodies prepared by immunization with the whole molecule of $T\beta_4$. Taking into consideration the high homology in the primary structure of β-thymosins, one cannot exclude a possible cross-reactivity of antibodies raised against a β-thymosin against other members of this peptide family. In fact, anti-$T\beta_4$ antibodies show a cross-reactivity of 10% against $T\beta_9$ and anti-$T\beta_9$ antibodies cross-react 35% with $T\beta_4$. This kind of antibodies are useless for immunohistochemical or quantitative tissue extract investigations, especially in tissues from species containing two β-thymosins. Therefore, an ELISA was developed using antibodies against the N-terminal fragment 1–14 of $T\beta_9$ respectively $T\beta_4$ (Mihelić et al., 1990; Livaniou et al., 1992). By this method, the cross-reactivity is diminished up to 1% and the antibodies are able to detect the native β-thymosin with approximately the same affinity as its fragment 1–14. This fragment can be obtained either by enzymatic digestion of the native molecule or by solid phase synthesis (Mihelić and Voelter, 1993). For the development of an ELISA for the specific detection of $T\beta_{10}$, the fragment 1–12 of $T\beta_{10}$ was synthesized and used for immunization (Hörger et al., 1992). The antigenic index, calculated according to Jameson and Wolf (1988) as well as epitope mapping studies with polyclonal anti-$T\beta_4$ antibodies revealed that regions with high antigenicity are located mainly in the N-terminal part of the molecule (Voelter et al., 1990a, 1992a). Using anti-$T\beta_9$[1–14] antibodies in immunohistochemical studies, the localization of $T\beta_9$ was specifically detected in frozen sections of

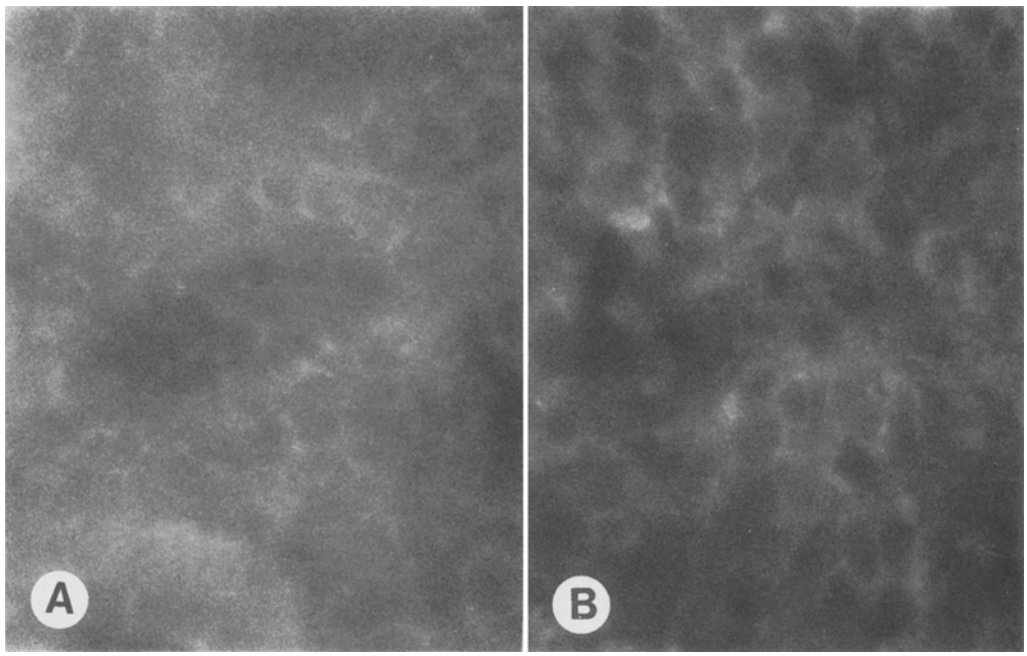

Fig. 2. Immunohistochemical localization of Tβ_9 in frozen sections of bovine thymus (**A**) and spleen (**B**), using an indirect antibody fluorescent technique. The antibodies against Tβ_9 were prepared by immunization with its fragment 1–14. Positive reaction is visible in the cell lumen, and a relatively strong one at the cell borders. Magnification: **A**, **B** × 900

