Immunohistochemical Detection of Wheat Protein in Model Samples

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Abstract

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The study focused on the optimisation of immunohistochemical examination for gluten content detection in model samples (pork meat with wheat semi-smooth flour, pork meat with wheat protein edible vital). The best results were achieved with immunohistochemical method based on ABC (avidin–biotin complex) method utilising polyclonal antibodies diluted 1:1000. The results demonstrate that for pure wheat protein detection, the utilisation of immunohistochemical detection, which can detect as little as 0.1% of the added wheat protein, is more advantageous, while the commonly used ELISA method reliably proves this additive approximately from 0.4% upwards.

Keywords: food allergens; gluten; celiac disease; identification

Wheat gluten, consisting of prolamine proteins and glutelins which swell in water and make highly viscous colloid gel, is commonly added into meat products (Tatam & Sherry 2008). Gluten in meat products improves viscoelastic parameters, colour stability, solidity, sappiness, and humidity retention of the product (Day *et al.* 2006). It also decreases the cooking losses and positively influences the structural and sensory characteristics of the product.

Proteins in the grain gluten can induce celiac symptoms in hypersensitive consumers. Celiac disease is a life-long autoimmune disease which can result in symptoms such as urticaria, atopic dermatitis, diarrhea, tiredness, bone pains, stomachaches, loss of appetite, loss of weight, anemia, osteoporosis, infertility, and mental problems (Hischenhuber *et al.* 2005). This disease striking both young and adult is incurable. The only effective remedy is a lifelong gluten-free diet (Setty *et al.* 2008). For consumers suffering from celiac disease is it therefore vital to obtain gluten-free food. Though there are no conclusive data on the threshold of gluten sensitivity of celiac patients (Stern *et al.* 2001), it is necessary to develop sensitive and accurate procedures for gluten detection in food products for quality control (Poms *et al.* 2004). The aim of this paper was the optimisation of immunohistochemical examination, its comparison to ELISA reference method, and suitability evaluation at routine examination of food products for gluten content. With the immunohistochemical

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method, the procedure achieving the best results from the point of view of later-on quantitative examination was also searched for.

MATERIAL AND METHODS

Preparation of model samples. Model samples of pork meat without any additive and with 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1%, 2.5%, and 5% of pure wheat protein edible vital additives were used. Other model samples were pork meat with 0.5%, 1%, 2.5%, and 5% of wheat semi-smooth flour additives.

Sample treatment and preparation. The samples were processed for immunohistochemical examination according to the procedure described by Pospiech *et al.* (2009). The samples (5 g) were fixed in 10% water solution of neutral formalin (RNDr. Jan Kulich, Ltd., Prague, Czech Republic) for 24 hours. After fixation, the samples were dehydrated in an ascending sequence of alcohol in the autotechnicon apparatus AT-4 and embedded into paraffin blocks in Paraplaste (RNDr. Jan Kulich, Ltd., Prague, Czech Republic); these were cut to 4 µm sections on a rotation microtome (Mikrom HM 400, Carl Zeiss, Jena, Germany). The sections were spread on the water surface and mounted on a slide SuperFrost plus (Menzel-Glaser, Braunschweig, Germany). For each sample, four paraffin blocks were prepared from which 50 µm sections were cut. The samples for immunohistochemical examination were simultaneously processed in the shortened way by means of freezing without fixation. The frozen samples (1 × 1 cm) were cut to 4 µm sections on a freezing microtome HM 550 (Microm International GmbH, 69190 Walldorf, Germany) and mounted on a slide SuperFrost plus (Menzel-Glaser, Braunschweig, Germany).

Examination procedure. From each sample, 72 sections were immunohistochemically processed and subsequently microscopically examined at ×40, ×100 and ×400 magnifications in a light microscope Nikon ECLIPSE E200 (Nikon-Alphaphot-2 YS 2, Nikon Type 119, Japan). For the results documentation, a set of digital photos was captured by means of Canon PowerShot G9 camera (Canon Inc., Japan) utilising the image capturing software PSRemote Version 1.5.2 (Breeze systems, Bagshot, UK). The samples with and without wheat proteins were processed utilising the ELISA method as well.

Immunohistochemical examination of samples. For the immunohistochemical examination of the samples, the procedure was used based on avidin – biotin complex (ABC) method for immunohistochemical detection of soya protein according to Pospiech *et al.* (2009), modified for wheat protein detection. It is an amplifying indirect three-stage method which uses high bond affinity between avidin and biotin for antibody detection. At first, a bond occurs between the primary specific antibody and secondary antibody conjugated with biotin. The third stage is the bond between avidin – biotin complex – peroxidase on the secondary biotinylated antibody which significantly strengthens the signal.

