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Research article

Development of a specific IgY-based ELISA for prothymosin alpha, a bioactive polypeptide with diagnostic and therapeutic potential☆

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ABSTRACT

Prothymosin alpha (ProTα) is a highly conserved polypeptide (109 amino acids in humans) with diagnostic and therapeutic potential; ProTα exerts intra- and extra-cellular biological functions associated with cell proliferation, apoptosis and immune regulation, while it has been suggested to act as a damage-associated molecular pattern (DAMP) or alarmin. In this work, chicken polyclonal anti-ProTα antibodies that had been developed several years ago were immunochemically evaluated and proven to retain immunoreactivity for ProTα, with remarkable thermal and pH stability. Moreover, the antibodies showed practically no cross-reactivity with a series of ProTα-fragments, eventually intracellularly produced -such as ProTα[1-28] (also known as Tα1) and ProTα[100-109], which exert *per se* biological activity and might be present in biological samples along with the intact molecule, being therefore highly specific for whole-length ProTα. Based on the above antibodies (IgYs-3*e*), a highly specific competitive ProTα-ELISA with well-studied analytical characteristics (intra- and inter-assay CVs: ≤5% and ≤12%, respectively, limit of detection: 2.1 ng/mL, recovery: 88–104%) was developed. The new ProTα-ELISA was applied to the analysis of supernatants of HeLa cells driven to necrosis; intact ProTα was measured in cell culture supernatants, at levels that seemed to depend on % cell necrosis.

1. Introduction

Prothymosin alpha (ProTα) is a polypeptide highly conserved and widely distributed in mammalian cells and tissues. The biological role of ProTα is dual, associated with promotion of cell proliferation and inhibition of apoptosis intracellularly and with pleiotropic regulation of immune responses extracellularly; due to its involvement in pivotal intracellular and extracellular functions, human ProTα has been considered as target for developing new diagnostic and/or therapeutic strategies, mainly for immune-related diseases and cancer [1, 2]. The dual functional profile of ProTα, in and out of cells, is a characteristic feature shared among a wide family of biomolecules termed damage-associated molecular patterns (DAMPs) or alarmins; under homeostatic conditions these molecules are confined within the cell, but, under survival-threatening circumstances, they are released from dying cells, usually through mechanisms that are not fully clarified, communicating in this way a danger/alarm signal to the immune system [3, 4]. Although ProTα has been suggested to act as an alarmin [1], much more data should be gathered elucidating the precise conditions and the mechanisms through which ProTα (either intact or fragments thereof) may be driven out of cells.

According to literature reports, ProTα may be processed by intracellular enzymes to shorter peptidyl fragments (Fig. 1), some of which have shown biological activity *per se*, mainly as immune-response modifiers; these fragments include the N-terminal 28-mer peptide ProTα[1-28], also known as thymosin alpha 1 (Tα1), which may be formed after proteolytic cleavage of ProTα by a lysosomal asparaginyl endopeptidase [5], and the C-terminal decapeptide ProTα[100-109], containing a sequence necessary for transportation of the polypeptide to the cell nucleus (Nuclear Localization Signal, NLS) [6], which may be produced after cleavage of

☆ This article is dedicated to the memory of Dr. Gregory P. Evangelatos, who first developed antibodies Y against highly conserved alpha- and beta- thymosins.

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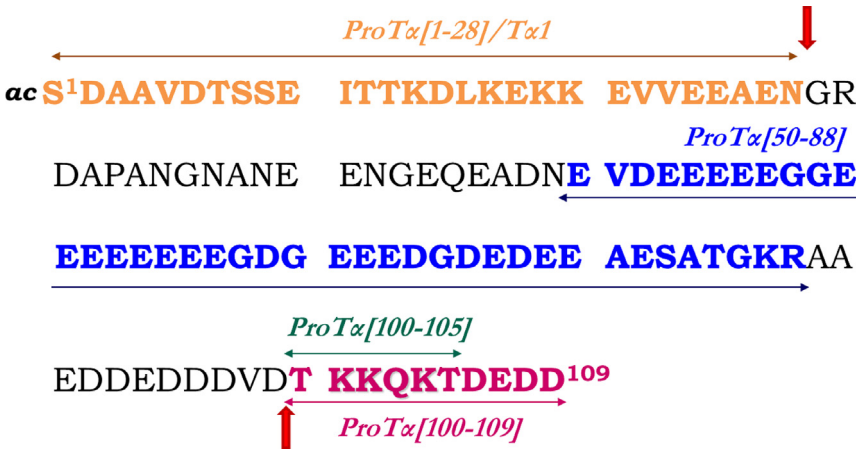


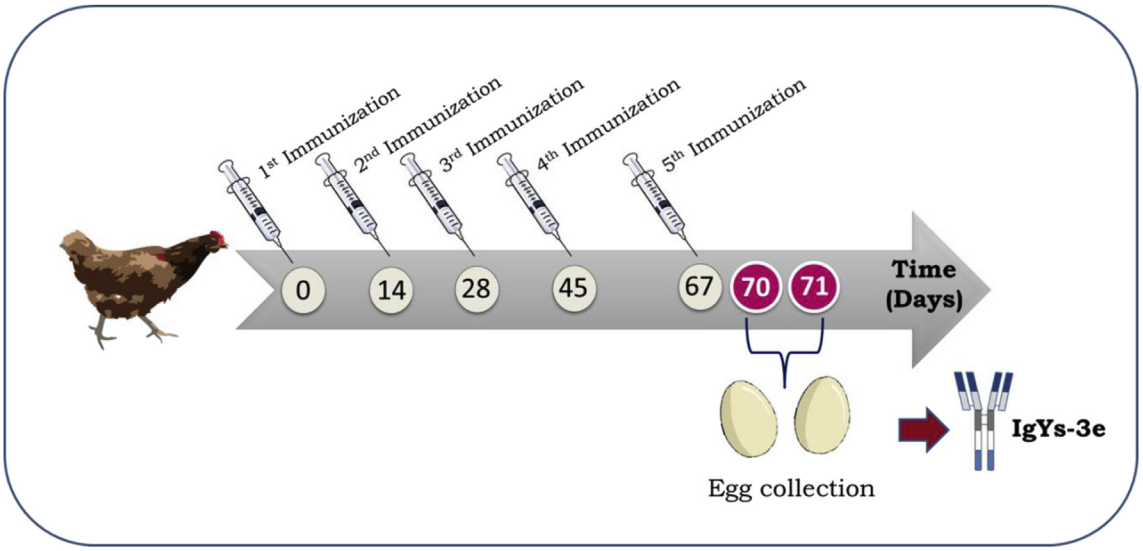
Fig. 1. Primary structure of human prothymosin alpha (isoform 2, NCBI reference sequence: NP_002814.3). Amino acid sequences of the fragments [1-28] (orange), [50-88] (blue), [100-105] (green), and [100-109] (magenda) are marked with horizontal arrows. Vertical arrows indicate the so far reported proteolytic cleavage sites of the molecule.

ProTα by caspase-3 in cells undergoing apoptosis [7]. As obvious, distinguishing between parental ProTα and its bioactive fragments, especially those that may be concomitantly present in biological samples, can provide valuable information on the precise biological function(s) of the polypeptide under normal and pathological conditions. Such discriminating ability may be achieved with immunochemical assays based on antibodies highly specific for intact ProTα.

