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Characterization of Bovine Thymus Extract: Implications for Quality Assessment of Animal-Derived Ingredients

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Abstract

Thymus extracts are regarded as promising and commercially relevant natural ingredients of food products with reported tolerability and therapeutic potential, although their safety profile varies by preparation and context. Animal-derived ingredients used in food supplements are complex biological materials whose composition and properties depend on source and processing, making characterization essential for product standardization and quality assessment. The aim of this study was to investigate a new commercially available bovine thymus extract using complementary physicochemical, biochemical, molecular, and cell-based analyses to provide an integrated approach for future assessment of thymus-derived extract composition and biological properties. The extract contained approximately 41% protein, 10% reducing sugars, and a negligible lipid fraction (<0.02%) and showed a heterogeneous molecular profile with prominent low-molecular-mass components. Reproducible chromatographic patterns support chromatographic profiling as a tool for batch comparison and evaluation of extracts from different sources and batches. Immunochemical characterization of immunomodulatory components was considered as a complementary approach. However, the reliability of such tools was limited, and definitive identification requires further investigation. The extract also induced TNF-α and IL-1β release in THP-1-derived macrophages, indicating a measurable cellular response. Overall, these findings support future studies to define compositional markers, validate analytical procedures, and strengthen standardization of thymus-derived preparations using complementary approaches.

Keywords: thymus extract; immunomodulatory compounds; size exclusion chromatography; quality control

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1. Introduction

The thymic gland has recently regained its significance as an important organ in sustaining immune competence and overall health and its removal has been associated with higher mortality and cancer risk [1]. The organ is mainly responsible for the development of the immune system mediated by secreted peptide hormones, including thymosin alpha-1, beta-4, beta-10 (Tα1, Tβ4, Tβ10), thymulin, thymopoetin, and cytokines. The age-related immunosenescence of the thymus is associated with higher susceptibility to infectious diseases and cancer in the elderly [2]. A recent report showed clear evidence of inflammaging suppression driven by circulating thymulin from young subjects [3]. Since the early 1980s animal-derived thymus extracts (thymomodulin) containing numerous thymic-derived factors have gained interest following the description of their biological

activity [4,5]. Driven by the thymus's essential role in T-cell development and immune regulation, continuous interest in thymus extracts reflects the lack of clear evidence for the efficacy of the individual synthetic factors as well as the growing demand for natural immune-supporting products. Numerous studies have been published over the years supporting thymomodulin's association with improved immune function in immunodeficient and immunocompromised states, including enhanced T-cell maturation and activity, increased phagocytic function, and normalization of disturbed immune parameters [6–11]. Clinical studies further suggest potential benefit in recurrent respiratory infections, chronic bronchitis, atopic disease, and cancer-related immune dysfunction, where reductions in infectious episodes and chemotherapy-related myelosuppression have been reported [7–11], documenting immunomodulatory properties of those preparations and their use in supportive therapy in different disorders, including cancer [6,12–22]. Thymomodulin, among other immunostimulants, appears to be safe and well-tolerated and is not associated with an increased risk of adverse events [17,23,24], regardless of administration route. However, thymomodulin, as a complex mixture of thymus-derived peptides, remains incompletely characterized and requires better standardization to ensure reproducible results.

Animal-derived thymus extracts are used in food supplements and other products intended for human consumption. Their composition varies depending on the methods of preparation, including aqueous or organic solvent extraction, fractionation, and purification [2,16–18]. Unlike preparations containing a single defined compound, tissue-derived extracts may comprise complex mixtures of proteins, peptides, carbohydrates, lipids, and other constituents [3,17–20]. Their characterization is therefore important for establishing compositional profiles and identifying analytical parameters that may support product consistency and quality assessment. Recent research has demonstrated substantial differences in the physicochemical and molecular profiles of bovine thymic peptide preparations obtained using different isolation procedures [25]. Standardization and guidelines on the composition and use of food products and supplements containing animal-derived thymus extracts are important for the credibility of products [19] and reproducibility of clinical results. The assessment of such ingredients, especially in complex mixtures such as thymus extracts, may benefit from combining basic compositional measurements with electrophoretic and chromatographic profiling, investigation of selected molecular constituents, and exploratory cell-based assays, such as the induction of NF-κB reporter activity [25]. However, candidate parameters must be evaluated for their specificity, reproducibility, and relevance to the intended purpose before they can be adopted as validated quality-control criteria.

Since the detailed composition of complex thymus-derived preparations remains insufficiently defined, further work is needed to establish analytical parameters suitable for their systematic comparison. The present study aimed to characterize a commercially available bovine thymus extract using an integrated approach containing complementary physicochemical, biochemical, chromatographic, immunochemical, and cell-based analyses. The study examined its major compositional features, molecular profile, selected peptide-related signals, and ability to induce cytokine secretion in THP-1-derived macrophages. Based on these findings, we discuss the potential contribution of complementary analytical methods to the future quality assessment of bovine thymus extract as an animal-derived ingredient, while identifying the limitations and further validation required before specific quality criteria can be established.