The sections were immersed in: (1) xylene (RNDr. Jan Kulich Ltd., Prague, Czech Republic), twice for 7 min; (2) absolute ethanol (Moravský Lihovar, Kojetín, Czech Republic), twice for 7 min, and then 90% aqueous etanol followed by 70% aqueous ethanol (v/v), 7 min each bath; (3) tap water for 7 min; (4) distilled water for 7 min; (5) PBS – Phosphate Buffered Saline, 80 g/l NaCl (RNDr. Jan Kulich Ltd., Prague, Czech Republic), 2 g/l KCl, 2 g/l KH_2PO_4, 23,4 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.16 g/l NaOH adjusted to pH 7,4; (6) citrate buffer 21 g/l $C_6H_8O_7$, 9 g l-1 NaOH adjusted to pH 6 for 5 min at 650 W in microwave (we started in this point with the sections processed by freezing microtome); (6) PBS for 5 min; (7) 3% (v/v) H_2O_2 (Conlac peroxides); PBS for 30 min; and then (8) PBS twice for 5 minutes. The sections were then incubated successively: (9) for 30 min at 25°C with 5% (v/v) powdered milk diluted in TBS (Dako TBS, Glostrup, Denmark) with additive 5 ŋl Tween® 20 (Sigma-Aldrich Corp., St. Louis, USA); (10) for 12 h at 8°C with an undiluted monoclonal anti-wheat antibody of own provenance, acquired by immunisation of laboratory mice or with an polyclonal anti-wheat antibody isolated from a rabbit (Sigma-Aldrich Corp., St. Louis, USA) in three different dilutions (1:500, 1:1000, 1:1500) diluted with antibody diluent (DakoCytomation ref. S0809, Glostrup, Denmark). In the negative control, the primary antibody was replaced by antibody diluent (DakoCytomation ref. S0809, Glostrup, Denmark), and washed in PBS twice for 5 min; (11) for 30 min at 25°C with 25 µl per section of anti-rabbit (Vector Laboratories, PK 6101, Burlingtone, USA)/anti-mouse biotinylated antibody (Vector Laboratories, PK 6101, Burlingtone, USA) (anti-rabbit for sample processing utilising

polyclonal antibodies and anti-mouse for a procedure where monoclonal antibodies were used) containing 10 ml TBS, 3 drops of normal blocking serum stock, and 1 drop of biotinylated antibody stock, and washed in PBS twice for 5 min; (12) for 30 min at 25°C with 25 µl per section of ABC (avidin – biotin complex) reagent (VectorLaboratories, PK 6101, Burlingtone, USA) containing 5 ml TBS, 2 drops of reagent A and 2 drops of reagent B, and washed in PBS for 5 minutes.

Antibody binding was visualised by incubation in 25 µl per section of 3,3'-diaminobenzidine (DAB) (DakoCytomation, Glostrup, Denmark) for 3 min, the reaction was stopped by washing in a water bath for 5 minutes. The background was visualised in Calleja bath (Calleja 1897) or in toluidine bath (Flint 1994) for 5 min and washed in water bath and 6 sections were left without staining, then in 96% aqueous (v/v) and finally absolute ethanol twice for 5 min each, and in xylene p.a. (RNDr. Jan Kulich Ltd., Prague, Czech Republic) twice for 5 minutes. A drop of solacryl (RNDr. Jan Kulich Ltd., Prague, Czech Republic) and a micro cover slip were laid onto each section.

RESULTS AND DISCUSSION

Plant proteins in various forms, differing in the ratio of proteins and polysaccharides – flour, concentrate, isolate – or in character – textured protein – are added into meat products. Every protein type has certain characteristics and specific use (Pedersen 1995) as well as the way of detection. Utilising transparent staining methods, one can rely only on their typical appearance and additive structure (Tremlová & Štarha 2002), on the other hand, when immunohistochemical methods are used, wheat protein can be identified quite easily since it is highlighted by DAB chromogen (Figure 1). In the samples containing wheat flour, this highlighting occurs only slightly (Figure 2). This results from a relatively low concentration of wheat protein in wheat flour (7–13% of proteins), nevertheless, it can be demonstrated by histological methods. On the other hand, these cannot distinguish the specific type of flour.