Antibodies G (IgGs) for ProTα raised in mammals are often broadly specific and of low affinity/avidity/titer [8]. The evolutionary high conservation of the polypeptide across mammalian species may possibly explain its poor immunogenicity [9]. Nevertheless, a series of IgGs have been successfully raised against fragments of ProTα, mainly from the N-terminal and C-terminal regions, showing adequately high titers, excellent specificity and no cross-reactivity with other cellular proteins, including members of the beta-thymosin family [10, 11]. However, these antibodies recognize, apart from intact ProTα, the corresponding N- or C-terminal fragments of the polypeptide used for immunization; thus, they cannot be considered to be solely specific for intact ProTα.

During the last decades, the approach of developing antibodies against highly conserved mammalian proteins in avian species -also known as antibodies Y or IgYs [12]- has been widely used, since these antibodies are expected to exhibit superior affinity characteristics due to the phylogenetic differences between mammalian and avian classes [13]. Moreover, IgYs can be easily isolated at large quantities from the yolk of "immune" eggs (*i.e.* eggs obtained from immunized chickens), instead of "immune" serum (*i.e.* serum obtained from immunized animals), while a series of other advantages have also been reported [14]. In this context, polyclonal IgYs for ProTα were previously developed in chickens and isolated from the immune egg yolk by our team [10, 15].

In the present work, we evaluated a preparation of previously developed IgYs, specified as IgYs-3*e*. IgYs-3*e* had been raised against a conjugate of ProTα with KLH prepared *via* glutaraldehyde (ProTα/KLH) as previously described [15], isolated from immune eggs (collected on two consecutive days after the fifth immunization, Scheme 1) *via* the acidified water dilution method as previously described [15] and then stored as a lyophilized powder (-30 °C) for several years. IgYs-3*e* were evaluated herein for the first time in terms of their purity, thermal and pH stability, titer and cross-reactivity with a series of synthetic ProTα fragments; moreover, they were applied to the development of a competitive ProTα-ELISA specific for determining intact ProTα in biological samples. The newly developed ProTα-ELISA was thoroughly validated in terms of assay characteristics and finally applied to the analysis of culture supernatants of HeLa cells *in vitro* led to necrosis.



Scheme 1. Schematic representation of the immunization protocol leading to production of polyclonal antibodies Y under evaluation (IgYs-3*e*).

2. Materials and Methods

2.1. Peptides and commercial antibodies

ProTα (isolated from bovine thymus) was a product of Thymoorgan GmbH. Bovine ProTα was used as a low-cost, fully compatible substitute for human ProTα, since both peptides consist of 109 amino acids differing just in two amino acid residues, at positions 31 and 83 [9,15]. The fragments ProTα[1-28], ProTα[50-88], ProTα[100-105], and ProTα [100-109] (Fig. 1) were in-house synthesized according to the Fmoc/*t*Bu solid-phase peptide synthesis strategy and purified with semi-preparative reversed phase HPLC, following protocols previously described by our group [16, 17]. Yield of peptide synthesis was often low, due to "difficult peptide sequences" existing especially in ProTα[50-88] [18]. Nevertheless, purity of the final products (after HPLC-purification) exceeded 95% and identity of the synthetic peptides was confirmed by ESI-MS analysis (data not shown).

Commercially available non-immune IgYs from pooled chicken serum (n-IgYs, product I-4881), enzyme-labeled anti-chicken antibody (rabbit anti-chicken IgY/HRP, product A-9046) and enzyme-labeled streptavidin (streptavidin/HRP, product S-5512) were purchased from Sigma. Biotinylated anti-chicken antibody (anti-chicken IgY/biotin) was a product of Chemicon.

2.2. Other reagents, solutions and instruments

Salts, other chemicals and BSA were purchased from Sigma or Merck. All solutions were prepared in ultrapure water.

SDS-PAGE: SDS-loading buffer: 0.06 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 1% β-mercaptoethanol, 0.1 M dithiothreitol (DTT); running buffer: 3 g/L Tris base, 14.4 g/L glycine, 1 g/L SDS; coomassie staining solution: 2% (w/v) coomassie brilliant blue R-250 in a MeOH/H_2O/glacial acetic acid (4.5/4.5/1 v/v/v) solution. The experiments were performed with the Mini-PROTEAN Tetra Cell (BioRad) electrophoresis device.

ELISA: High-binding 96-well ELISA plates (Costar microplates, #3590) were used for the ELISAs along with the following solutions: coating solution 1: phosphate buffered saline 0.01 M, pH 7.4 (PBS); coating solution 2: citrate buffer 0.01 M, pH 5.0; blocking solution 1: PBS with 2% (w/v) BSA; blocking solution 2: PBS with 6% (w/v) non-fat powdered milk; washing solution: PBS with 0.05 % (v/v) Tween-20 (PBS-T); diluting solution 1: PBS-T with 0.2% (w/v) BSA; diluting solution 2: phosphate buffered saline 0.001 M, pH 7.4, with 0.05% (v/v) Tween-20 and 0.5% (w/v) BSA; chromogenic solution 1: ABTS (1 mg/mL)/H_2O_2 (0.003%) in citrate/phosphate buffer 0.1 M, pH 4.4. The plates were washed in an ELISA plate washer (DIA Source) and absorbance values (A_{405} nm) were measured in a microtiter plate reader (Seak, Model Sirio S).

Dot-Blot: washing solution: PBS with 0.05 % (v/v) Tween-20 (PBS-T);

