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Proteomic Characterization of IgY Preparations Purified with a Water Dilution Method

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Antigen-specific chicken IgY antibodies have been used for oral immunotherapy as an alternative or complement to antibiotics in several studies. The water dilution (WD) method has several advantages for purifying IgY. It is rapid, efficient, suitable for large-scale production, and nothing but water is added. The water-soluble fraction contains other proteins and lipids besides IgY. The protein content was characterized by two-dimensional gel electrophoresis (2DGE) and nanoflow liquid chromatography coupled offline to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (nanoLC-MALDI TOF/TOF MS). Protein analysis was complicated due to the large dynamic concentration range, but 26 proteins could be identified. The relative protein concentrations in different batches were very similar according to protein patterns on 1D gels and protein concentration determinations. Thus, the purification method has a high reproducibility. The concentrations of cholesterols and triglycerides were low and should not have an effect on the plasma levels of treated patients. Purification of IgY for oral use with WD is therefore a recommended method.

KEYWORDS: Egg yolk; water dilution method; two-dimensional gel electrophoresis; nanoLC-MALDI TOF/TOF MS

INTRODUCTION

There is increasing interest in the use of immunoglobulin Y (IgY) from chicken eggs for immunotherapeutic and immunodiagnostic purposes, and the importance of eggs as a source of antibodies is well recognized. The egg yolk contains high concentrations of antibodies, but the high concentration of lipids in the egg yolk is a major problem in the purification of the antibodies. The egg yolk of the hen egg is mainly composed of proteins and lipids and can be separated into a granular and a plasma fraction (*1*). The plasma fraction can be further divided into low-density lipoproteins and a water-soluble part. The water-soluble part contains livetins including IgY. IgY is the most abundant serum antibody in the chicken and functionally similar to both mammalian IgG and IgE (*2*). IgY antibodies are actively transferred from the hen serum to the egg yolk where they are found in high concentration (*3*). Specific antibodies are obtained by immunizing the hen with the antigen of interest. There are several ways to purify IgY from the egg yolk, for example, salt, dextran sulfate, xanthan, gum, ethanol, and polyethylene glycol precipitation, thiophilic chromatography, or water dilution (WD) (*4*−*6*). The WD method yields preparations containing water-soluble egg yolk proteins and some lipids (*4*). This method is simple, rapid, suitable for large-scale production, and efficient for obtaining IgY with high activity (*5*).

IgY is attractive for oral immunotherapy because it neither activates the human complement system nor reacts with rheumatoid factors, human anti-mouse antibodies, or human Fc receptors, which are all well-known cell activators and mediators of inflammation (*7*). The WD method does not include any toxic compounds or any other additives, but water. Therefore, orally administered IgY treatment is comparable to eating eggs and is as such nontoxic as long as the subject is not egg allergic.

Specific IgY antibodies are an alternative to antibiotics for humans and animals. Several animal studies and a number of human studies on IgY against different pathogens have proven IgY to effectively treat and prevent infections. For example, IgY against rotavirus was protective against bovine rotavirus both in calves and in mice, whereas anti-*Escherichia coli* IgY reduced mortality in newborn piglets (*8*). Salmonellosis has been prevented both in neonatal calves and in a mice model. In humans IgY against *Streptococcus mutans* decreased caries when used as a mouth rinse (*9*) and anti-*Helicobacter pylori* IgY reduced *Helicobacter* infections (*10, 11*). The latter has been tested as a supplement in drinking yogurt (*11*), which shows the possibility of using IgY in functional food. Our group has performed the longest study of oral IgY treatment to humans by studying anti-*Pseudomonas* IgY to cystic fibrosis (CF) patients. They have been treated for up to 13 years. The treatment prevents *Pseudomonas aeruginosa* infections and

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delays the time to chronic infections (*12, 13*). Chronic *Pseudomonas aeruginosa* lung infections are one of the major causes of morbidity and mortality in CF patients (*14*).

We used the WD method for the purification of IgY as we considered it to be the most suitable for large-scale production of yolk antibodies for immunotherapeutic purposes. One aim of this study was to characterize the antibody preparations purified with the WD method and to identify proteins other than IgY present in the preparations. Other aims were to investigate the reproducibility of the WD method and determine cholesterol and triglyceride levels.

