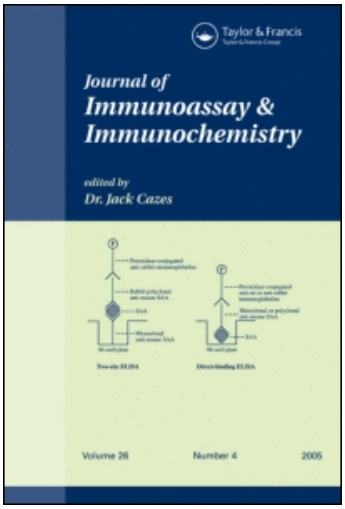

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CONCENTRATIONS OF THYMULIN IN UNEXTRACTED SERUM FROM PIGS, SHEEP AND CATTLE AS MEASURED BY ELISA

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ABSTRACT

These studies were conducted to develop an ELISA for measurement of thymulin concentrations in unextracted blood serum (or plasma) from domestic animal species (pigs, sheep and cows). This assay was quite variable (intraassay C.V. of 13.3 and 6.4% at 12.6 and 50.5 pg/mL and interassay C.V. of 24.2%). Serial dilutions of serum from these species produced inhibition curves parallel to the reference standard, suggesting that there were no substances in serum causing non-specific interference in the assay. In addition, none of the other thymic peptides tested resulted in problematic displacement of thymulin binding to the antiserum. Using this assay, it was found that somatotropin (ST) treatment had no effect on serum thymulin concentrations in either pigs or cows. Chromatographic separation of thymulin activity in sheep serum showed three peaks with approximate MW estimates of 95, 80 and 1 kDa. Serum thymulin concentrations in a sheep injected with thymulin was cleared from blood with a half-life ($t_{1/2}$) of 10.3 ± 0.6 min. Serum thymulin concentrations increased between birth and 6 mo old in pigs. These data indicate that a rapid and reliable ELISA has been developed to measure thymulin in blood of these domestic animals. This assay should be of value in the study of thymulin function and factors regulating its secretion.

KEY WORDS: Thymulin, ELISA, pig, sheep, cow

INTRODUCTION

It has been well established that the thymus is a true endocrine gland. As such, it secretes peptide hormones thymulin and thymosin (1,2,3). Thymulin is a nonapeptide (1) which was originally called FTS (Facteur Thymique Serique) by the group who isolated and characterized it (4,5). It is thought that thymulin is directly involved in intrathymic T-lymphocyte differentiation and in enhancing the activity of circulating T-lymphocytes (6,7). Factors influencing thymulin concentrations in serum of rats and humans have been studied using bioassay (8), radioimmunoassay (9,10,11) and immunoassay (12). We were interested in developing a sensitive ELISA for thymulin with which to study the regulation of its secretion in domestic animals (pig, sheep, cattle). Furthermore, we hoped the assay could be used to quantitate thymulin in unextracted serum. Finally, we used this assay to test the hypothesis that somatotropin (ST) treatment would increase serum concentrations of thymulin.

MATERIALS AND METHODS

The Assay. Antiserum against synthetic thymulin coupled to bovine serum albumin was generated in rabbits as described by Pleau et al. (13). The ELISA is a competition assay as depicted schematically in Fig. 1. In this procedure, equal volumes (60 μL) of assay buffer (0.1 M PBS, 1% BSA, 0.1% Triton X-100, 0.1% Tween-20), serum or reference standard in assay buffer

