ARTICLE

Screening of by-products from the food industry as growth promoting agents in serum-free media for skeletal muscle cell culture

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R. Christel Andreassen, [ab] Mona E. Pedersen,[a] Kenneth A. Kristoffersen,[a] Sissel B. Rønning [a]*

The most significant cost driver for efficient bio-production of edible animal proteins is the cell culture media, where growth factors account for up to 96 % of the total cost. The culture media must be serum-free, affordable, contain only food-grade ingredients, be efficient to promote cell growth and available in massive quantities. The commercially available serum substitutes are expensive and not necessarily food-grade. Identifying inexpensive food-safe alternatives to serum is crucial. By-products from food production are available in massive quantities, contain potential factors that can promote growth and are promising ingredients for serum replacement. The main goal of this study was to explore if food-grade by-product materials can be used as growth promoting agents in skeletal muscle cell culture to develop a tailor-made serum free media. Different by-products, including chicken carcass, cod backbone, eggshell membrane, egg white powder and pork plasma were enzymatically or chemically hydrolyzed. The hydrolysates in addition to lyophilized pork plasma and yeast extract were further characterized by size-exclusion chromatography, elemental combustion analysis and degree of hydrolysis. The materials were used as supplement to or replacement of commercial serum and further evaluated for their effect on metabolic activity, cell proliferation and cell cytotoxicity in muscle cells cultured *in vitro*. Our results indicate that none of the materials were cytotoxic to the skeletal muscle cells. Hydrolysates rich in peptides with approximately 2-15 amino acids in length were shown to improve cell growth and metabolic activity. Of all the materials tested pork plasma hydrolysates and yeast extract were the most promising. Pork plasma hydrolysates increased metabolic activity by 110 % and cell proliferation with 48 % when cultured in serum-free conditions for 3 days compared with control cells cultured with full serum conditions. Most interestingly, this response was dependent on both material and choice of enzyme used. We suggest that these materials have the potential to replace serum during cultivation and as such be included in a tailor-made serum-free media.

[a.] *Nofima - Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 210, N-1431, Ås, Norway*
[b.] *Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine University of Oslo, Oslo, Norway*
* *Corresponding author: sissel.ronning@nofima.no.*
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Introduction

The world's livestock sector is developing at an extraordinary rate to meet the growing demand for high-value animal protein. Livestock products are an excellent protein source with high nutritional value and an important source of essential micronutrients. However, the increasing animal production is not sustainable. The industry is compelled to look for alternative and more environmentally friendly ways to produce animal proteins. A groundbreaking new technology and promising alternative to traditional meat production is cultured meat. This meat will bypass animal production and can in theory be produced faster and more efficiently than conventional meat.[1] Cultured meat is made by harvesting a small biopsy of skeletal muscle cells from a living mammal, these cells are multiplied and grown to produce muscle tissue (*i.e.*, meat). A major challenge with this technology is the serum required for cell growth. In cell culture, fetal bovine serum (FBS) typically provide the necessary nutrient supplementation in culture media. FBS is the supernatant of clotted blood from a bovine fetus mainly collected by cardiac puncture. This serum contains an undefined cocktail of stimulating factors required to sustain cell growth and maintenance of most mammalian cells. Serum is expensive, the cost can be up to 95% of the total cost of the cell media, and the supply is lower than the demand.[2] In addition to being expensive, there are biosafety and ethical issues due to the nature of how FBS is harvested. As such, serum supplementation is a limiting factor and cannot support sustainable large-scale protein production intended for human consumption.

Extensive research the last decade has been focused on the reduction or replacement of FBS as part of good cell culture practice (GCCP). [3] The challenge of producing a serum-free media (SFM) is to identify and replace the specific components in serum that promote cell growth and determine the optimal media composition to each cell type. Today, defined serum replacements are available for cell types commonly used in medical and industrial applications. These culture media compositions are not published for commercial reasons. However, typical components of serum include serum proteins (Albumin, Globulins), transport proteins (Transferrin, Transcortin, α1- and β1-Lipoprotein), attachment factors (Fibronectin, Laminin), enzymes, hormones (Insulin, Glucagon, Corticosteroids, Prostaglandins), growth factors and cytokines (EGF, FGF, NGF, ECCGF, PDGF, IGFs, Interleukins, Interferons, TGFs), fatty acids and lipids, vitamins, trace elements, carbohydrates, and nonprotein nitrogen.[4] To the best of our knowledge the commercially available serum substitute alternatives for primary skeletal muscle cells are not food-grade and the cost is still an issue.[3, 5] Therefore, finding an inexpensive food-safe replacement for serum is of great interest.

By-products from food production are available in massive quantities and are promising ingredients for serum replacement. It is estimated that nearly 40-60% of farmed fish and animals total mass are classified as residual products with food-grade quality, including carcasses, blood and skin.[6] These residues have excellent nutritional value and contain proteins and other essential nutrients with potential bioactive properties.[7] Bioactive peptides can be released from by-products via hydrolysis and can exert beneficial effects on physiological functions beyond nutritional value, including cell growth regulation and promoting high cell culture performance.[6] Protein hydrolysates are a mixture of peptides with varying length and free amino acids. Hydrolysis is a process that involves breaking down proteins into smaller and more water-soluble peptides. Such hydrolysis can be performed using chemical or enzymatic processes.[8] Protein hydrolysates are reported to have growth promoting effects in mammalian cell culture.[9] Studies demonstrate that protein hydrolysates of eggshell membrane (ESM) regulate cellular functions and have anti-inflammatory properties.[8] Results from several other studies have indicated that peptides from blood sources have a wide range of bioactive effects, including blood sugar regulation, lowering blood pressure, as well as antioxidant and antimicrobial properties.[10-14] For many decades yeast extract have been used in microbiology as a stimulator of bacterial growth,[15] and the benefits of yeast extract in enhancing protein production and cell growth in some cell types are also documented.[16, 17]

Based on the current knowledge, our goal was to investigate if hydrolyzed proteins from food-grade by-product materials and yeast extract could be used as growth promoting agents in skeletal muscle cell culture.

Results and discussion

Nine by-product hydrolysates produced by enzymatic or chemical hydrolysis, in addition to lyophilized pork plasma and yeast extract were characterized for their content of total nitrogen, molecular weight distribution (MWD) and degree of hydrolysis (DH) in dry material mass. Residual components such as lipids, sugars or ashes were not analyzed. The processed materials were further assessed for their effect on cell metabolic activity, proliferation, cytotoxicity and evaluated for their suitability to support cell growth during serum starvation in skeletal muscle cells.

Choice of by-product material and enzyme affected protein degradation during hydrolysis

The total nitrogen content (Fig. 1A) ranged from 52-95 %. The degree of hydrolysis (DH) ranged between 20-60 % (Fig. 1B), except chemically hydrolyzed ESM at 11%. The latter is probably due to the extra processing steps performed to remove salt compounds after chemical hydrolysis (*i.e.*, dialysis). The dialysis cutoff was set to 100-500 Da and all molecules of smaller sizes were eliminated. The DH of this material is therefore not directly comparable with the other crude hydrolysates. The choice of peptidase and the combination of exopeptidases and endopeptidases influence the DH. Alcalase is a nonspecific serine-type protease derived from *Bacillus licheniformis* and contains manly endopeptidases. Flavourzyme contains an exopeptidase-endopeptidase enzyme mix with mainly exopeptidases produced from *Aspergillus orizae*.[18] The differences in enzyme activity and selectivity under the reaction conditions used can explain the observed differences in DH of the same raw material (Fig. 1B).[19] This could be substrate dependent, for example, egg white powder digested with Alcalase or Flavourzyme showed approximately the same DH percentage.

In the current study, the results from the size exclusion chromatography (SEC) analysis with UV detection (214 nm) demonstrated that the 11 different materials tested had distinct MWD profiles (Fig. 1C). Peptide bonds absorbs strongly at this wavelength, SEC in combination with UV detection is therefore one of the more commonly used instrumental setups in the study of food-grade hydrolysates.[20] There are however some limitations to this setup which influence how well the detected MWD profile reflect the actual MWD profile. Free amino acids are not well detected at this wavelength, while proteins and peptides are detected by absorption contributions from both peptide bonds and their side-groups resulting in scaling errors.[21] Despite these limitations, this method provides useful information to differentiate the materials used in this bio screening. The chromatograms were sectioned into four different size ranges (F1, F2, F3 and F4) and the relative area under the curve for each size rage were calculated (see Fig. 1C and supporting information (SI) Fig. S-1). Hydrolysates produced using Flavourzyme generally contain larger fractions of free amino acids (F4) (2-fold or greater) compared to Alcalase digestion of the same raw material (SI table. 1). These results are consistent with previously published studies of hydrolysates prepared using these enzymes (*i.e.*, Alcalase and Flavourzyme).[18,22,23] Enzymatically hydrolyzed pork plasma had a very different MWD compared to the intact pork plasma powder, the latter predominantly consisted of peptides larger than 15 amino acids (95%, F1). The pork plasma hydrolysates produced in this study mainly contained peptides with approximately 2-5 amino acids in length (F3), this fraction was greater for plasma digested with Alcalase (75,7%) then with Flavourzyme (56,6%). The SEC results show that hydrolysates produced by enzymatic hydrolysis contained mainly large fractions of short peptides and less than 20% larger peptides (>15 amino acids) with two exceptions (chicken carcass and cod backbone digested with Alcalase).

Serum starvation dramatically impacted cell metabolism and proliferation

Serum starvation can cause cellular stress as serum is necessary for optimal cell growth.[24] Skeletal muscle cells cultured for 48 hours in either serum-free or serum-reduced media conditions (serum starvation) showed decreased metabolic activity and cell proliferation (Fig. 2). The metabolic activity was reduced by 18.4% in serum-reduced conditions, while a 52.2% reduction was observed in serum-free media compared to control cells cultivated in normal serum conditions. Likewise, reducing or removing serum decreased cell proliferation by 17.3% and 46%, respectively.

