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Proteomic Identification of Proteins as Potential Biomarkers of Nonmeat Components in Meat Products

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Abstract—Reference and commercial samples of meat products (boiled and canned) were analyzed via classical, two-dimensional electrophoresis according to O'Farrell. Ten different protein products or their fragments were identified in the final stage of the procedure. They turned to be components of soy, milk, egg, pumpkin, and sunflower. They can be used as the targets of undeclared additives to develop faster and more sensitive quality control methods.

Keywords: control of the composition of meat products, targets of non-muscle supplements, proteomics

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INTRODUCTION

A number of protein supplements of nonmuscle origin are used in some cases in the manufacture of various types of meat products. These include proteins of eggs and milk and vegetable products of soy, wheat, rice, oats, pumpkin, etc.

The composition of a commercial product is always officially regulated by the relevant government standards or manufacturer's specifications. They set the limit for all components, including nonmuscle ones, the presence or excess of which can be considered falsification or violation of the production conditions.

Quality control of such food products involves the use of methods that allow the identification of specific components of nonmuscle origin and the assessment of their qualitative and quantitative content in the manufactured product.

The majority of such protein supplements currently undergo additional preparation stages, which ultimately leads to the loss of part of the amino acid sequence, and further heat treatment (boiled sausages up to 90°C, and canned food up to 115°C and a pressure of 0.23 MPa) in the presence of a curing-nitrite mixture can also cause thermochemical modifications in the intact amino acid structures of additives, which further complicates the choice of bioindicators for control.

One of these most common herbal supplements is soybean supplement. As a rule, they are currently used in the form of soy isolate or texturate due to their greater manufacturability. At the same time, soy, like many other nonmuscle additives (wheat flour, milk, eggs and their processed products) is included in the list of allergens, the presence of which in products must be strictly controlled and put on the label in accordance with TR CU 022/2011 ("Food products in terms of their labeling") [1].

There have been repeated attempts to use soy proteins as targets for nonstandard additives have been made, in particular, for β-conglycinin [2], various types of glycinins [3], and trypsin inhibitors such as Bauman-Birk and Kunitz [4]. The development of such methods for the control of nonstandard additives in meat products continues to this day. At present, the top two competing methods in terms of performance and price are "rapid-fire" (or panoramic) proteomics, termed "shotgun," [5] and enzyme immunoassay [6]. For panoramic proteomics, the m/z values are important (mass-to-charge ratio) with confirmation of the amino acid sequence of the corresponding tryptic peptides. For enzyme immunoassay, the main priority is the part of the protein that remains intact and can serve as a target for antibodies.

Classical, two-dimensional electrophoresis according to O'Farrell is not the most productive method of quality control of such samples, but it allows the identification of preserved foreign proteins and/or their fragments in meat products, although the development of quantitative characteristics requires the use of more sophisticated research methods [7].

The goal of the work is to identify and identify proteins/fragments of non-muscle origin in two types of heat-treated meat products (cooked sausages and

canned food such as stews) as bioindicators to determine their compliance with labeling and to developing faster, more economical, and more productive control methods.

EXPERIMENTAL

Samples of two types of meat products were presented: Doktor and Lyubitel cooked sausages and canned products, stewed beef and stewed pork, which had a more severe heat-treatment regime.

Of the total number of samples of cooked sausages studied ($n = 40$), three samples were made based on the the experimental production of the Federal Research Center of Food Systems (Russia) with strict control of the recipe and the amount of raw meat pledged, and they were considered a standard. The rest were purchased in retail chains of the Russian Federation and countries of the Commonwealth of Independent States (CIS). In addition to beef and pork, some samples contained poultry meat, beef, soy supplements, whey milk proteins, wheat fiber, and pumpkin flour.

The second batch of samples ($n = 15$) represented canned pork and beef products with a more severe heat-treatment regime. Three samples were produced based on the Food System of the Federal Research Center, with strict quality control of the used meat raw materials, and they were also considered a standard. The rest were purchased in retail chains of the Russian Federation, the CIS, and the European Union. Soy protein was detected in two samples.

