Food Research International 45 (2012) 39–44

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Use of lysozyme as an indicator of protein cross-contact in fresh-cut vegetables via wash waters

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ARTICLE INFO

Article history:
Received 10 June 2011
Accepted 5 October 2011

Keywords:
Allergen-indicator
Lysozyme
Cross-contact
Fresh-cut vegetables

ABSTRACT

To evaluate risks linked to cross-contact of allergenic proteins in food production sites via carry-over, it is necessary to gain more insight in the behavior of proteins during processing. A typical case example of cross-contact is related to the re-use of wash water in the fresh-cut vegetable processing industry. In this study, the carry-over of allergenic proteins via wash water was quantified by applying an allergen-indicator, lysozyme. The adsorption of the allergen to the fresh-cut vegetables could be characterized by a Langmuir adsorption isotherm. From the adsorption characteristics, it was observed that the carry-over was significantly stronger on carrots compared to lettuce and leek. This was moreover observed from the mass balance which illustrated that the total amount of lysozyme transferred from the wash water to the fresh-cut vegetables was in average 77.4, 25.1 and 22.2% for respectively carrots, lettuce and leek. From a deterministic risk assessment, it could be concluded that allergenic proteins can be transferred via wash water to fresh-cut vegetables in the next production batch in such quantities that they pose a risk towards allergic consumers. The proposed methodology enables the food industry to validate designed preventive measures in the framework of their allergen management.

1. Introduction

Food allergies are mainly provoked by naturally present allergenic proteins in food products (Deibel et al., 1997; Hefle, 1996; Taylor & Hefle, 2005). The Codex Alimentarius Commission of the World Health Organization recognized a series of plant and animal products as important food allergies e.g. milk, egg, fish, crustacean, cereals containing gluten, peanuts, soybean, tree nuts (Codex Alimentarius Commission (CAC), 2008). The European Food Safety Authority identified additionally celery, sesame, mustard, lupine, and molluscs as allergenic food products (European Food Safety Authority (EFSA), 2004). Thus allergenic proteins are identified in the agri-food chain as an important food safety hazard. Food allergies affect about 1–3% of the adults and up to 4–6% of infants and children in Europe (European Food Safety Authority (EFSA), 2004). To prevent allergic reactions, the allergic patient should avoid allergen containing foods and therefore they depend upon correct food labeling. The food industry is responsible for such proper labeling. However, up till now different recalls of foods containing undeclared allergens were reported by the European Rapid Alert System for Food and Feed (RASFF) and the US Food and Drug Administration (FDA) (RASFF, 2010; Vierk, Falci, Wolyniak, & Klontz, 2002). In order to avoid such recalls, a management system to control food allergen related hazards should be established and implemented in a company specific setting. Quality assurance guidelines have been published to guide companies in setting up an allergen management (Food Standards Agency, 2010; USDA, 2010; Vital, 2010). However, important aspects in the control of allergens are not yet considered in these manuals such as the behavior of allergens in cleaning and control procedures in food production facilities. Moreover, the current scientific research focuses mainly on the analytical or medical aspects of food allergies (Breiteneder et al., 1995; European Food Safety Authority (EFSA), 2004; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008; USDA, 2010; Wang, Li, Zhao, Chen, & Ge, 2011). In the framework of a Hazard Analysis Critical Control Plan (HACCP) approach, it is absolutely necessary to gain more insight in the behavior of allergens during processing in an industrial setting, allowing one to evaluate risks linked to cross-contact in production sites. Cross-contact can occur due to carry-over via media applied in typical food-processing operations such as water during washing steps or oil during frying (Jackson et al., 2008). The vegetable processing industry is facing challenges in reducing water volumes and therefore re-use of water is commonly applied. Extensive research has been conducted to the microbiological and chemical consequences of re-use of water (Leifert, Ball, Volakakis, & Cooper, 2008; Selma, Allende, Lopez-Galvez, Conesa, & Gil, 2008; Tiangang, Gilbert Yuk, Jiguo, Chongyu, & Thomas Wai, 2007; Vandekinderen, Devlieghere, De Meulenaer, Ragaert, & Van Camp, 2009) but not yet the possibility of carry-over of allergenic proteins. The risk of carry-over of allergenic proteins from for example celery to the

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doi:10.1016/j.foodres.2011.10.007

water and from the water to other vegetables is however relevant and should be evaluated.

Celery is an important food allergen in Europe and is frequently present as an ingredient in processed vegetables (Breiteneder et al., 1995; Faeste et al., 2010; Vieths et al., 2002). A sensitive PCR analysis and ELISA method were developed to analyze celery allergens however both methods had their drawbacks (Faeste et al., 2010; Mustorp et al., 2008). While the DNA based method did not detect the allergen as such, the ELISA showed cross reactivity to carrot, potato and parsnip. These disadvantages were also seen for the detection of other allergens and therefore confirmatory methods based on mass spectrometry are required to increase the fidelity of the analytical results obtained by PCR or ELISA (van Hengel, 2007). Since there is yet no such reliable and robust detection method for celery allergens available, problems arise when evaluating celery carry-over. In general, the lack in robust and reliable detection methods for processed allergens limits a correct assessment of cross-contact in the food industry (Cucu, 2011). As a consequence, more research is also required to evaluate the prevention strategies for cross-contact (Jackson et al., 2008).