2. Results

2.1. Physicochemical Properties of Thymus Extract

Bovine thymus extract is produced through tissue processing without the use of alcohol or other solvents. It appears as a yellowish, highly hygroscopic powder, which is easily soluble in water and PBS. The aqueous solution of 1 mg/mL shows a mean pH of 5.7. The solubility in water and in PBS is similar and reaches 450 mg/mL (solubility limit). The concentrated solution (300 mg/mL) is transparent and presents a yellow color (Figure 1A), while at a low concentration (1 mg/mL) it is colorless (Figure 1B).

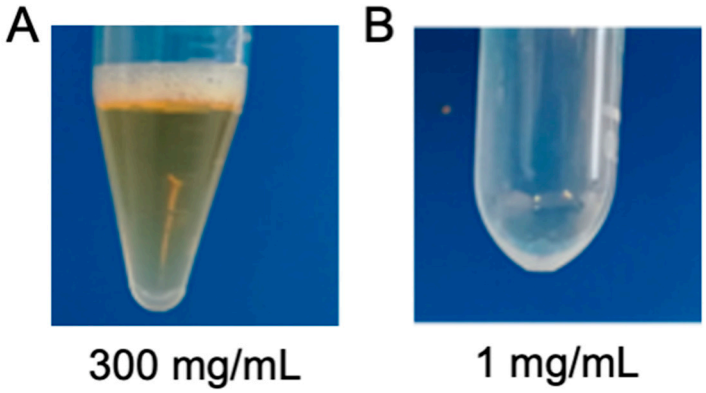


Figure 1. Solubility and appearance of the bovine thymus extract. (**A**) The solution in PBS at 300 mg/mL and (**B**) 1 mg/mL is shown.

The recorded spectrum of a 1 mg/mL aqueous solution showed intensive absorbance characteristic of nucleic acids (260 nm) and aromatic amino acids (280 nm) falling within 230–300 nm. There was also a noticeable peak observed at ~360 nm, although with weak intensity.

The analysis of fluorescence within the range of 400–750 nm revealed an intense emission maximum at 460 nm when the mixture was excited with light of 360 nm (Figure 2B).

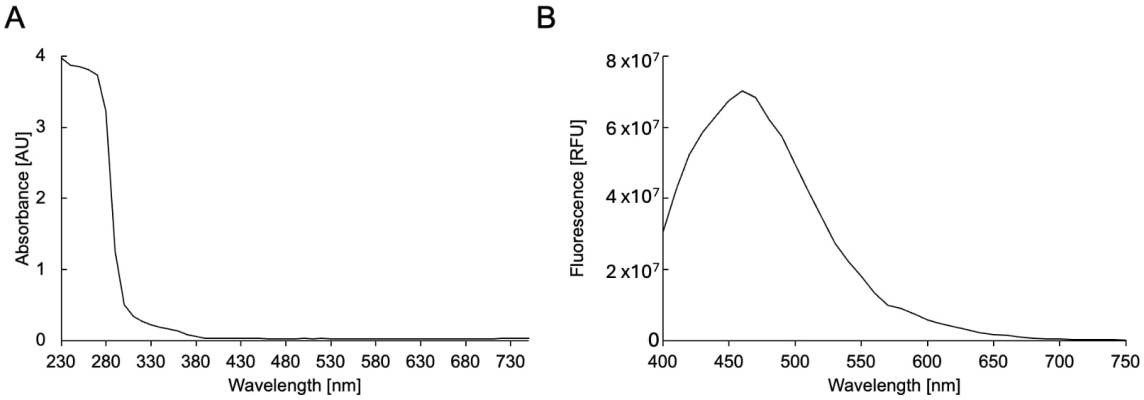


Figure 2. Spectroscopic analysis of the bovine thymus extract. (**A**) The aqueous solution at 1 mg/mL concentration was analyzed for absorbance within 230 nm–750 nm and (**B**) fluorescence emission within 400–750 nm at excitation of 360 nm.

The obtained results indicate the complex composition of the thymus extract, which warrants further, more detailed investigation into specific absorbance and fluorescence biomarkers.

2.2. Protein, Carbohydrate, and Lipid Content of Thymus Extract

The nutritional composition of consumed products, especially protein, carbohydrate, and lipid levels, is considered in standardization guidelines for recommended uptake, in-

cluding in food supplements [26]. These nutrients are present in animal tissue-derived food products at various levels, depending on the tissue type, i.e., meat (75% water, 19% protein, 2.5% lipid, 1% carbohydrate, 1.5% non-protein nitrogenous compounds, 1% inorganic matter) [27]. Thus, the analyses of three important organic components were performed to characterize the bovine thymus extract. The analysis of dry thymus extract (presenting a loss on drying of 2.1% within 4 h at 105 °C) was performed to determine the composition of individual nutrients, which was expressed relative to the dry mass of the samples. The summarized data is shown in Table 1.

Table 1. Summary of biochemical parameters.