MATERIAL AND METHODS

IgY Preparation. IgY was prepared from immunized eggs by a water dilution method (*4*). Briefly, the egg yolk from immunized eggs was separated from the white and diluted 1:10 in deionized water. Then the solution was left to settle at 4 °C for at least 6 h to form a lipid-containing precipitate and a supernatant containing the water solubilized proteins/compounds, including IgY. The supernatant was then easily removed from the precipitate by decanting.

SDS-PAGE. The protein compositions of 25 samples of IgY preparations were determined by 1D-SDS-PAGE performed with Invitrogen's (Carlsbad, CA) system and 4−12% polyacrylamide gels (Tris-HCl) according to their instructions. SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used as MW marker. Electrophoresis was performed at 200 V for 35 min. Protein bands were detected by staining with Colloidal Blue Kit (Invitrogen) and performed according to the manufacturer's instructions. The total protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as standard.

2D Sample Preparation. The IgY preparation was dialyzed and then either used directly or freeze-dried in a Speedvac and the product dissolved in MQ-H_2O (0.5−4 mg/mL). Proteins (400 μL) were precipitated by adding 3 volumes (1200 μL) of −20 °C cold acetone and followed by incubation overnight at −20 °C. The next day the samples were centrifuged for 10 min at 16000*g* at 4 °C. The protein pellet was washed twice in (1200 μL) ice -cold acetone. The final pellet was reconstituted in ReadyPrep 2-D starter kit rehydration/sample buffer (Bio-Rad) to a final concentration in the range of 1−2 mg/mL. The sample was incubated for 1 h to allow proteins to solubilize. Thereafter, the protein sample was centrifuged at 16000*g* for 10 min at 20 °C to remove unsolubilized products. The final supernatant was filtered and immediately used for strip rehydration.

2D Gel Electrophoresis. Seventeen centimeter long isoelectric focusing strips, pH 4−7 (Bio-Rad), were passively rehydrated overnight with 300 μL (300−600 μg) of protein preparation under mineral oil at room temperature. Isoelectric focusing was carried out the next day for a total of 40 kV·h using an IPG-phor unit (Bio-Rad) (20 °C). Focused strips were stored at −70 °C. Before the second electrophoresis step, strips were equilibrated for 10 min in ReadyPrep 2-D starter kit equilibration buffer I containing dithiothreitol and ReadyPrep 2-D starter kit equilibration buffer II containing iodoacetamide (Bio-Rad). Thereafter, the strips were attached to 8−16% SDS gels (Tris-HCl, 193 × 183 × 1 mm^3) Protean II Ready Gel, Precast gel (Bio-Rad). Electrophoresis was performed in a Protean II XI cell (Bio-Rad) and run for 30 min at 16 mA/gel, to allow proteins to enter the gel, and then the current was increased to 24 mA/gel. Electrophoresis continued for nearly 5 h. Protein spots were visualized with a Colloidal Blue Kit (Invitrogen) according to the manufacturer's instructions. Spots were excised from the gel and analyzed by MALDI-TOF MS at the WCN Expression Proteomics Facility (Department of Medical Biochemistry and Microbiology, Uppsala University) (*15*).

Immediate Protein Digestion Procedure for Shotgun Proteomics. An aliquot of the IgY preparation, corresponding to approximately 240 μg of total protein content, was dried in a Speedvac and redissolved in 100 μL of digestion buffer (8 M urea, 400 mM NH_4HCO_3). A volume of 10 μL of 45 mM dithiothreitol (GE Healthcare, Uppsala, Sweden) was added before incubation for 15 min (50 °C). Furthermore, 10 μL of 100 mM iodoacetamide (Sigma Chemical Co., St. Louis, MO) was added, followed by incubation at room temperature (15 min, darkness). Afterward, 10 μg of trypsin (1:24 w/w) from bovine pancreas (1418475) (Roche Diagnostics, Penzberg, Germany), dissolved in 100 μL of water, was added, and the samples were incubated for 24 h at 37 °C in darkness. The samples were desalted on ZipTip C_{18} columns (Millipore, Bedford, MA) (*16*).