Supplementation with by-product hydrolysates and yeast extract to cell culture media enhanced cell growth when serum was present and restored cell growth when serum was reduced and depleted

By-product supplementation to cell culture media promoted muscle cell metabolic activity and proliferation (Fig. 3 and 4). This effect was dose-dependent, with an upper limit of 1 mg/mL, while media supplementation with 10 mg/mL nearly depleted cell growth and was highly cytotoxic to the cells. Interestingly, none of the materials were harmful to the skeletal muscle cells below the upper limit. Hydrolysates rich in small peptides with approximately 2-15 amino acids in length increased cell growth depending on the combination of enzyme and raw material. Of all the materials tested in this study, pork plasma hydrolysates and yeast extract were the most promising (Fig. 3A and B). Hydrolysates from pork plasma generated with Alcalase enzyme had the most potent effect on cell growth, with more than 150% increase in metabolic activity and 50% increase in cell proliferation compared to cells grown in normal serum conditions (Fig. 3A). To our knowledge, this is the first report of pork plasma by-product hydrolysates as a promoter of cell growth. However, a study reports a promising animal cell culture supplement containing lysate of porcine platelets.[25] Plasma contains hormones, antibodies, antigens, nutrients and proteins. The main plasma proteins are Albumins (50%), Globulins (alpha 15%, beta 15% and gamma 15%) and Fibrin (5%).[26-28] Fibrin plasma proteins have previously been shown to contain specific sequences that interacts with growth factors (GFs) and enzymatic cleavage of fibrin(ogens) can promote cell proliferation of both endothelial cells and fibroblasts.[29, 30] In addition, fibrin(ogens) participate in cell-matrix interactions, forming a provisional matrix that is suggested to act as a reservoir for secreted GFs.[30, 31] Interestingly, in this study neither intact pork plasma itself nor pork plasma hydrolysates generated with Flavourzyme had similar positive effects compared with pork plasma digested with Alcalase (Fig. 3A). In the presented experiments, Yeast extract enhanced cell metabolic activity more than 100 % and cell proliferation with almost 50 % (Fig. 3B). Yeast extract is the water-soluble portion of autolyzed yeast. The MWD profile of the yeast extract used in this study shows that it consists of a large fraction of small peptides (63.9%), 31.7% free amino acids and less than 5% (4.4%) large peptides (>15 amino acids), based on the relative area. Like FBS, it is not fully understood which components of yeast extract are responsible for the growth-promoting effects.[16] Unlike FBS, yeast extract is food-grade with no ethical challenges of use. Cod backbone and chicken carcass hydrolysates did not influence cell activity in normal serum conditions (Fig. 3C), these hydrolysates contained 23.7-29.7 % less fractions of small peptides and 23.6-31.4 % more large peptides compared to pork plasma hydrolyzed with Alcalase. A similar pattern was observed with pork plasma and egg white powder, where treatment with Alcalase increased metabolic activity more compared with Flavourzyme digestion (Fig. 4A). Interestingly, the opposite trend was observed with eggshell membrane, where Flavourzyme hydrolysis increased the metabolic activity more compared with Alcalase and NaOH digestion (Fig. 4B). Previous reports show that animal derived hydrolysate mixtures of low molecular weight compounds (peptones) improved cell growth to a higher degree then a defined mixture of amino acids.[9, 32] The SEC analysis data (SI table. 2) show that Flavourzyme digested raw material contained 2-fold larger fractions of free amino acids and 10-25% fewer small peptides (2-15 amino acids in length) compared to hydrolysates of the same by-product material produced with Alcalase. Bioactive peptides

share common features, such as peptides of 2-20 amino acids in length.[7] The physiological functions of hydrolysates can be regulated by the amino acid sequence composition and length, which is dependent on by-product origin and protein degradation during hydrolysis. This demonstrate that not only the source of by-product, but also the choice of enzyme is important when generating hydrolysates with cell growth enhancing capabilities.

When serum was reduced by 50%, both pork plasma hydrolysates (Fig. 5A), yeast extract (Fig. 5B) and chicken carcass hydrolysate (Fig. 5C) digested with Alcalase were able to restore cellular function and enhance cell growth. In contrast, cod backbone hydrolysates were not able to restore cell function compared to cells grown in normal serum conditions (Fig. 5C). Likewise, egg white and eggshell membrane digested with Alcalase also restored cell function (Fig. 6A and B). Pork plasma and yeast extract were the only materials that were able to completely restore cell function in serum-free conditions. Plasma digested with Alcalase and yeast extract enhanced cell metabolic activity by over 100% and 50%, respectively (Fig. 7A and B). This was dose dependent and high concentrations (1 mg/mL and 10 mg/mL) was cytotoxic to the cells. Egg white hydrolysate restored cell metabolic activity to 86% (Fig. 8A), while the other hydrolysates had little or no effect on cellular function (Figs. 7 and 8).

The results showed that pork plasma hydrolysates and yeast extract can recover cell growth (both metabolism and proliferation) in reduced and serum-free conditions. It is possible that additional effects could be obtained by mixing different protein hydrolysates as the results presented in figure 7 suggest that pork plasma digested with Alcalase and yeast extract can replace serum during bovine muscle cell cultivation. In this study, the complete chemical profile of the tested materials was not analyzed, and the molecular mechanisms of the observed effects are not yet elucidated. The dose-response curves (Figs. 3-8) reach a plateau, which may be explained by the complex nature of the crude hydrolysates and materials that were used. The materials tested contain a mixture of peptides, some of which could inhibit cell growth as well as peptides that can promote cell growth.[33]

According to an analysis performed by the Good Food Institute, growth factors account for over 99% of the total cell culture medium cost. [34] An optimal media should be formulated with reduced raw materials costs without compromising the cellular yield. None of the materials tested in our study were cytotoxic to the skeletal muscle cells. In fact, the hydrolysates rich in peptides with approximately 2-15 amino acids in length improved cellular growth and metabolic activity. Most importantly, the materials tested are food-grade, inexpensive, easy to produce and presents a higher ethical quality compared to FBS. Since by-products are available in massive quantities, ingredients from such material will undoubtedly reduce the cost of cell culture media which is the most significant cost driver for cultured meat production. Secondly, this represents an industrial opportunity for the food industry to increase the value of by-products and at the same time contribute to the circular economy by reducing waste. Finally, discovery of bioactive peptides can create new business opportunities for other markets such as the pharmaceutical and nutraceutical industry. Research groups are continuously working to optimise big scale hydrolysis production to ensure minimal batch to batch variation and high-quality yield while reducing costs. Among the modern analytical approaches for discovery of bioactive constituents in complex mixtures is the use of chromatography-coupled bioassay where eluents of a separation (fractions) are directed to high-throughput bio-screening, an approach used to identify and characterize bioactive peptides from chicken hydrolysates.[35] Further studies are necessary to evaluate the chemical profile and elucidate the effects of specific hydrolysate fractions and yeast extract on cellular response in bovine muscle cells to optimize and formulate a tailor-made serum-free media for bovine muscle cells.

Materials and methods

Raw-material and chemicals

The cod backbone was provided by Sjømat AS (Oslo, Norway), and the mechanical chicken carcass residue was provided by Nortura (Hærland, Norway). Industrially made and patented avian egg white powder and eggshell membrane were provided by Norilia (Oslo, Norway), and the whole pork blood was collected at the time of slaughter from a commercial abattoir Flesland (Hvalstad), Norway. Yeast extract was purchased from Duchefa Biochemies B.V (Haarlem, The Netherlands). The two food-grade enzymes used in this study were Alcalase and Flavourzyme (*Aspergillus oryzae*) purchased from Novozymes A/S (Bagsværd, Denmark). Ultroser G serum substitute was purchased from Pall Corporation (Port Washington, NY, USA). Dulbecco`s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin solution 10 000 units per mL (P/S), Amphotericin B, and 0.05% trypsin/EDTA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Entactin-Collagen-Laminin (ECL) was purchased form Millipore Sigma (Burlington, MA, USA) and Collagenase from Sigma Aldrich (Merck KGaA, St.Lois, MO, USA). All other reagents were from Sigma Chemicals Co. (St. Lois, MO, USA) unless otherwise noted.

Preparation of protein hydrolysates

A total of nine different hydrolysates were produced with either Alcalase, Flavourzyme or by chemical hydrolysis with sodium hydroxide (NaOH). Cod and chicken by-products were hydrolyzed according to previously published protocol by G. Wubshet *et al*.[36] In short, samples were homogenized using a food processor. Hydrolysis was carried out in a Reactor-Radley™ jacketed vessel (Radleys, Saffron Walden, Essex, United Kingdom) under stirring (300 rpm, Radley Torque Value 200) at 50°C, run for 60 minutes and terminated by heat inactivation (95 °C for 15 minutes). The mixture was centrifuged for 15 min at 4600 *xg*, resulting in a water phase supernatant, a fat phase and solid residue. The water phase supernatant was lyophilized to produce a hydrolysate protein powder. The eggshell membrane (ESM) was produced and harvested using a patented process by Biovotec and washed with 0.1 M hydrochloric acid (HCL) for 10 minutes under 300 rpm stirring and washed twice with dH_2O before it was freeze dried and milled to a powder with an average particle size of 0.25 nm (Helium-Neon Laser Optical System, Sympatic Inc., Clausthal-Zellerfeld, Germany). 10 g ESM powder was mixed with 200 mL dH_2O in a 0.2 L pyrex flasks under stirring (300 rpm) at 50°C and run for approximately 12 hours. When the temperature reached 50°C, 2% enzyme (Alcalase or Flavourzyme) or 5% w/v NaOH was added to initiate the reaction. Enzymatic hydrolysis was inactivated by heating (95°C) for 15 min. Only the NaOH hydrolyzed ESM reaction mixture was dialyzed (Spectrum laboratories, Inc. Biotech CE tubing MWCO 100-500 Da, Repligen Europe B.V, The Netherlands) for five days with two to three daily water changes (dH2O) and inactivated by pH neutralizing with HCL. The hydrolysate mixtures were centrifugated at 4600 *xg* for 15 minutes before the water phase supernatant was vacuum filtrated and lyophilized.

Plasma is the liquid cell-free part of blood that has been treated with anticoagulants. Blood from six-month-old pigs were collected during slaughter by open draining and added sodium citrate (0.6% w/v) to prevent blood clotting. The blood was fractionated by 30 min centrifugation (10 000 *xg*), resulting in a liquid phase supernatant of plasma and a solid phase of cells. The cell fraction was discarded, and the liquid phase was lyophilized by freeze drying. The pork plasma and egg white dry powder were hydrolyzed with Alcalase and Flavourzyme using the same protocol previously described for enzymatically hydrolyzed ESM power. The hydrolysates and materials used in this study are listed in table 1.