Specification (Tested Parameter)		Result
Appearance		Yellowish to light brown powder
pH (1 mg/mL in H_2O)		5.70 (±0.01)
Solubility [mg/mL]		≤450
Protein content [%]	Kjeldahl method	40.69 (±0.05) *; LOD = 0.04%; CV < 5%
	NanoDrop	40.16 (±0.78) #
Reducing sugars [%]		10.07 (±2.64) #; LOD ≤ 0.1%; CV < 10%
Lipids [%]		0.07 (±0.07) *; LOD = 0.01%; CV < 5%

* Based on 2 measurements; # based on 3 measurements.

The Kjeldahl method has been formerly accepted and standardized by regulatory agencies for protein content analysis in food matrices as a reference method [28]. It is based on measuring the total nitrogen content, which is used as an indicator of protein quantification in organic material. We used this method to characterize the thymus extract and showed that protein makes up 40.69% of its composition. As a complementary method, the measurement at 280 nm was performed using NanoDrop, yielding a protein content of 40.16%.

Carbohydrate level is utilized for the characterization of marketed products aimed at human consumption, including wine [29] or cereals [30]. The thymus extract studied in this manuscript showed a carbohydrate content of 10.07% of (reducing sugars) as determined by analysis employing the Luff–Schoorl reagent.

Finally, lipid analysis, assessed by using Soxhlet extraction, showed a negligible <1% amount in the thymus tissue extract.

2.3. Characterization of the Proteins Present in the Thymus Extract

At first, the thymus extract was analyzed by SDS-PAGE. The tested material consisted mainly of proteins and peptides migrating as molecules with a molecular weight below 37 kDa (Figure 3, lane 2). The major band visible on the Coomassie-stained gel showed a protein smaller than 10 kDa.

Size-exclusion chromatography was next used to confirm the composition of the thymus extract. The column was calibrated with a mixture containing standards of known molecular weights (ranging from 1.35 to 670 kDa) and eluting from the column within 40–170 mL (Figure 4A, orange chromatogram). The loaded thymus extract consisted of molecules eluting within 40 and 180 mL, similarly to the employed molecular standards of mass below 44 kDa (Figure 4A, blue chromatogram). The protein nature of the material eluted between 26 and 80 mL was confirmed in the individual fractions using a polyacrylamide gel stained with Coomassie Blue (Figure 4B, lanes 2–11). In contrast, fractions eluted between 80 and 128 mL (Figure 4B, lanes 12–19) and further up to 200 mL contained molecules migrating out of the 15% polyacrylamide gel.

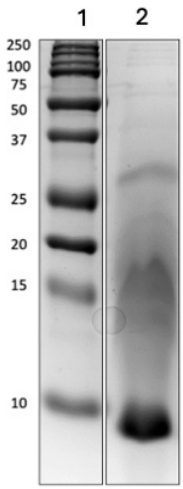


Figure 3. Electrophoretic analysis of thymus extract. The protein standard (lane 1) was subjected to SDS-PAGE on a 15% polyacrylamide gel along with 1 mg/mL of the initial thymus extract solution (lane 2). The gel was photographed after staining with Coomassie Blue.

In addition to proteins, there was also a substantial amount of material corresponding to small molecules with a size less than 1.35 kDa (Figure 4A, blue chromatogram). It likely consists of short peptides, amino acids, carbohydrates, and lipids detected in biochemical analyses (Table 1). Moreover, the repeatable pattern was observed for consecutive chromatographic runs (Figure 4C). We collected the material eluted within 44–115 mL and pooled it into four fractions, namely FR1: 44–49 mL, FR2: 50–70 mL, FR3: 71–97 mL, and FR4: 98–115 mL (indicated on the chromatogram in Figure 4C). FR1–FR4 contained polypeptides retained on the 20% gel with no distinct protein bands, suggesting complex mixtures of compounds (Figure 4D).

It is important to note that chromatographic separation was carried out in native conditions (using PBS as an elution buffer), allowing for the retention of existing aggregates of molecules that migrated through the column as larger compounds (Figure 4A, elution volume of 38–80 mL, corresponding to the elution of standard proteins of 670–17 kDa), while electrophoresis performed under reducing conditions revealed smaller molecules migrating as bands of ~30–10 kDa (Figure 4B, lanes 4–11).

2.4. Biological Activity of the Thymus Extract

We performed several complementary assays, i.e., Western blotting, ELISA, and mass spectrometry, to identify and quantify the immune regulatory factors known to be generated by thymus tissue. In addition, the functional assays were also performed to verify the activity of the thymus extract to induce a cellular response by quantifying the released cytokines, like TNF-α and IL-1β, in the in vitro assays.

In an attempt to check the presence of components with known biological activity in the separated fractions, immunochemical methods were employed for the determination of the most well-known thymic immunomodulators, like thymosins. At least two antibodies for the specific target were tested in Western blot analysis for Tα1, Tβ4, and Tβ10 level assessment. Unfortunately, we faced difficulties in reliably determining these molecules. All attempts returned negative results for the identification of both reference samples, i.e., the commercially acquired, pure thymosins as well as the whole bovine thymus extract. These surprising results were observed, although the reference peptides and the tested antibody were obtained from the same supplier.