Shotgun Proteomic Analysis Using NanoLC-MALDI TOF/TOF MS. NanoRP-HPLC was performed with a 1100 nanoLC system (Agilent Technologies, Waldbronn, Germany), equipped with a fraction collector capable of direct fractionation onto a MALDI target plate. A volume of 8 μL, corresponding to approximately 400 ng of digestion products, was injected into a 10 μL sample loop. A 15 cm × 180 μm, C18 column (Thermo Electron, Waltham, MA) with 5 μm particle size and an H_2O/ACN/TFA solvent system [H_2O, 0.1% TFA (A); ACN, 0.1% TFA (B)] for separating the enzymatic cleavage products was used. A flow rate of 2 μL/min starting with isocratic elution at 2% B for 20 min, then gradient elution from 2 to 8% B in 5 min, then from 8 to 32% B within 86 min, then from 32 to 40% B in 5 min, and finally from 40 to 80% B in 1 min was applied. The peptide elution was followed by online fractionation onto a MALDI target with a collection rate of four fractions a minute for 96 min within the elution period from 20 min (2% B) to 116 min (40% B), resulting in 384 fractions. For optimal MS results disposable prespotted anchorchip targets (PAC-targets, Bruker Daltonics, Bremen, Germany) were chosen. Mass data were acquired with an Ultraflex II MALDI-TOF/TOF (Bruker Daltonics) in reflector mode. The acquisition was assisted by applying WarpLC software (Bruker Daltonics) for automatic TOF-MS spectra acquisition followed by optimized precursor selection for subsequent MS/MS experiments.

Data Analysis. For final protein identification all collected MS/MS data were run in a combined Mascot database search. The following specified parameters were applied for database search: database (SwissProt v. 51.6); taxonomy (Metozoa); proteolytic enzyme (trypsin); peptide mass tolerance (±50 ppm); fragment mass tolerance (±0.5 Da); global modification [carbamidomethyl (Cys)]; variable modification [oxidation (Met)]; peptide charge state (1+); maximum missed cleavage (1). The offline setup is beneficial for optimal precursor ion selection for subsequent MS/MS experiments. First, TOF-MS data from all 384 collected fractions were acquired. Afterward, a compound list of all contingent precursor candidates was created, and a ranking of each detected ion for each spot was performed. Hereby, the distribution and abundance of each mass are taken into consideration to evaluate which precursor ion should be fragmented on which spot to best cover as many compounds as possible for the following MS/MS experiments. The acquired MS/MS data were combined in one data file and subjected to comprehensive database search following the Mascot algorithm. Here, every MS/MS spectrum was searched individually, and the results were merged afterward, resulting in one protein list without redundancy. In LC-MS/MS data base proteomics, large sets of fragmentation spectra are created, and final protein identification by only a single peptide is not uncommon. Therefore, harsh peptide identification criteria are necessary to prevent false-positive protein matches. Each protein was considered to be a positive match if it was identified by at least one MS/MS that fulfilled criteria of significance. The significance threshold was set to at least 95% ($p \leq 0.05$), and peptide uniqueness was required, indicating identity or extensive homology. To avoid accumulation of low-scoring peptide identities to false-positive total ion scores, the result import settings were set accordingly to allow only unique peptides with individual ion scores beyond the significance threshold to contribute to the total proteinscore. These limitations for tandem MS-based protein identification allow database searching of large MS/MS data sets, where typically low-scoring peptide matches are observed due to statistical distribution also known as Poison distribution (www.matrixscience.com/help/interpretation_help.html).

Cholesterol and Triglyceride. Cholesterol (reagent 7D62-20) and triglyceride (reagent 7D74-20) measurements were performed on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL) according to the recommendations of the manufacturer and reported using SI units. The total coefficient of variation (CV) for the cholesterol

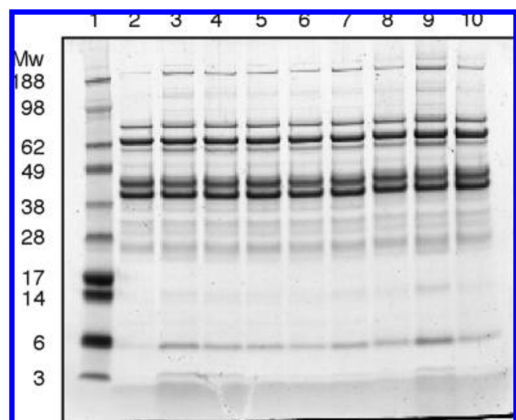

Figure 1. 1D gel of nine IgY preparations: lane 1, protein standard; lanes 2−10, nine IgY batches purified from egg yolk with the water dilution method. The protein pattern was the same for all batches.