TABLE 1. RAW MATERIALS AND HYDROLYSATES USED

MATERIAL	Hydrolysis/enzyme	Abbreviations
PORK PLASMA	-	PBP
	Alcalase	PBP-A
	Flavourzyme	PBP-F
CHICKEN CARCASS	Alcalase	K1-A
COD BACKBONE	Alcalase	T1-A
EGG WHITE POWDER	Alcalase	EW-A

	Flavourzyme	EW-F
EGGSHELL MEMBRANE	Alcalase	ESM-A
	Flavourzyme	ESM-F
	NaOH	ESM-NaOH
YEAST EXTRACT	-	YE

Total nitrogen, carbon and sulfur determination

Nitrogen, Carbon and Sulfur was determined using elemental combustion analysis (varioEL CER 2019, CHNS system, Elementar, Langenselbold, Germany) from solid dry powder samples that are converted to N_2, CO_2 and SO_2, the concentrations were measured by gas chromatography. The calibration curve was generated using 3 aliquots of pure sulfanilamide standards. The Kjeldhal method was used to estimate total nitrogen content with a conversion factor of 6.25.[37]

Degree of hydrolysis (DH)

The DH was measured using the trinitrobenzene sulfonate (TNBS) method described by Kristoffersen *et al*. [38] The buffer (0.21 M sodium phosphate buffer; pH 8.2) was prepared and stirred for 60 minutes at room temperature. Calibration solutions were prepared by a dilution series containing 0, 0.075, 0.15, 0.3, 0.6, 0.9, 1.2 and 1.5 mM Leucine in 1% SDS solution. The samples were prepared by dissolving 10 mg/mL hydrolysate powder in 0.1 M Tris-HCl pH 8.0 buffer followed by a dilution in 1% SDS-solution to 0.5 mg/mL. All samples and calibration solutions were measured in triplicate in Pierce™ 96-Well Polystyrene Plates, Corner Notch (Thermo Fisher Scientific, Waltham, MA, USA). 15 µL sample (reference or calibration solution) was added per well followed by the addition of 45 µL 0.21 M sodium phosphate buffer (pH 8.2) and 45 µL TNBS solution (0.05% w/v in water). The plate was sealed with a sticker and wrapped in aluminum foil to avoid UV degradation during the one-hour incubation time at 50 °C. After incubation, 90 µL 0.1 M HCl was added to all wells before absorbance was measured at 340 nm using a BioTek Synergy™ H1 spectrophotometer (BioTek Instruments, Winooski, VT, USA). The DH% values were then calculated according to Equation 1, using h_{tot} estimated from total nitrogen estimations from elemental combustion analysis (h is the number of cleaved peptide bonds).[39] Protein content analysis data is presented in figure 1A.

Equation 1 $$\mathrm{DH} = \frac{h}{h_{tot}} \times 100\%$$

Size exclusion chromatography

The SEC was preformed according to Wubshet *et al*.[36] 2 mg/mL injection solutions of standards and rehydrated hydrolysates (1% w/v), filtrated using Millex-HV PVDF 0.45 µm 33 mm filter (Millipore Sigma, Burlington, MA, USA) of the supernatants were directly used as injection solution without further modifications. Chromatographic separation of standards and samples was performed with a Thermo Scientific Dionex UltiMate 3000 Standard System (Thermo Fisher Scientific, Waltham, MA, USA). The injection volume was 10 µL for the standards and 15 µL for samples. Separation was performed at 25 °C using a BioSep-SEC-s2000 column (300 × 7.8 mm, Phenomenex, Torrence, CA, USA). The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion 30:70 (v/v), containing 0.05 % trifluoroacetic acid. Isocratic elution was carried out using a flow rate of 0.9 mL/min for 20.0 minutes. Between 20.0 and 20.1 minutes the mobile phase was changed to NaH_2PO_4 (0.10 M) and maintained until 23.0 minute for column cleaning. Elution conditions were restored between minute 23.0 and 23.1 and the column was equilibrated for an additional 27 minutes. Chromatographic runs were controlled from Chromeleon™ Chromatography Data System (CDS) software (Thermo Fisher Scientific, Waltham, MA, USA). From chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was used in the M_w calculations. The retention times of analytical standards were obtained from manual peak picking Chromeleon (CDS). The retention times of the standards were used to construct a third polynomial (r^2=0.97) fitted calibration curve.[40] The retention times for the standards are presented in supporting information (SI) Table S-2. Finally, M_w were calculated using PSS winGPC UniChrom V 8.00 (Polymer Standards Service, Mainz, Germany) for each chromatogram. The calculation from the software was based on a slicing method, similar to those previously used for analysis of protein hydrolysates. [41]

Bovine primary skeletal muscle cell isolation

The muscle cells were extracted from *Longissimus thoracis* (beef sirloin, Nortura AS, Rudshøgda, Norway) as previously described by.[42, 43] In short, muscle biopsy samples (1-2 g) were digested with 0.72 mg/mL collagenase in 10 mL DMEM containing 10 000 units per mL P/S and 250 µg/mL amphotericin B for 1 hour at 37°C with 70 rpm shaking. The tissue was further digested for 25 minutes with 0.05% trypsin/EDTA and added 10% FBS for enzyme inactivation, this step was repeated three times before the cells were pooled. To purify the myogenic cells (*i.e.*, fibroblast removal), the cells were incubated in uncoated 25 cm^2 cell culture flasks for one hour at 37°C and 5% CO_2 in maintenance culture media containing DMEM medium supplemented with 2% fetal bovine serum (FBS), 2% Ultroser G, 250 µg/mL fungizone , and 10 000 units per mL penicillin/streptomycin (P/S. Fibroblasts adhere to the plastic and the non-adhering primary muscle cells were collected and seeded into cell flasks coated with 1 mg/mL Entactin-Collagen-Laminin (ECL). When the cells reached 70-80% confluence they were harvested in freezing media (8% dimethyl sulfoxide in DMEM media) and stored in a liquid nitrogen tank. [42, 43]

Cell culture and treatment

Primary skeletal muscle cells were kept at 37°C and 5% CO_2 in maintenance culture media containing DMEM medium supplemented with 2% fetal bovine serum (FBS), 2% Ultroser G, 250 µg/mL fungizone, and 10 000 units per mL penicillin/streptomycin (P/S). Ultroser G is a commercially available serum substitute with semi-defined composition and is considered to have a concentration five times higher than fetal calf serum. The experimental culture media had three different serum conditions (serum free, reduced serum and normal serum) and did not contain P/S or amphotericin B. The constituents of the different culture media used in this study are listed in table 2. all experiments were performed in the 2nd or 3rd cell culture passage.

TABLE 2. CONTENT OF MAINTENANCE AND EXPERIMENTAL MEDIA

	Maintenance	Experimental		
MATERIAL		*Normal serum (S)*	*Reduced serum (RS)*	*Serum free (SF)*
DMEM	500 mL	50 mL	50 mL	50 mL
FBS	2 %	2 %	1 %	0 %
ULTROSER G	2 %	2 %	1 %	0 %
P/S	10 000 units per mL	0 units per mL	0 units per mL	0 units per mL
FUNGIZONE	250 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL

Determination of cell proliferation, viability and cytotoxicity

The cells were seeded 3000 cells per well grown in ECL coated 96-well plates (BD Falcon, Franklin Lakes, NJ, USA) in triplicates and kept at 37°C and 5% CO_2 in maintenance culture medium. 24 hours after seeding, the cells were treated with increasing concentrations (0.0001-10 mg/mL) of hydrolysates in experimental media with normal serum, reduced serum or serum free conditions (table 2). 48 hours after treatment cell viability and proliferation was analyzed using CellTiter-Glo ®Luminescent assay (Promega, Madison, WI, US) and CyQuant™ Assay (Invitrogen, Carlsbad, CA, US), respectively. Muscle cell cytotoxicity was measured as lactate dehydrogenase (LDH) leakage into the media using Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). Luminescent, fluorescent and absorbance signals, respectively, were measured using Synergy H1 hybrid multi-mode microplate reader (Biotek, Winooski, VT, USA).

Data treatment

Each cell culture experiment was seeded out in triplicates and repeated at least three individual biological replicates. The experiments (cell metabolic activity, proliferation and cytotoxicity) were performed in triplicates and repeated three to four times. Data are presented as mean ± SEM. Significant variance by treatments in comparison to the control sample (cells grown in normal serum conditions) was determined by one-way ANOVA using Dunnett`s multiple comparison test. Differences were considered significant at $p<0.05$. All statistical analysis was performed in Graph Pad Prism version 7.04 (GraphPadSoftware, La Jolla, CA, USA), and presented in detail in tables S-3 to S-13.

