Active Packaging of Cheese with Allyl Isothiocyanate, an Alternative to Modified Atmosphere Packaging

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ABSTRACT

The natural antimicrobial compound allyl isothiocyanate (AITC), found in mustard oil, is effective against cheese-related fungi both on laboratory media and cheese. *Penicillium commune*, *Penicillium roqueforti*, and *Aspergillus flavus* were more sensitive to AITC when it was added just after the spores had completed 100% germination and branching had started on Czapek yeast extract agar than were spores in the dormant phase. The use of 1 AITC label (Wasaouro interior labels, LD30D, 20 by 20 mm) in combination with atmospheric air in the packaging extended the shelf life of Danish Danbo cheese from 4½ to 13 weeks. Two AITC labels extended the shelf life from 4½ to 28 weeks. Both 1 and 2 labels in combination with modified atmosphere packaging extended the shelf life of the cheese from 18 to 28 weeks. This study showed that AITC was absorbed in the cheese, but it was not possible to detect any volatile breakdown products from AITC in the cheese. Cheese stored for up to 12 weeks with an AITC label had an unacceptable mustard flavor. The mustard flavor decreased to an acceptable level between weeks 12 and 28. Cheese stored in atmospheric air had a fresher taste without a CO2 off-flavor than did cheese stored in modified atmosphere packaging. AITC may be a good alternative to modified atmosphere packaging for cheese. The extended shelf life of cheese in the package is very desirable: the cheese can be transported longer distances, and the packaging can be used for the final maturing of the cheese. Furthermore, AITC can address problems such as pinholes and leaking seals in cheese packaging.

Conventional packaging of semihard cheeses is typically performed in a modified atmosphere (MA) at 25 to 50% CO2 and 75 to 50% N2 (9, 19). CO2 extends the shelf life of cheese by hindering the growth of spoilage microorganisms, but the gas does not eliminate them. If there are pinholes or leaking seals in the package, microorganisms will grow and result in discoloration and the formation of off-flavors and mycotoxins. MA packaging also gives cheese a CO2 off-flavor (19). Therefore, MA packaging is not optimal, and there is growing interest in new packaging technologies. The desire for expanded use of natural antimicrobial compounds is increasing in light of consumer demands for minimally processed, safe foods that are convenient to use and have an adequate shelf life (25).

Several authors have reported a broad spectrum of antimicrobial active compounds in thyme, clove, cinnamon, bay leaves, oregano, garlic, lemongrass, and mustard, and the MICs for these active substances have been established (2, 18, 24, 27, 34). Several of these active compounds are volatile; they therefore have the potential to contaminate foods during active packaging, when full contact with the packaging material cannot be attained. There are a number of potential methods of incorporating naturally occurring antimicrobials into cheese. They may be added to the cheese milk or applied to the cheese by spraying, immersing, or dusting the products. Antimicrobials may be applied to the packaging material that comes in contact with the food or incorporated into the plastic films used for packaging (10, 25, 30).

The active compound allyl isothiocyanate (AITC), which is from plant members of *Brassicaceae* and found in mustard, horseradish, and Japanese wasabi, has proved to be an effective antimicrobial component (3, 4, 12, 20, 27). Early in the 20th century, the preservative effect of essential oil from mustard was reported (11). Several authors have suggested the use of the volatile AITC in active packaging, e.g., in combination with MA packaging. Good results of preservation with AITC in the gas phase have been reported with respect to raw beef, cured pork, sliced raw tuna, cheese, egg sandwich, noodles, pasta, rye bread, and pears (12, 16, 20). Suhr and Nielsen (27) reported that the MIC of AITC was 250 times lower in the gas phase than in the water phase. Nielsen and Rios (20) reported that AITC could be both fungicidal and fungistatic, depending on the concentration of AITC and the number of fungus spores. Problems that occur when mustard oil is used are related to its very pungent taste and its extreme reactivity toward nucleophiles such as water and SH- and OH-groups in food (5). This pungent taste may produce a new flavor in the food, which could be either desirable or undesirable, depending on the concentration and the food matrix.

Naturally extracted AITC is permitted in Japan as a preservative (12), and the U.S. Food and Drug Administration has no objection to the claimed GRAS (generally recognized as safe) status of this compound in meat, fish, shellfish, and poultry products and in baked pies (29). To our knowledge, Europe has not yet passed specific legislation.
that addresses active packaging. Agents used in active packaging are considered additives. The migration of antimicrobial compounds from the packaging to the product still has to follow "Legislation for migration of components from the packaging material to the product." This limits migration to 60 mg of packaging material per kg of food (8). In November 2004, a revision of the regulation regarding "materials and articles intended to come into contact with food" opened the way for the introduction of active and intelligent food packaging in Europe (8). The allowed daily intake of AITC is 0.06 mg/kg of body weight per day (7). Delaquis and Mazza (3) reported the MIC for AITC to be 0.034 to 600 ppm, which is below the recommended total daily intake.