This study developed a methodology to quantify cross-contact in wash water by using an allergen-indicator. Lysozyme was chosen as indicator protein since it is fully characterized (Proctor & Cunningham, 1988) and it is detectable by a reliable, robust HPLC method (Kerkaert, Mestdagh, & De Meulenaer, 2010; Pellegrino & Tirelli, 2000). From the quantification of the lysozyme carry-over, a deterministic risk assessment was made to evaluate the risk of celery protein carry-over via wash water. This proposed methodology enables the food industry to validate designed preventive measures in the framework of allergen management.

2. Materials and methods

2.1. Chemicals

A freeze-dried egg white lysozyme standard (p.a.), Fluka 62971, was obtained from Sigma Aldrich (Bornem, Belgium). NaCl (p.a.), potassium phosphate (p.a.), sodium dihydrogenphosphate (p.a.) and HPLC grade water were supplied by Chemlab (Zedelgem, Belgium). HPLC grade acetonitrile and trifluoroacetic acid (p.a.) were from VWR International (Leuven, Belgium) and Sigma Aldrich (Bornem, Belgium), respectively.

2.2. Plant material

Carrots (*Daucus carota* L.), lettuce (*Lactuca sativa* L.) and leek (*Allium porrum* L.) were obtained from the local supermarket (Delhaize, Belgium). The carrots were topped and rasped with a Multi Pro Food Processor (Kenwood, Vilvoorde, Belgium). The lettuce was manually processed by removing the outer leaves and the inner core and cutting the lettuce in shreds. The leek was treated similarly, the roots and decayed leaves were removed, the stalks were longitudinally cut and subsequently transversally with a sharp knife.

2.3. Analysis of industrial wash waters

Samples of industrial wash waters were taken at Allgro, a vegetable processing industry (Sint-Lievens-Houtem, Belgium). Different fresh-cut vegetables (leek, celeriac, celery, lettuce, carrots and soup greens) were washed and samples were taken from the wash water at several time intervals. The pH of the samples was monitored using a LAB 850 pH-meter (Schott Instruments, LSB, Kontich, Belgium) and the crude and net protein content was analyzed. To determine the crude protein content, the wash water was concentrated prior to Kjeldahl analysis (AOAC International Official Method 981.10, 1981). The net protein was calculated by subtracting the non protein nitrogen content from the crude protein content. The non protein nitrogen content was determined by precipitating the proteins with 15% TCA (final concentration) and analyzing the supernatant by Kjeldahl analysis.

2.4. Quantification of the allergen carry-over

To quantify the carry-over of allergens to vegetables in wash water, lysozyme was used as an allergen indicator. Three vegetables (carrots, leek, lettuce) were washed at RT during 15 min in a 0.1 M phosphate buffer at pH 5.8 containing varying concentrations of lysozyme (0; 150; 300; 600; 900; 1200; 1500; 1800; 2100 μg/mL). These concentrations were comparable with the protein concentrations found in industrial wash waters. The ratio of vegetable over wash water applied in industry depends on the vegetable, while 0.5 kg/L is applied for leek and lettuce, 2 kg/L is used for carrots. In our study a ratio of 0.5 kg/L was used for all vegetables, however for leek the 0.5 and 2 kg/L were compared in order to analyze the effect of the vegetable to wash water ratio. After 15 min of washing, the vegetables were dried with a manual kitchen centrifuge (Zyliss, Bern, Switzerland). Subsequently, the vegetables and remaining wash waters were analyzed on their lysozyme content. For the vegetables, an extraction procedure was followed as specified below while the wash water was filtered over a 0.45 μm HPLC filter (FP30-0.45CA, Novolab, Belgium) and injected on HPLC. For each vegetable, each lysozyme concentration and each vegetable to wash water ratio, three batches were washed and further analyzed.

2.5. Lysozyme analysis

The chromatographic method of Pellegrino and Tirelli (2000) was adapted for vegetables. About 2 g of homogenized vegetables was brought into a beaker to which 20 mL of 1 M NaCl was added. The sample was mixed with the Ultra Turrax (CAT, UAF-25R) for 2 min at 25,000 rpm, which was rinsed with 10 mL NaCl. The mixtures were placed on a shaker for 1 h, diluted to 50 mL with 1 M NaCl and filtered over a paper filter (Schleicher & Schuell, Germany) and a 0.45 μm HPLC filter (FP30-0.45CA, Novolab, Belgium). The final extract was analyzed by an HPLC (1100 system, Agilent Technologies, Switzerland) which was equipped with a reversed-phase polymeric column (PLRP-S 250×4.6 mm, 300 pore size, 5 μm particle size) from Varian Inc. (Belgium). The mobile phase consisted of a gradient of water and acetonitrile both containing 0.1% trifluoroacetic acid (v/v). Eluting conditions expressed as proportion of water with 0.1% TFA: 0–10 min:69%, 19 min:49.3%, 20 min:25%, 20–21 min:25%, 22 min:69%, 22–30 min:69%. The flow rate was 1 mL min^{-1}, the column temperature 45 °C and the injected volume 50 μL. Detection was carried out with a fluorescence detector (FLD, G1321, Agilent Technologies, Switzerland) set at 280 nm ex. and 340 nm em. An emission spectrum was taken between 300 and 500 nm in order to confirm the identity of the peak and as such guarantee the specificity of the analysis. From a recovery study it was observed that the modified method was able to extract and detect 100% of the lysozyme spiked on carrot and leek in a concentration range of 25 to 200 μg/g. For lettuce however, a slightly lower recovery of 87% was detected in the same concentration range. The results of the carry-over on lettuce were corrected for this recovery.

2.6. Statistical analysis

Each data point in the carry-over study was a result of three independent determinations. The data of the carry-over study was fitted to a non-linear regression curve using the SPSS 16 statistics package.