Conclusion

In this study we show that choice of by-product material and enzyme have impact on protein degradation during hydrolysis. The results show that none of the hydrolysates were harmful to the skeletal muscle cells. In fact, hydrolysates rich in peptides with approximately 2-15 amino acids in length enhanced cell growth. Most interestingly, this response was dependent on both material and choice of enzyme used. Of all the by-products tested pork plasma hydrolysates and yeast extract were the most promising materials. We suggest that these materials have the potential to replace serum during cultivation and as such be included in a tailor-made serum-free media.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1. I. T. Kadim, O. Mahgoub, S. Baqir, B. Faye and R. Purchas, Cultured meat from muscle stem cells: A review of challenges and prospects, *Journal of Integrative Agriculture*, 2015, **14**, 222-233.
2. C. E. Jochems, J. B. van der Valk, F. R. Stafleu and V. Baumans, The use of fetal bovine serum: ethical or scientific problem?, *Altern Lab Anim*, 2002, **30**, 219-227.
3. J. van der Valk, D. Brunner, K. De Smet, A. Fex Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price, M. L. Scarino and G. Gstraunthaler, Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods, *Toxicol In Vitro*, 2010, **24**, 1053-1063.
4. D. Brunner, J. Frank, H. Appl, H. Schoffl, W. Pfaller and G. Gstraunthaler, Serum-free cell culture: the serum-free media interactive online database, *Altex*, 2010, **27**, 53-62.
5. C. Rauch, E. Feifel, E. M. Amann, H. P. Spotl, H. Schennach, W. Pfaller and G. Gstraunthaler, Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media, *Altex*, 2011, **28**, 305-316.
6. T. Aspevik, A. Oterhals, S. B. Ronning, T. Altintzoglou, S. G. Wubshet, A. Gildberg, N. K. Afseth, R. D. Whitaker and D. Lindberg, Valorization of Proteins from Co- and By-Products from the Fish and Meat Industry, *Topics in current chemistry (Journal)*, 2017, **375**, 53.
7. S. Chakrabarti, S. Guha and K. Majumder, Food-Derived Bioactive Peptides in Human Health: Challenges and Opportunities, *Nutrients*, 2018, **10**.
8. T. T. Vuong, S. B. Ronning, T. A. E. Ahmed, K. Brathagen, V. Host, M. T. Hincke, H. P. Suso and M. E. Pedersen, Processed eggshell membrane powder regulates cellular functions and increase MMP-activity important in early wound healing processes, *PLoS One*, 2018, **13**, e0201975.
9. A. Mizrahi, Primatone RL in mammalian cell culture media, *Biotechnol Bioeng*, 1977, **19**, 1557-1561.
10. C. S. Bah, A. Carne, M. A. McConnell, S. Mros and D. Bekhit Ael, Production of bioactive peptide hydrolysates from deer, sheep, pig and cattle red blood cell fractions using plant and fungal protease preparations, *Food Chem*, 2016, **202**, 458-466.
11. T. Lafarga and M. Hayes, Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients, *Meat Science*, 2014, **98**, 227-239.
12. J. T. Wei and B.-H. Chiang, Bioactive peptide production by hydrolysis of porcine blood proteins in a continuous enzymatic membrane reactor, *Journal of the Science of Food and Agriculture*, 2009, **89**, 372-378.
13. Y. Yu, J. Hu, Y. Miyaguchi, X. Bai, Y. Du and B. Lin, Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived

from porcine hemoglobin, *Peptides*, 2006, **27**, 2950-2956.
14. Q. Liu, B. Kong, L. Jiang, X. Cui and J. Liu, Free radical scavenging activity of porcine plasma protein hydrolysates determined by electron spin resonance spectrometer, *LWT - Food Science and Technology*, 2009, **42**, 956-962.
15. O. Zarei, S. Dastmalchi and M. Hamzeh-Mivehroud, A Simple and Rapid Protocol for Producing Yeast Extract from Saccharomyces cerevisiae Suitable for Preparing Bacterial Culture Media, *Iran J Pharm Res*, 2016, **15**, 907-913.
16. M. Mosser, R. Kapel, I. Chevalot, E. Olmos, I. Marc, A. Marc and E. Oriol, Fractionation of yeast extract by nanofiltration process to assess key compounds involved in CHO cell culture improvement, *Biotechnol Prog*, 2015, **31**, 875-882.
17. C. F. Shen, T. Kiyota, B. Jardin, Y. Konishi and A. Kamen, Characterization of yeastolate fractions that promote insect cell growth and recombinant protein production, *Cytotechnology*, 2007, **54**, 25-34.
18. M. Merz, W. Claaßen, D. Appel, P. Berends, S. Rabe, I. Blank, T. Stressler and L. Fischer, Characterization of commercially available peptidases in respect of the production of protein hydrolysates with defined compositions using a three-step methodology, *Journal of Molecular Catalysis B: Enzymatic*, 2016, **127**, 1-10.
19. C.-Y. Chang, K.-C. Wu and S.-H. Chiang, Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates, *Food Chem*, 2007, **100**, 1537-1543.
20. S. G. Wubshet, D. Lindberg, E. Veiseth-Kent, K. A. Kristoffersen, U. Böcker, K. E. Washburn and N. K. Afseth, Bioanalytical aspects in Enzymatic protein hydrolysis of by-products, *Proteins: Sustainable Source, Processing and Applications*, ed. C. M. Galanakis, Academic Press, 2019, **8**, 225-258. DOI: https://doi.org/10.1016/B978-0-12-816695-6.00008-8,
21. E. M. Fujinari and J. D. Manes, Determination of molecular-mass distribution of food-grade protein hydrolyzates by size-exclusion chromatography and chemiluminescent nitrogen detection, *J Chromatogr A*, 1997, **763**, 323-329.
22. M. Smyth and R. J. FitzGerald, Relationship between Some Characteristics of WPC Hydrolysates and the Enzyme Complement in Commercially Available Proteinase Preparations, *International Dairy Journal*, 1998, **8**, 819-827.
23. H. A. Nchienzia, R. O. Morawicki and V. P. Gadang, Enzymatic hydrolysis of poultry meal with endo- and exopeptidases, *Poult Sci*, 2010, **89**, 2273-2280.
24. M. U. Rashid and K. M. Coombs, Serum-reduced media impacts on cell viability and protein expression in human lung epithelial cells, *J Cell Physiol*, 2019, **234**, 7718-7724.
25. A. Aldén, L. Gonzalez, A. Persson, K. Christensson, O. Holmqvist and S. Ohlson, Porcine platelet lysate as a supplement for animal cell culture, *Cytotechnology*, 2007, **55**, 3-8.
26. E. Donnelly and R. Delaney, The fractionation of porcine plasma by potential food industrial techniques, *International Journal of Food Science & Technology*, 2007, **12**, 493-503.
27. N. K. Howell and R. A. Lawrie, Functional aspects of blood plasma proteins, *International Journal of Food Science & Technology*, 1983, **18**, 747-762.
28. A. Carne, B. csf, A. Bekhit, A. Carne and M. McConnell, Slaughterhouse Blood: An Emerging Source of Bioactive Compounds, *Comprehensive Reviews in Food Science and Food Safety*, 2013, **12**, 314-331.
29. T. M. Odrljin, J. R. Shainoff, S. O. Lawrence and P. J. Simpson-Haidaris, Thrombin cleavage enhances exposure of a heparin binding domain in the N-terminus of the fibrin beta chain, *Blood*, 1996, **88**, 2050-2061.
30. M. M. Martino, P. S. Briquez, A. Ranga, M. P. Lutolf and J. A. Hubbell, Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix, *Proc Natl Acad Sci U S A*, 2013, **110**, 4563-4568.
31. M. W. Mosesson, Fibrinogen and fibrin structure and functions, *J Thromb Haemost*, 2005, **3**, 1894-1904.
32. D. C. Jan, S. J. Jones, A. N. Emery and M. al-Rubeai, Peptone, a low-cost growth-promoting nutrient for intensive animal cell culture, *Cytotechnology*, 1994, **16**, 17-26.

33. J. Girón-Calle, J. Vioque, J. Pedroche, M. Alaiz, M. M. Yust, C. Megías and F. Millán, Chickpea protein hydrolysate as a substitute for serum in cell culture, *Cytotechnology*, 2008, **57**, 263-272.
34. L. Specht, *An analysis of culture medium costs and production volumes for cell-beased meat*, The Good Food Institute, 2019, **1**, 1-29.
35. R. C. L. Lima, R. S. Berg, S. B. Ronning, N. K. Afseth, S. H. Knutsen, D. Staerk and S. G. Wubshet, Peptides from chicken processing by-product inhibit DPP-IV and promote cellular glucose uptake: potential ingredients for T2D management, *Food & function*, 2019, **10**, 1619-1628.
36. S. G. Wubshet, I. Mage, U. Bocker, D. Lindberg, S. H. Knutsen, A. Rieder, D. A. Rodriguez and N. K. Afseth, FTIR as a rapid tool for monitoring molecular weight distribution during enzymatic protein hydrolysis of food processing by-products, *Analytical Methods*, 2017, **9**, 4247-4254.
37. P. Sáez-Plaza, M. Navas, S. Wybraniec, T. Michałowski and A. Garcia Asuero, An Overview of the Kjeldahl Method of Nitrogen Determination. Part II. Sample Preparation, Working Scale, Instrumental Finish, and Quality Control, *Critical Reviews in Analytical Chemistry*, 2013, **43**.
38. K. Aase Kristoffersen, N. Kristian Afseth, U. Böcker, D. Lindberg, H. de Vogel-van den Bosch, M. Linnéa Ruud and S. Gizachew Wubshet, Average molecular weight, degree of hydrolysis and dry-film FTIR fingerprint of milk protein hydrolysates: Intercorrelation and application in process monitoring, *Food Chemistry*, 2020, DOI: https://doi.org/10.1016/j.foodchem.2019.1258
0039. A. H. Simonne, E. H. Simonne, R. R. Eitenmiller, H. A. Mills and C. P. Cresman, Could the dumas method replace the Kjeldahl digestion for nitrogen and crude protein determinations in foods?, *Journal of the Science of Food and Agriculture*, 1997, **73**, 39-45.
40. Y. Vander Heyden, S. T. Popovici and P. J. Schoenmakers, Evaluation of size-exclusion chromatography and size-exclusion electrochromatography calibration curves, *Journal of Chromatography A*, 2002, **957**, 127-137.
41. D. S. T. Hsieh, C. Lin, E. R. Lang, N. Catsimpoolas and C. K. Rha, Molecular-weight distribution of soybean globulin peptides produced by peptic hydrolysis, *Cereal Chemistry*, 1979, **56**, 227-231.
42. S. B. Ronning, M. E. Pedersen, P. V. Andersen and K. Hollung, The combination of glycosaminoglycans and fibrous proteins improves cell proliferation and early differentiation of bovine primary skeletal muscle cells, *Differentiation*, 2013, **86**, 13-22.
43. E. Veiseth-Kent, M. E. Pedersen, S. B. Ronning and R. Rodbotten, Can postmortem proteolysis explain tenderness differences in various bovine muscles?, *Meat Sci*, 2018, **137**, 114-122.

