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Traditional Baking Enzymes - Proteases

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My topic for today is proteases. I will cover what they are and what they do, how to test them, compare them, and how to use them in various applications.

First off, we need to define what a protease is. As many other enzymes used in the food industry, proteases take a long polymer and break it into small bits. In this case, proteases hydrolyze proteins and their decomposition products into simpler compounds such as peptides and amino acids. A protease may be functional on a particular protein only if the protein is in a specific form. For instance, a protease may hydrolyze egg albumin in a raw egg but be inactive on denatured albumin in a cooked egg. The same can be true of meat products, soy, and gluten. The physical form of the protein can determine if a protease will be functional. As an example, think of trying to cut a ball of yarn with a pair of fingernail clippers. Unless the ball of yarn is fully unraveled, it is almost impossible to make a significant cut in the yarn length. However, unravel it and the nail clippers can easily cut anywhere in the chain.

There are two basic types of enzyme action on the protein polymer, endo and exo. Endo action is defined as random splitting of the polymer anywhere along the chain. This action contributes most to dough relaxing, preventing dough shrinkback, better bread volume and pan flow and faster bakery throughput.

Bakery output is really why proteases are used. You can make any baked good without a protease. All you need to do is keep on mixing and mixing. Proteases are minor, inexpensive ingredients that can have a major impact on profits. For example, assume you have a bakery that is optimized using two mixers for finished doughs and kicking out one dough every ten minutes, that's six doughs per hour. Assume you run 18 hours per day. That's 108 doughs per day. Flour is changeable. For the sake of argument, assume you get in a load or two that is very strong. Mix time has been extended to accommodate the stronger flour. Now you are doing one dough every 12 minutes instead of every 10 minutes. Instead of 108 doughs you are only producing 90 in that same 18 hours. That is nearly a 20% loss in productivity. Put another way, to make the same amount of bread, you would need to pay your plant 3.6 hours of overtime, your oven and proof box would be running an extra 3.6 hours as well. It could be a very expensive proposition. The problem is resolved by the simple addition of a few protease tablets or packets toeach dough, either at the sponge or the dough side. The added expense of a few dollars per hour in protease costs is more than justified by the alternate loss in production.

The other type of action associated with enzymes is exo action. This is defined as specific cleaving of a terminal group on the polymer. In the case of proteins, an amino acid or a di- or tri- peptide is cleaved. The most common application is "debittering" a protein hydrolyzate such as cleaving leucine. The free amino acids also contribute to browning and to flavor of the bread.

In this next section, I will discuss the sources for proteases and how they are generally assayed.

The proteases with the most history of use are the animal derived and botanical. The animal proteases are pancreatin, trypsin, and chymotrypsin. These are rarely used in the baking industry. The primary reasons are cost and the presence of side activities. Pancreatin for instance contains protease, lipase, and amylase. The lipase could cause significant flavor problems because it will produce free fatty acids and could lead to rancid or soapy flavors. Even if a baker should find a unique application, there is still a problem. These enzymes are derived from porcine and bovine sources. There is no way anything from a porcine source could ever be kosher. Further, any finished product in which pancreatin is used could never be kosher. Plus, if you do run a kosher facility, the cleanout would be very labor intensive.

The botanical proteases are papain, bromelain, and ficin. Of the three, bromelain has the widest use in the baking industry. Over the course of the last 30 years since it was available, it has tended to replace papain in the baking industry because it does the same job but does it faster. On a per unit of activity basis, bromelain is generally a little more expensive than papain, but the cost is negligible compared to the improvement in the rate of the reaction. Ficin has not been used because of high cost and intermittent availability. The other advantages of the botanical proteases have been that they were free of side activities such as amylase. This advantage is diminishing as techniques in gene deletion or gene doubling have lead to microbial proteases that are essentially free of other activities.

When you buy botanical proteases, you will find the activities often quoted as MCU, CDU, BTU, TU, and GDU. The assay parameters are outlined on the slide. The typical substrates used are NFDM, hemoglobin, and casein.

The most common sources of fungal proteases are *Aspergillus oryzae* and *Aspergillus niger*. These also have a very long history of use. As an example, these proteases were in commercial use prior to 1958, and are part of the GRAS affirmation petition on enzymes filed in 1972. There are patents on these proteases in the 1930's, 40', and 50's.