The objective of this study was to investigate the potential use of AITC in the active packaging of cheese. Thus, it was investigated whether AITC could control the growth of cheese spoilage fungi. The effect of application time was also tested, as different active packaging systems have different release characteristics. Finally, the performance in a real packaging situation was investigated with respect to spoilage-free periods, chemical changes, and flavor development.

MATERIALS AND METHODS

Microorganisms. For inoculation, Penicillium roqueforti, IBT 12845, Penicillium commune IBT 10253, Penicillium nalgiovense IBT 12105, Geotrichum candidum IBT 9283, Aspergillus flavus IBT 15606, and Debaryomyces hansenii IBT 307 were obtained from the culture collection of BioCentrum-DTU (Kgs. Lyngby, Denmark). All microorganisms originated from the spoilage of cheese or from cheese production facilities, except for A. flavus, which had been isolated from rye bread.

The fungi were kept on silica gel at 9°C and 50% relative humidity until use. Silica gels were sprinkled onto Czapek yeast extract agar (CYA) (22) and incubated at 25°C for 7 days. Sponges were harvested and used as inocula for a second incubation on CYA at 25°C. Seven-day-old sponges were harvested and transferred to a test tube with a spore suspension (0.5% Tween 80 and 0.5% agar in distilled water). D. hansenii was incubated in yeast broth (21 g of YM broth [Difco Bacto] and 1,000 ml of Milli-Q water, autoclaved for 15 min at 121°C) until a dense solution evolved (24 to 48 h). After 20% glycerol was added to the yeast cell suspension, the solution was transferred to vials and kept at −80°C until use. The spore suspensions and the yeast cell suspension were adjusted to a concentration of 105 to 106 spores per ml as determined by a counting chamber.

Allyl isothiocyanate. Wasawo interior AITC labels (20 by 20 mm; Carex Inc., Osaka, Japan) had AITC incorporated in the glue and were placed inside the packaging.

The mustard oil used was obtained from Extract Mex (San Luis Potosi, Mexico) with AITC at 95% purity. This mustard oil was used to prepare the standard curve for the gas chromatograph (GC).

Adding AITC labels at different times after inoculation on CYA. Petri dishes with CYA were spot inoculated with 10-μl spore suspensions of P. commune, P. roqueforti, P. nalgiovense, D. hansenii, and A. flavus. Three sizes of labels (¼, ½, and 1) were placed inside the petri dishes after incubation for exactly 0, 1, 2, and 3 days at 25°C, and they were then sealed with Parafilm. A comparative study was carried out at 5°C with the AITC labels added exactly 0, 3, 6, and 9 days after inoculation, as compensation for the slower germination of the microorganisms at the lower temperature. Reference plates were prepared in the same way without AITC labels. All tests were performed in duplicate, and the diameters of the colonies were measured regularly with a ruler over a 28-day period. The diameter was corrected for the size of the inoculation spot. The Parafilms were removed from the petri dishes after 28 days to test whether the microorganisms were able to recover.

Application of active packaging of semihard cheese with AITC labels. Semihard Danish Danbo cheese 45+ was supplied by Arla Foods amba (Viby, Denmark). The cheese was cut into pieces measuring 8.5 by 6.0 by 3.0 cm with an average weight of 163.8 ± 5.1 g. The composition of the cheese was as follows: fat, 27.0%; protein, 25.6%; solids, 57.6%; fat in solids, 46.9%; salt, 1.6%; and pH, 5.12.

The packaging material consisted of thermoformed deep-drawn amorphous polyethylene terephthalate containers made of 550 μm of foil (Færch Plast A/S, Holstebro, Denmark) with a volume of 334 ml. The containers were sealed at 140°C with polyethylene terephthalate film that had antifog properties (Galaxy TS 355, Multivac, Wolfertschwenden, Germany).

This experiment consisted of three types of packaging contents: (i) noninoculated cheese (see below), (ii) inoculated cheese, and (iii) empty packaging. Packaging conditions were 0, 1, and 2 AITC labels in combination with atmospheric air (AA) or MA (20% O2 and 25 to 27% CO2). The AITC labels were placed inside the packaging prior to sealing. During packaging, the composition of MA was measured by a gas analyzer (Check Mate 9900, PBI Dansensor A/S, Ringsted, Denmark). Packaging was stored for up to 28 weeks.