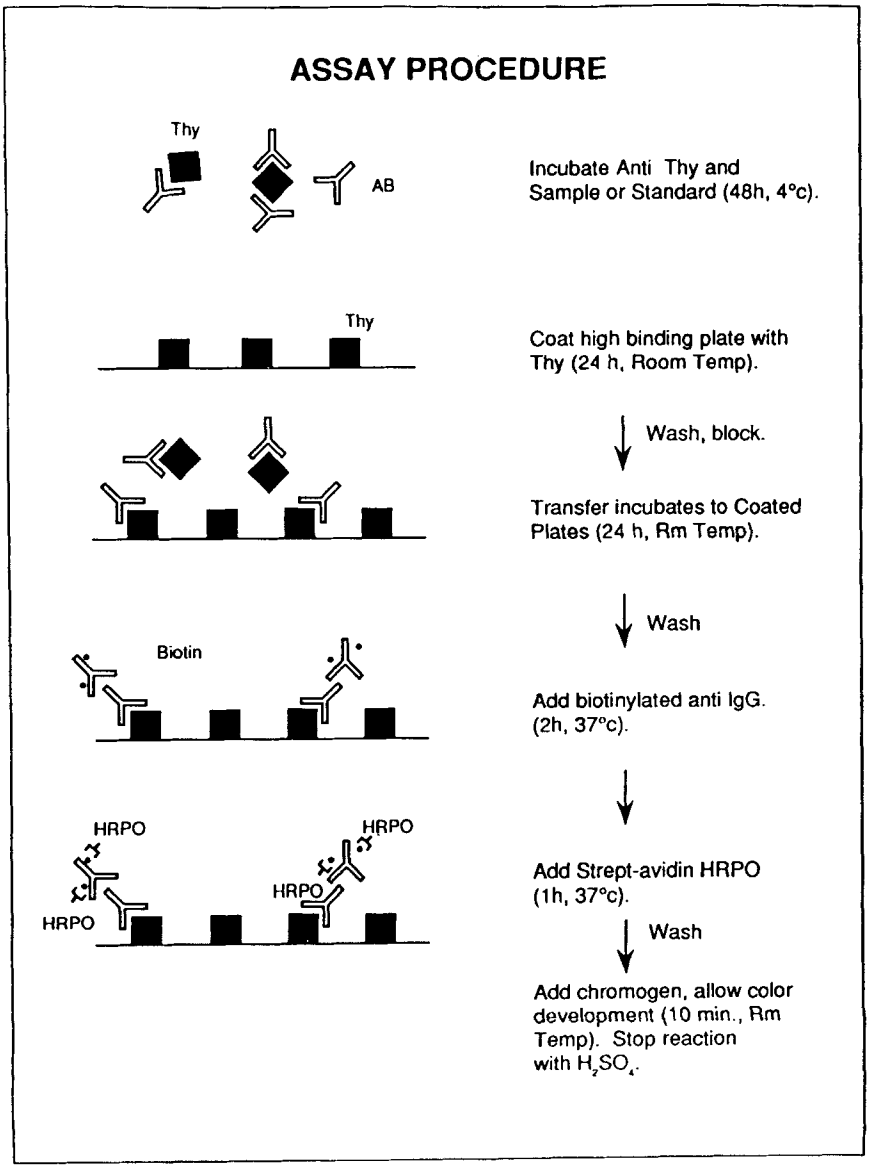

FIGURE 1: Schematic diagram depicting the various steps in the thymulin ELISA. Abbreviations: Thymulin = Thy, Antibody = AB, Horseradish peroxidase (HRPO).

and antiserum (diluted to 1:50,000 in assay buffer) are added on d 1 to wells in a low-binding Costar (Cambridge, MA) microtiter plate and incubated for 48 h at 4° C. On d 2, thymulin (25 ng/well in 100 μL of carbonate buffer, 0.1 M, pH 9.6) is added to Costar high-binding EIA plates and incubated for 24 h at room temperature to coat the wells with thymulin. On d 3, the thymulin coated, high binding plate is washed (and blotted on absorbent paper) 3 times with nanopure water containing .1% Tween 20. The thymulin coated plates are then blocked by adding 100 μL of assay buffer and incubating at room temperature for 30 min. The plates are again washed 3 times and then 100 μL of incubate mixtures from the low-binding plates are added to the thymulin coated and blocked plates and incubated for 24 h at 4° C. On d 4, the plates are washed 3 times with deionized water + .1% Tween 20 and 100 μL of biotin conjugated anti-IgG (Amersham, RPN 004) diluted 1:2500 in assay buffer are added to each well. The plates are incubated for 2 h at 37° C and washed 3 times. Then 100 μL of a 1:1000 dilution of Streptavidin conjugated to Horseradish Peroxidase (Amersham RPN 1231) is added. Plates are incubated at 37° C for 1 h and washed 3 times. Then 100 μL of chromogen solution are added. The chromogen used is TMB (3,3,5,5-tetramethylbenzadine (Sigma T2885). The chromogen solution is made by adding 100 μL of TMB stock solution (120 mg TMB/ml in DMSO) and 1 μL hydrogen peroxide to 10 mL of 0.1 M acetate buffer, pH 5.5. Color development proceeds for 10-15 min, and then is stopped by adding 100 μL