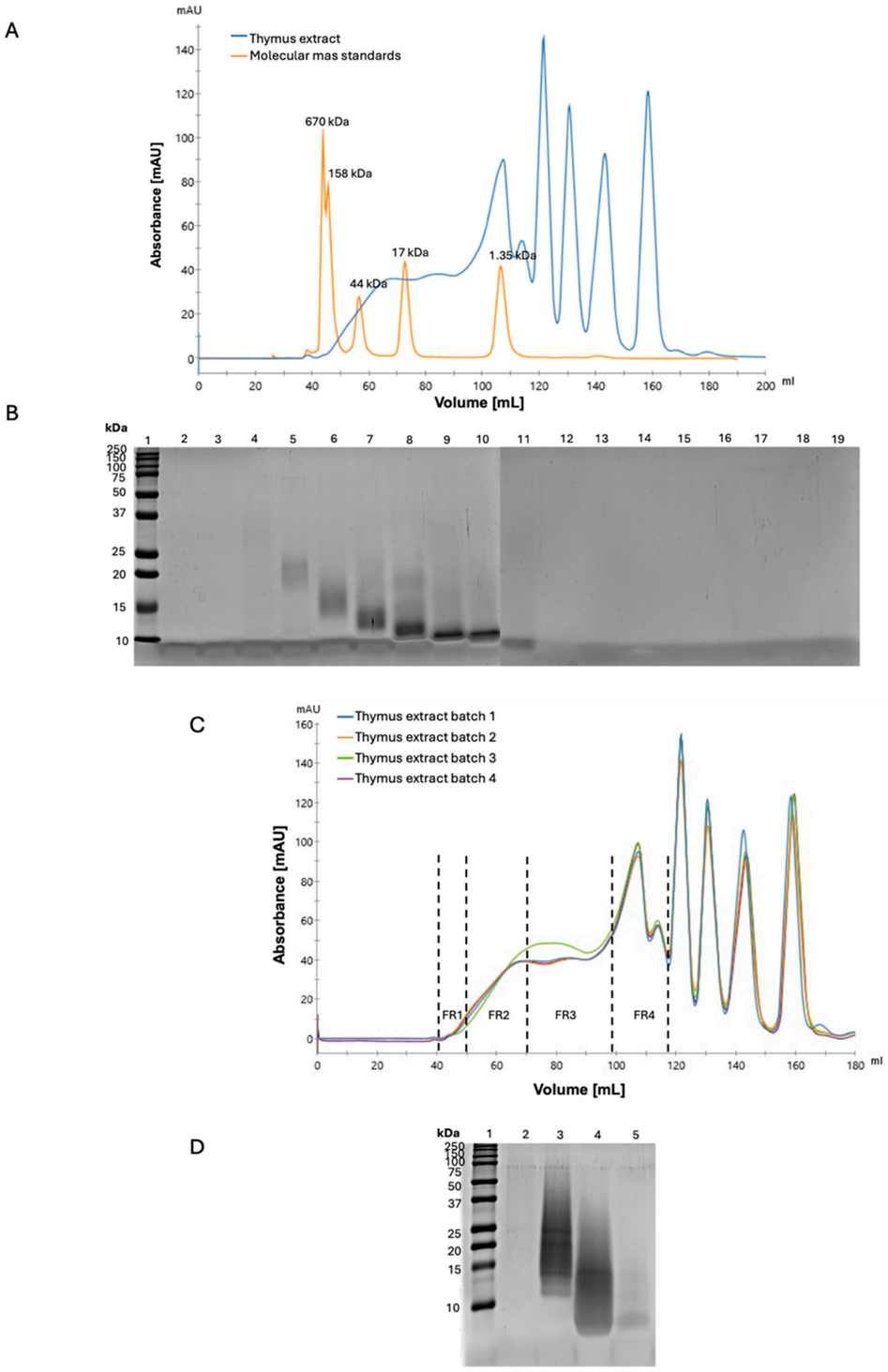


Figure 4. Chromatographic separation and electrophoretic analysis of thymus extract fractions. (**A**) The thymus extract solution was loaded on a HiLoad® 16/600 Superdex® 75 pg column calibrated using a mixture of standard compounds with a defined molecular-mass within 1.35–670 kDa. (**B**) The material eluted at 26 mL (lane 2), 32 mL (lane 3), 38 mL (lane 4), 44 mL (lane 5), 50 mL (lane 6), 56 mL (lane 7), 62 mL (lane 8), 68 mL (lane 9), 74 mL (lane 10), 80 mL (lane 11), 86 mL (lane 12), 92 mL (lane 13), 98 mL (lane 14), 104 mL (lane 15), 110 mL (lane 16), 116 mL (lane 17), 122 mL (lane 18), and 128 mL (lane 19) was analyzed on 15% gel along with the molecular-mass standard (lane 1). (**C**) Four consecutive chromatographic runs of the same production batch were compared for stability of the elution profile. The material pooled into fractions FR1–FR4 (as indicated by dashed lines) was concentrated on Centricon filters and (**D**) analyzed by SDS-PAGE on 20% gel, lane 1: molecular mass standard; lane 2: FR1; lane 3: FR2; lane 4: FR3; lane 5: FR4.