The results achieved by immunohistochemical processing were compared to the results achieved by ELISA method (Table 1). According to these results, ELISA method detects reliably the concentrations above 0.4% of wheat protein additive while the concentration of 0.1% of wheat protein additive can be detected immunohistochemically. However, as reported by Horn (1987), in the histological finding evaluation one has to take into account the fact that the results of histological and immunological evidence for foreign protein do not exclude each other. Therefore, in spite of negative immunological findings, or possibly histological findings as well, the processing of plant protein ingredients cannot be excluded. Because of that, chemical, electrophoretic, and

Table 1. Comparison of results achieved by ELISA method and immunohistochemical method with application of polyclonal and monoclonal antibodies

Sample No.	% of wheat protein	ELISA	Polyclonal antibodies diluted to 1:1000		Nonoclonal antibodies undiluted	
			paraffin blocks	freezing microtome	paraffin blocks	freezing microtome
1	0	–	–	–	–	–
2	0.1	–	+	+	+	+
3	0.2	+/–	+	+	+	+
4	0.3	+/–	+	+	+	+
5	0.4	+	+	+	+	+
6	0.5	+	+	+	+	+
7	1	+	+	+	+	+
8	2.5	+	+	+	+	+
9	5	+	+	+	+	+

+ positive result; +/– dubious result; – negative result

Table 2. Results with application of polyclonal antibodies

Sample No.	% of wheat protein	Paraffin blocks			Freezing microtome		
		polyclonal antibodies dilution					
		1:500	1:1000	1:1500	1:500	1:1000	1:1500
1	0	–	–	–	–	–	–
2	0.1	+	+	+	+	+	+
3	0.2	+	+	+	+	+	+
4	0.3	+	+	+	+	+	+
5	0.4	+	+	+	+	+	+
6	0.5	+	+	–	+	+	+
7	1	+	+	–	+	+	+
8	2.5	+	+	–	+	+	+
9	5	+	+	–	+	+	+/–

+ positive result; +/– dubious result; – negative result

histological and immunological testing methods should be used simultaneously if we want to present evidence for ingredients rich in nitrogen and having a character different from that of meat.

Optimal procedure for wheat protein demonstration was sought for during immunohistochemical examination. The results achieved from the samples sections in freezing microtome were compared to the results achieved with the samples processed in paraffin blocks. Next, the use of monoclonal and polyclonal antibodies was compared. As obvious from Tables 1 and 2, positive results were achieved with all samples prepared in freezing microtome in contrast to paraffin blocks. In the case of sections provided by means of freezing microtome, the bond between antibodies and antigens is influenced neither by thermal changes nor chemical denaturation induced by fixative solution. Thus, the detection of wheat protein in this way is more reliable.

With polyclonal antibodies, the impact of various antibody dilution degrees was also observed (Table 2). Positive results with the dilution of 1:500 were achieved even with the sample containing the smallest amount of wheat protein, however, with high unspecific bond in the sample background. The best results were achieved with 1:1000 dilution in the sections obtained from freezing

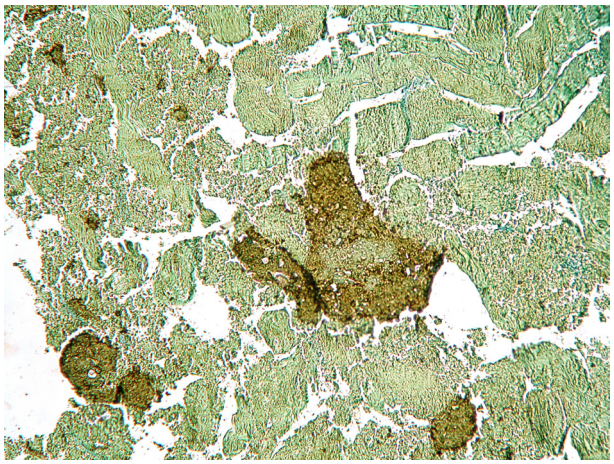

Figure 1. Model sample: meat with gluten, imunohistochemical method, highlighted by DAB chromogen, staining according to Calleja, examined at ×100