Two-dimensional electrophoresis (2DE) was conducted according to O'Farrell with isoelectric focusing in an ampholine (IEF-PAGE) pH gradient, as described previously [8]. Proteins were detected on two-dimensional electrophoregrams via staining with Coomassie blue R-250 (SVB R-250) and then sequentially with silver nitrate [9].

To identify proteins, individual fractions were excised from dry 2DE, the excised fragments were ground, and trypsinolysis was performed as described earlier [10]. Next, the corresponding sets of peptides were studied via matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry and tandem mass spectrometry (MS/MS) on a MALDI-Ultraflex TOF mass spectrometer (Bruker, Germany) with a UV laser (336 nm) in positive ion mode in mass range of 500–8000 Da, and they were calibrated according to the known peaks of trypsin autolysis. The obtained mass spectra ("peptide fingerprints") were deciphered with traditional bioinformation technologies.

Bioinformatics analysis of mass-spectrometric peptide fingerprints with mascot and other bioinformatics technologies. Analysis of the obtained mass spectra of tryptic peptides was carried out with the Mascot program, the Peptide Fingerprint option (Matrix Science, United States); the accuracy of the determination of the MN^+ mass from a search of the databases of the U.S. National Center for Biotechnology Information (NCBI) is equal to 0.01%. Equipment of the Industrial Biotechnology Center for Collective Use of a Federal State Institution, the Fundamental Foundations of Biotechnology Federal Research Center of the Russian Academy of Sciences, was used in the research.

A comparative analysis was conducted on the proteomic profiles of the presented samples, the information modules "Proteins of skeletal muscle of cows (*Bos taurus*)" and "Pig skeletal muscle proteins (*Sus scrofa*)," and the Proteomics of Muscle Organs database (http://mp.inbi.ras.ru).

RESULTS AND DISCUSSION

Violation of the formulation of meat products during its 2DE analysis according to O'Farrell is reflected in the appearance of atypical protein fractions, which, as a rule, are immediately visible on two-dimensional electropherograms during the analysis of commercial products (Fig. 1).

Boiled sausages. Comparative analysis of reference samples and cooked sausages with labels declaring the presence of protein supplements of nonmuscle origin reveals a corresponding panel of potential biomarkers. All proteins detected in the reference samples were identified and turned out to be pork and/or beef proteins, with the exception of the fraction of almost full-size ovotransferrin (no. 1), which is a biomarker of the amount of egg proteins used in the preparation of reference samples of these cooked sausages. Ovotransferrin is quite resistant to heat treatment. Mass-spectrometric identification showed that a large fragment of the amino acid sequence (a.s.) from position 44 to 673 of 705 remains intact.

Milk proteins are another type of animal supplement often used in the production of these types of sausages. Subject to the requirements of the Government Standard/Technical Conditions (GOST/TU), it is usually not detected with 2DE due to the small amount of addition of dairy raw materials. However, the manufacturer may overestimate the amount of milk added to increase the level of total protein in the product, which has been observed in some cases in commercial products from different manufacturers. Additional fractions appear, which, according to the results of mass-spectrometric identification, turned out to be fragments of two milk proteins: kappa casein, a *CSN3* gene product from 25th to 105th position of 190 a.s., and *CSN2* casein, in which two fractions were detected and identified as fragments of 64–224 a.s. and 121–224 of 224 a.s.