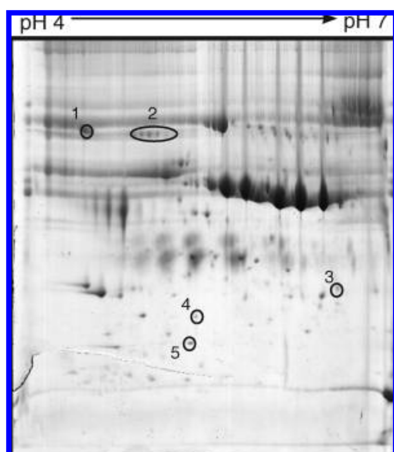

Figure 2. 2D gel of proteins in an IgY preparation. Due to the high dynamic range of the proteins it was hard to find an optimal concentration. Thus, some proteins are overrepresented, causing streaking. Five protein spots were identified from the gel: PIT-54 (1); hemopexin (2); Ig γ chain (3); similar to Niemann−Pick disease (type 2) (4); and transthyretin (chain A) (5).

method was 0.7% at 3.3 mmol/L and 0.5% at 5.7 mmol/L. The total CV for the triglyceride method was 1.1% at 0.9 mmol/L and 1.1% at 2.2 mmol/L.

RESULTS

Reproducibility of Protein Content. The protein content of 25 IgY samples was investigated by SDS-PAGE and colloidal blue staining (**Figure 1**). The same pattern was found both for the content and for the amount of proteins, independent of storage for different numbers of months at −20 °C. The protein concentration for all batches was around 1.8 mg of proteins/mL (C.V. 7%), as determined by Bradford assay.

Protein Characterization by 2DGE. An initial screening with a 2D gel with a pH range of 3−10 showed that almost all proteins were in the pH range of 4−7, and therefore this range was chosen for further analysis (data not shown). Many spots appeared on the gels, but there were also problems with streaking that disturbed the picture (**Figure 2**). Gels with samples from different IgY batches had a similar protein pattern.

Five proteins were identified with MS analyses of excised and trypsinated gel spots from 2DGE experiments. These findings include hemopexin, transthyretin (chain A), PIT-54, similar to Niemann−Pick disease (type 2), and Ig γ chain.

However, protein identification was hampered due to the large dynamic range of protein abundances. Therefore, additional analytical strategies for protein identification were necessary.

Protein Identification by NanoLC-MALDI TOF/TOF MS. To gain further knowledge about the protein content of the here-described IgY preparations, we decided to apply another proteomic bottom-up approach based on nanoLC MALDI TOF/TOF MS. The results show identification of 21 additional proteins. The most abundant protein identities according to this method were α-livetin (a serum albumin precursor), conalbumin (ovotransferrin precursor), ovalbumin, and vitellogenins 1 and 2 (**Table 1**).

Lipids. In the 16 batches investigated none contained >0.6 mM of cholesterol, and all but two contained <0.5 mM. Triglyceride levels were in the range of 0.43−2.68 mM (medium = 1.39 mM ± 0.68). Cholesterol levels are well below the reference interval for human cholesterol.

DISCUSSION

Previous comparison between different purification methods for IgY has proved the water dilution method to be effective (*5*). The highest recovery and the highest yield were obtained by this method, and it was also the best for large-scale production, which is important for medicinal use when large quantities are needed. We have treated CF patients with anti-*Pseudomonas* IgY with promising results (*12, 13*). Because the antibodies were purified without the addition of any toxic products, anti-*Pseudomonas* IgY treatment is somewhere between a medical agent and a functional food. The WD method gives a high yield of IgY, and also other water-soluble proteins and some lipids. In this work the IgY preparations were characterized to get a better picture of what the treatment contains. The evaluation of components present after water dilution purification of egg yolks may help in identifying useful proteins in addition to IgY. The only precaution that should be taken for IgY as oral treatment is egg allergy. By defining the proteins in the water-soluble fraction, the risks are characterized.

