Determining Antimicrobial Activity in Hydrolysates Obtained from Buttermilk Substrates by Trypsin and Pepsin

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Introduction:

Milk is an emulsion, composed of various nutrients such as fat, sugar (lactose), proteins, minerals and other organic substances that are dispersed in water.[1] It is passed on from mother to a young infant in all mammals as a primary source of nutrition. Milk is also an important source of antibodies and antimicrobial substances to otherwise vulnerable infant. There are numerous well-documented and studied antimicrobial proteins such as lactoferrin and α_{s2}-casein.[2] Within the structures and sequences of milk proteins, there are numerous bioactive peptides that exhibit antimicrobial properties.

Recently, studies have shown that these antimicrobial peptides are found in other dairy proteins such as milk fat globule membrane (MFGM) which can be found in buttermilk and whey buttermilk.[3] Gastrointestinal enzymes, namely pepsin and trypsin, can hydrolyze these milk proteins to release the peptides.

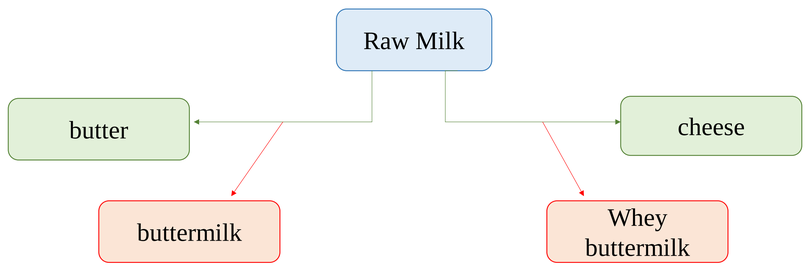
Buttermilk and Whey Buttermilk:

Buttermilk and whey buttermilk are by-product of butter and cheese production.

Composition of:

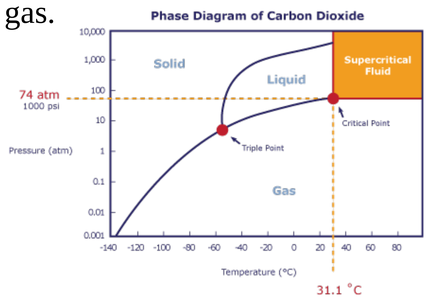
buttermilk = casein +whey proteins + MFGM proteins

whey buttermilk = whey proteins + MFGM proteins



Supercritical Fluid Extraction (SFE):

Supercritical fluid is a fluid beyond its critical point where it is not liquid nor gas.



Supercritical carbon dioxide works as a solvent to remove phospholipid from buttermilk and whey buttermilk, changing the structure and conformation of these substrates. Due to the changes in conformation, SFE treated substrates might be hydrolyzed at different sequences, thus producing different peptides.

References:
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3. Rasmussen, J.T. (2009). Bioactivity of milk fat globule membrane proteins. The Australian Journal of Dairy Technology. 64:63-67
Graph of supercritical CO_2 http://www.novasterilis.com/ourcompany/index.php/technology/scco2

Methods:

Pepsin: Acidic Enzyme, optimum pH 2, found in stomach
Trypsin: Basic Enzyme optimum pH 8, found in intestine

Hydrolysis With Trypsin or Pepsin at 37℃

Time of hydrolysis for all substrates: 0, 0.5, 1, 2, 4, 6, and 24 hours
Heat hydrolysates at 80℃ for 30 minutes to denature the enzyme

Centrifuge and filter to remove intact proteins

Lyophilize (freeze-dry) for at least 12 hours

- **Escherichia coli (K12** and OP50)
- **Bacillus licheniformis**
- Staphylococcus epidermidis
- Pseudomonas fluorescens
- Listeria innocua

Put hydrolysates on filter disks and inoculate on Mueller Hinton Agar overnight

No zone

B +T at 4 hr

SWB + P 0 hr C

Zone of inhibition

Penicillin 100mg

Both plates had a slight zone of inhibition, but the zone was not prominent.

Results and Discussion:

Due to the time constraint, only E.coli K12 was fully tested. Generally SFE treated substrates showed some sign of zone of inhibition. However, the concentration of these peptides was too low, therefore the measurements of the zones were negligible. Also, the two plates that showed clear zone of inhibition were hydrolyzed at 0 hour. This indicates that the inhibition might have been created by intact proteins or hydrolysates created during the processing of the substrates.

Substrate	E.coli Concentration	Zone of Inhibition Plate 1	Plate 2
SB+P 0hr	10^6	1.1 mm	
SB+P 4hr	10^6	1.1 mm	
SB+P 4hr	10^7	1.1 mm	
SWB+T 0hr	10^7	1.1 mm	1.2 mm
SWB+T 0hr	10^6	1.1 mm	
SWB+P 0hr	10^7	1.0 mm	0.9 mm
SWB+P 0hr	10^6	1.3 mm	1.0 mm
SWB+P 4hr	10^7	1.1 mm	1.1 mm
SWB+P 4hr	10^6	1.0 mm	

Table 1.Only the substrates that had a zone of inhibition are reported. Out of all the substrate and enzyme combination, SFE treated whey buttermilk hydrolyzed with pepsin showed the most zone of inhibition.

Future work :

This research is in its preliminary stage. The purpose of this lab was to determine the presence of the antimicrobial peptides in buttermilk and whey buttermilk, not to locate exact sequences of the peptides.

In order to improve the results, the following steps can be done in the future:

- Centrifuge and filter hydrolysates by size (i.e. 10 kDa and 3 kDa)
- Concentrate hydrolysates more efficiently
- Test on other bacteria
- Use spectrometer to read the growth/inhibition instead of plating method.
- Compare the results of non-SFE treated and SFE treated substrates

Application :

Bacillus is a type of bacteria that can create an endospore when the condition is too harsh for it to remain in vegetative state. Endospores are very sturdy and hard to eliminate completely. This poses a problem in milk industry, because bacillus spores can remain in milk even through pasteurization and can cause spoilage. If there are microbial peptides that are able to kill these bacteria, they can be added as a natural additive to improve shelf life of not only dairy products but other processed food as well.

Antimicrobial peptides can also have pharmaceutical application. Instead of using antibiotics, which are overused, these antimicrobial peptides can be used together with the antibiotics to produce synergistic effects.[2]

All of these applications can potentially appraise the value of buttermilk and whey buttermilk

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