Herbal additives are widely used in the production of cooked sausages. The most common supplement is soy protein derivatives. Figure 1b shows the pres-

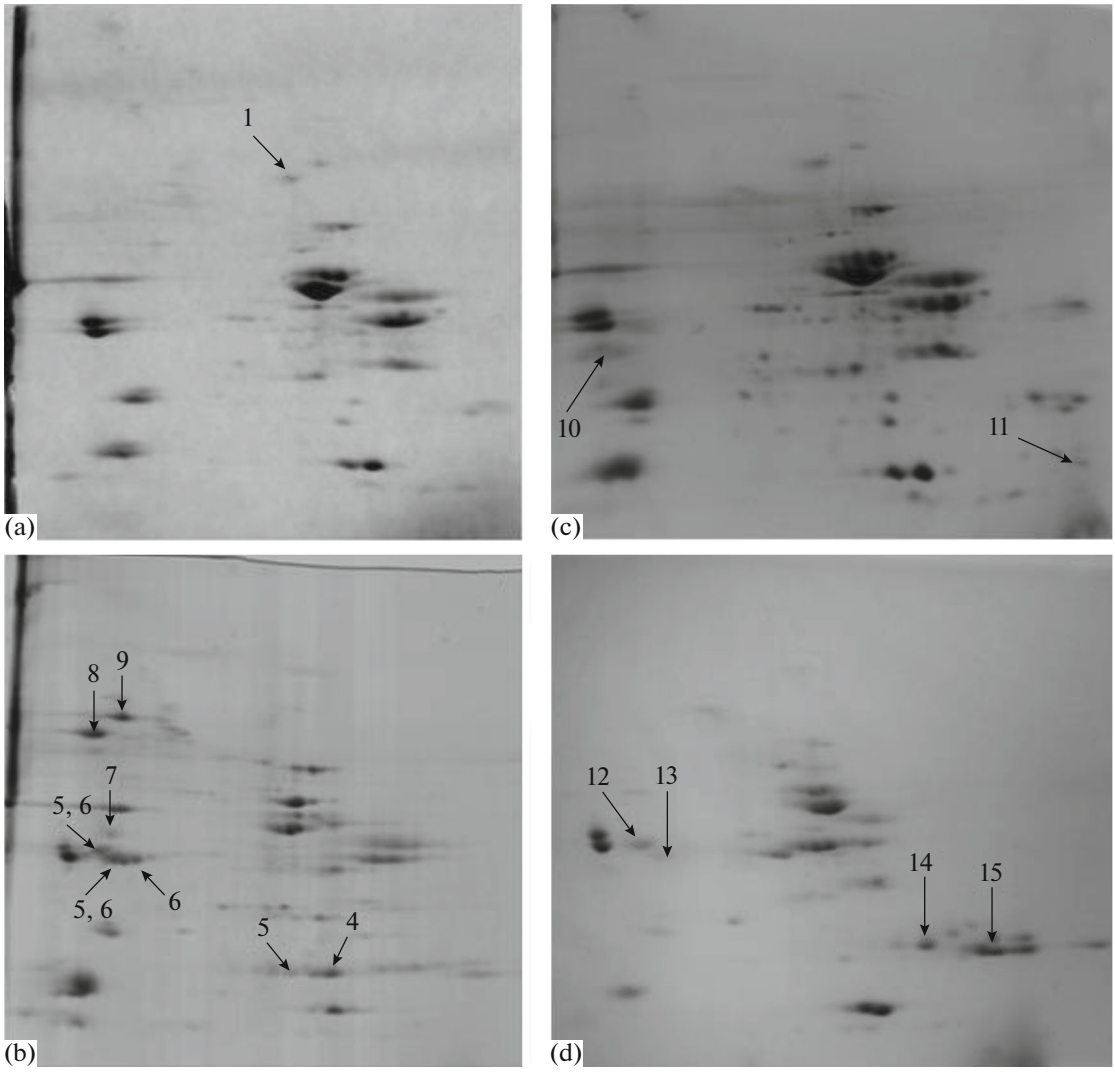

Fig. 1. Two-dimensional electrophoregrams of boiled sausage proteins: (a) reference sample according to the recipe of a Doktor sausage; (b) with declared nonmuscle, soybean additives; (c) with an excess of milk additive; (d) with declared additives of pumpkin flour. Fraction numbering is according to Table 1.