There was a high reproducibility in protein content between the batches, with regard to protein pattern on 1D gels (**Figure 1**) as well as protein concentration. This confirms that the water dilution method for purification of IgY yields a consistent formula. IgY is very stable over time, and here it was also confirmed that the other proteins in the 1D gels were intact. In addition, the protein content did not change after freeze-drying or freeze-drying followed by storage at 37 °C for 8 weeks.

In the IgY solutions there is a huge dynamic range concerning the protein concentrations that was problematic both for 2D electrophoresis and for nanoLC-MALDI TOF/TOF MS analysis. This is clearly seen on colloidal blue stained gels (**Figure 2**). It was therefore difficult to find an optimal concentration at which low-abundant proteins are visualized and high-abundant ones do not lead to streaking or hide the less abundant ones. Streaking could also be due to lipid and salt contamination.

Due to the large dynamic range concerning the protein concentrations, suppression of low-abundant analytes is a major challenge in proteomic analysis. Therefore, highly resolving and highly sensitive separation and detection methods are essential to increase the level of protein identification. The presented bottom-up strategy, based on enzymatic digestion of the whole sample followed by nanoLC coupled offline to MALDI TOF/TOF MS, has proven to be a powerful proteomic approach for profiling egg yolk proteins. In total, 21 proteins could be identified (**Table 1**). To obtain the best possible separation of the enzymatic cleavage products, a gradient elution optimization

Table 1. Proteins Identified with NanoLC-MALDI TOF/TOF MS

no.	protein name	database entry[a]	Mascot score[b]	MW[c]	no. of peptides[d]
1	serum albumin	ALBU_CHICK	2034	71867.69	49
2	ovotransferrin	TRFE_CHICK	1117	79551.09	20
3	ovalbumin	OVAL_CHICK	1061	43195.63	17
4	vitellogenin-1	VIT1_CHICK	783	212608.02	13
5	vitellogenin-2	VIT2_CHICK	769	206731.51	16
6	ovalbumin related protein Y	OVALY_CHICK	415	44028.85	10
7	apolipoprotein B (fragment)	APOB_CHICK	362	50872.65	9
8	ovoinhibitor	IOV7_CHICK	293	54394.10	6
9	apovitellenin-1	APOV1_CHICK	259	12015.55	9
10	ovomucoid	IOVO_CHICK	180	23659.68	2
11	riboflavin-binding protein	RBP_CHICK	130	28276.62	3
12	apolipoprotein A-I	APOA1_CHICK	122	30640.25	4
13	Ig λ chain C region	LAC_CHICK	114	11524.60	2
14	ovalbumin-related protein X	OVALX_CHICK	79	26331.26	2
15	fibrinogen α chain	FIBA_CHICK	62	83242.38	1
16	coagulation factor IX	FA9_CHICK	77	53082.68	1
17	complement factor B-like protease	CFBL_CHICK	77	27719.08	1
18	Ig λ chain V-1 region	LV1_CHICK	41	11801.72	1
19	vitelline membrane outer layer protein 1	VMO1_CHICK	32	20677.28	1
20	filensin	BFSP1_CHICK	30	76457.74	1
21	cystatin	CYT_CHICK	29	15561.90	1

[a] Uniprot knowledgebase entry. [b] Mascot protein score revealed by MudPIT scoring. Proteins were found and identified by integrated Mascot database batch search of all MS/MS in SwissProt v 51.6. All matches are identified significantly. Identified proteins are considered as positive match on at least a 95% significance level ($p < 0.05$), corresponding to a significance threshold ion score of 29. [c] Molecular mass in daltons. [d] Number of tryptic peptides that match the identified protein. At least one matching peptide for each identified protein must fulfill criteria of significance ($p < 0.01$) and uniqueness.