of 2 M H_2SO_4. Plates are read on a Titertek (Multiscan Plus) (Flow Laboratories) plate reader at 450 nM. The computer is programmed to plot the reference standard curve and to calculate the relative concentration of thymulin in samples by extrapolation from the standard curve.

Assay Reliability.

1. Assay precision was assessed by determining the within and between assay coefficient of variation (C.V.). Interassay C.V. was based on the addition of 5 replicates of the same sheep and cow serum pools. Intraassay C.V. was determined by including a sheep serum pool in 10 different assays over time.
2. Recovery was determined by assay of a constant volume of pig, sheep and cow serum to which were added increasing amounts of thymulin (3.0, 6.0, 12.0 and 25.0 pg). This was done to demonstrate that thymulin could be quantitated in unextracted serum samples and is a test of accuracy.
3. Parallelism between the thymulin reference standard curve and dilutions of sera was studied to further document the specificity of the assay for thymulin. For this purpose, 7.5, 15.0, 30.0 and 60 μL of a sheep serum pool (containing sera from 24 female sheep) or 15, 30 and 60 μL of sera from several pigs and cows were assayed.
4. Specificity. Specificity was tested by adding increasing amounts (from .25 to 1 ng/mL) of thymosin α.1, thymosin β-4 and thymopoietin to the assay. These thymic peptides were obtained from Sigma Chemical Co.

The second means by which assay specificity was tested was by examination of species specificity. To do this, 60 μL of sera from animals of several different species were added to the assay.

Chromatographic Characterization of Thymulin Activity in Serum. Ten mL of a sheep serum pool (made from adult female sheep) were concentrated to 3.5 mL using an Amicon (Amicon, Inc., Beverly, MA) stirred cell concentrator (model 8050) with a YM-1 filter (1000 MW cutoff). This sample was then applied to a column containing Ultrogel (IBF Biotechnics, France) ACA 44 (3.0 x 60 cm, MW range 10,000 to 130,000) and eluted with .01 M phosphate buffer (pH 7.5) with .15 M NaCl. The flow rate of the column was approximately 20 mL/h. Fraction volume was about 4.0 mL. Blue dextran, bovine serum albumin, ribonuclease A and L-Tyrosine were used to develop a standard molecular weight curve. Fractions were assayed for thymulin and protein elution pattern was determined by spectrophotometer (OD 280).

To determine if thymulin injected into a sheep would remain in low MW condition or bind to a serum protein(s), 2 mL of serum from thymulin injected sheep (thymulin concentration was 155 pg/mL, 5 min after thymulin was injected) subsequently were applied to the same column.

Thymulin in Unextracted Sera.

a. Effect of Sex and Age. Blood samples were obtained from crossbred male and female pigs as described (14). These samples were frozen

and kept at -20° C for approximately 1 year before they were assayed for thymulin.

b. Effect of Growth Hormone (GH) or Somatotropin (ST) Treatment. Blood serum samples were obtained from female pigs of 2 lines (lean and obese) which were treated with 0, 2 and 4 mg porcine ST (PST)/d as described by Klindt et al. (15). In another study with pigs, blood was obtained from castrated male pigs (45 kg at start of treatment) treated with 0 or 100 μg PST$\cdot$kg $BW^{-1}\cdot d^{-1}$ for 40 d. Blood samples were obtained on d 30 of treatment (16). In an additional experiment, blood samples were obtained from mature, lactating dairy cows (5) treated with bovine somatotropin (29 mg/d) at 0, 30 and 60 d of treatment and from another group of control cows not injected (17).