We have also tested several commercially available ELISA kits from different vendors targeting thymosins of interest, including Tα1, Tβ4, and Tβ10. With this approach, conclusive, reliable, and repeatable results were only obtained for Tβ4. However, none of the tests were able to quantify samples of commercially acquired, pure, standard thymosin. In the Tβ4 ELISA the highest signal for the tested samples of bovine thymus extract was found in FR3 with 27.73 ng/mL (±6.23 ng/mL), followed by FR4 with 5.53 ng/mL (±3.79 ng/mL). The original thymus extract solution (10 mg/mL) showed a signal corresponding to 3.6 ng/mL of Tβ4 based on the ELISA calibration curve derived from the reference protein included in the ELISA kit. Several attempts to determine Tα1 and Tβ10 using the commercial ELISA kits of different origins generated no conclusive results. On the other hand, the untargeted mass spectrometry showed a very complex protein/peptide mixture (Tables S1 and S2); however, no hits for any type of thymosin (*Bos taurus* and *Mammalian* databases) were returned in the employed experimental settings.

To complement the characterization of the thymus extract, we employed the THP-1 cell line as a model of macrophages differentiated from human monocytes [31] to test the induction of cytokine secretion. The cells treated with varying concentrations (0.1–5.0 mg/mL) of thymus extract were tested for the secretion of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). In three independent experiments, the analyzed thymus extract sample induced the production of TNF-α and IL-1β in a concentration-dependent manner (Figure 5). The highest values obtained following cell stimulation with a concentration of 5 mg/mL were 2295.3 ± 1052.3 and 2237.3 ± 275.1 pg/mL for TNF-α and IL-1β, respectively. The results also indicated that the mean level of TNF-α was higher in media derived from cells stimulated with high concentrations of thymus extract compared to media derived from cells treated with bacterial LPS (10^3 EU/mL), which induced, on average, 1949.5 ± 1267.6 pg/mL of this cytokine. In addition, minimal impact on cell viability (8.5% decrease) was confirmed by MTT assay on the same THP-1 cells treated with the thymus extract at 0.1–5.0 mg/mL (Figure S1).

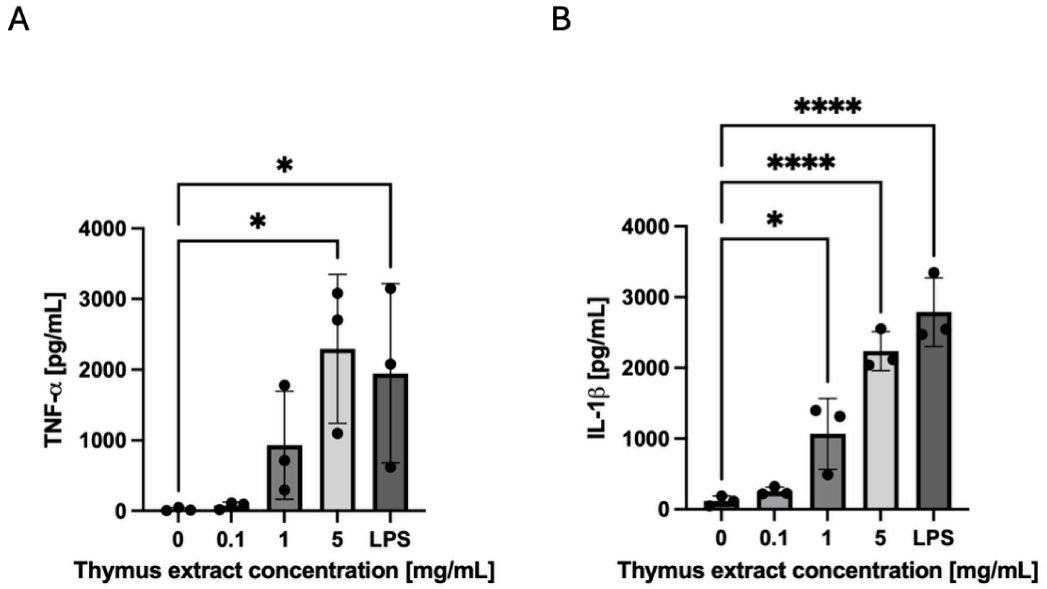


Figure 5. Effect of thymus extract on the level of (**A**) TNF-α and (**B**) IL-1β secreted into culture medium by THP-1-derived macrophages. The cells were incubated for 72 h with different concentrations of thymus extract (0–5.0 mg/mL) and compared with cells incubated in the presence of bacterial LPS (10^3 EU/mL). Ordinary one-way ANOVA analysis was performed, where **** indicates $p < 0.0001$, * indicates $p < 0.05$, and $n = 3$.

3. Discussion

Bovine thymus extract is a promising natural ingredient for food applications, but its variable composition makes standardization and quality control difficult. Accordingly, quality control should rely on a multi-level analytical approach that combines compositional, molecular, physicochemical, and biological evaluation, because complex extracts cannot be adequately described by a single readout [32–34].