Figure Captions

Fig. 1 Chemical characterization of tested materials and hydrolysates including, chicken carcass (K), cod backbone (T), egg whites (EW), eggshell membrane (ESM), pork blood plasma (PBP) and Yeast extract (YE). A: Total nitrogen content was analyzed with elemental combustion analysis and estimated by using the Kjeldhal method with a conversion factor of 6.25. B: Degree of hydrolysis was determined by the TNBS method. C: Size exclusion chromatography (SEC) at 214 nm (UV) was used to determine the molecular weight distribution (MWD) profile of the different materials and hydrolyzed by-products. The raw materials were either lyophilized, or hydrolysed with enzymes (Alcalase (A) or Flavourzyme (F)) or NaOH. The different fractions (F1-4) display the percentage of peptides with approximate amino acid length; >15 (F1), 5-15 (F2), and 2-5 (F3), in addition to free amino acids (aa)(F4).

Fig 2. Cell metabolic activity (ATP) and proliferation (DNA) were analyzed in bovine skeletal muscle cells using CellTiter-Glo ®Luminescent assay (Promega) and CyQuant™ Assay (Invitrogen), respectively. The muscle cells were cultivated for 48 hours in normal serum conditions (2% FBS and 2% Ultroser G), reduced serum (1% FBS and 1% Ultroser G) or serum-free conditions (0% FBS and 0% Ultroser G). The results are presented as mean ± SEM (n= 66 independent cell experiments seeded out in triplicates). Asterix indicate significant differences compared to control (cells grown in normal serum conditions). ****p<0.0001 determined by one-way ANOVA using Dunnett`s multiple comparison test).

Fig 3. **Supplementation of hydrolysates from pork plasma by-products (A), yeast extract (B), chicken and cod by-products (C) to cell culture media enhanced cell growth in normal serum conditions.** The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded out at density 3000 cells / well in 96-well plates and cultivated in media with normal serum conditions supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).

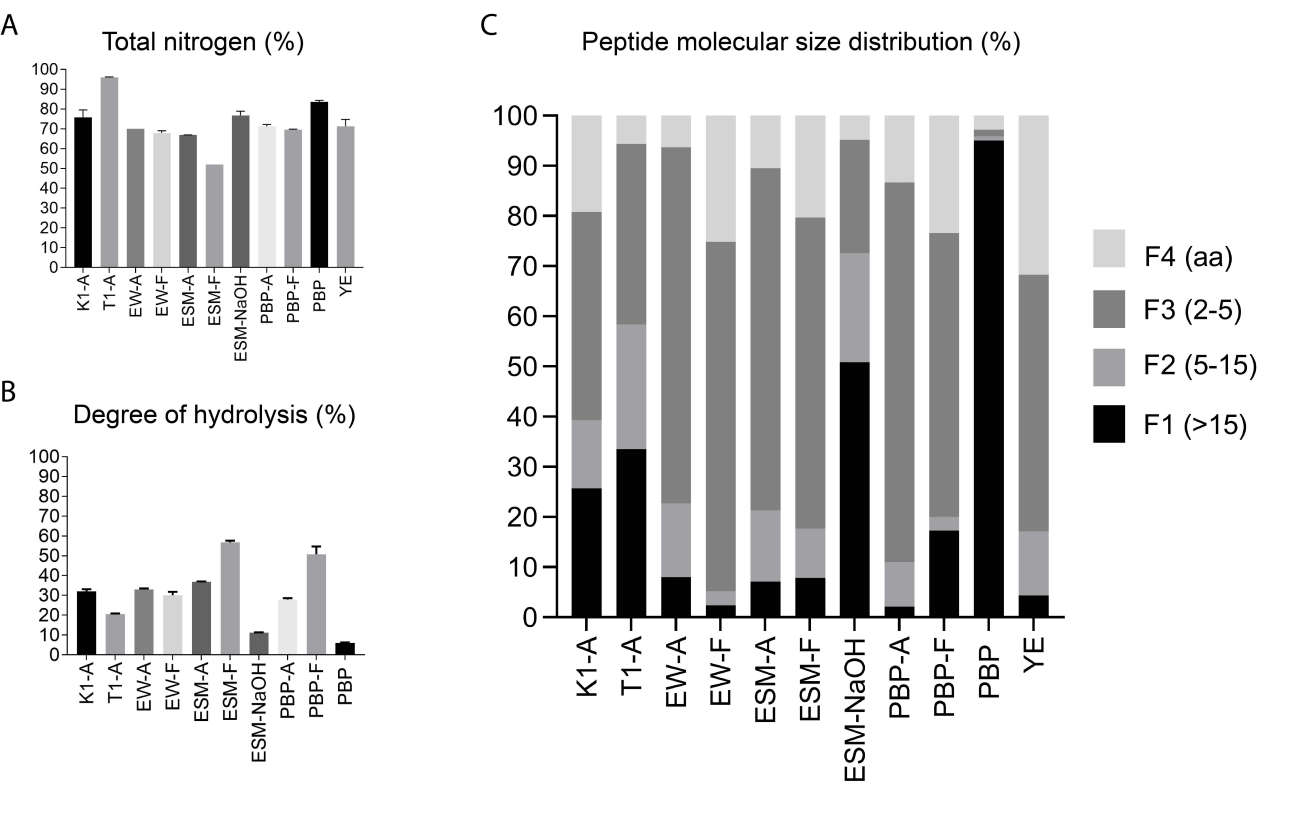
Fig 4. **Supplementation of hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) to cell culture media enhanced cell growth in normal serum conditions.** The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded out at density 3000 cells / well in 96-well plates and cultivated in media with normal serum conditions supplemented with protein hydrolysates, extract in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).

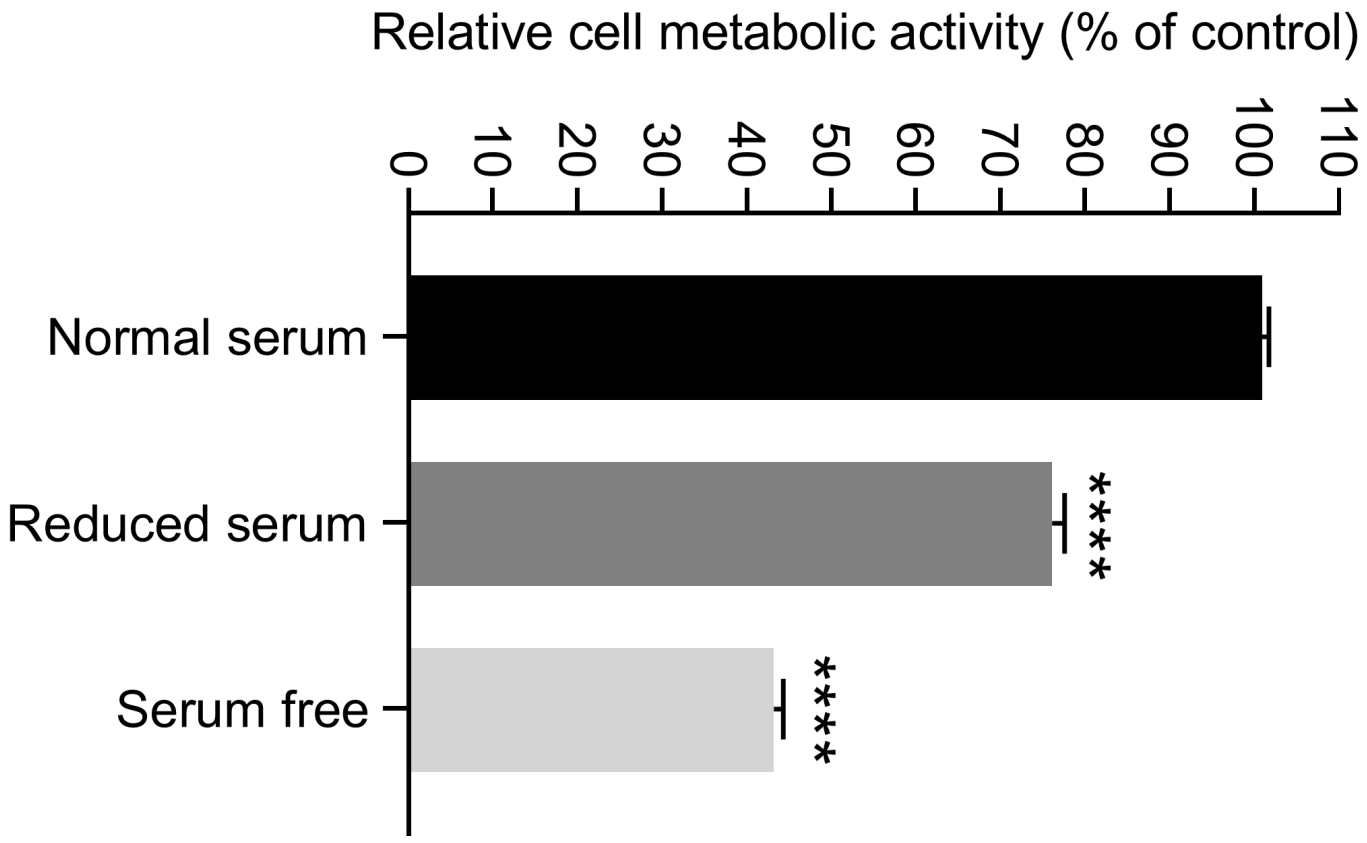
Fig 5. By-product hydrolysates from pork plasma by-products (A), yeast extract (B), chicken and cod by-products (C) recover and enhance muscle cell metabolic activity and proliferation in reduced serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in DMEM media with 2% FBS and 2% Ultroser G (normal serum conditions). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with reduced serum conditions (1% FBS and 1% Ultroser G) supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).

Fig 6. By-product hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) recover and enhance muscle cell metabolic activity and proliferation in reduced serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in DMEM media with 2% FBS and 2% Ultroser G (normal serum conditions). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with reduced serum conditions (1% FBS and 1% Ultroser G) supplemented with protein hydrolysates in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).

Fig 7. Alcalase digested pork plasma (A), yeast extract (B) and chicken and cod by-products (C) recover and boost cell growth in serum-free conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with serum-free conditions (0% FBS and % Ultroser G) supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).

Fig 8. By-product hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) recover muscle cell metabolic activity and proliferation in serum-free conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (y-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with serum-free conditions (0% FBS and % Ultroser G) supplemented with protein hydrolysates in dilution series with concentrations ranging from 0.0001-10 mg/ml (x-axis). Luminescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n=3-4 independent cell experiments seeded out in triplicates).