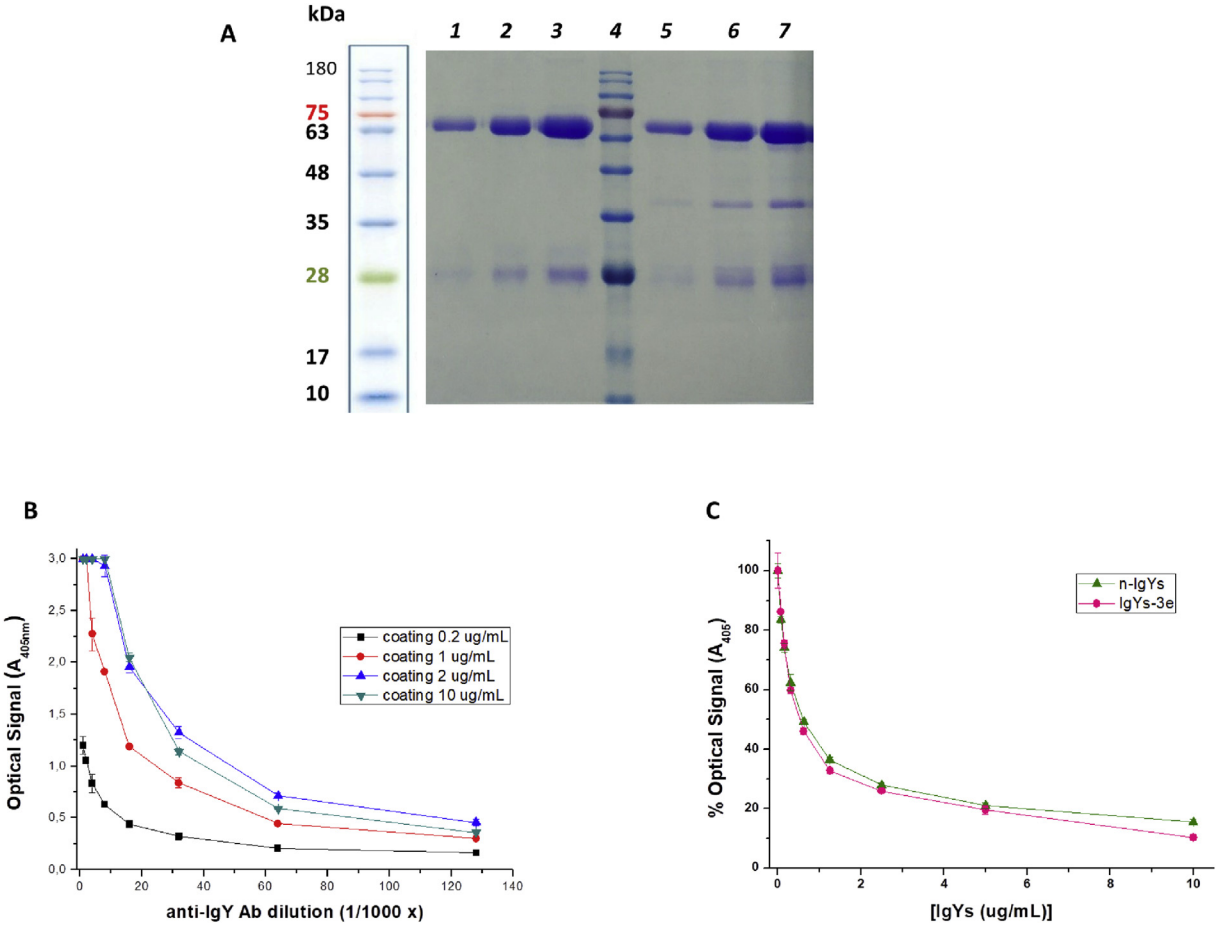


Fig. 2. IgY purity (A): IgYs-3*e* were analyzed with SDS-PAGE, on a 12% polyacrylamide gel with coomassie brilliant blue R-250 staining. Lanes 1-3: commercially available n-IgYs (2.5, 5.0 and 7.5 μg, respectively) as control; lane 4: molecular weight markers; lanes 5-7: IgYs-3*e* (2.5, 5.0 and 7.5 μg, respectively). IgY measurement (B, C): Titration IgY-ELISA (B): Titer curves obtained in the presence of increasing concentrations of n-IgYs (0.2–10 μg/mL) as coating antigen. A coating concentration of 2 μg/mL and a 1:32,000 dilution of the commercially available, enzyme-labeled anti-chicken antibody were the conditions selected for setting-up the competitive IgY-ELISA finally applied to the analysis of IgYs-3*e*. Competitive IgY-ELISA (C): The displacement curves obtained with increasing concentrations of n-IgYs, *i.e.* commercially available non-immune chicken IgYs, and with increasing concentrations of IgYs-3*e* are shown.

blocking solution 3: PBS-T with 3% (w/v) BSA; diluting solution 1: PBS-T with 0.2% (w/v) BSA; chromogenic solution 2: DAB (0.7 mg/mL)/H_2O_2 (0.03%) in PBS. Nitrocellulose membranes (Protran BA 83, Schleicher & Schuell) were used.

2.3. Evaluation of IgYs-3e

2.3.1. IgY purity: SDS-PAGE analysis

IgYs-3*e* along with commercially available n-IgYs samples (20 μL each) containing 2.5, 5.0 and 7.5 μg of protein, were treated for 5 min at 95 °C in SDS-loading buffer and then subjected to SDS-PAGE on 12% polyacrylamide gel slabs. Gels were finally stained with coomassie brilliant blue R-250 (Fig. 2A).

2.3.2. IgY measurement: in-house developed competitive IgY-ELISA

IgY concentration was measured in an in-house developed IgY-ELISA, based on commercially available n-IgYs and enzyme-labeled anti-chicken antibody. Before use, IgYs-3*e* along with n-IgYs were reconstituted in a 1:1 (v/v) mixture of PBS: glycerol.

Protocol for titration IgY-ELISA: ELISA microwells were coated with n-IgYs (0.2, 1, 2, or 10 μg/mL in coating solution 1; 100 μL/well) and left overnight at 4 °C. The following day, after washing with PBS (x2), wells were blocked with blocking solution 1 (200 μL/well) for 1 h at room temperature (RT) and washed again with washing solution (x3). Next, rabbit anti-chicken IgY/HRP (1:1,000–1:128,000 in diluting solution 1; 100 μL/well) was added to the wells and incubated for 90 min at 37 °C. Then, wells were washed with washing solution (x3) and incubated with chromogenic solution 1 (100 μL/well; 30 min; RT). Finally, the absorbance was measured at 405 nm and titration curves were plotted using Origin Pro 8.0 (Fig. 2B).

Protocol for competitive IgY-ELISA: Based on the results from titration experiments, ELISA microwells were coated with n-IgYs (2 μg/mL in coating solution 1; 100 μL/well) and left overnight at 4 °C. The following day, wells were washed, blocked and washed again as described above. Then, n-IgYs or IgYs-3*e* at increasing concentrations (0.078–10 μg/mL in diluting solution 1; 50 μL/well) and rabbit anti-chicken IgY/HRP (1:16,000 in diluting solution 1; 50 μL/well) were added to the wells and incubated for 90 min, at 37 °C. Washing, incubation with the chromogenic solution 1, and absorbance measurement were performed as above; finally, displacement curves were plotted using Origin Pro 8.0 (Fig. 2C).

2.3.3. IgY titer and stability: in-house developed titration ProTα-ELISA

2.3.3.1. Titer of IgYs-3e. ELISA microwells were coated with ProTα (0.1 μg/mL in coating solution 2; 100 μL/well) and left overnight at 4 °C. The following day, wells were washed, blocked and washed again as described in 2.3.2. Then, wells were incubated with IgYs-3*e* at increasing concentrations (10–200 μg/mL in diluting solution 1) for 2 h at 37 °C, washed with washing solution (x3), incubated with rabbit anti-chicken IgY/HRP (1:2,000 in diluting solution 1) for 90 min, at 37 °C, washed again with washing solution (x3), and incubated with chromogenic solution 1 (100 μL/well; 30 min; RT). Finally, absorbance was measured at 405 nm and titration curves were plotted. In parallel, n-IgYs were used as a negative control.

2.3.3.2. Thermal stability of IgYs-3e. A series of stock solutions of IgYs-3*e* (2 mg/mL in PBS) were prepared and left for 90 min in a water bath, at 37, 50, 60, 70, 75 and 80 °C, respectively. Then, they were allowed to reach RT, diluted with PBS to three different concentrations, *i.e.* 10, 25 and 50 μg/mL, and analyzed in the titration ProTα-ELISA, in comparison with untreated IgYs-3*e* of the same concentration (10, 25 and 50 μg/mL). No precipitate formation could be observed in any of the IgYs-3*e* solutions during their treatment at different temperatures. Experimental conditions selected were based on similar previous studies with other immunoglobulins Y [19, 20].

2.3.3.3. pH stability of IgYs-3e. A series of stock solutions of IgYs-3*e* (2 mg/mL in PBS) were prepared, their pH was adjusted to 3–10 with 1N HCl or 1N NaOH and then they were left for 90 min in a water bath at 37 °C. Afterwards, the IgYs-3*e* solutions were allowed to reach RT, diluted with PBS to three different concentrations, *i.e.* 10, 25 and 50 μg/mL and analyzed in the titration ProTα-ELISA, in comparison with untreated IgYs-3*e* of the same concentration (10, 25 and 50 μg/mL). No precipitate formation could be observed in any of the IgYs-3*e* solutions during their treatment at different pH conditions. Experimental conditions selected were based on similar previous studies with other immunoglobulins Y [19, 20].