As a class, these enzymes typically are used in neutral and acid pH applications. On gluten, they are slow acting relative to other proteases and may have significant side activities such as amylase or pentosanase. As an example, three different *A. oryzae* proteases were analyzed for amylase side activity. Each is from a different production strain. The enzymes were adjusted to have the same HUT activity per gram. As you can see, #1 has the highest activity. #3 was below the level of detection. The HU(T) method is the most common method of analysis for fungal proteases used in the baking industry. Hemoglobin is the substrate used. You will often see tablets or packets with a stated activity of so many HUT per tablet. For instance, one tablet may contain 50,000 HUT, with one or two tablets per cwt. as the suggested dose. As a point of reference, the raw material used to produce that tablet will be 500,000 + HUT/g. The tablet is the best method to dose in this case because the baker would have to weigh out 0.1 to 0.2 grams per cwt of our enzyme concentrate.

The other common sources for microbial proteases are *Bacillus subtilis* and *licheniformis*. These are used in neutral / mildly alkaline applications. They are faster than the fungal proteases but a little easier to control than bromelain or L-cysteine. They may or may not have significant amounts of amylase. Pentosanase activity is fairly rare. Historically, the most common assays are the Anson, Delft Unit (DU), - *caution* this is also the notation for a starch degrading assay, the Dextrinizing Unit-, the Northrup unit and the neutral protease also known as the PC assay method. The substrates used are hemoglobin and casein.

The biggest problem for the baker is that it is difficult to compare the protease performance on gluten because none of these assays measure activity on gluten. None reflect actual dough conditions. The best way to compare them is in the bakery / bake lab with some type of device to measure the amount of energy used to bring the dough to optimum development. An example would be a farinograph in the lab or a Mixotron hooked up directly to your mixer. For the baker, there must be a practical solution in the plant. It must be easy to use by both trained and untrained personnel. In a production plant, the best solution is to have a mixotron on related device hooked up to the mixer. There is visual proof of dough development. With the mixotron, you will see the peak dough development on a graph and know exactly when to kick the dough out.

A different wet chemistry approach was developed by TNO in the Netherlands. They presented the information at an AACC convention five or six years back. They developed a system using gluten as a substrate. At the time the details were a little vague because they were (and still are) selling the service to compare the proteases. Therefore, they did not identify the specific fungal proteases evaluated. They only stated that they were all from different production organisms. With the data generated, and assuming a linear relationship between the various relationships, I have presented the nine proteases equalized on the hemoglobin activity. Each has been established as having a factor of 100. TNO has reported the reaction rates on glutenin, gliadin, and casein on each of these nine fungal proteases.

Glutenin can be classed as a heterogenous mixture of proteins. It has a molecular weight of 100,000 to several million. It is a multiple chain protein with crosslinked intermolecular disulfide bonds. It has moderate adhesiveness and high elasticity. As you can see, (slide 16), proteases 8 and 9 have the highest rate when 5 has no appreciable activity.

Gliadin is considered a heterogeneous mixture of prolamines with a molecular weight of 25- 60,000. It is a single chain protein containing intramolecular disulfide bonds. It has high adhesiveness and low elasticity. In this case, 8 and 9 have low activity, whereas 1 has very high activity. Again #5 has no apparent activity.

Finally, TNO tested the same nine proteases on casein. This is a milk protein as a common substrate in many enzyme assays. It is interesting to note that #5 seems to only function on hemoglobin and nothing else. Number one and number six had the highest activity. Based on this work, a blend of #1 and # 8 or #9 would be a very effective protease blend to hydrolyze gluten.

Deciding which enzyme to use in a baked good will depend on the rate and the ability to control the rate of the reaction. Many times the choice is made for you by history of use. There may be other enzymes that will work as well or better but to make a change is expensive in terms of labor and test doughs. In addition, it is unlikely that there will be huge savings. Proteases as a class of enzymes tend to be inexpensive in relation to other enzymes.

As we go through the rest of the presentation, I will speak briefly on various applications such as breads, crackers, cookies, tortillas/ pizzas, and other products.

In breads, the traditional sponge dough system can usually get the most effect by adding the protease to the sponge. A typical dose is 50,000 HUT/ cwt flour. If the bakery philosophy is to have a plain sponge, then the dose to reduce mix time at the dough side may be 250,000 HUT/cwt. In a straight dough, protease is generally used to improve handling properties and give a mellower less bucky dough rather than as a mix time reducer. In a no time dough better pan flow and improved gas retention. In continuous mix systems and extruded doughs, proteases will give better dough handling with less wear and tear on the machines. The dose will be dependent on the amount of time and if it was a brew system with or without flour. As far as I am aware, continuous mix bread is the only bread production method that has used bromelain. The other production methods all use the fungal protease.