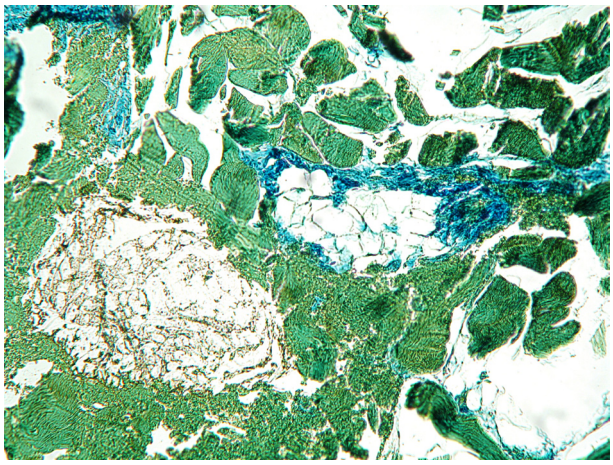

Figure 2. Model sample: meat with weat flover, imunohistochemical method, highlighted by DAB chromogen, staining according to Calleja, examined at ×10

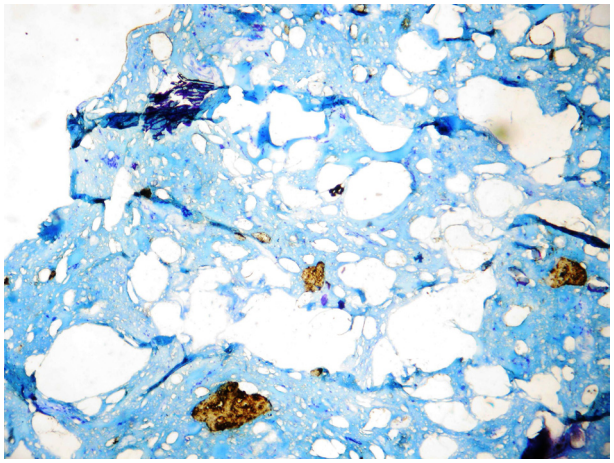

Figure 3. Model sample: meat with gluten, imunohistochemical method, highlighted by DAB chromogen, toluidine blue staining, examined at ×100

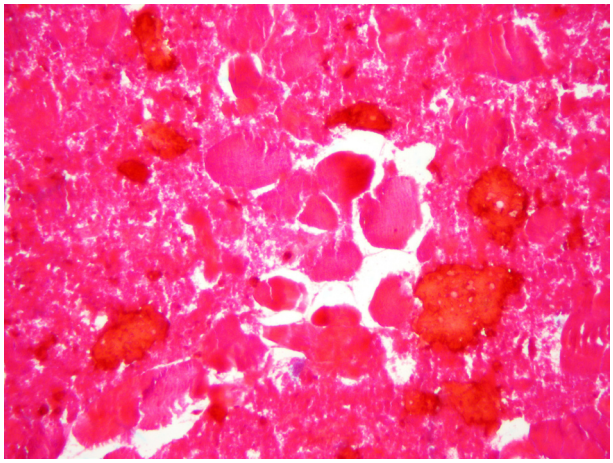

Figure 4. Model sample: meat with gluten, imunohistochemical method, highlighted by DAB chromogen, eosin staining, examined at ×100

microtome. Concerning the samples processed in paraffin blocks, polyclonal antibodies diluted 1:1500 achieved positive results only in the samples with up to 0.5% wheat protein additive. This was caused probably by a higher number of the binding positions than the amount of antibody offered and thus no observable indication of wheat protein in the sample occurred. Regarding monoclonal antibodies, positive results were achieved already in the samples with 0.1% of additive (Table 1) but, due to a high specificity of monoclonal antibodies, the colour contrast was lower than in polyclonal antibodies.

We searched for optimum contrast between the wheat protein observed and the background in staining immunohistochemically processed samples for qualitative evaluation as well as for possible sample quantification. In our experiment, only qualitative sample examination was performed, however, the individual preparation staining or non-staining methods were compared from the point of view of subsequent quantification by image analysis or stereology, too. Toluidine blue staining (Flint 1994) (Figure 3) seems to be the best for image analysis while other possibilities can be used for qualitative examination and stereology, too, the best of them seems to be the staining according to Calleja (Calleja 1897) (Figure 1). In contrast, eosin staining (Figure 4) is not appropriate because during this staining method, brown wheat protein is covered in a hue of red, which covers the result of immunohistochemical bond of antibodies on the observed protein.

CONCLUSION

Immunohistochemical method seems to be an appropriate diagnostic method for wheat protein detection in meat products. The best results were achieved with the sections provided on freezing microtome and subsequently processed by using polyclonal antibodies diluted 1:1000. If the sections are stained with toluidine blue after immunohistochemical processing, the amount of wheat protein in the sample can be subsequently quantified as well. On the other hand, the sample processing by means of immunohistochemical methods is time consuming.

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