Figure 3. Mass chromatogram of one nanoLC-MALDI TOF/TOF MS experiment: two-dimensional survey of all mass spectra [*x*-axis; *m*/*z*, mass over charge ratio) acquired from each consecutive collected fraction during the peptide elution time (*y*-axis, number of fraction corresponding to the acquired mass spectra)]. The values on the *z*-axis (rainbow scale) indicate mass peak intensities in arbitrary units.

was necessary to prevent suppression effects during TOF MS analysis. A satisfactory distribution of contingent precursor candidates for subsequent MS/MS experiments was essential to cover as many compounds as possible in the sample digest and to enhance the protein identification rate (**Figure 3**). In total, 26 proteins in the IgY preparations were identified. Mann and Mann recently identified 100 proteins in the egg yolk water-soluble fraction with 1D SDS-PAGE, LC-MS/MS, and MS^3, including most of the proteins found in this work (*17*). They set the pH to 5 during purification, which yields more proteins than if the pH is not adjusted. The latter means a pH around 6, and this was used for purification of IgY in the present work, which in part explains why fewer proteins were identified here. We used nonadjusted pH because that enables decanting of the supernatant, whereas sedimentation at pH 5 requires centrifugation, which is impractical when large volumes are purified.

Before MS analyses, the proteins were reduced (and alkylated). Thereby, IgY is degraded into heavy and light chains (*18*). Thus, intact IgY could not be detected with MS analyses, but Ig λ chain regions were identified with nanoLC-MALDI TOF/TOF MS and Ig γ chain as spots on 2D gels. Specific IgY has been found with immunological methods (data not shown). Nilsson et al. have identified the most abundant proteins in the egg yolk (*19*) and the water-soluble part of the egg yolk plasma (*20*) by 2D SDS-PAGE, and MS analyses included the IgY heavy chain as a number of spots with a high variation in isoelectric point. The heterogeneity of the isoelectric point of IgY, which varies between 5.7 and 7.6 (*21*) is due to phosphorylations and differences in the amino acid sequences.

Mann and Mann claim that they are the first to identify some plasma proteins in the egg yolk (*17*). We also found some: fibrinogen, hemopexin, and coagulation factor IX. Some of the other proteins identified are known as egg white proteins. However, these proteins have been found in the egg yolk by others (*17, 19, 20*). Examples of proteins found in the egg white as well as in the water-soluble fraction of the egg yolk were ovalbumin, ovomucoid, ovotransferrin, cystatin, and ovoinhibitor. Interestingly, ovotransferrin and cystatin have antibacterial activity, whereas ovomucoid and ovoinhibitor have antiviral activity (*1*). Some of the above-mentioned proteins are also immunostimulatory (*1*). Moreover, lipoproteins of the egg yolk have also been suggested to have antibacterial effect (*1*), and a number of apolipoproteins were detected in the IgY preparations: apolipoprotein A1, apolipoprotein B, vitellogenins 1 and 2, and apovitellenin 1. This indicates that the other proteins in the IgY preparation could have additional positive effects in IgY treatment besides the primary effect of the antibodies. It is noteworthy that chicken ovomucoid protects to some extent against proteolytic degradation by digestive enzymes and therefore might protect IgY when given as an oral treatment for gastrointestinal infections (*1, 22*). Oral delivery of insulin has been improved by the addition of ovomucoid (*23, 24*).

It was of interest to determine the concentration of cholesterol in the IgY preparations for purposes when IgY is given orally. The amounts of cholesterols and triglycerides were low. The concentration of cholesterol was around 10 times lower than the normal human serum levels. The CF patients on anti-*Pseudomonas* IgY receive every day a dose of 70 mL, corresponding to half an egg. Accordingly, daily intake of IgY should not affect the cholesterol and triglycerides levels. Moreover, it has been shown that egg consumption does not necessarily affect serum cholesterol levels (*25, 26*) and that egg eating might even decrease serum cholesterol levels (*27*).

In conclusion, purification of IgY from the egg yolk with the water dilution method is of high reproducibility with low levels of cholesterols and triglycerides, which is of great importance not only for medicinal use but also for other applications. Moreover, 26 of the most abundant proteins in the IgY preparations were identified. Identification of proteins is valuable for prediction of allergic reactions. Some of the identified proteins have antibacterial effect, which might imply an additive effect in IgY treatment.

ABBREVIATIONS USED

2DGE, two-dimensional gel electrophoresis; ACN, acetonitrile; BSA, bovine serum albumin; IgY, immunoglobulin Y; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; nanoLC, nanoflow liquid chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; WD, water dilution.

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