Time Course Disappearance of Exogenous Thymulin from Sheep Blood In Vivo. Fifteen adult, non-pregnant, non-cyclic (anestrous) sheep were fitted with indwelling jugular catheters (16 G, 3 1/4") (Becton Dickenson, USA). The following d, blood samples were obtained from the catheters at 15 min intervals for 2.5 h. Thymulin was injected into 5 sheep (each group) at 0, 450 ng and 4500 ng in 5 mL PBS. The heparinized blood was allowed to stand overnight at 4° C, after which plasma was harvested and stored at -20° C until assayed.

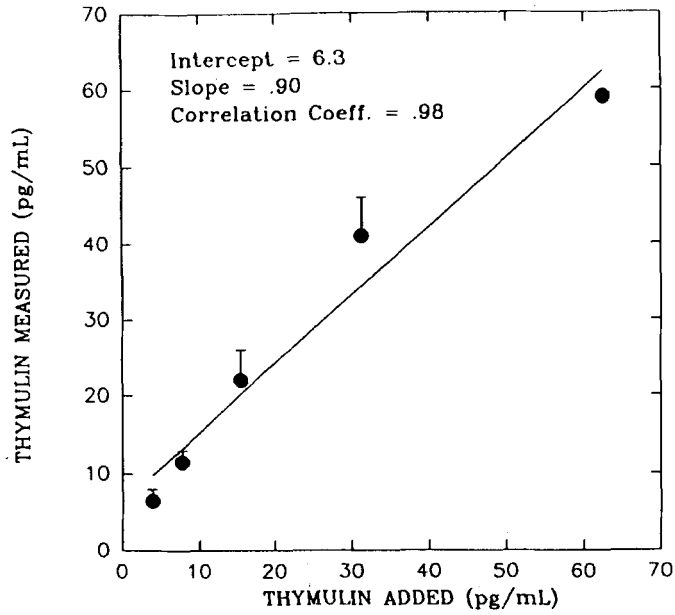

FIGURE 2: Recovery of known quantities of thymulin added to pig serum. The values shown are means ± SEM of two separate determinations.

Because some of the sheep had lost their catheters by the following day, the sheep used for high dose injection included 2 animals not previously injected and 3 animals previously injected with the low dose of thymulin.

RESULTS

Assay Reliability.

1. Precision

Assay variability is known to be related to sample concentration with variation being highest at both ends of the reference standard curve and

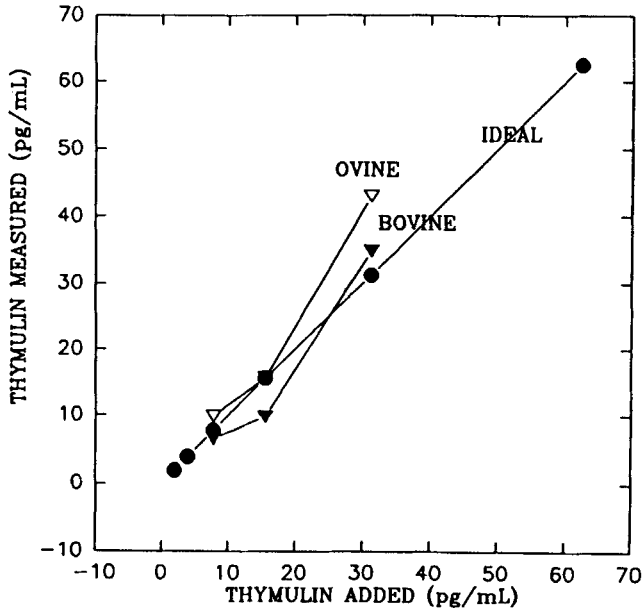

FIGURE 3: Recovery of thymulin added to sheep and pig serum. These curves are plotted in comparison to an "ideal" curve.

lowest in the middle (18). Accordingly, estimates of intraassay C.V. were 13.3% and 6.4% with sheep (12.6 ng/mL) and cow (50.5 pg/mL), respectively. The intraassay C.V. below 10 and above 80 pg/mL is over 25%; therefore, the aceptable working range of the standard curve was considered to be between 10 and 80 pg/mL. At a concentration of 30 to 40 pg/mL, interassay C.V. was 24.2%.