The present study provides an initial compositional and biological characterization of a commercially available bovine thymus extract, showing a substantial protein fraction, measurable reducing carbohydrates, and a low lipid fraction, while electrophoretic and size-exclusion analyses indicate a heterogeneous molecular profile with many low-molecular-mass components similarly to previous reports [25,35]. These complementary techniques capture different but related aspects of the sample: electrophoresis reflects species detectable under denaturing and reducing conditions, whereas size-exclusion chromatography reflects apparent hydrodynamic size distribution under native conditions. This distinction is important when characterizing complex animal-derived ingredients, for which total protein content cannot adequately describe molecular composition or structural heterogeneity. Similar variability in the physicochemical properties and molecular-mass distributions of bovine thymic peptide preparations obtained using different isolation procedures was previously reported by Besman et al. [25]. Together, these observations support the use of multiple analytical techniques rather than reliance on a single compositional measurement, especially when the goal is to interpret a complex extract in a way that is comparable across preparations.

An important methodological observation concerned protein quantification. The Kjeldahl method indicated a relatively high protein content, in contrast to the routinely used Bradford assay in our laboratory [36], which did not produce reliable results. This likely reflects the assay's dependence on interactions with proteins rich in basic amino acids and its limited sensitivity toward peptides and proteins with molecular masses below 3 kDa [37–39]. Despite electrophoresis demonstrating the presence of proteins in the 10–20 kDa range, the abundance of smaller peptides may have interfered with Bradford measurements, as the assay is driven mainly by interactions with basic and aromatic residues. Thus, samples enriched in short peptides or proteins with an unfavorable amino-acid profile can give underestimated or otherwise unreliable values [40]. This interpretation fits the present material, because the extract also showed a heterogeneous molecular profile with a substantial low-molecular-mass fraction, which would be expected to affect Bradford readout more strongly than Kjeldahl-based protein estimates [41]. Taken together, the results suggest that the higher Kjeldahl value likely reflects the broader nitrogen-containing fraction, while the lower Bradford signal more narrowly reflects the subset of proteins and peptides that respond efficiently to the dye. The close agreement between NanoDrop (280 nm) and Kjeldahl supports its use as a practical preliminary or routine screen for thymus extracts, but not as a universal replacement for reference protein determination [42,43]. More broadly, the extract's heterogeneous molecular profile and the known variability of thymic peptide preparations across isolation procedures show why total protein content alone cannot adequately describe molecular composition or structural heterogeneity. Together, these observations support using multiple analytical techniques rather than relying on a single compositional measurement, especially when characterizing complex animal-derived ingredients.

The chromatographic profiles observed across the four runs examined in the present study were comparable (Figure 4C). This observation supports further investigation of size-exclusion chromatography as a potential tool for comparing batches from the same product source [44]. Nevertheless, the limited number of batches does not establish the extent of variability across manufacturers, raw-material sources, or production processes. The

chromatographic profile and reference molecular standards (Figure 4A) should therefore be regarded as a candidate analytical parameter rather than a validated specification. Further studies should establish method reproducibility, identify informative profile characteristics including peak integration and quantification, and define acceptable ranges of and the extent of variation across independent batches, manufacturers, raw-material sources, and production processes.

The observed discrepancy in chromatographic and electrophoretic migration of the compounds present in the separated thymus extract might be explained by the methodological conditions [45], i.e., conditions allowing for the retention of native aggregates (Figure 4A) or reducing conditions, resulting in dissociation of aggregates into single molecules that migrate on the gel in the form of low-molecular-weight peptides (Figure 4B). Since the compounds eluted from the column with a volume higher than 80 mL (molecules of molecular mass <1.35 kDa) were not visible on SDS-PAGE, fractionation and characterization, potentially involving mass spectrometry and other suitable analytical techniques, will be necessary to determine whether these components represent short peptides or non-protein compounds [46,47]. Such information would help clarify the molecular complexity of the extract and improve the interpretation of its chromatographic profile.

Spectroscopic analysis provided an additional layer of information about the physicochemical characteristics of the preparation. Absorbance and fluorescence analyses are suitable methods for the characterization of dairy-derived food and food supplement ingredients because they can sensitively capture compositional and structural changes in complex matrices [48]. It can be considered when tracking the stability of the biological components of thymus tissue preparations, especially by monitoring changes in characteristic absorbance maximum seen at 360 nm (Figure 2A) and fluorescence emission at 460 nm observed under excitation at 360 nm, although this link is still an inference from the spectral behavior rather than a thymus-specific validation of stability [45]. Since protein-derived material, especially collagen, was identified as the most abundant component of thymus extract (Tables S1 and S2), it is not surprising to observe the absorbance maximum at 280 nm and characteristic fluorescence emission at ~460 nm (Ex = 360 nm) resulting from tryptophan residues [49] like in other animal proteins [50]. However, the observed intensity of the non-tryptophan fluorescence beyond 440 nm visible in the fluorescence spectrum of the thymus extract should be considered for monitoring. It includes numerous posttranslational protein modifications, like different advanced glycation end-products [51] and kynurenine-derived modifications [52,53]. Before their application in routine quality assessment, the specificity, sensitivity, and robustness of these spectral parameters should be investigated, together with their relationship with processing conditions, storage stability, and independently characterized chemical components.