There are two types of cracker production, fermented soda cracker and chemically leavened. The soda cracker will usually use a bacterial protease that is neutral to slightly alkaline in pH optimum. The soda cracker will have a 10 to 20 hours fermentation for the sponge. The sponge is brought out and the rest of the flour and other ingredients are added. Typically this is when the protease is also added. The dose rate will be in the range of 200 to 400 Northrup per cwt or 150,000 PC/ cwt. The protease has plenty of time to mellow the flour as the dough will rest for up to four hours before further processing. The protease allows for easier working of the dough into thin sheets without shrink back or wrinkling on the band oven. Chemically leavened crackers are generally a no time type dough. If a protease is used then it must be very aggressive on gluten. Bromelain is usually the product of choice with dosing of 100 to 200 BTU per cwt. On an equal activity basis, bromelain is much more aggressive that papain. Cookies can use proteases but they are not often used as far as I know. The enzymes can be used if you are stuck with hard wheat flour and generally try and use a soft wheat flour. Bacterial proteases, bromelain, and papain have all been used. There is an interesting article that was published Cereal Chemistry, Vol. 66, No. 2, 1989 by Gaines and Finney. They evaluated quite a number of different proteases and found that papain gave the best spread

A protease can be used in tortillas or in pizzas. Bromelain is usually the product of choice. However, due to short processing times, L-cysteine or sodium metabisulfite are the products of choice.

Masa production offers a new opportunity for proteases. The traditional production method is to add 2 – 3% lime (KOH) to corn and add hot water and let the whole thing cook for 12 to 24 hours. The purpose is to soften the corn and allow easy separation of the outer husk. The corn is drained and rinsed and then ground. The masa is then used for corn tortilla production or corn chips. EDC has done several trials that indicate the processes may be able to be reduced to 3 or 4 hours. Rather than adding a fixed amount of lime, the pH was adjusted to 9 – 10. The enzyme was added at this time and the temperature was kept at 160° F max. It appeared that the corn was ready for grinding in 3+ hours rather than the normal. It was also interesting that the corn was white rather than yellow. To get the yellow, additional lime had to be added.

There are other technologies that can be used to accomplish the same thing as a protease. They are L-cysteine , glutathione from inactive yeast, and sodium metabisulfite. Where they tend to be used is when a very rapid breakdown is needed but that the reaction stops. Unfortunately, when a protease is added, it will continue to work until the substrate, gluten in this case, is used up. If your ovens go

down for an extended period of time, an enzyme treated dough could end up totally broken down. The other compounds may give you a dough that is recoverable.

In the area of future applications, perhaps there will be a greater examination of what is "bread flavor". Can the use of proteases from enzymes or yeasts modify the flavor of the bread? Proteases are used to enhance flavor production in a number of other industries. The best known example is enzyme modified cheese. Selective proteases and lipases can speed flavor production. Cheddar cheese can take up to a year to age. Under the proper conditions, enzymes can speed the aging process to one or two months or even shorter. Proteases play an important part in flavor development. Generally the aminopeptidase and carboxypeptidase activity are important factors. However, the relationship can vary dramatically between various enzyme preparations. For example:

Enzyme	Protease Conc.	Protease 400	Protease 100	Exo-Protease
GTG-ase	3	30	100	N/A
LAP-ase	5	30	100	100

In summary, proteases remain an important and an inexpensive tool to improve production output and product quality.

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Definition

- Hydrolyze/degrade proteins and their decomposition products into simpler compounds such as peptides and amino acids.