2. Recovery from serum

To demonstrate the ability of the assay to quantitate thymulin in sera, constant volumes of pig, sheep and cow serum were "spiked" with increasing

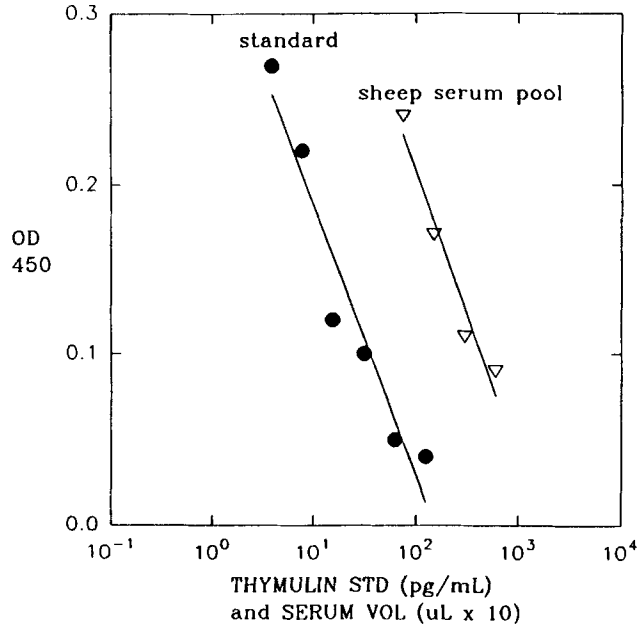

FIGURE 4: Reference standard curve and inhibition curve obtained by adding 7.5, 15, 30 and 60 μL of a sheep serum pool (made by pooling sera from 24 adult female sheep).

amounts of thymulin. Percentage of added thymulin which was recovered was 124% in pig serum, 126% in sheep serum and 87% in cow serum. The recovery curve for thymulin in porcine serum is shown in Fig. 2. The line has a slope of 0.90 and y-intercept of 6.3 pg/ml. The line plotted in Fig. 2 was determined using the assumption that the recovery was linear. However, visually the data appear to be curvilinear in form and suggest that the values of thymulin in pig serum are overestimated in the mid range of the curve and underestimated at both ends. Recovery curves of thymulin in sheep and cow

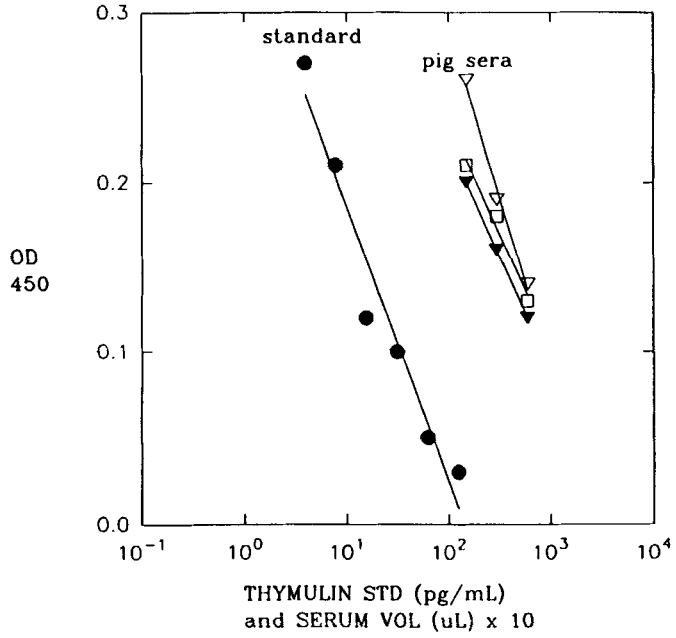

FIGURE 5: Reference standard curve and inhibition curve obtained by adding 15, 30 and 60 μL of three different pig sera.

sera are shown in Fig. 3. Similar to the results with pig serum, it appears that the assay may slightly overestimate actual concentrations in the range of 30 pg/mL.