The analysis of the thymus extract also generated significant difficulties in immunochemical analyses used within this study, namely in Western blot and ELISA. We tested several antibodies and commercially available ELISAs. The only assay that showed reactivity with the analyzed extract was directed against Tβ4. However, immunoreactivity attributed to thymosin β4 should be distinguished from definitive molecular identification [54], especially because the assays for other thymosins, including Tα1 and Tβ10, were negative under the conditions used. Therefore, as the immunoassay-based detection of thymosins was not sufficiently validated under the present experimental conditions, it should be interpreted with caution. Further analyses require more optimal immunochemical tools [55,56].

The extensive protein fragmentation occurring during the extraction process may eliminate epitopes required for recognition by antibodies used in commercial ELISA kits [56]. Notably, none of the tested assays detected commercially available thymosin reference proteins, regardless of whether they were synthetic or tissue-derived. It therefore remains

unclear whether the negative results reflect the absence of target analytes or inadequate antibody recognition. Potential explanations include epitope masking, degradation, or aggregation of the reference peptides [36,57–59]. Furthermore, limited information provided by manufacturers hindered the evaluation of assay suitability.

In addition to compositional and physicochemical characterization, the present study explored the potential contribution of a cell-based assay as a complementary source of biological information. Regardless of the precise identification of the complete protein composition, the tested thymus extract induced cytokine (TNF-α and IL-1β) secretion in well-accepted THP-1-derived macrophage model cells [31] in vitro (Figure 5). Besman et al. [25] reported modulation of NF-κB reporter activity by other bovine thymic peptide preparations in THP-1 cells. Although both studies investigate biological responses to thymus-derived materials, the different preparations and endpoints preclude direct comparison. NF-κB reporter activity and cytokine secretion are distinct readouts and should not be interpreted as interchangeable evidence of a common mechanism. The findings instead support further investigation of biological assays as complementary tools for characterization. The use of cell-based assays for quality assessment would require additional validation, including assessment of reproducibility, appropriate controls, and the ability to distinguish relevant differences between preparations. At present, the cytokine response observed here is best regarded as an exploratory biological characteristic of the tested extract rather than a validated indicator of product quality or efficacy [60].

Finally, the assessment of animal-derived ingredients intended for food applications must also address microbiological and other safety-related characteristics. Although the present study focused primarily on compositional, physicochemical, and exploratory biological analyses, in food and supplement contexts, safety frameworks consistently emphasize that microbial contamination testing is a necessary part of quality control, especially for materials of animal origin and other complex natural ingredients [61,62]. Studies of dietary supplements and animal-derived foods show that microbiological monitoring commonly includes indicators such as total aerobic microbial count, yeast and mold, Enterobacteriaceae or coliforms, and specified pathogens including *Salmonella*, *E. coli*, and *S. aureus* [62]. Finally, the assessment of animal-derived ingredients intended for food applications must also address microbiological and other safety-related characteristics. Although the present study focused primarily on compositional, physicochemical, and exploratory biological analyses, these parameters alone cannot establish the overall safety of the preparation, and testing for microbial contamination should not be omitted from the QC process [63].

4. Materials and Methods

4.1. Chemicals

All chemicals, acids, bases or buffers were purchased from Sigma Aldrich (St. Louis, IL, USA) unless otherwise stated.

4.2. Biochemical Composition Analysis of Thymus Extract

Protein content of bovine thymus extract (Biofac A/S, Kastrup, Denmark) was determined by the standard Kjeldahl method [64].

In addition, an alternative method to determine protein concentration was employed based on absorbance measurements at 280 nm using NanoDrop One (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The content of reducing carbohydrates in the thymus extract sample was assessed using the Luff–Schorl method [65].

Lipid content was determined according to an established protocol [66], including hexane extraction carried out for up to 5 h, following the Soxhlet test. Lipid content was calculated as a (%) of the initial sample.

4.3. Absorbance and Fluorescence Analyses

The absorbance spectrum of 1 mg/mL thymus extract solution in milliQ water was recorded within the wavelength range of 230–750 nm with 10 nm intervals using SpectraMax iD3 (Molecular Devices, San Jose, CA, USA). The same device was used for the analysis of fluorescence properties of the thymus extract solution dissolved in the MilliQ water to a concentration of 1 mg/mL. The fluorescence emission was recorded within 400–750 nm at 10 nm intervals upon exposure to light at a wavelength of 360 nm. Each analysis was performed twice ($n = 2$).

4.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein pattern of thymus extract was assessed using SDS polyacrylamide gel electrophoresis according to the standard Laemmli method [67] described in Supplementary Materials S1.2.