2.3.4. IgY specificity for intact ProTα: Dot-Blot analysis

Serially diluted solutions (10 μL) of intact ProTα and its synthetic fragments ProTα[1-28] (Tα1), ProTα[50-88], and ProTα[100-109] (1 mg/mL, 0.2 mg/mL, 0.05 mg/mL and 0.01 mg/mL) were separately spotted onto a nitrocellulose membrane. The membrane was blocked with blocking solution 3 for 1 h at RT on a shaker, washed twice (5 min each) with PBS and then incubated for 2 h at RT on a shaker with IgYs-3*e*, 50 μg/mL in diluting solution 1. The membrane was washed three times (5 min each) with PBS-T and incubated for 90 min at RT on a shaker with a solution of rabbit anti-chicken IgY/HRP, diluted 1:750 in diluting solution 1. The membrane was washed three times (5 min each) with PBS-T and stained with the chromogenic solution 2.

2.4. Competitive ProTα-ELISA and sample analysis

2.4.1. Working protocol

ELISA microwells were coated with ProTα (0.1 μg/mL in coating solution 2; 100 μL/well) and left overnight at 4 °C. The following day, wells were washed with PBS (x2), blocked with blocking solution 2 (200 μL/well) for 1 h, at RT and washed again with washing solution (x3). Then, wells were incubated (2 h, 37 °C) with a 1:1 mixture of ProTα standard solutions (1-1,000 ng/mL in diluting solution 1; 50 μL/well) along with IgYs-3*e* (5 μg/mL in diluting solution 2; 50 μL/well), pre-incubated overnight at 4 °C. Afterwards, wells were washed (x3), incubated with biotinylated anti-chicken antibody (1:5,000, 90 min, 37 °C), washed again and incubated with streptavidin/HRP (200 ng/mL; 60 min, 37 °C). Following a last washing step (x3), chromogenic solution 1 (100 μL/well; 30 min, RT) was added, the absorbance read at 405 nm (A_{405}), and the standard curve plotted.

2.4.2. Specificity for intact ProTα

Specificity of the competitive ProTα-ELISA for intact ProTα was evaluated as follows: ELISA microwells were coated, blocked, and washed as described in 2.4.1. Then, the wells were incubated (2 h, 37 °C) with a 1:1 mixture of intact ProTα, or its synthetic fragments ProTα[1-28] (Tα1), ProTα[50-88], ProTα[100-109], and ProTα[100-105], at increasing concentrations (1-1,000 nM in diluting solution 1; 50 μL/well) along with IgYs-3*e* (5 μg/mL in diluting solution 2; 50 μL/well), pre-incubated overnight at 4 °C. Wells were washed, incubated with biotinylated anti-chicken antibody, streptavidin/HRP and chromogenic solution 1, as described in 2.4.1. Finally, the absorbance was measured at 405 nm and the displacement curves were plotted.

2.4.3. Cell culture and sample preparation

The human cell line HeLa (cervical adenocarcinoma) was obtained from the American Tissue Culture Collection (ATCC CCL-2). HeLa cells were maintained in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM Hepes, 5 μg/mL gentamycin, 10 U/mL penicillin, and 10 U/mL streptomycin (all from Lonza, Cologne, Germany) (thereafter referred to as complete medium) at 37°C, in a humidified 5% CO_2 incubator.

HeLa cells were led *in vitro* to cell death *via* a series of stress stimuli: temperature-induced stress by heating at 56 °C for 1 h, repetitive multiple (x4) cycles of freeze (-80 °C)/thaw (37 °C), serum starvation for 6 h,

or *via* exogenous drug administration, *i.e.*, 6 h incubation with 6 ng/mL TNF-α and 5 μg/mL emetine or 6 ng/mL TNF-α and 50 μg/mL actinomycin D (all from Sigma-Aldrich) diluted in serum-free medium. Selection of incubation times and concentrations of drug stimuli were determined from preliminary titration and kinetic studies (data not shown).

In detail, HeLa cells were seeded to 6-well plates (500,000 cells/well) and left to adhere overnight in complete medium. The following day, culture medium was discarded, cells were washed twice with Hank's balanced salt solution (HBSS, Lonza) followed by the addition of serum-free medium or serum-free medium supplemented with the above-mentioned drugs and further incubated for 6 h at 37 °C. For induction of temperature stress, after aspiration of culture medium and washing with HBSS, cells were harvested from the plates and re-suspended in serum-free medium followed by heating or freeze/thaw cycles at the indicated temperatures for the indicated time points. Control cells were detached and harvested from plates, washed twice with HBSS, and resuspended in FACS buffer (PBS with 0.5% (w/v) BSA). Culture supernatants were collected from all treatment conditions, clarified from cell debris by centrifugation (9,300 g for 10 min), a protease inhibitor cocktail (Complete® Mini, Roche Diagnostics GmbH, Manheim, Germany*)* was added (1 tablet per 10 mL supernatant) and stored at -80 °C until measurement for ProTα content with the optimized ProTα-ELISA. Cells from the same cultures were stained with propidium iodide (PI) (10 min, RT, in the dark) and cell viability was assessed by flow cytometry, using a FACSCanto II flow cytometer (Becton-Dickinson Biosciences, Erembodegem, Belgium) equipped with FACSDiva software (Becton-Dickinson Biosciences). The percentage of necrotic cells (PI positive, PI+) was calculated for each sample.

2.4.4. Assay characteristics

Matrix effect: To investigate possible effects of cell culture matrix on the assay results, ProTα solutions of known concentration (standard solutions) were prepared in diluting solution 1, plain RPMI or complete medium and applied to the working protocol. The corresponding standard curves were plotted and analysis of variance (ANOVA) was performed to assess any statistically significant differences due to matrix interference.

Recovery: HeLa supernatants were spiked with ProTα as follows: 7.5 μL of a ProTα solution in RPMI (2 μg/mL, 1 μg/mL, or 0.2 μg/mL) were added to 142.5 μL of the HeLa supernatant (spiked ProTα concentration: 100, 50 and 10 ng/mL). The samples were analyzed with the competitive ProTα-ELISA and the percent recovery was calculated as the ratio of the ProTα concentration determined to that theoretically expected x 100.