Part of the cheeses was inoculated with a 10-μl spore suspension of P. roqueforti, P. commune, P. nalgiovense, G. candidum, and D. hansenii, as in the inoculation on CYA agar. To reduce the number of cheeses, all five spore suspensions were inoculated on the same cheese surface—one spot in each corner and one in the middle. Because of the potential interaction between the microorganisms, which could not be controlled in this experiment or in the dairy industry, only the initial sign of microbial growth on the cheese surfaces was studied on (i) noninoculated cheese and (ii) inoculated cheese twice a week for the first 3 weeks of storage, once a week during weeks 3 to 12 of storage, and every third week during weeks 12 to 28 of storage.

Analysis of the AITC in the headspace of the packaging and identification of volatile compounds were carried out in (i) noninoculated cheese and (ii) empty packaging. These studies were performed on three cheese packages under each packaging condition after 1, 3, 7, and 12 weeks of storage. At the same time, a sensory evaluation was made of the noninoculated cheese. The analysis methods are described below.

Measurements of AITC in the headspace. A standard curve of released AITC was identified by static headspace analysis. Samples of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.5, and 5.0 μl of mustard oil diluted in 100 μl of 96% ethanol were transferred to a 22-ml GC vial. The samples were prepared in triplicates and kept at room temperature (21°C) for a minimum of 8 h.

The headspace sample was automatically withdrawn with an autosampler (HS 40 XL, Perkin-Elmer, Norwalk, Conn.) and injected into a GC (model XL, Perkin-Elmer) with an injection time of 0.06 min. The GC was equipped with a DB-5 column (J & W Scientific, Folsom, Calif.) having dimensions of 60-m length, 0.25-mm diameter, and 1.0-μm film thickness, and a Flame Ion-
Identification of volatile compounds. The volatile compounds were identified by static headspace analysis. A tube packed with 225 mg of Tenax was put in each package through a hole in the top film. The packaging was sealed inside a stomacher bag (65 μm of polyethylene), and the volatiles were trapped on Tenax for 24 h at 5°C. The GC–mass spectrometry program was carried out according to Sønsen et al. (28). The volatile compounds were identified by matching their mass spectra with those from a commercial database (Wiley 275.L, 5972 series, Hewlett-Packard Co., Palo Alto, Calif.) in ChemStation (G1701DA MSD). The applied procedure was semiquantitative, and the relative percentages in relation to the total peak area were calculated for the identified compounds. Ethanol was kept out of the calculation. A partial least-squares regression model was used for a descriptive modeling of data in the multivariate data analysis program Unscrambler (version 7.8, Camo Process, Oslo, Norway).

RESULTS AND DISCUSSION

Adding AITC labels at different times after inoculation on CYA. Figure 1A through 1C shows the linear growth of P. commune, P. roqueforti, and A. flavus colonies when ¼ AITC label was added 0, 1, 2, and 3 days after incubation at 25°C. Adding ¼ AITC label right after inoculation, when the spores were in the inactive phase, caused an inhibition of 4 to 9 days. The spores were completely inhibited when ¼ AITC label was added 1 day after inoculation. At this time, the spores had completed germination, and branching had started. Adding ¼ AITC label in the vegetative growth phase (2 and 3 days after inoculation) resulted in an inhibition of up to 9 days for P. commune and P. roqueforti, whereas A. flavus was not inhibited when AITC was added 2 and 3 days after inoculation. Total inhibition was observed when ½ or 1 AITC label was added 0, 1, 2, and 3 days after inoculation.

The growth curves of P. commune, P. roqueforti, and A. flavus when ¼ AITC label was added on three different days were displaced parallel and had approximately the same slope as when ¼ AITC label was added 0, 2, and 3 days after inoculation at 25°C. This indicates that AITC extended the lag phase of the surviving microorganisms but not the growth rate. Several authors have suggested that the antimicrobial mechanism of AITC is due to the inhibition of the intracellular enzymes in the microorganism (3, 4, 15, 32). This experiment indicated that spores of P. commune, P. roqueforti, and A. flavus in the lag phase were capable of changing their metabolic pattern and surviving when they were exposed to lower concentrations of AITC than the MIC. Identical results for the germination were not obtained at 5°C. However, this could be due to differences in the microbial growth stages of the spores at the time AITC labels were added or to differences in the enzymatic activities at the lower temperature.