A
Total nitrogen (%)
100
90
80
70
60
50
40
30
20
10
0
K1-A
T1-A
EW-A
EW-F
ESM-A
ESM-F
ESM-NaOH
PBP-A
PBP-F
PBP
YE
B
Degree of hydrolysis (%)
100
90
80
70
60
50
40
30
20
10
0
K1-A
T1-A
EW-A
EW-F
ESM-A
ESM-F
ESM-NaOH
PBP-A
PBP-F
PBP
C
Peptide molecular size distribution (%)
100
90
80
70
60
50
40
30
20
10
0
K1-A
T1-A
EW-A
EW-F
ESM-A
ESM-F
ESM-NaOH
PBP-A
PBP-F
PBP
YE
F4 (aa)
F3 (2-5)
F2 (5-15)
F1 (>15)


Relative cell metabolic activity (% of control)
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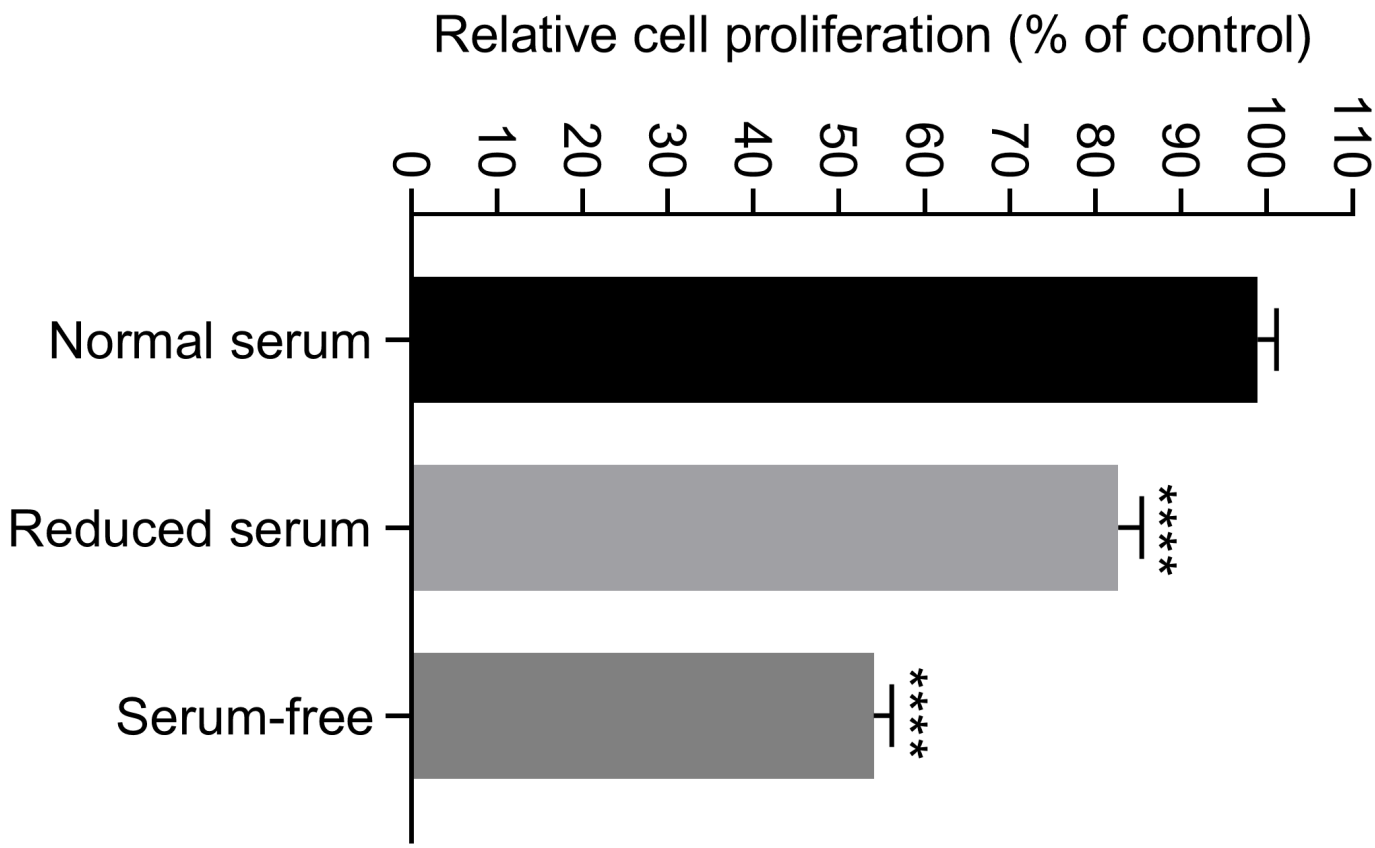
20
30
40
50
60
70
80
90
100
110
Normal serum
Reduced serum
Serum free
****
****


Relative cell proliferation (% of control)
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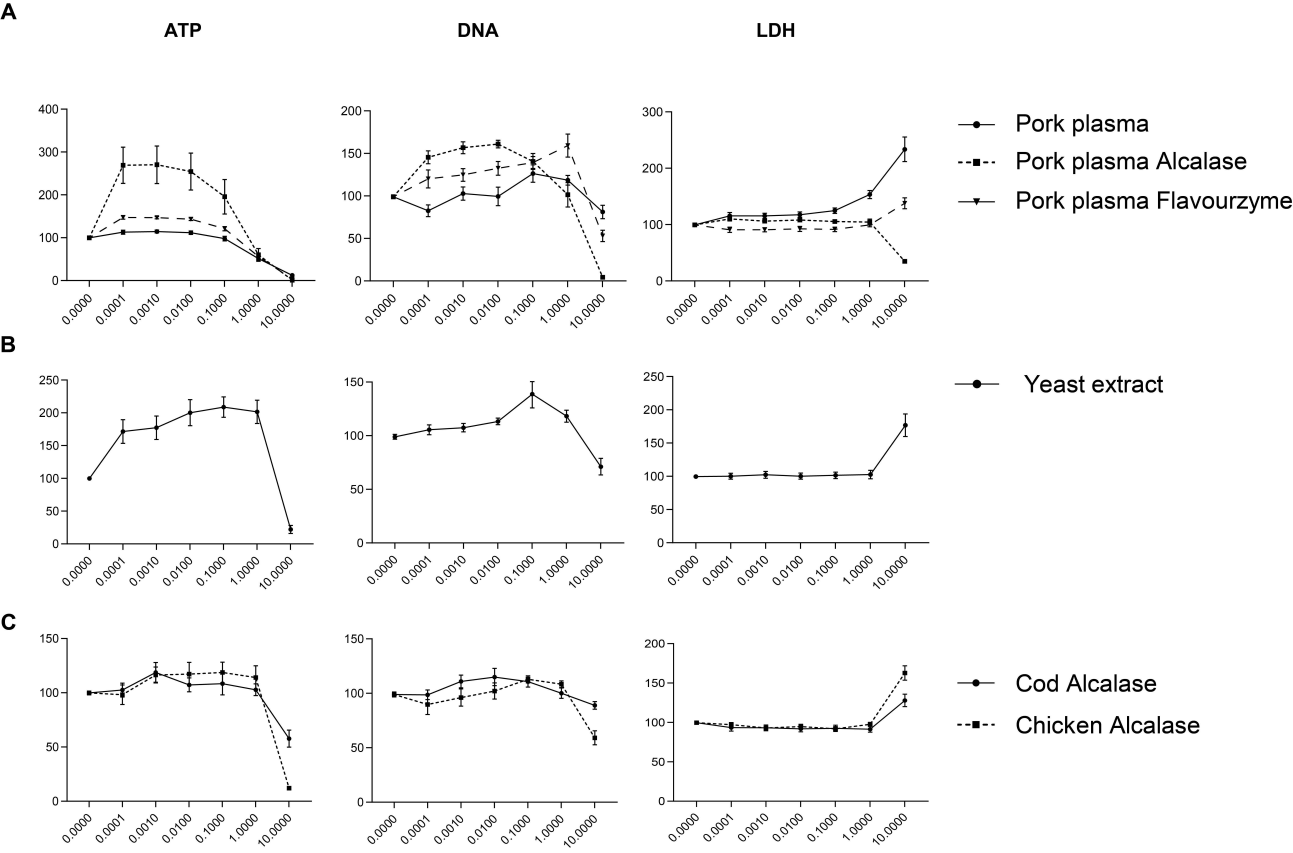
10
20
30
40
50
60
70
80
90
100
110
Normal serum
Reduced serum
Serum-free
****
****


A
ATP
DNA
LDH
Pork plasma
Pork plasma Alcalase
Pork plasma Flavourzyme
B
Yeast extract
C
Cod Alcalase
Chicken Alcalase
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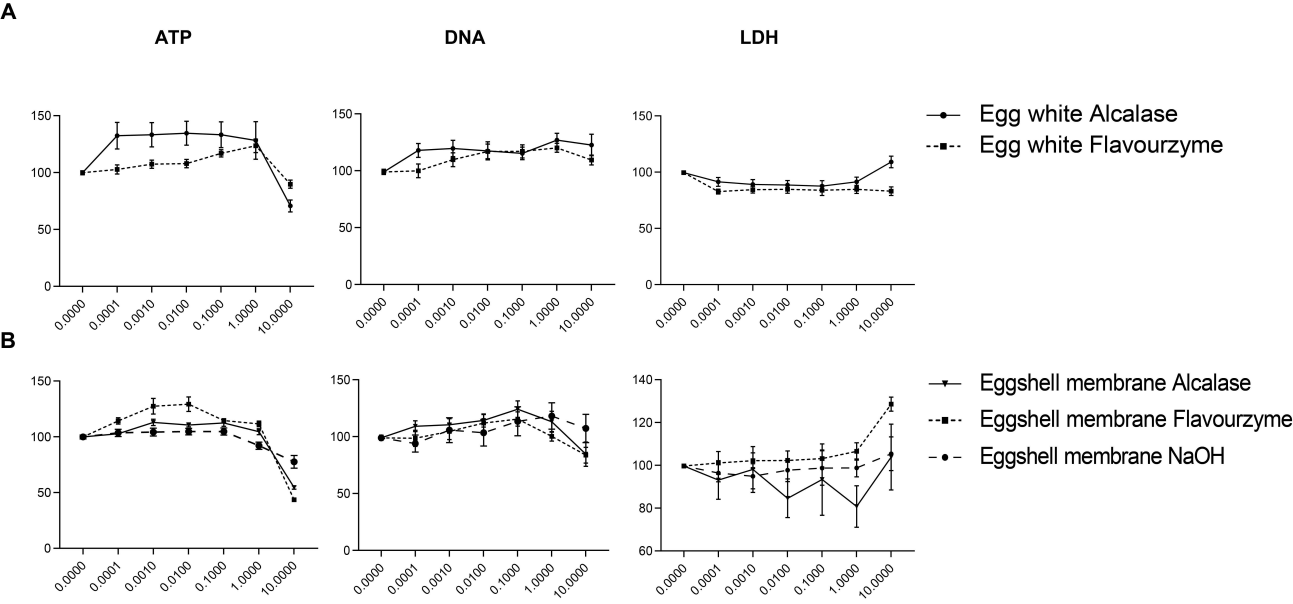
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000