4.5. Size-Exclusion Chromatography

Aqueous solution of thymus extract was analyzed on HiLoad Superdex 75 16/600 column (Cytiva, Uppsala, Sweden) by loading a 2 mL sample of 50 mg/mL solution in phosphate-buffered saline, pH 7.4, as a mobile phase. A flow rate of 1 mL/min was applied during a separation employing the ÄKTA GO system (Cytiva, Marlborough, MA, USA). Absorbance at 215 nm, characteristic for molecules containing peptide bonds, was recorded to monitor eluted molecules with high sensitivity. The separated mixture was abundant in short peptides, with some potentially lacking tryptophan, thus making them undetectable at a wavelength of 280 nm.

Gel filtration standard (Bio-Rad Laboratories, Hercules, CA, USA), containing bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.35 kDa), was used to estimate the protein mass of the eluted molecules based on the elution volume. The material was recovered by collecting 3.0 mL samples within the elution volume range of 40 to 115 mL and pooled into 4 fractions (44–49 mL—FR1; 50–70 mL—FR2; 71–97 mL—FR3; 98–115 mL—FR4). Next, the pooled fractions were concentrated ten-fold using a Centricon Plus-70 Centrifugal Filter, 3 kDa cutoff (Merck KGaA, Darmstadt, Germany), and stored at −20 °C until further analysis.

4.6. ELISA for the Determination of Thymosins and Cytokines

The thymosin β4 (Tβ4) level in thymus extract was quantified using an ELISA kit (G-Bioscience, St. Louis, IL, USA). The commercial ELISAs for Tβ10, Tα1, and pro-Tα1, claimed to have human or bovine specificity and provided by several vendors (BlueGene (Shanghai, China), G-Bioscience (St. Louis, MO, USA), Assay Genie/ELISA Genie (Dublin, Ireland), CloudClone (Wuhan, Chin), MyBioSource (San Diego, CA, USA), Cusabio (Wuhan, China)), were used in this study.

Cytokine quantification (tumor necrosis factor alpha and interleukin 1 beta) was performed with Quantikine™ ELISA (R&D Systems, Minneapolis, MN, USA). The commercial assays were performed according to the manufacturer's instructions with details described in Supplementary Materials S1.3.

4.7. In Vitro Bioactivity Assay

The human monocytic THP-1 TIB-202 cell line (American Tissue Culture Collection, Manassas, VA, USA) was used to assess the biological activity of the thymus extract. Cells were cultured in RPMI 1640 medium (PanBiotech GmbH, Aidenbach, Germany) supplemented with 10% heat-inactivated FBS (*v*/*v*) (PanBiotech GmbH, Aidenbach, Germany) at 37 °C in a 5% CO2 atmosphere. When required, the culture density was assessed. Next, cells were centrifuged at 200× *g*, 5 min, and room temp. and reconstituted in complete growth medium containing 320 nM PMA (phorbol 12-myristate 13-acetate) (InvivoGen, San Diego, CA, USA). Next, cells were seeded on 6-well plates at a density of 1.4×10^6 cells/well and incubated for 48 h at 37 °C, in 5% CO2, to allow differentiation toward macrophages. On the day of the experiment, medium from each well was discarded, and the cells were washed with the culture medium warmed to 37 °C. Dilutions of thymus extract in complete growth medium were prepared in concentrations of 0.1, 1.0, and 5.0 mg/mL and applied to macrophage cultures (2.8 mL per well). Lipopolysaccharide (LPS, *E. coli* 0111:B4) (InvivoGen, San Diego, CA, USA) solution in growth medium (10^3 EU/mL) was used as the positive control of macrophage stimulation, and complete growth medium was used as the culture control. Experimental plates containing cells were further incubated for 72 h (37 °C, 5% CO2). Finally, the growth medium was collected and centrifuged at 4600× *g* for 15 min to remove cell debris. The supernatant was aliquoted and stored at −80 °C for further analysis of TNF-α and IL-1β levels by ELISA.

5. Conclusions

Due to the growing number and variety of food products and dietary supplements containing thymus extracts on the market, control tests are needed to ensure product quality, safety, and consistency. As shown in the present study, selecting appropriate analytical methods for a complex natural matrix such as bovine thymus extract is challenging, so the characterization strategy should remain complementary and combine basic compositional measurements, molecular profiling, selected constituent analysis, and exploratory biological testing. However, these parameters should not yet be regarded as a complete or validated quality-control standard, and further work is needed to examine more batches and sources, validate the analytical methods, and identify the most informative measures of product consistency. The findings also indicate that new immunochemical tools, including specific antibodies and analytical methods, are still needed to improve detection and quantification of thymosins in biological samples and commercial products. At the same time, microbiological quality and other relevant safety requirements should be assessed separately, since total microbial counts, specified pathogens, endotoxin levels, residual DNA, and species authentication may all contribute additional information that is not captured by the methods used here. In addition, the molecular identity of several chromatographic and spectroscopic signals remains unresolved, and the immunochemical and cell-based results were obtained under limited conditions, so they should not be extrapolated to human health effects or product efficacy. Overall, these results provide a methodological basis for future studies using multiple batches, standardized reference materials, validated procedures, and correlations between physicochemical characteristics and biological responses, with the longer-term goal of building a more reliable quality-assessment framework for bovine thymus extract.

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