Design Article

Using Antifreeze Proteins to Develop an Alternative to Road Salts

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Road salting is the practice of sprinkling chloride salts (usually sodium chloride) on roadways before snowfall, to prevent the formation of hard and slippery layer of ice on roads. Road salting is done to provide safer driving conditions by lowering the freezing point of salt. However, there are several adverse ecological effects such as extinction of land and sea species, land salinization, airborne abrasives, and roadway erosion. Chloride salts have potential public health effects as "10% to 60% of applied sodium chloride enters shallow subsurface waters and accumulates until steady-state concentrations are attained." These ice melts when the season changes or during rainfall, the salt dissolve in water and flow towards rivers and aquifers. The mixing of chloride salts in water pose a major threat to drinking water sources. This paper presents an alternative to the road salts with none of the negative effects of chloride salts. Some microorganisms produce Antifreeze Proteins (AFPs). These proteins contribute in lowering the freezing point of water, thus preventing freezing. AFPs prevent the freezing of the bloodstream and water inside of animals and plants, respectively, living in cold environments. Isolated AFPs may be able to operate as de-icers. In 2009, the Tokyo Tech iGEM team successfully facilitated expression of theTenebrio molitor AFP in the *Escherichia coli* strain Origami 2. Unfortunately, the Tokyo Tech team had issues with the expression and functionality of the protein due to the formation of inclusion bodies. This paper follows the methods of expression used by the Tokyo Tech iGEM team, but, due to the issues encountered with inclusion bodies, we propose different methods for isolating the AFPs. In order to isolate AFPs from inclusion bodies, methods proposed by Anupam Singh and his colleagues were used. These methods include purification, solubilization, and refolding. Such methods will allow the AFPs to be expressed with correct folding and full functionality enabling them to be used as an alternative to road salts.

Keywords: Antifreeze Proteins, *Tenebrio molitor*, road salts, driving conditions, environment friendly

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Background

In winters, road salting is critical to maintaining the safety of drivers in cold-weather regions. Salting has become a dominant method for avoiding dangerous road conditions due to the formation of snow and ice in the winter. Road salting results in lowering the freezing point of ice (Dai et al. 2012). Chloride salts have been used for many decades as deicers (Dai et al. 2012). The Virginia Department of Transportation (VDOT) uses salt as an abrasive and dry sodium chloride (NaCl) as their primary deicer. VDOT also uses other salts such as calcium chloride, magnesium chloride, calcium magnesium acetate, and potassium acetate (Virginia Department of Transportation 2009). Concerningly, Chloride salts, as well as other additives and abrasives used in addition with chloride salts, have been found to have potentially adverse environmental impacts and public health effects. These salts also damage the infrastructures.

Both Chloride salts and other additives can substantially harm marine life . Additives are also added in the salt which have shown adverse ecological effects such as decrease in species diversity. For example, the excess nitrogen and increased phosphorus content in waterways cause overgrowth of aquatic organisms and plants. The sudden growth of plants in the water increases the biological oxygen demand in water. which disrupts the aquatic environment and decreases species diversity (Fay 2012). Abrasives are added during road salting to increase friction. Abrasives also negatively affect the aquatic environments. Abrasives enters in the streams during runoff. They will form a layer above the water surface thus depriving off the water from fresh oxygen. The biological oxygen demand increases which inhibits fish reproduction as well as the growth of macroinvertebrates (Fay 2012). The salt can remain in the soil for a long time, and cause soil sealing which decreases the ability of soil to absorb water.. The other effects of presence of salt in the soil is that it causes land salinization which affects the metabolism of plants, reduces soil productivity. It can also kill the soil organisms and decrease it's fertility ("Soil Quality," 1998). The chlorine and bromine radicals, which are highly reactive due to unpaired electrons in the salt, can disturb metabolism in plants as well as have adverse effects on the atmosphere (Dai et al. 2012).