P. nalgiovense and D. hansenii were completely inhibited when ¼ AITC label was added 0 and 1 day after the inoculation at 25°C (data not shown). This indicates that P. nalgiovense and D. hansenii were more sensitive to AITC than were P. commune, P. roqueforti, and A. flavus when the compound was added 1 day after inoculation. The addition of ¼ AITC label 2 and 3 days after the inoculation of P. nalgiovense or D. hansenii resulted in growth patterns similar to P. commune and P. roqueforti. A total inhibition was seen when adding ½ or 1 AITC label at 25°C as well as when adding AITC labels at 5°C in each case.

Application of active packaging of semihard cheese with AITC labels: microbiological growth on the cheese
surface. The application study showed that AITC was an effective antimicrobial compound in a food matrix such as cheese. The tested AITC concentration was increased to 1 and 2 AITC labels, as the volume of the package was larger than the petri dish, and the MIC of antimicrobial compounds was higher in a food matrix than in a laboratory medium (27, 34).

Figure 2 shows the first day of visible microbiological growth on the cheese surface for both noninoculated and inoculated cheese. The shelf life of the cheese was estimated from the noninoculated cheese. The shelf life of cheese stored in AA without an AITC label was 4½ weeks. One AITC label was able to extend the shelf life from 4½ to 13 weeks, and 2 AITC labels extended the shelf life from 4½ to 28 weeks. The shelf life of cheese stored in MA packaging without AITC labels was 18 weeks. Both 1 and 2 AITC labels extended the shelf life from 18 to 28 weeks. The observed microorganisms on cheese stored without AITC labels were dominated by penicillia. The microorganisms on noninoculated cheese were dominated by pinpoint yeasts like colonies, which were not further identified. Extended shelf life of packaged cheese is very desirable, because the cheese can be transported longer distances, and the packaging can be used for the final maturing of the cheese.

All inoculated microorganisms were inhibited on the cheese surface when AITC labels were placed in the packaging. Yeast and molds started to appear in places on the cheese surface other than the inoculated spot after 12 and 28 weeks, when the cheeses were stored with 1 and 2 AITC labels in combination with AA. Likewise, yeast and molds started to appear after 28 weeks of storage when the cheeses were stored with 1 and 2 AITC labels in combination with MA packaging.

Concentration of AITC in the headspace. The concentration of AITC in the headspace of cheese packaging was very low compared to the empty packaging with an AITC label (see Fig. 3). On the basis of this difference, the amount of AITC bound to the cheese was estimated to be $1.55 \pm 0.20 \mu\text{l per 334-ml volume packaging stored with 1 AITC label}$ and $3.12 \pm 0.47 \mu\text{l per 334-ml volume packaging stored with 2 AITC labels}$ after 1 week of storage. This indicates that equilibrium was not established between the AITC-in-the-glue phase and the AITC-in-the-gas phase. For equilibrium, the concentration of AITC in the headspace with 2 AITC labels would have been less than twice the concentration with 1 AITC label. Furthermore, the maximum absorbed amount of AITC from 2 AITC labels in the package was $163.8 \text{ g of cheese stored in 334 ml of packaging corresponds to } 37\% \text{ of the legally allowed AITC on the list of flavoring substances authorized for use in foodstuffs at the national level that has been listed by the European Commission (7).}$

A decrease of AITC in the empty packaging was observed from weeks 1 to 3; thereafter, the concentration remained stable between weeks 3 and 7 and decreased again between weeks 7 and 12. The decrease of AITC during storage could have been due to the absorption of AITC in the inner surface of the packaging (26) or to the decomposition of AITC. Sekiyama et al. (23) did not find that AITC could penetrate polyethylene terephthalate film, but amorphous polyethylene terephthalate film (the packaging material in this study) was not tested.

Only a small concentration of AITC was observed in the headspace of the cheese packaging compared with the concentration observed in the empty packaging with AITC labels. This indicated that the released AITC had been absorbed in the cheese.

Identification of volatile compounds in the headspace. The content of volatile organic compounds in the headspace of the cheese packaging is illustrated in Figure 4 as a partial least-squares regression loading plot, showing the relationship between packaging conditions and volatile components detected in the cheese. Principal component (PC) 1 correlates with the concentration of AITC and storage time, whereas PC2 correlates with AA and MA. Three PCs gave the optimal model explaining 45.25% of the validation variance and 35, 20, and 10%, respectively, of the

**FIGURE 2.** Illustration of the first sign of microbiological growth on the surface of noninoculated (■) and inoculated (□) cheese. Stored with 0, 1, and 2 AITC labels in atmospheric air (AA) or modified atmosphere (MA) at 5°C.