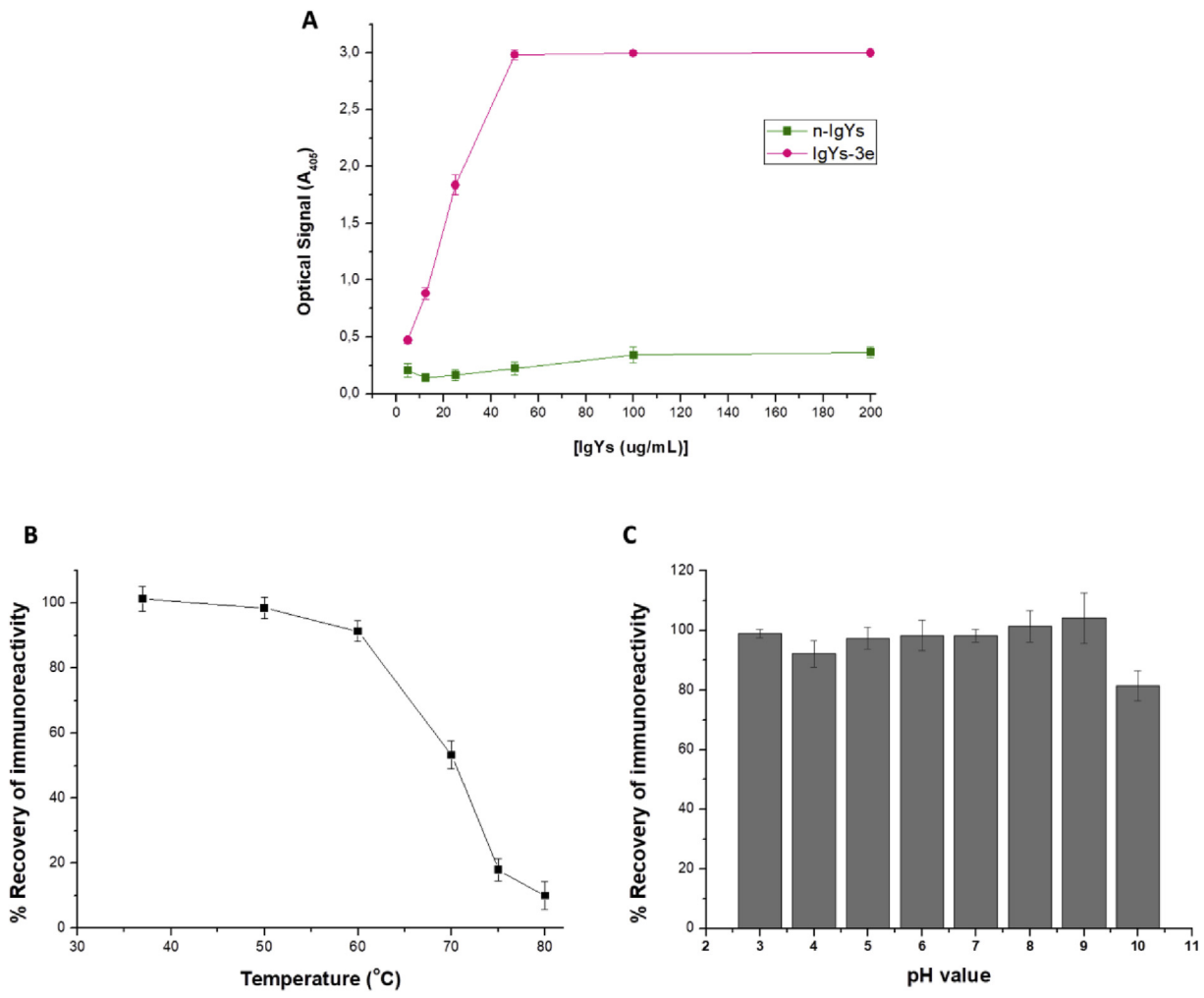


Fig. 3. IgY titer and stability: Titer curve (A) obtained with increasing concentrations of IgYs-3*e* in the titration ProTα-ELISA; commercially available non-immune chicken IgYs (n-IgYs) were used as negative control. Thermal stability (B) of IgYs-3*e* was assessed in the titration ProTα-ELISA and expressed as % immunoreactivity of IgYs-3*e* treated at various temperatures, in comparison with untreated IgYs-3*e* (100% immunoreactivity). pH stability (C) of IgYs-3*e* was assessed in the titration ProTα-ELISA and expressed as % immunoreactivity of IgYs-3*e* treated at various pH values, in comparison with untreated IgYs-3*e* (100% immunoreactivity). Treated and untreated IgYs-3*e* were used at three different concentrations (10, 25 and 50 μg/mL); the results shown in B and C correspond to 25 μg/mL. Mean values ± SDs from 3 experiments are shown (A, B, C).

The ProTα standard solutions in the recovery experiments were prepared in RPMI.

2.5. Statistical analysis

Data were analyzed with Origin Pro 8.0 and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Values in the standard curves represent mean of six replicates ± SD; for zero calibrator/blank, 10 replicates were included; the biological samples of unknown ProTα concentration were analyzed in duplicates.

3. Results

3.1. Evaluation of IgYs-3e

3.1.1. IgY purity

The purity of IgYs-3*e* was evaluated with SDS-PAGE analysis, as described in Materials and Methods. As revealed, two main bands were obtained, with molecular weights of ~63 and ~28 kDa, corresponding to the heavy and light chains of immunoglobulins Y, respectively (Fig. 2A). Additionally, one protein migrating at ~35 kDa co-existed with the IgYs; based on similar analyses previously carried out by our team that included standard proteomic technology, this band may be attributed to the egg yolk protein vitellogenin II precursor [15].

3.1.2. IgY measurement

Total IgY concentration in the lyophilized IgYs-3*e* preparation was evaluated with the in-house developed competitive IgY-ELISA. More specifically, a series of IgYs-3*e* samples were prepared by diluting a stock solution of IgYs-3*e* (2 mg of the in-house prepared lyophilized powder/mL) first 1:200 (10 μg of in-house prepared lyophilized powder/mL) and then 1:2, serially. The IgYs-3*e* samples were analyzed in the competitive IgY-ELISA; as revealed, the displacement curve thus obtained was almost superimposed to the curve obtained with the standard solutions of n-IgYs (Fig. 2C). Thus, the lyophilized IgYs-3*e* preparation seems to consist mainly of IgY immunoglobulins, while the protein-band shown in the SDS-PAGE analysis and/or other impurities seem to represent a rather low percentage (<10%) of its content. Overall, it is assumed that almost 200 mg of IgYs were isolated from the two immune yolks. The above results were also confirmed with spectrophotometry experiments [21].

3.1.3. IgY thermal and pH stability

IgYs-3*e* were evaluated in the titration ProTα-ELISA, in parallel with n-IgYs as a negative control, and shown to be still immunoreactive to ProTα, despite their long-lasting storage (Fig. 3A). Moreover, the titration ProTα-ELISA was used for evaluating the thermal and pH stability of IgYs-3*e*: As shown in Fig. 3B, IgYs-3*e* were highly stable at temperatures up to 60 °C, since their immunoreactivity in the titration ProTα-ELISA was 91–101%, in comparison with that of untreated IgYs-3*e* (100% immunoreactivity). At 70 °C, IgYs-3*e* retained 50% of their immunoreactivity, whereas at higher temperatures immunoreactivity was substantially reduced (to 18% and 10%, when treated at 75 °C and 80 °C, respectively). Moreover, as shown in Fig. 3C, the stability of IgYs-3*e* treated at a broad pH range (3–9) was excellent, since their immunoreactivity in the titration ProTα-ELISA was in the range 92–104% as compared with that of untreated IgYs-3*e*. IgYs-3*e* treated at pH 10 showed relatively, but not dramatically-decreased immunoreactivity (82% in comparison with untreated IgYs-3*e*). Overall, IgYs-3*e* were exceptionally stable at a wide range of temperature (37–60 °C) and pH (3–9) values.

3.1.4. IgY specificity for intact ProTα

IgY specificity for intact ProTα was evaluated with Dot-Blot analysis. As shown in Fig. 4, intact ProTα was well recognized by IgYs-3*e* in all concentrations tested (0.01–1 mg/mL). On the contrary, IgYs-3*e* could just marginally recognize ProTα[1-28] at the highest concentration used (1 mg/mL), while ProTα[50-88] and ProTα[100-109] were not recognized.