There are several potential health effects associated with salting roads. Abrasives consist of fine particles, which can become airborne. These abrasives can be inhaled causing respiratory irritation. Some of the abrasives may be classified as carcinogens when inhaled in substantial amounts (Fay 2012). The salt could get infused in the environment and will cause skin irritation to humans and animals. The salt-water solution will form on the road when the temperature rises. It water will evaporate and fine salt on the roads will be carried away by moving vehicles or air. Consequently, the salt will be inhaled by humans and other species.. This affects the oxygen levels and results in dead cones in lakes and water bodies (Plumer 2015). Road salting has a severe impact on pets and animals, such as dogs. They walk on these roads or reside near the mountain highways causing chemical burns on their paws (Cotroneo 2017).

Apart from mixing salt in the air, a small amount of it may be ionized into the sodium and chloride ions. These ions are absorbed by the plants. Overdosing of these ions in plant will affect them adversely causing them to prematurely die. The high level of salts on roads kills microorganisms and insects by reducing the amount of available food sources. Various species such as snails, clams and fish will die due to shortage of food which is essential for symbiotic relations in the biodiversity (Learn 2017). The result is destabilization of the biodiversity and affected growth of certain plants.

The illnesses caused by abrasives is yet not studied extensively, however, the effects of this factor should be taken into account while considering the adverse effects of salting. Chloride salts, commonly used for road salting, also pose health risks as they can pollute water sources (Fay 2012). The Chloride salts can enter into the aquifers. The roadside wells are at the highest risk. Residues of salt have been found in aquifers year-round, which are increasing continuously due to continuous exposure of an aquifer to salts from roads (Fay 2012). A study pointed out that about 60 to 90% of the salt in water comes from road salting (Kelly 2010). These alarming conditions are a severe threat to the purity of well water. The neighbouring communities relying on the well water may be affected. Moreover, these salts could have damaging short and long term public health effects.

In addition to the adverse environmental and health effects, it also causes deterioration of roads. The salt creates cracks and erosion of the pavements due to water that seeps into the pavement and asphalt cracks. This water expands leading to deterioration of roads and formation of potholes (Fay et al. 2012). The current method of road salting is not only harmful to the environment, but also expensive due to the costs of fixing the damaged roads. The chloride content in salt pollutes the water that is supplied to residential areas. According to a study by the Minnesota Pollution Controlcontrol Agency, one teaspoon of road salt can pollute 5 gallons of water (Minnesota Pollution Control Agency 2015). Conclusively, road salting has major environmental consequences, therefore, It is required to search for an alternative to the road salt. The proposed solution must be cost effective leading to less road repairs (Fay et al. 2012).

The solution to road icing problem needs to be mitigated while avoiding the potential negative effects of salting.

In nature, some organisms have developed antifreeze systems of their own. Antifreeze Proteins (AFP) are produced by vertebrates, invertebrates, plants, fungi, and lichen (Sharma et al. 2014). These proteins lower the temperature of the organism through Thermal Hysteresis (TH) and Ice Recrystallization Inhibition (IRI). These proteins tend to absorb the surface layer of the ice crystals when the organisms begin to freeze. AFPs have already been used by humans to help in the storage of cold food, cold tolerance in plants, cancer cell destruction, and as a de-icing agent (Sharma et al. 2014). In this paper, a genetic engineering technique will be proposed that favors the production of AFPs in order to mitigate road ice.