A
ATP
DNA
LDH
Egg white Alcalase
Egg white Flavourzyme
B
Eggshell membrane Alcalase
Eggshell membrane Flavourzyme
Eggshell membrane NaOH
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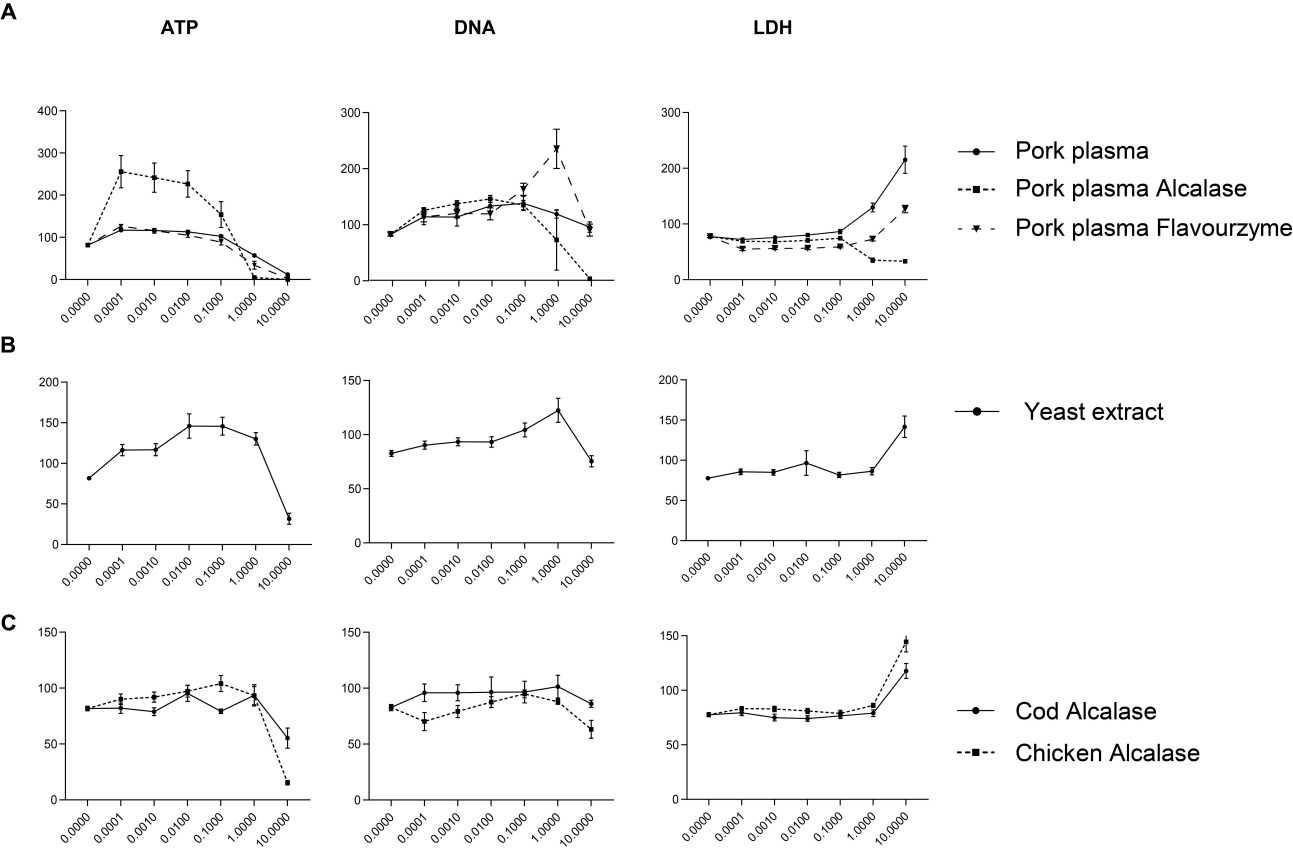
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000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ATP
DNA
LDH
Pork plasma
Pork plasma Alcalase
Pork plasma Flavourzyme
B
Yeast extract
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Chicken Alcalase
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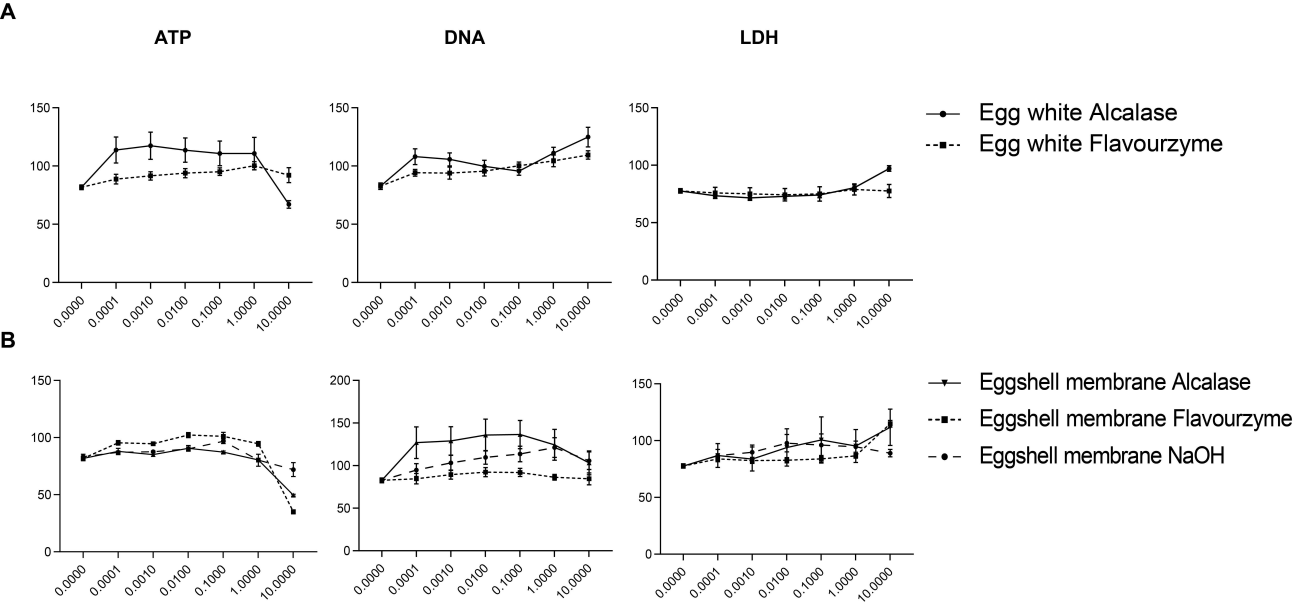
1.0000
10.0000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DNA
LDH
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Egg white Flavourzyme
B
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Eggshell membrane NaOH
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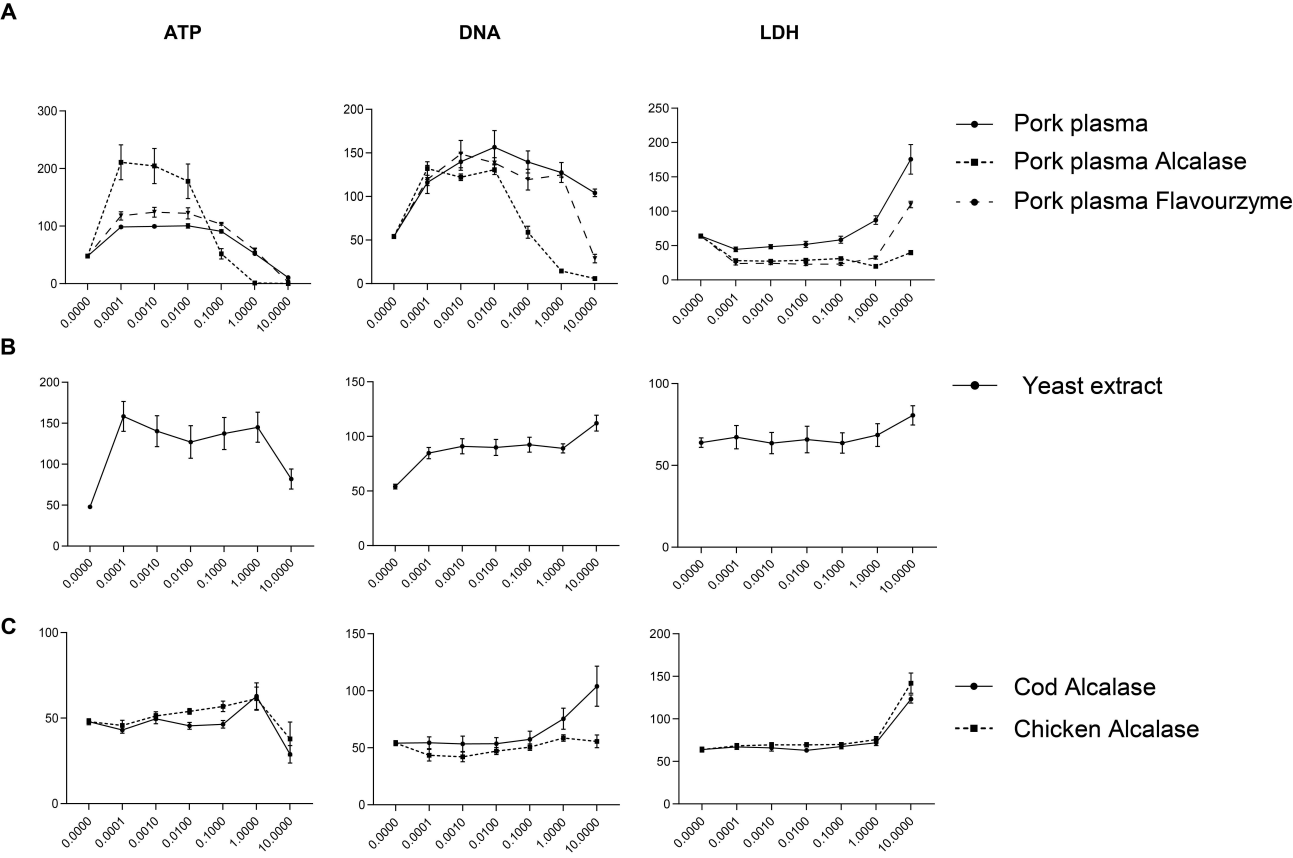
0.0010
0.0100
0.1000
1.0000
10.0000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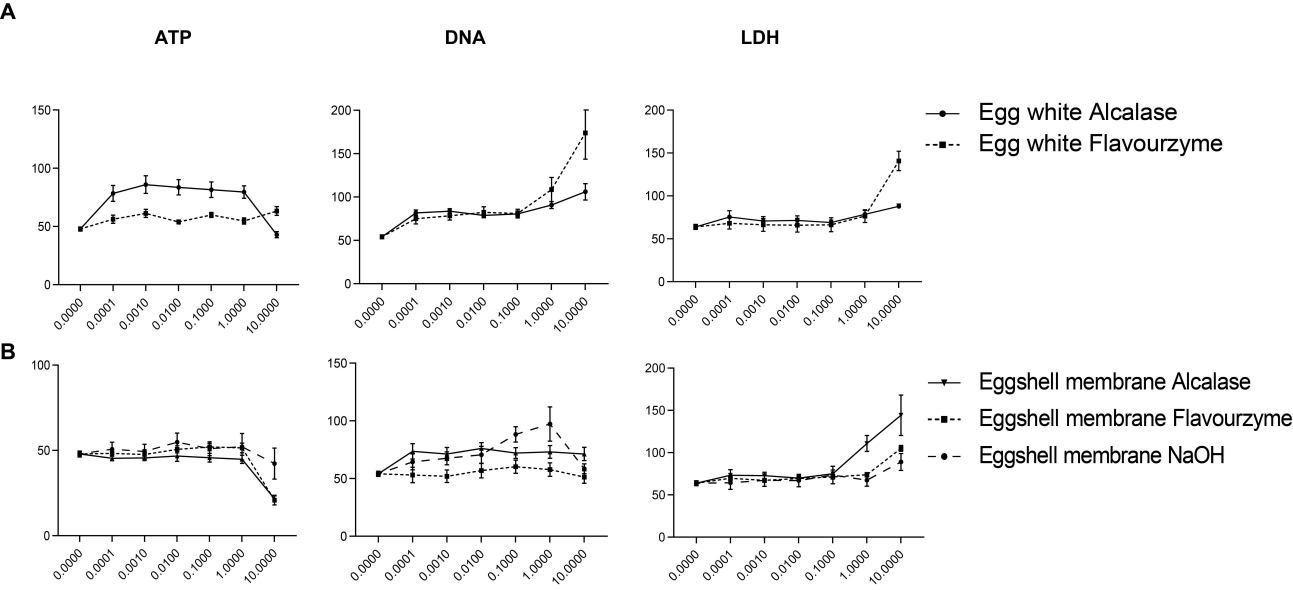
10.0000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ATP
DNA
LDH
Egg white Alcalase
Egg white Flavourzyme
B
Eggshell membrane Alcalase
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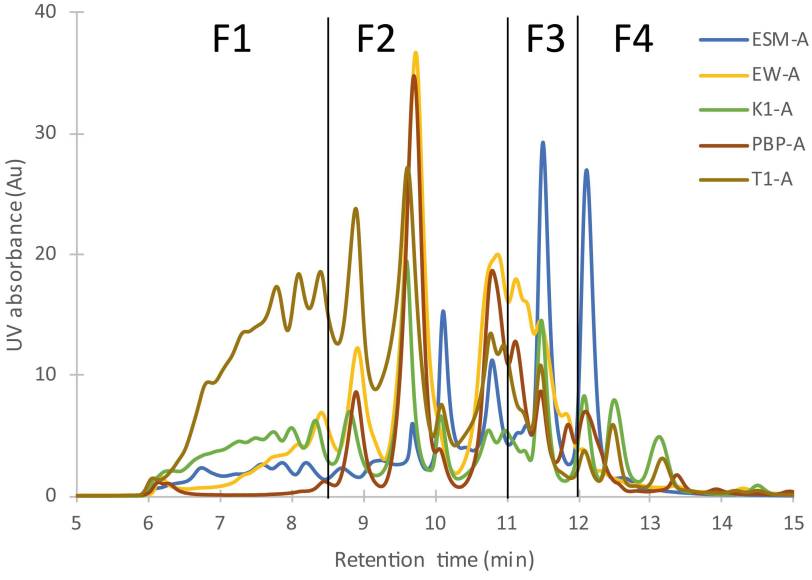
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000