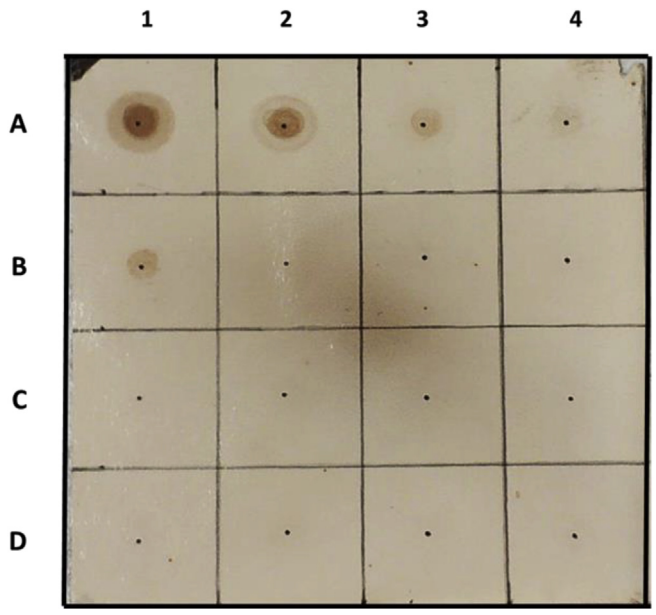


Fig. 4. IgY specificity: the specificity of IgYs-3*e* for intact ProTα was evaluated in Dot-Blot analysis. ProTα (A), ProTα[1-28] (B), ProTα[50-88] (C), ProTα[100-109] (D), were spotted on nitrocellulose membrane at decreasing concentrations (1 mg/mL, 0.2 mg/mL, 0.05 mg/mL and 0.01 mg/mL, from left to right).

3.2. Competitive ProTα-ELISA

The assay set-up is shown in Fig. 5A.

3.2.1. Specificity for intact ProTα

Specificity of the competitive ProTα-ELISA for intact ProTα was evaluated with cross-reactivity experiments carried out in the presence of increasing concentrations (1–1,000 nM) of either intact ProTα or various synthetic fragments of the polypeptide, *i.e.* ProTα[1-28] (Tα1), ProTα[50-88], ProTα[100-105], and ProTα[100-109]. A 100% optical signal corresponds to the absorbance value obtained when not any solution of ProTα or ProTα-fragments is present. Absence of cross-reactivity is explicitly clear (Fig. 5B) in the case of the bioactive fragment ProTα[100-109] as well as the shorter fragment ProTα[100-105], which still includes the NLS sequence of the polypeptide [6]. The long central fragment ProTα[50-88] has exhibited no cross reaction either (Fig. 5B). On the other hand, IgYs-3*e* seemed to marginally cross-react with ProTα[1-28], but even in this case no exact % cross-reactivity could be calculated, since none of the ProTα[1-28] solutions tested including that of the highest concentration (1,000 nM) could lead to optical signal lower or equal to 50%. These results fall well in line with the ones of the Dot-Blot analysis.

3.2.2. Working range, matrix effect, accuracy and precision

Working range: the working range of the competitive ProTα-ELISA was assessed using a series of ProTα standard solutions in diluting solution 1 at a wide concentration range (1–10,000 ng/mL) run in quadruplicate and determined at 2.5–1,000 ng/mL. The detection limit (LoD) of the assay based on the equation: *LoD=mean value ± 3SDs of zero calibrator* [22], was calculated to 2.109 ng/mL, and the quantitation limit (LoQ) following the equation: *LoQ = mean value ± 10SDs of zero calibrator* [22], to 11.740 ng/mL. LoD and LoQ values may be further decreased by using enzyme substrates generating a fluorescent or chemiluminescent signal.

Matrix effect: Standard solutions of ProTα were prepared in PBS (dilution buffer 1), plain RPMI, and complete medium. As shown in Fig. 5C, the corresponding standard curves are almost superimposable, indicating lack of matrix interference in the assay working range. The

assay detection limit was not matrix-affected, either.

Accuracy: the accuracy of the competitive ProTα-ELISA was determined through "spike and recovery" experiments, using three different concentrations of ProTα, low (10 ng/mL), medium (50 ng/mL) and high (100 ng/mL), spiked in a HeLa cell culture supernatant; as shown in Table 1, recovery values ranged from 88 to 104%.

Precision: Intra-assay CV of the competitive ProTα-ELISA was less than 5% as assessed by applying 10 replicates of zero standard and 8 replicates of the other standard solutions in the same plate; inter-assay CV was less than 12%, as assessed by applying 6 replicates of each standard solution to five individual plates, run in five consecutive days.

3.2.3. Analysis of biological samples (HeLa cell culture supernatants)

Culture supernatants of HeLa cells led to necrosis were selected as putative biological source of intact ProTα and analyzed for ProTα content with the competitive ProTα-ELISA. Cell necrosis was induced with various treatment conditions, as described in Materials and Methods, and the percentage of necrotic cells was determined by flow cytometry using standard PI staining; indicative histograms of each treatment condition used are shown in Fig. 6A.

A total of 15 samples (HeLa cell culture supernatants) were included in the study. ProTα concentration of each HeLa cell culture supernatant in relation to the corresponding percentage of cell necrosis is presented in Fig. 6B. Furthermore, samples were classified into 3 groups, according to percentage of cell necrosis, as follows: Group I (n = 3): samples with <10% necrotic (PI+) cells; Group II (n = 7): samples with 10–90% PI+ cells; Group III (n = 5): samples with >90% PI+ cells. The concentrations of ProTα measured in each group of samples are shown in Fig. 6C; low levels of ProTα (mean value 7.4 ng/mL), were detected in samples of Group I, whereas higher levels were measured in samples of Group II and even higher ones in samples of Group III with mean values of 47.3 ng/mL and 127.7 ng/mL, respectively. One-way analysis of variance showed that mean values of Groups II and III differed significantly from the mean value of Group I ($p < 0.05$ and $p < 0.0001$, respectively). It is therefore suggested that there is a trend of gradual increase in ProTα levels as the

Table 1
Recovery values of ProTα in supernatants of HeLa cells spiked with the indicated concentrations of the polypeptide.

ProTα spiked	Expected ProTα concentration in HeLa supernatants (ng/mL)	Detected ProTα concentration in HeLa supernatants (ng/mL)	Recovery %
-	N/A*	5.5 ± 0.3	N/A
100 ng/mL	105.5	109.8 ± 6.9	104.1
50 ng/mL	55.5	54.5 ± 2.6	98.2
10 ng/mL	15.5	13.6 ± 0.8	87.7

* N/A, not applicable.