Systems

To create an environmentally friendly de-icing alternative using naturally occurring Antifreeze Proteins, the AFPs must be expressed using the *E. coli* strain Origami 2. *Tenebrio molitor*, a species of mealworm, produces the AFP that is most viable for deicing as a replacement of salt. This is due to the unique strength of *Tenebrio Molitor* AFP*'s* that allows for a specific TH activity that is 300-500 times greater than AFPs of other chemical compounds (Tahara 2009). Tenebrio *molitor* AFP is made up of 84 amino acids that are right-handed β-helix containing 12 residues per coil, multiple intramolecular disulfide bonds and rigid threonine side chains (Tahara 2009). To express the *Tenebrio Molitor* AFP*,* the *E. coli* strain Origami 2 will be used as it has been proven to successfully produce this AFP by an IGEM team in Tokyo in 2009 (Tahara 2009). To facilitate this expression, the DNA of Tenebrio *Molitor* AFP will be inserted as a plasmid (Figure 1) into the Origami 2 DNA, and allow it to facilitate the expression of the AFP. The AFP gene will be attached with a His-tag to the *Tenebrio molitor* AFP protein to facilitate in-protein purification. His-tags allow protein purification because they made possible for proteins to be easily detected due to the string of histidine residues that bind to immobilized metal ions, thus allowing the proteins to be easily isolated and purified (Jansen 2011). Using the His-tag, the*Tenebrio Molitor* AFP will be purified and separated from Origami 2. It will allow the AFP to be applied directly on the roads. The AFP will prevent the formation of ice by lowering the freezing point of forming ice crystals.

Device Level

To replace the salts and use AFPs on roads, the protein must be expressed by a bacterial cell and should be isolated from the bacteria. Considerable amount of research has been done on isolating certain AFPs. In 2009, the Tokyo Tech iGEM team successfully facilitated expression of Tenebrio *Molitor* AFP in the *E. coli* strain Origami 2. This AFP gene has been synthesised and is available via GENEART. The team was able to confirm both the expression and function of the AFP in *E. coli* (Tahara 2009). Due to its availability and success in expression, Tenebrio *Molitor* AFP is a viable option for this project.

The Origami 2 strain is a promising option because it has shown expressing *Tenebrio molitor* AFP. Origami 2 is an *E. Coli strain* trademarked by Novagen. According to materials provided by Novagen, Origami 2 was designed to solve the problem of misfolded proteins (EMD Chemicals n.d.). For most proteins to fold correctly, they must have intact disulfide linkages in between cysteine residues (Soderberg 2015). Glutathione, a redox agent regulates the formation and disintegration of such linkages. Glutathione operates with an important group called thiol and is abbreviated as GDH when lacking thiol. Without thiol, Glutathione does not have full functionality (Soderberg 2015). For a protein to be properly expressed and folded correctly, the cell that it is expressed in must be able to facilitate disulfide linkages that rely on functional glutathione. Novagen's Origami 2 strain has been mutated to fight reduced disulfide bonds and protein misfolding. Origami 2 is desirable because it is chemically competent cell. There is no need to further treat it in the lab. Chemically competent cell is mutated to be competent thus enhancing efficiency (EMD Chemicals n.d.). Novagen's Origami 2 is the most promising option for the correct expression of Tenebrio *molitor* AFP due to its property of mitigating protein misfolding.

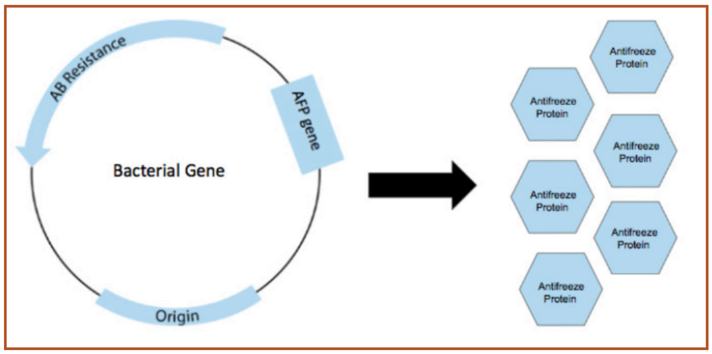

Figure 1

Parts Level

Although,the research of Tokyo Tech iGEM Team in expressing the *Tenebrio molitor* AFP provides a foundation to the future expression of the AFP for de-icing needs, however, the discrepancies in the procedure must be remedied before using the AFP for commercial purposes. While the Tokyo Tech iGEM team was able to express the AFP, however, the proteins formed inclusion bodies (Tahara 2009). Inclusion bodies make it harder to isolate individual proteins from their host (Singh 2015). To isolate the *Tenebrio molitor* AFP, the AFP must be separated from the host, *E. coli*, without the aggregated inclusion bodies.