**FIGURE 3.** AITC concentration in the headspace of cheese packages (volume = 334 ml) stored over time. Empty packaging with 2 labels and AA (■); empty packaging with 2 labels and MA (△); empty packaging with 1 label and AA (●); cheese packaging with 2 labels and AA (□); cheese packaging with 2 labels and MA (△); cheese packaging with 1 label and AA (○); and cheese packaging with 1 label and MA (○), for 12 weeks at 5°C.
voluntary component variance in PC1, PC2, and PC3. The loading plot shows that AITC, 1-cyano-2,3 epithiopropane, 4-isothiocyanate, 2-buty1-2-propenoate, and allyl cyanide correlated positively with the increasing concentrations of AITC and short storage times. These four compounds were also found in the empty packaging with AITC labels (data not shown) and involved the myrosinase-glucosinolate system where AITC is formed from sinigrin (33).

Oxidation products (from cheese), such as 3-hydroxy-2-butanal, 2,3-butanon, hexanal, dimethyl disulfide, nonanal, 2-methyl-butanal, and 3-methyl-butanal, correlated well with a long storage time and no AITC label. This indicates that AITC is a natural antioxidant as well as a natural antimicrobial compound. The antioxidative effect of AITC in food is not well established in the literature. Furthermore, the oxidation products correlated better with MA than with AA, except for 3-hydroxy-2-butanal.

It was not possible to detect the volatile breakdown products from AITC. The literature reports that AITC reacts with disulfide in proteins and free amino acids such as arginine, lysine, alanine, and glycine. Under these reactions, compounds such as N-allyl thiocarbamoyl amino acid, 2-thiodyrantione, 2-thiohydantoin, allyl amine, and allyl thiourea were produced. These compounds were not detected in this experiment (1, 13, 14, 21).

**Indication of the sensory profile of the cheese.** Two untrained assessors recognized a change in the sensory profile of the cheese after storage with AITC, but the chosen concentration of AITC in the cheese packaging did not give an acceptable sensory profile within 12 weeks. After 1 and 3 weeks of storage, the cheese packed with AITC labels had a very strong mustard flavor, which increased with the number of AITC labels. The mustard flavor in cheese stored with 2 AITC labels decreased from 5 to 4 on a sensory scale during the 12 weeks of storage, and cheese stored with 1 AITC label decreased from 4 to 3, which was still not acceptable. After 28 weeks, only cheese packed with 2 AITC labels was evaluated. The mustard flavor of the cheese had decreased dramatically to 1 on the sensory scale, as had the sensor evaluation of the oxidation products, both of which were acceptable. Drobaica et al. (5) found that AITC reacted strongly with nucleophiles such as water and SH- and OH-groups, all of which can be found in cheese. This could be the reason for the changes in the cheese flavor.

The untrained assessors recognized a fresher taste without CO2 in cheese stored in AA with AITC than in the cheese stored in MA. This could be due to less oxidation and no CO2 in the packaging. Therefore, the packaging of cheese with AA and AITC may be a good alternative to the MA packaging of cheese.

The tested amount of microorganisms on the cheese surface and on CYA (a five-spot, 10-μl spore suspension each containing 104 to 105 spores) was high compared with the natural contamination level in a dairy and on a packaging line. According to Nielsen and Rios (20), the antimicrobial effect of AITC depends on both the concentration of AITC and the number of fungus spores. AITC can sanitize the cheese and thereby hinder the contamination of microorganisms in leaking seals of the packaging or pinholes. The latter is occasionally seen in the packaging of special shredded or cubed cheese.

There are, however, still some challenges before the use of AITC is optimal. The amount of AITC has to be reduced, or alternatively, the AITC has to migrate out of the cheese packaging. A reduced amount of AITC should be supplied sometime after the packaging of the cheese, depending on the storage temperature. Cheese-related fungi, such as *P. commune* and *P. roqueforti*, are more sensitive to AITC when the spores have just started branching. Developing a slow-release method can delay the release of AITC to the packaging atmosphere. A slow-release method based on the incorporation of AITC into cyclodextrin has been developed in the European Union project Biopack (31). Another way of reducing the AITC concentration, and thereby the off-flavor formation, may be through the use of a combination of several natural antimicrobials with different mechanisms. For a more extensive use of natural antimicrobial compounds, it is necessary to examine their efficiency and functionality in real foods, to evaluate toxicological aspects and interactions with the food components, and, finally, to review the mode of action against...
microorganisms and the nutritional and sensory influences on the food.

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