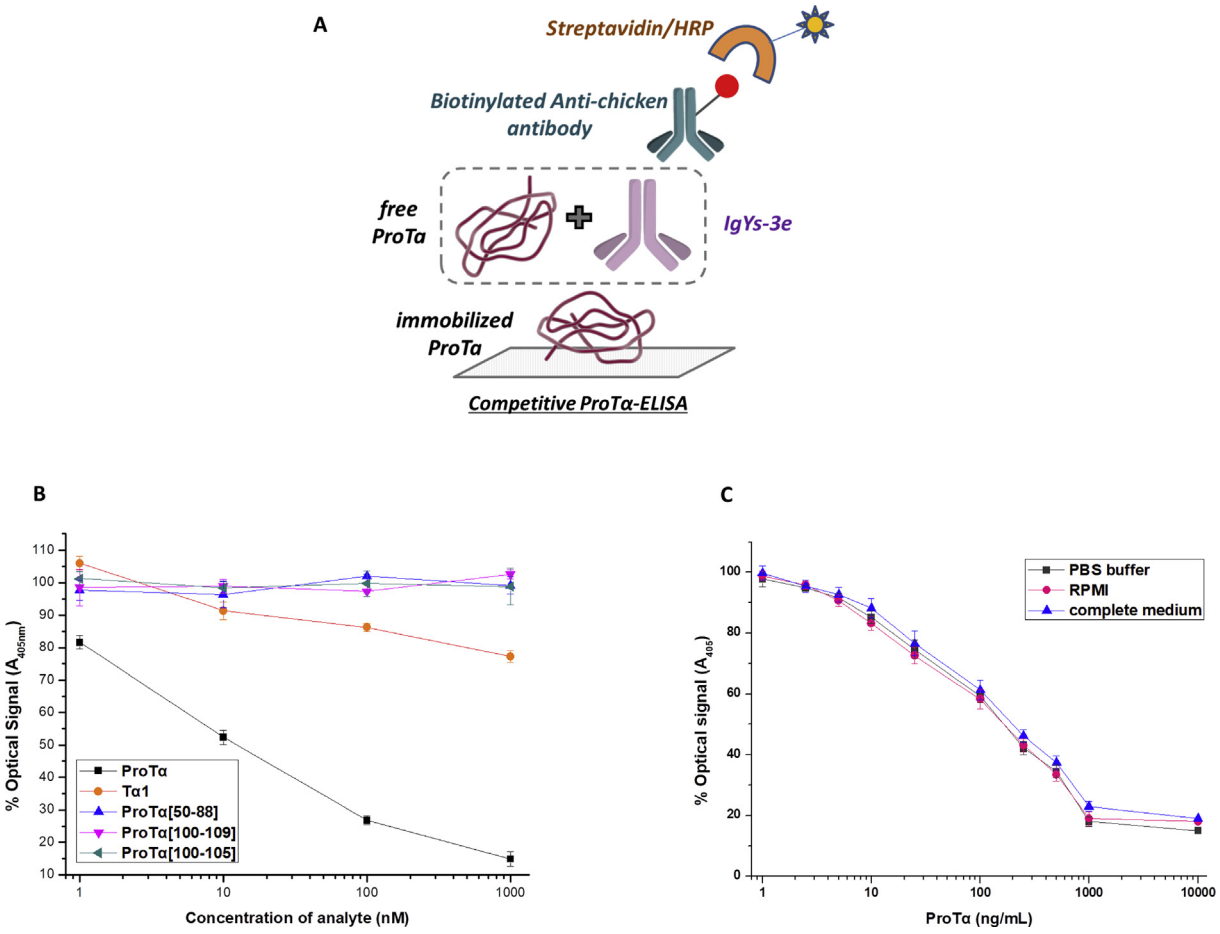


Fig. 5. A. Schematic presentation of assay set-up and main reagents used in the competitive ProTα-ELISA. B. Specificity of the competitive ProTα-ELISA for intact ProTα was evaluated with cross-reactivity experiments. Solutions of intact ProTα, ProTα[1-28] (Tα1), ProTα[50-88], ProTα[100-109], and ProTα[100-105] (Analytes) at increasing concentrations (1–1,000 nM) were used. A 100% optical signal corresponds to the absorbance value obtained in the absence of any analyte. Mean values ± SDs from 3 experiments are shown. C. Standard curves of the competitive ProTα-ELISA obtained with ProTα in PBS buffer, RPMI, and RPMI supplemented with 10% FBS (complete medium); the three superimposable curves indicate no matrix interference. A 100% optical signal corresponds to the absorbance value obtained in the absence of any solution of ProTα. Mean values ± SDs from 3 experiments are shown.