In 2015, Anupam Singh and colleagues proposed methods to successfully remove inclusion bodies. Inclusion bodies are usually circular, dense particles that accumulate in the cytoplasm or periplasmic space of *E. coli* (Singh 2015). Singh and colleagues proposed four main steps to decrease interference by inclusion bodies by separating them from proteins. First, the inclusion bodies must be purified by lysing the cells with precision, making sure to isolate the inclusion bodies. In the second step, a solubilization agent of moderate strength must be used to solubilize the inclusion bodies. An "organic solvent based buffer" may be the best agent to use due to its high efficiency and mitigation of aggregation taking place when refolding the protein (Singh 2015). The third step is to use a buffer to refold the proteins into the correct formation. Tenebrio *molitor* AFP contains many intramolecular disulfide bonds that may be cause protein misfolding (Tahara 2009). In order to mitigate the difficulties posed by higher concentrations of disulfide bonds, Stepwise dialysis should be used as the protein folding method. Dialysis technique has shown positive results for proteins containing disulfide bonds (Singh 2015). Finally, the protein must be purified (Singh 2015). These methods should mitigate interference due to inclusion bodies and increase the efficiency of isolating functional *Tenebrio molitor* AFPs. Once the Tenebrio molitor has been isolated, it can be sprayed on the roads for deicing purposes.

Safety

The AFPs will be sprayed on roadways to lower the freezing point of water and inhibiting ice formation. Although this will increase road safety in cold environments, however, near roadways, some ecosystems will be affected by the sprayedAFPs. AFPs will contaminate runoff from roadways to the surrounding environment including nearby vegetation and waterways. The melting water can pollute the groundwater near the road and surrounding ecosystem. AFPs that are released into the environment will be taken up by plants which can lower the freezing temperature of the plants, thus,disrupting the natural dormance systems of plants during the colder seasons. This dormancy allows the plants to drop leaves, cease or slow down the growth, and reduce the water in roots, tissue, and branches. The freezing of water inside the plant will allow it to take colder conditions while the surrounding ground freezes. The frozen plant remains safe during the cold (Botts 2016). The uptake of foreign AFPs by the plants will result in lowering the freezing point of water, thuss disrupting the dormancy system in plants. When plant is unable to go into the dormant state, it may cause premature bloom during the spring season. Early bloom due to presence of melted water in the plants will result in the death of the plant due to unfit external conditions.

Natural AFPs in other organisms   prevent freezing of the blood of animals in subzero environments. It is primarily found in fish where it keeps arctic fish resistant to the subzero water temperatures, by preventing the formation of ice crystals in the blood (Duman 2015). The AP's can affect animals living near roadside areas, fish which lives in run-off water ponds, and animals that drink from road runoff to excess AFPs. The AFPs may potentially raise the blood temperature of such organisms and cause adverse health effects. It is unlikely that the presence of AFPs would have adverse effects in animals unless they entered their bodies in excess. However, in the case of a severe freezing environment that required the use of large volumes of AFP followed by a thaw can cause increased runoff, AFPs may enter the surrounding environment in substantial amounts high enough to be harmful to organisms living roadside areas.

Discussion

The use of the present invention i.e. AFPs can solve numerous issues caused by conventional road salting. The use of *Tenebrio molitor* AFP's in *E. coli* helps shielding the roads from further damage. The AFP,s are less dangerous to natural biodiversity. The *E. coli* will be used as a bio-spray to reduce the freezing temperature of water to prevent ice from sticking to the roads during winter. As mentioned previously, the Tokyo Tech iGem team were unable to fix the protein folding issue This paper proposes the addition of Origami 2 to solve the issue at hand. Creating a bio-spray will prove to be an effective and eco-friendly solution. The solution presented in this paper also has other benefits such as accident risk-reduction on the roads and less damage to the roads compared to road salts.

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