ence of six fractions of soy proteins (in the sample with the manufacturer's declared presence of soy), which turned out to be fragments (Table 1) of four soy proteins There were products of four genes in the form of monomorphic or mixed fractions: glycinin A1aBx (*GY1*), a.s. 321–425 of 495; glycinin A1aB1b mutant subunit (*GY1*), a.s. 36–259 of 386; glycine G2 (*GY2*), a.s. 33–256; glycine (*A3B4*), a.s. 79–236, α β-conglycinin subunit (*CG-4*), a.s. 24–522 of 543, and the α 'β-conglycinin subunit (*CG-1*) as a full-length protein. Products of the *GY1* gene were identified in two transcription variants that may overestimate the amount of soybeans in the sample with quantitative methods of assessment. These fractions were also detected in some samples of commercial products from different manufacturers in varying quantities but were not declared in the composition.

Eleven fractions of pumpkin/pumpkin meal proteins were identified in a sample of a national product from CIS countries. They turned out to be different fragments of the N- (47–282) and C-terminus (306-468) β 11S globulin subunits (480 amino acid residues (aar)); one fraction of storage protein 2, which is similar to 11S globulin (11S globulin seed storage protein 2-like), the fragments 291–412 of 465 and 257–475 of 511 aar with subunit β, which is similar to 11S globulin. The pumpkin genome is not yet sufficiently annotated in international databases and is presented only in the form of specific loci, but the corresponding target fragments of the amino acid sequence are already pre-

Table 1. Nonmuscle markers detected via mass spectrometry in meat products

no.	Protein name (*gene symbol*)	Mass-to-charge ratio (m/z) of the identified tryptic peptides in ascending order of mass	Revealed preserved fragments of the amino acid sequence of proteins in meat products
		Boiled sausages	
		Egg-white markers (*Gallus gallus*)	
1	Ovotransferrin (*TRFE*)	831.4; 888.4; 912.5; 1046.5; 1066.5; 1226.1; 1307.6; 1328.7; 1330.7; 1429.7; 1443.8; 1454.8; 1515.8; 1533.8; 1651.8; 1694.8; 1745.9; 1830.1; 1893.0; 1959.0; 2055.0; 2377.2; 2569.3; 2633.3; 2953.5; 3284.6	44–673 275–421 266–421
2	Ovotransferrin (*TRFE*)	1066.5; 1328.7; 1694.8; 1745.9; 1893.0; 2055.0	275–421
3	Ovotransferrin (*TRFE*)	1066.5; 1694.9; 1429.7; 1745.9; 1830.1; 1893.0; 2055.0; 2569.3	266–421
		Soy-protein markers (*Glycine max*)	
4	Glycinin A1aBx (*GY1*)	588.3; 978.5; 1101.6; 1148.6; 1424.9; 1568.9; 2619.3; 3912.0; 4853.4	321–425
5	Glycinin A1aB1b mutant subunit (*Gy1*)	711.4; 1029.5; 1039.6; 1899.9; 2340.2; 2432.3; 3140.5; 3461.6; 3901.9; 4800.3	36–259
6	Glycinin G2 (*GY2*)	1039.6; 1098.6; 1278.7; 1930.9; 3217.6; 3794.9	33–256
7	Glycinin (*A3B4*)	1325.6; 2287.1; 2622.4; 2340.2; 2728.3; 947.4; 3042.2; 4580.0	79–236

Table 1. (Contd.)