3. Parallelism

Comparisons of the inhibition curves obtained with increasing volumes of sera from sheep, pig and cow serum are shown in Fig. 4, 5 and 6, respectively. Although the slopes of the curves were not tested statistically for differences, visually they appear to be virtually the same. These results indicate that

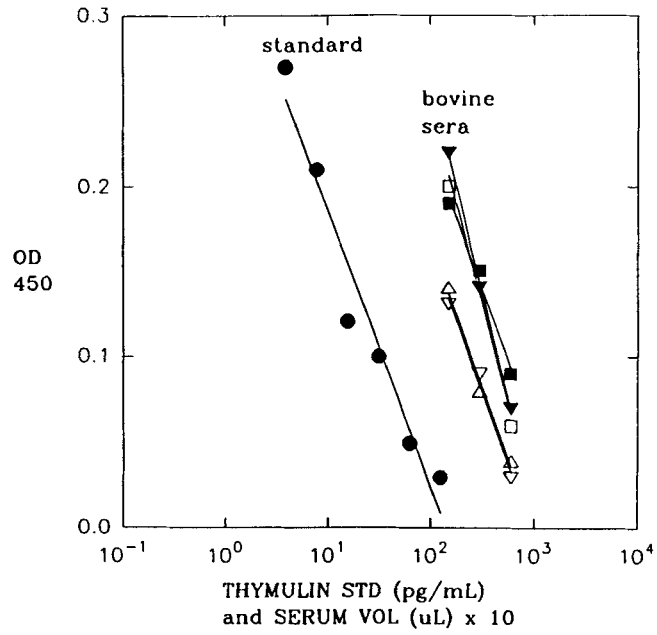

FIGURE 6: Reference standard curve and inhibition curve obtained by adding 15, 30 and 60 μL of five different cow sera.

synthetic thymulin standard and the thymulin in these sera compete for binding on the antisera with comparable binding affinities. This would, in turn, imply that these sera did not contain any nonspecific factors which would likely bind to the antibodies with a different affinity.

4. Specificity

Of the hormones tested, only thymosin-α1 showed any degree of competition in the assay and that was not observed below 500 pg/mL (Fig. 7). The other peptides had no effect at concentrations up to 1000 pg/mL. Serum

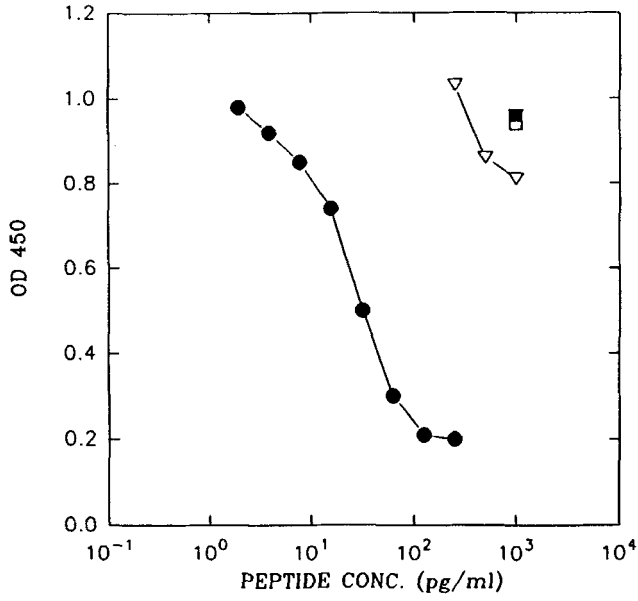

FIGURE 7. Representative thymulin reference standard curve (• - •). Also shown are the inhibition curves by 250 to 1000 pg/mL of thymosin α-1 (▽ - ▽), thymosin β-4 (□) and thymopoeitin 13 and 6 (■).

concentrations of thymosin-α1 in the cow seldom get as high as 500 pg/ml (19). Therefore, thymosin-α1 likely does not present a problem of non-specific interference in the assay.