Endo Action

- Random internal
- Contributes most to dough relaxing
- Prevents "shrink back"
- Faster bakery throughput
- Better volume / Gas Retention

Value of Endo Action

- 2 mixers,
- 1 dough every 10 min, 18 hrs

108 doughs per 18 hours

New Flour, higher protein

- Adds 2 mins per dough
  90 doughs in 18 hours
- *Need 3.6 more hours for 108 doughs*

Exo Action

- Specific to the terminal group
- Produces free amino acids
- Contributes to browning reactions
- May reduce bitter peptides or modify flavor

Sources, Animal

- pancreatin
- contains amylase, protease, lipase
- rarely used
- expensive
- non - kosher
- problems with side activity

Sources, Botanical

- papain, bromelain, ficin
- long history of use
- rapid rate of reaction
- broad pH and temperature optimums
- no amylase or pentosanase side activity

- malted barley flour
- amylase, protease side activity

Common Botanical Protease Assay Methods

- Milk Clot Unit MCU/mg
- NFDM :pH 6.0 , 40° C, 3 to 4 minutes
- Casein Digestion Unit CDU/mg
- Casein: pH 7, 37° C, 1 minute
- Tyrosine Unit TU/mg
- Casein: pH 6.0, 40° C, 60 minutes
- Bromelain Tyrosine Unit BTU/g
- Hemoglobin: pH 5.0, 30° C 30 min; 23 C 30 min
- Gelatin Digestion Unit GDU/g
- Gelatin: pH 4.5, 45° C, 20 minutes

Sources, Microbial, Fungal

- *Aspergillus oryzae /Aspergillus niger*
- Neutral to acid pH applications
- Relatively slow acting
- May have significant amylase side activity
- May have significant pentosanase side activity

Comparison of HUT vs. SKB

- Protease 1,
  - 100,000 HUT/g, 5,000 SKB/g
- Protease 2,
  - 100,000 HUT/g, 400 SKB/g
- Protease 3,
  - 100,000 HUT/g, >10 SKB/g

Comparison of HUT vs. LAP

- All 100,000 HUT, *A. oryzae*
- Protease 1 < 50 LAP/g
- Protease 2 ~125 LAP/g
- Protease 3 > 300 LAP/g

HU(T)

- Most common fungal protease assay method
- Substrate hemoglobin
- 30 minutes, pH 4.7, 40° C

Sources, Microbial, Bacterial

- *Bacillus subtilis, licheniformis*
- Neutral to alkaline pH applications
- Faster than fungal
- Slower than bromelain or L-cysteine
- May have amylase
- Unlikely to have pentosanase

Common Assay Methods, Bacterial Protease

- Anson, A/g
- Hemoglobin: pH 7.5, 25° C, 10 min
- Delft DU/g
- Casein: pH 8.5, 40° C, 40 min
- Northrup NU/g
- Casein: pH 7.4, 40° C, 35 min
- Neutral Protease PC/g
- Casein: pH 7.0, 37° C, 60 min

Assay Method Problem for Baking

- None use gluten or flour
- None reflect dough conditions

A Practical Solution

- Must be easy to use in plant
- Must be easy to use for all personnel
- *Mixitron on each mixer to optimize time and protease use levels*

Protease Action on Gluten

- From Report at TNO
- Enzymatic Modification of Wheat Gluten
- Weegels, Jager, Voorpostel, Harte, Hamer
- AACC Presentation
- Modified to reflect 100 units on Hemoglobin

Protease Reaction on Hemoglobin

Protease Reaction on Glutenin

Protease Reaction on Gliadin

Protease Reaction on Casein

Relative Reaction Rates

- Rule of thumb
- Fungal proteases slowest
- Moderate speed, bacterial protease
- Fastest bromelain
- Bromelain not as fast as L-cysteine

General Use in Baking

- Breads
- Crackers
- Cookies
- Tortillas/ pizzas
- Other ingredients

Breads

- Sponge dough
- Straight dough
- No-time
- Continuous mix

Crackers

- Yeast raised
- Chemically leavened

Cookies

- Protease may improve spread

Tortillas & Pizzas

- Limited use in flour tortillas
  - very short floor time
- Prevents shrinkback in pizza
  - competes with bisulfite and L-cysteine

Masa Production

- Possible use of alkaline protease
  - reduce time for soaking
  - reduce lime requirement
  - easier to produce light rather than yellow masa
  - patented by University of Nebraska

Other Technologies

- L-cysteine
- Glutathione
- Sodium metabisulfite

Future Applications

- What is bread flavor?
  - Can proteases boost or modify?

Peptidase Activity in Commercial Enzymes

- Protease Concentrate
  - GTG-ase 3, LAP-ase 5
- Protease 400
  - GTG-ase 30, LAP-ase 30
- Protease 100
  - GTG-ase 100, LAP-ase 100
- Exo-Protease
  - GTG-ase NA, LAP-ase 100