no.	Protein name (*gene symbol*)	Mass-to-charge ratio (*m/z*) of the identified tryptic peptides in ascending order of mass	Revealed preserved fragments of the amino acid sequence of proteins in meat products
8	α β-conglycinin subunit (*CG-4*)	563.3; 954.5; 1182.6; 1078.5; 1533.8; 1617.9; 1770.0; 2017.9; 2323.2; 2552.2; 2700.4; 2991.5; 3008.3; 3224.5; 3371.7; 3684.8; 3712.6; 3831.0; 4227.2	24–522
9	α' β-conglycinin subunit (*CG-4*)	563.3; 872.4; 938.5; 1078.5; 1141.6; 1222.7; 1510.8; 1533.8; 1652.9; 1740.8; 2136.0; 2449.1; 2761.5; 2935.3; 3074.5; 3114.6; 3655.8; 3770.0; 3820.0; 4188.9; 4272.2	1–559
Milk-protein markers (*Bos Taurus*)			
10	Casein kappa (*CSN3*)	970.5; 1250.7; 1979.1; 4990.1	25–105
11	Casein CSN2 (*CSN2*)	741.4; 779.5; 829.5; 1012.5; 1136.6; 2185.2; 2908.6; 5355.9	121–224 64–224
Pumpkin seed–protein markers (*Cucurbita pepo*)			
12	Subunit β 11S globulin (*LOC111464525*) *gi|112677	643.4; 687.4; 750.5; 903.4; 973.5; 1219.7; 1429.8; 1437.8; 1463.8; 1532.8; 2430.2; 2673.3; 2717.3; 3556.8	47–282
13	Subunit β 11S globulin (*LOC111464525*) *gi|112677	817.4; 1001.5; 1151.6; 1320.7; 1392.7; 1425.7; 1453.9; 1542.7; 1870.1; 1893.9; 1893.9; 2350.2; 2767.3; 2911.3; 3589.9	306–465 317–468 348–468

Table 1. (Contd.)

no.	Protein name (*gene symbol*)	Mass-to-charge ratio (*m/z*) of the identified tryptic peptides in ascending order of mass	Revealed preserved fragments of the amino acid sequence of proteins in meat products
14	Protein 2 similar to the storage protein 11S globulin (*LOC101217162*) *gi|449468676	610.4; 804.4; 838.4; 1126.5; 2469.0; 3204.5	291–412
15	Subunit β-like 11S globulin 1 (*LOC111464083*) *gi|659093215	1162.6; 1320.7; 1376.7; 1528.7; 1713.0; 2383.2; 3112.7	257–475
	Canned meat products		
	Soy protein markers		
16	Soy glycinin A3B4 (*A3B4*)	853.4; 1255.6; 1397.8; 542.8; 2061.0	361–499
17	Glycinin G1 (*GY1*)	1148.5; 1424.9; 1449.6	401–435
	Sunflower-protein markers (*Helianthus annuus*)		
18	11S globulin seed storage protein G3 (*LOC110881169*) *XP_021985214.1	760.3; 945.9; 1639.9; 1770.9; 2106.1; 2759.4	308–475

* Recording in NCBI Protein.

sented in the NCBI Protein database under the corresponding records.

Canned meat products. Comparative analysis of the protein composition of canned beef/pork stews with the controlled loading of raw materials and the declared addition of proteins of nonmuscle origin also revealed a panel of potential biomarkers of these additives, but the detection of such fractions turned out to be more difficult (Fig. 2). A stricter sterilization regime for canned food leads to the formation of a large number of fragments of muscle proteins, and thermochemical modifications cause in many proteins the formation of characteristic tracks of proteins of the same name [11] that differ in pI due to the blockade or disappearance of a part of, as a rule, alkaline charged groups, which introduces its own contribution to the proteomic profile of this product. Against this background, it is difficult to detect alien factions. Nevertheless, fragments of soy glycinins have been identified. These fractions are identified as a mixture of plant and muscle proteins. The plant component turned out to be fragments of glycine G1 (*GY1*)—401–435 and glycine (*A3B4*)—361–499. The molecular weight of the identified fragments was 17–18 kDa, which should correspond to fragments about 140 aa in length. The product of gene *A3B4* corresponds to this parameter, but the *GY1* product does not.

Mass spectrometry detects only tryptic peptides from the short (most thermochemically stable) segment, while the rest obviously change mass due to thermochemical modifications and are not recognized by the search program.