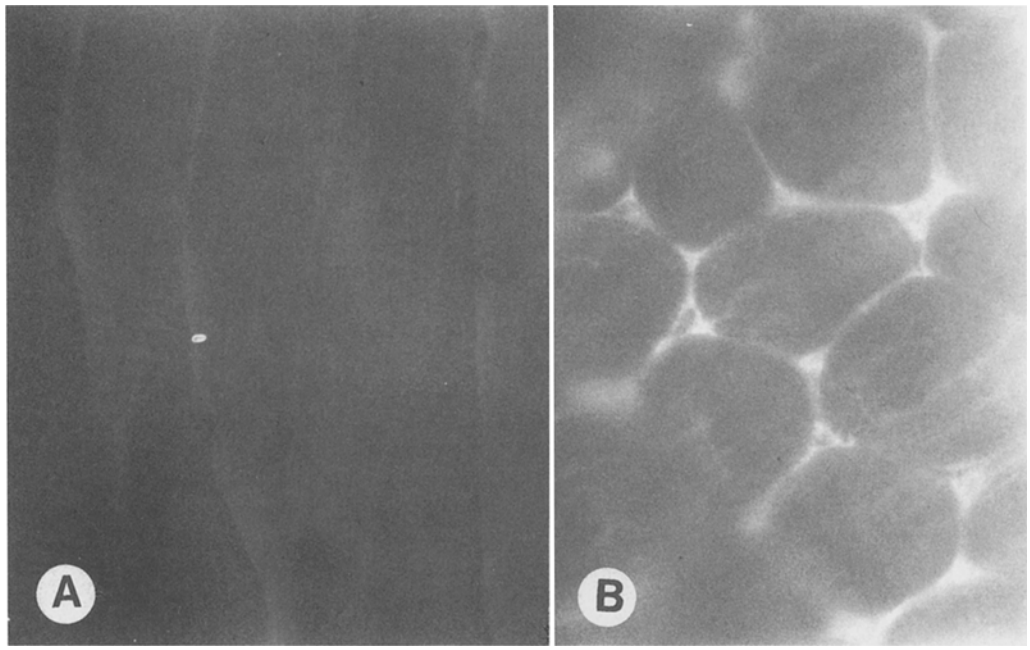

Fig. 3. Detection of Tβ_9 with anti-Tβ_9[1–14] antibodies in the sarcolemn of bovine muscle, **A**, longitudinal section; **B**, cross section. Magnification: **A** × 360, **B** × 900

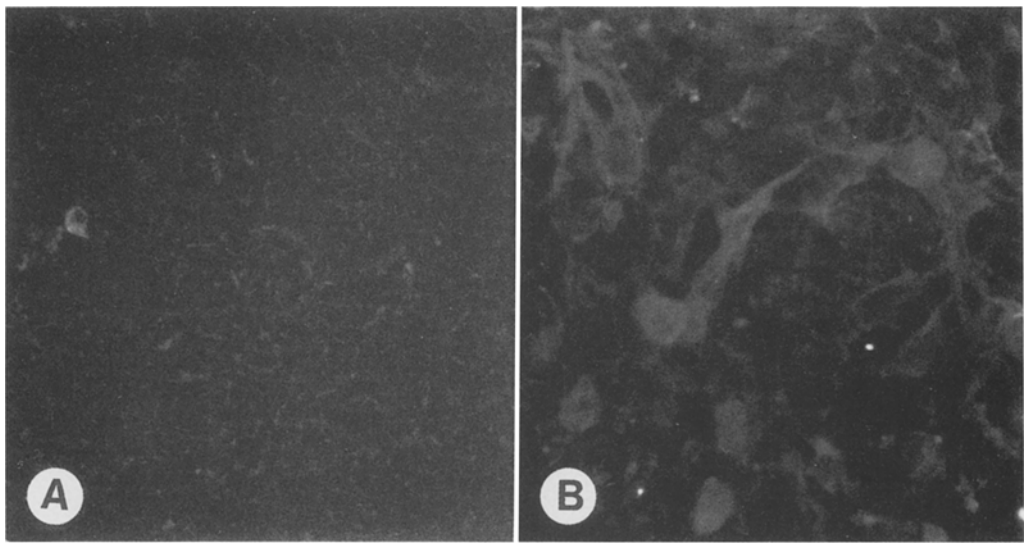

Fig. 4. Immunohistochemical staining of $T\beta_4$ in bovine thymus cortex (**A**) and medulla (**B**), using anti-$T\beta_4$ [1–14] antibodies. $T\beta_4$ is not only on organ-unspecific peptide, but is also distributed in different types of cells even in the same organ. Magnification: **A** × 360; **B** × 900

different bovine tissues besides $T\beta_4$ which is naturally present in 2–3 fold higher concentrations than $T\beta_9$ (Mihelić et al., 1989). In sections of the thymus, the positive reaction against $T\beta_9$ is relatively strong at cell borders (Fig 2). β-Thymosins seem to be present in the area of the cell lumen except the cell nucleus, as was also proven by RIA measurements of nuclear and extranuclear extracts of calf thymus (Tsitsiloni et al., 1992). $T\beta_9$ and its homologue $T\beta_4$ are located not only in the thymus, but in all other tissues investigated, i.e. in spleen (Fig. 2B), muscle (Fig. 3), kidney, liver, lung, connective tissue, with the highest intensity in spleen (Fig. 2B). Even in thymus, $T\beta_4$ is present in different types of cells (Fig. 4) which is not surprising with regard to its possible involvement with the cytoskeleton.

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