Chromatographic Characterization of Thymulin Activity in Serum. Thymulin activity was found in three peaks (Fig. 8). The first peak had an estimated MW of over 90 kDa, the second at approximately 80 kDa and the third eluted in the same fraction range as tyrosine. These results agree with those of

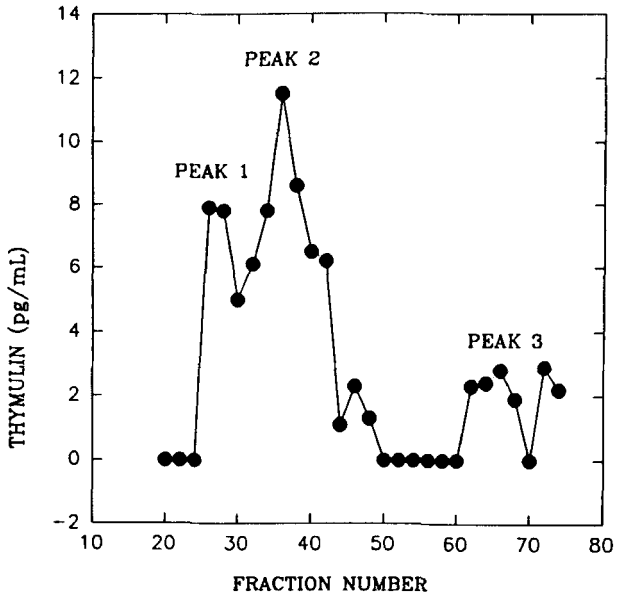

FIGURE 8. Thymulin activity of sheep serum fractionated on Ultrogel AcA 44 chromatography.

Dardenne et al. (20) indicating the presence of a serum carrier protein for thymulin.

In the serum sample from a thymulin injected sheep, there was increased thymulin activity in the Peak 1 range and the Peak 3 range. This observation suggests that some of the exogenously administered thymulin was bound to the Peak 1 protein within 5 min of its injection.

Thymulin in Unextracted Bovine sera

a. Effect of sex and age. Concentrations of thymulin increased ($P<.01$) in pigs (Fig. 9) between one and 6 mo of age. There was no difference

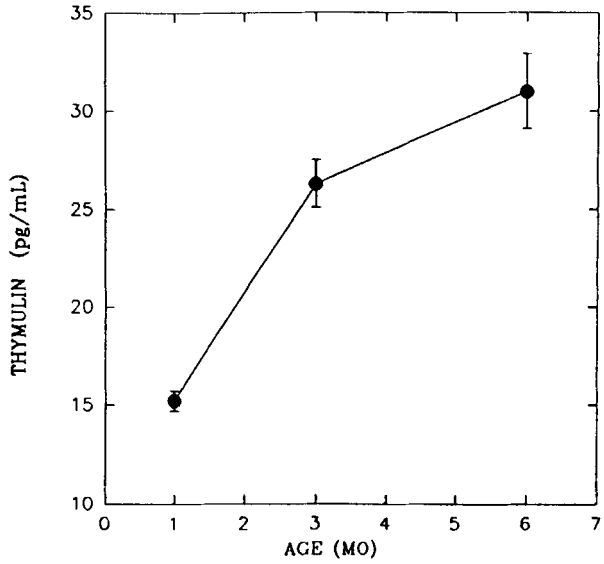

FIGURE 9. Serum thymulin concentrations in pigs between one and six months old (n=30 at each age).

(P>.05) between males and females. Therefore, the data shown represent an average of 15 males and 15 females at each age.

b. Effect of growth hormone. In the Klindt et al. (15) study, there was no effect of PST on serum thymulin (Table 1). Similarly, PST failed to cause a change in thymulin (P>05) in either the Evock-Clover et al. (16) study (Table 2) or in dairy cows treated with BST (Table 3) (17).