In canned beef stew produced in E.U. countries (France), the presence of the 308–475 fragment of the storage G3 protein 11S globulin from sunflower. The presence of such an additive is possible due to the addition of unrefined sunflower oil, but it is not indicated in the composition of the product and, therefore, is also a violation of the regulations. Only this part of the protein is the most resistant to thermochemical effects and can act as a marker for the presence of sunflower seed meal.

Table 1 presents a summary of the results of the mass-spectrometric identification of intact nonmuscle supplements (fragments of amino acid sequences and *m/z* tryptic peptides).

In general, the results showed that the spectrum of bioindicators to control the introduction of undeclared additives in meat products are quite limited. After heat treatment, only ovotransferrin or its frag-

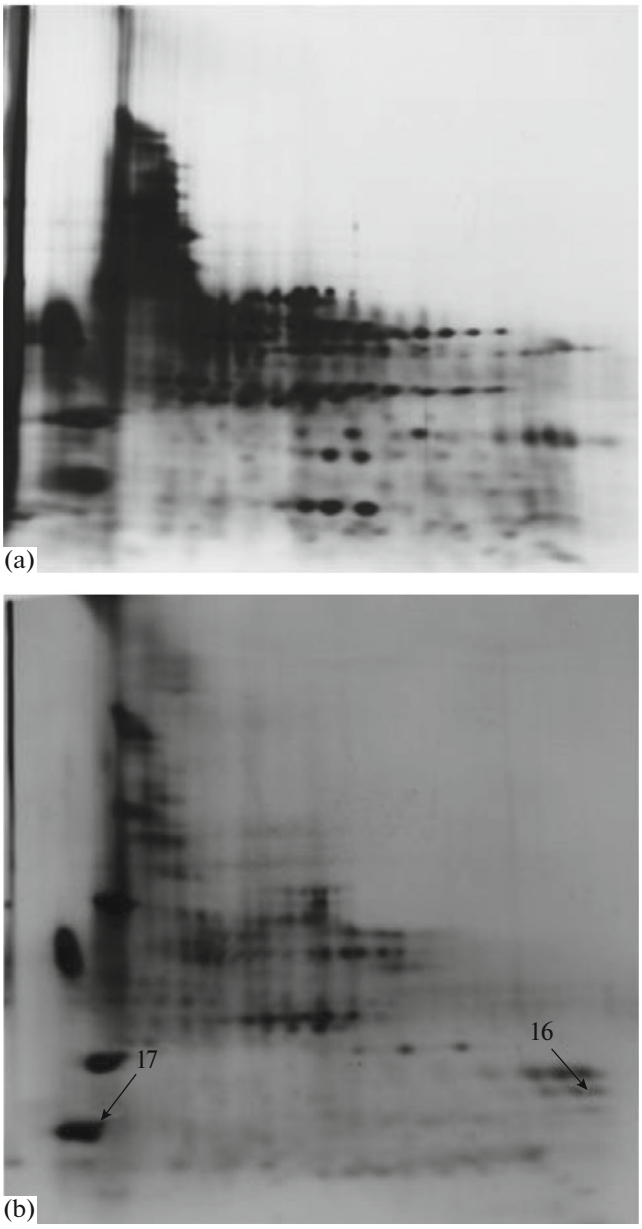

Fig. 2. Two-dimensional electrophoregrams of proteins in canned meat products: (a) reference sample; (b) with declared supplements of nonmuscle origin. Fraction numbering is according to Table 1.

ment, two types of milk caseins (with a predominance of the CSN3 gene product), and four derivatives of soybean glycinins and conglycinins (equally represented) are reliably detected, as well as three derivatives of proteins/fragments 11S globulin for pumpkin and sunflower.

The results enable a significant narrowing and detailing of the range of markers of such additives in meat products in order to improve the methods of monitoring its compliance with the declared composition and to prevent consumer confusion.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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