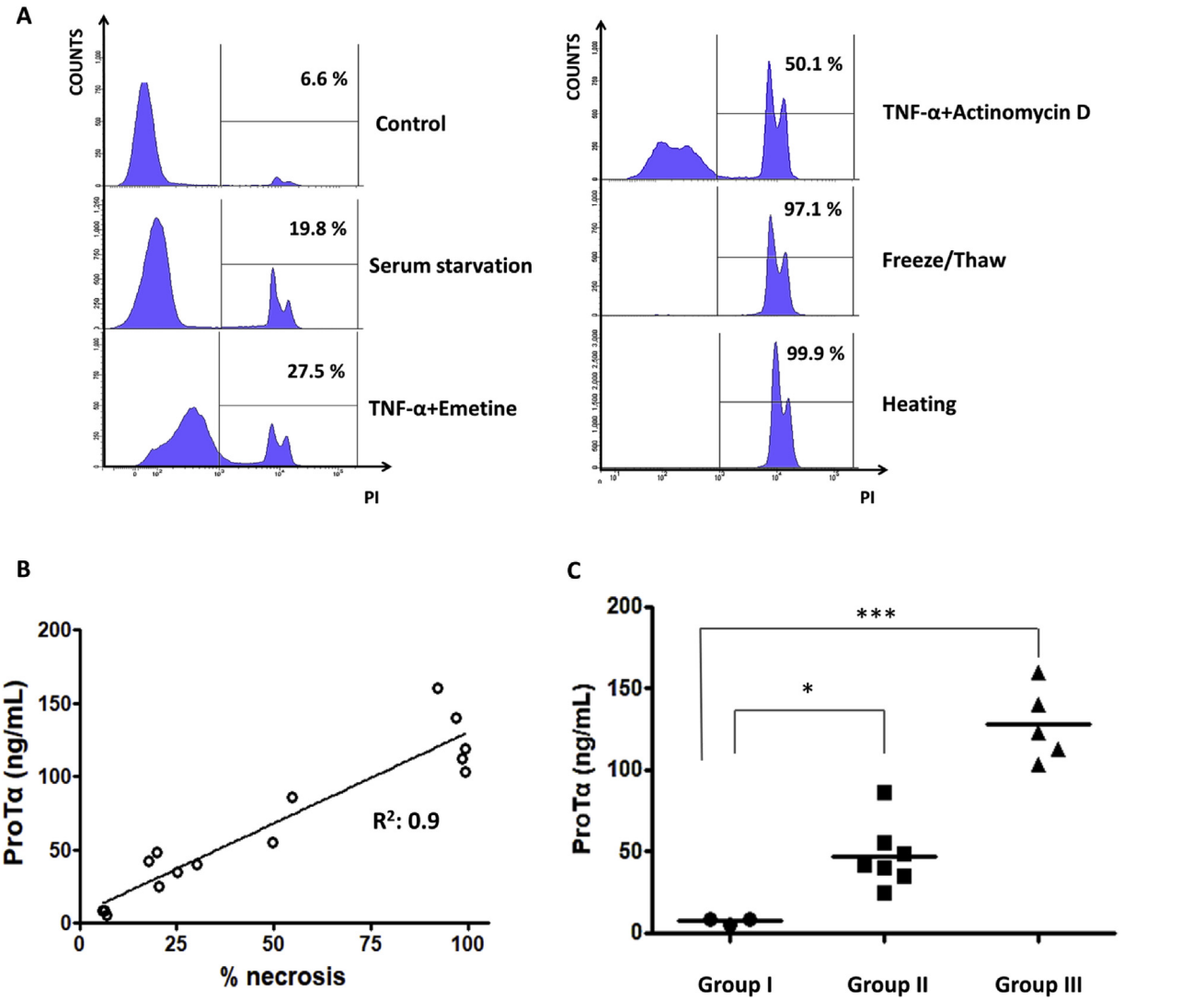


Fig. 6. A. Flow cytometry analysis of HeLa cells after induction of necrosis *via* various stimuli. Representative histograms of HeLa cells driven to necrosis with each of the stimuli used in the present study, *i.e.* serum starvation, TNF-α (6 ng/mL) + emetine (5 μg/mL), TNF-α (6 ng/mL) + actinomycin D (50 μg/mL), 4 cycles of freeze (-80 °C)/thaw (37 °C), and heating (56 °C for 1 h). Control cells were incubated in complete medium. The gate was set based on unstained control HeLa cells. Gated PI+ (necrotic) cells were analyzed and the corresponding percentages are shown. B. Correlation of ProTα concentration in HeLa cell culture supernatants and % of necrotic cells detected in the same cultures. Each symbol corresponds to supernatant and cells from one culture. Linear regression analysis showed a positive correlation between ProTα concentration and percentage of necrosis; R^2: 0.9. C. Concentration of ProTα in the supernatants of HeLa cells grouped as follows: <10 % necrosis (Group I), 10–90% necrosis (Group II), and >90 % necrosis (Group III). Each symbol corresponds to ProTα concentration value measured in a single sample. One-way ANOVA was used to compare mean values of Groups II and III with that of Group I (*, $p < 0.05$; ***, $p < 0.0001$).

percentage of necrotic cells increases, and this might be associated with increased extracellular release of ProTα due to extensive membrane rupture.

4. Discussion

Polyclonal IgYs for ProTα were raised by our team because avian organisms are likely to develop antibodies of higher affinity/specificity against highly conserved mammalian polypeptides, such as ProTα. In the present work, we have evaluated a specific preparation of IgY antibodies for ProTα (IgYs-3*e*), developed several years ago according to a method previously described by our team [15]. Impressively, IgYs-3*e* have retained their immunoreactivity after being stored for almost 15 years at -30 °C in lyophilized form. Moreover, the IgYs-3*e* proved to be highly stable in a wide range of temperature (37–60 °C) and pH (3–9) conditions. The above findings fully support other researchers' comments on the striking robustness of antibodies Y, which is a great advantage -especially for biotechnological applications that may include treatment of immunoglobulins under rather harsh conditions; this and other characteristics, such as low cost of production, high quantity and purity of IgYs isolated from immune egg yolks, absence of serological cross-reaction with rheumatoid factors and mammalian Fc receptors or inability of activating the mammalian complement system, have rendered IgYs valuable laboratory reagents [12, 14].

The IgYs-3*e* were highly specific for intact ProTα, since they did not cross-react with a series of ProTα-fragments including ProTα[1-28] (Tα1) and ProTα[100-109]. This finding confirms previously reported data of our team for similar IgY preparations [15]. The fragments ProTα[1-28] (Tα1) and ProTα[100-109] are of special interest, since they can be formed after enzymatic cleavage of the intact molecule under certain circumstances, have shown discrete biological activity in various *in vitro* and/or *in vivo* systems, and may be concomitantly present with intact ProTα in biological samples. In addition, the fragment ProTα[50-88], one of the largest regions of charged amino acids identified so far in

mammalian proteins [23] through which ProTα has been reported to interact with histone-1 in the cell nucleus [24, 25] was tested for the first time and did not exhibit any cross reaction with IgYs-3*e*, either.

Specific determination of intact ProTα in suitably-selected biological samples is considered important since it may shed more light on still obscure aspects of the biological role of the polypeptide. To this end, several competitive immunoassays, mainly ELISAs, have been reported to date, which usually employ in-house developed antibodies G raised against ProTα-fragments, mostly from the N-terminal region 1-28 (Tα1) [10,26,27,28,29] and the C-terminus [10, 11, 30, 31] of the polypeptide. As a consequence, these methods suffer from an "inbuilt" specificity limitation, since they can probably recognize, apart from the intact polypeptide, the corresponding antigenic ProTα-fragment as well. Moreover, one IgG-based sandwich ProTα-ELISA has been reported so far, at least to our knowledge; however, no data on the assay cross-reactivity with ProTα fragments are available [32].

By employing the IgYs-3*e*, we have developed a competitive ProTα-ELISA with high specificity for the intact polypeptide and desirable analytical characteristics (while a more complicated, IgG/IgY sandwich ProTα-ELISA is currently being evaluated for any potential analytical superiority, as mentioned in reference 2). The competitive ProTα-ELISA was applied to the analysis of specially-selected biological samples in an attempt to shed more light on the conditions under which intracellular ProTα can be released in the extracellular milieu, free to exert its pleiotropic extracellular activities. HeLa cells were selected as a model system, since they natively express increased amounts of ProTα and have been often employed as a cell system of choice in ProTα research [10, 33]. Cultures of HeLa cells were experimentally-exposed to multiple factors driving them to necrosis and cell culture supernatants were collected and analyzed for ProTα content. More specifically, by using a series of cell death stimuli, such as high temperature-, serum starvation-, and drug-induced stress, we managed to yield different levels of cell necrosis, spanning from very low (<10%) to very high (>90%), as determined through flow cytometric analysis following PI staining (Fig. 6A). Subsequently, by employing the competitive ProTα-ELISA, we determined the concentration of ProTα in the same cell culture supernatants. A total of 15 samples were analyzed and, interestingly, the results showed a positive correlation of ProTα levels with the extent of HeLa cell necrosis (Fig. 6B, C). The above results support the literature scenario suggesting that, under circumstances leading to survival-threatening unprogrammed cell death (herein necrosis), ProTα can be released outside the cells further acting as a DAMP/alarmin [1]. Moreover, these findings fall well in line with previously reported data from Ueda and colleagues, according to which ProTα was identified by chromatographic techniques to be present in the culture medium of cortical neurons undergoing serum starvation-induced necrosis [34]. In addition, the above findings support previous data suggesting that products of ProTα genes/pseudogenes are released from CD4+ T-cells in response to HIV infection and function thereafter as DAMPs [35]. Further studies employing more cell lines and more types of normal cells need to be performed in order to reach a solid conclusion on whether, when, under what conditions and how ProTα can undergo exocytosis.

4.1. Conclusion

Due to its high specificity for the intact molecule as well as its overall analytical characteristics, the newly developed IgY-based ProTα-ELISA may serve as a reliable *in vitro* tool, elucidating still obscure/controversial aspects of the biological role of this polypeptide and facilitating assessment of its diagnostic and therapeutic potential.

Declarations

Author contribution statement

Chrysoula-Evangelia Karachaliou: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ioannis Kostopoulos: Performed the experiments; Analyzed and interpreted the data.

Vyronia Vassilakopoulou: Performed the experiments.

Persefoni Klimentzou, Maria Paravatou-Petsotas, Wolfgang Voelter, Hubert Kalbacher, Christos Zikos: Contributed reagents, materials, analysis tools or data.

Ourania Tsitsilonis: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Evangelia Livaniou: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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