Time-course Disappearance of Exogenous Thymulin from Sheep Blood In Vivo.

Blood plasma concentrations of thymulin were increased in a dose-related manner (Fig. 10). The half-life ($t_{1/2}$) of thymulin was not different (P>.05)

TABLE 1

Serum concentrations of thymulin in female pigs (60 kg) treated with PST.

Treatment (mg PST/d)	n[a]	Thymulin (pg/ml)
0	6	27.4 ± 4.1
2	6	25.1 ± 1.5
4	6	25.3 ± 1.8

$P < .05$.

[a] Two serum pools each on d 14, 28 and 40 of treatment. Each pool contained sera from 4 pigs.

Klindt et al., 1992.

TABLE 2

Serum thymulin concentrations in pigs treated with chromium or PST.

Treatment	(n)	Thymulin (pg/ml)
Control	6	26.1 ± 2.1
Chromium	6	22.6 ± 2.5
PST	6	23.3 ± 1.3
Ch + PST	6	27.6 ± 2.5

$P > .05$.

Neutered males, 60 kg average body weight.

Evock-Clover et al., 1993.

TABLE 3

Serum concentrations of thymulin in dairy cows treated with BST (60 d treatment).

Treatment	(n)	Thymulin (pg/ml)
Control	5	45.6 ± 7.6
BST	5	32.7 ± 9.0

P>.05.
Binelli et al., 1993.

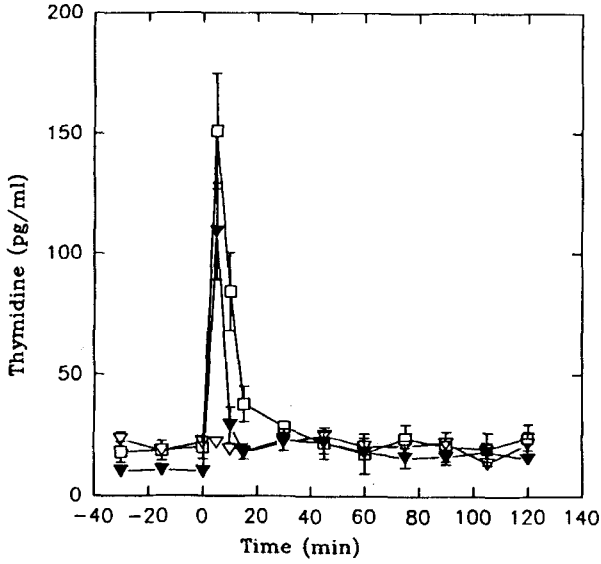

FIGURE 10. Serum concentrations of thymulin in sheep injected with 0 (o - o), 450 (• - •) and 4500 (▽ - ▽) ng of thymulin.

between the two injected groups. Therefore, all animals were used to estimate the $t_{1/2}$ (10.3 ± 0.6 min).

DISCUSSION

These results indicate that an ELISA for estimating the concentrations of thymulin in unextracted serum from pig, sheep and cow has been developed. Furthermore, they indicate that serum concentrations of thymulin increase from birth to puberty in pigs.

Previous researchers had suggested that growth hormone treatment stimulates thymic growth (21,22,15). We had hypothesized, therefore, that ST treatment would also stimulate secretion of thymulin. Thymulin did not respond in either porcine or bovine ST treatment. However, Dardenne et al. (23) reported that human GH but not ovine GH stimulated thymulin release in vitro. Accordingly, they attributed the stimulatory effect of human GH to the lactogenic activity of this hormone rather than to somatogenic activity. Our observations would suggest that neither PST nor BST directly stimulate thymulin secretion in these species even though ST did increase thymus size in pigs (24).

This assay provides the tool with which to study the regulation of the secretion of thymulin in various conditions of health and disease in domestic farm animal species. In turn, it is anticipated that such information could lead to the eventual use of the assay of thymulin in clinical medicine.

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