A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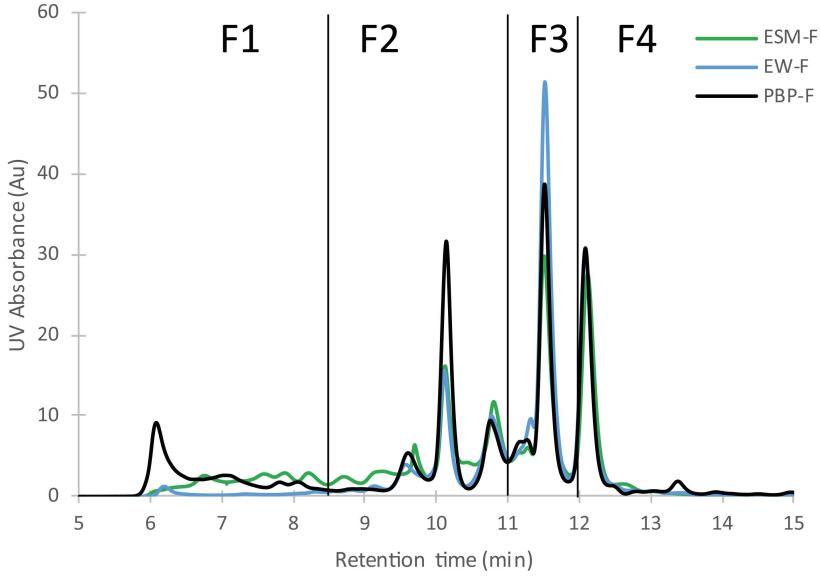
F2
F3
F4
ESM-A
EW-A
K1-A
PBP-A
T1-A
UV absorbance (Au)
Retention time (min)

B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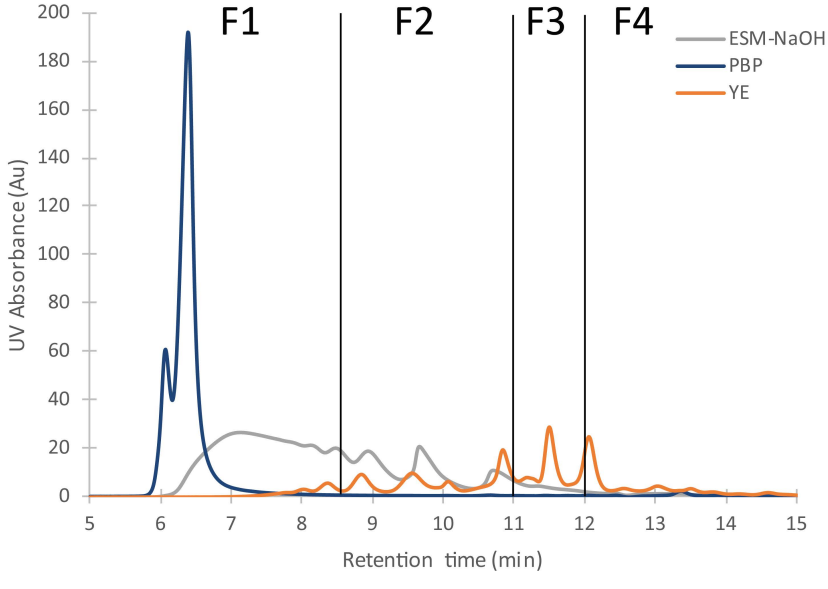
PBP-F
UV Absorbance (Au)
Retention time (min)

C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F3
F4
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PBP
YE
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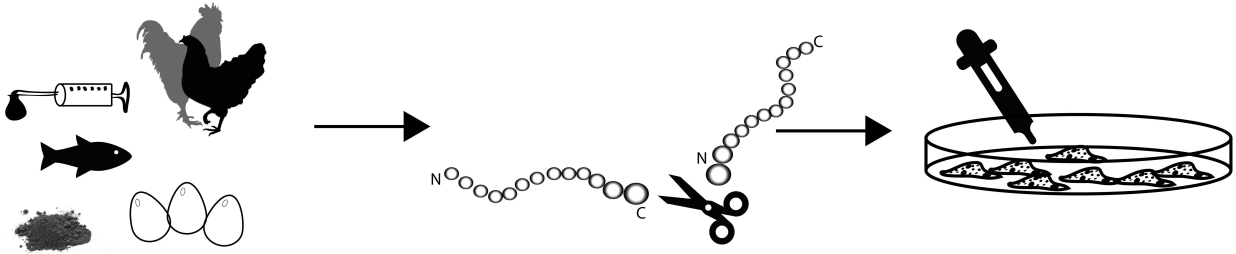
Retention time (min)



Hydrolysates from food by-products allow higher cell growth and metabolic activity than commercially available serum in skeletal muscle cell culture and can potentially be used to produce a tailor-made serum-free media.