PROTEINASE *A* SECRETION BY YEAST

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1. INTRODUCTION

Foaming property is one of the most conspicuous aspects of beer quality. Researches have been carried out to elucidate factors determining stability and several compounds have been pointed out. Various chemical compounds are positive factors of foam, including proteins, iso-α-acids, ß-glucan, melanoidins while others, like fats, fatty acids, alcohol or polyphenols have detrimental effects. All factors are connected with physical properties like viscosity...

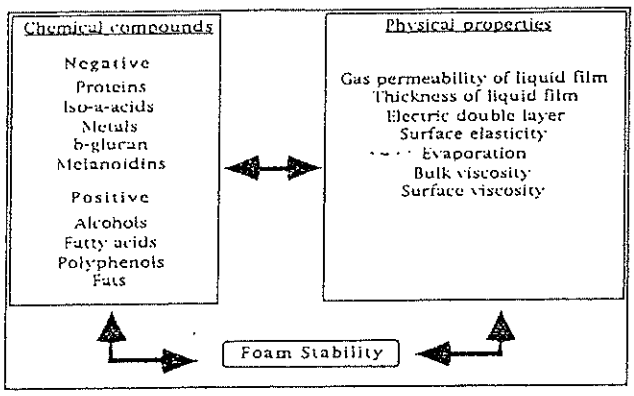

Figure 1: Overview of beer foam stability (Maeda et al., 1991)

Although the processes involved in foam formation are not well established, the latter can be controlled to a certain extent by retaining and enriching the beer in foam positive factors, and by reducing the negative ones (fats and fatty acids for example). Among the positive factors, polypeptide material is of prime importance for the formation and stabilisation of beer foam.
Beer contains 300 to 800 mg/l of proteinaceous material, mainly polypeptides, the majority of which is derived from barley. The polypeptides contribute not only to the beer foam (Pierce and Purssell, 1959; Omrod *et al*, 1991) but also to its body, mouthfeel, flavour, colour and haze. The quantity and the quality of barley-derived polypeptides depend on the activity of proteolytic enzymes operating during malting and mashing. The degree of proteolysis can be controlled by choosing an adequate program of temperature and timing during mashing, and the process is practically stopped after the final boil. In the past, it was assumed that no further proteolysis occurred in the subsequent fermentation and maturation steps (Hough *et al*, 1982).

However, Morris and Hough noted that *Saccharomyces cerevisiae* excretes proteolytic activity which allows gelatine degradation (Morris and Hough, 1956). Further investigations demonstrated a release of proteolytic enzymes from living brewer's yeast cell but also due to cell autolysis (Maddox and Hough, 1970; Dreyer *et al*, 1983).
One of these enzymes is of particular interest because of its high activity at the pH of the beer. The acid protease was isolated and its properties was similar to that of Proteinase A (Saheki *et al*, 1974).

2. PROTEINASE *A* AT THE BIOCHEMICAL LEVEL

Proteinase A is a vacuolar aspartic protease (PrA) encoded by the *PEP4* gene. The vacuole of *S.cerevisiae* has several physiological functions including the activities equivalent to that to lysosomes of higher eukaryotes, in particular with regard to proteolysis (Knop *et al*, 1993), post-translational modifications, degradation of specific enzymes and catalysis of a general protein turnover (Slaughter and Nomura, 1992). Vacuolar proteases are responsible for a large portion of the total cellular proteolysis in yeast, in particular under conditions of nutrient deprivation (Van Den Hazel *et al*, 1996).
With the development of new detection techniques using chromogenic substrates, the number of known proteases was increased to over 40 (Achsetter and Wolf, 1985). Among them Carboxypeptidase Y (CPY), Proteinase A (PrA) and Proteinase B (PrB) are enzymes which are transferred to the vacuole as soluble precursors.

The secretory pathway for *S.cerevisiae*

The secretory pathway of vacuolar proteases includes a number of functionally distinct organelles. The traffic is mediated by vesicles. Proteins that pass through the secretory pathway, are translocated across a membrane via the endoplasmatic reticulum. This translocation is promoted by a hydrophobic signal or prepeptide which may be removed by a peptidase activity. In the endoplasmic reticulum, the translocated protein undergoes a folding process. When folded, proteases are directed to the Golgi complex. The Golgi complex has several functions, the most relevant one being the modification by addition of carbohydrate side chains (protein glycosylation). An altered glycosylation of proteases seems not to result in mis-localisation of vacuolar and secreted proteins (Johnson *et al*, 1987). Vacuolar proteins are then sorted to the endosome and from there to the vacuole.

Endocytosed proteins are transported through vesicles and endosomal compartment from which they are carried to the vacuole (Van Den Hazel *et al*, 1996).

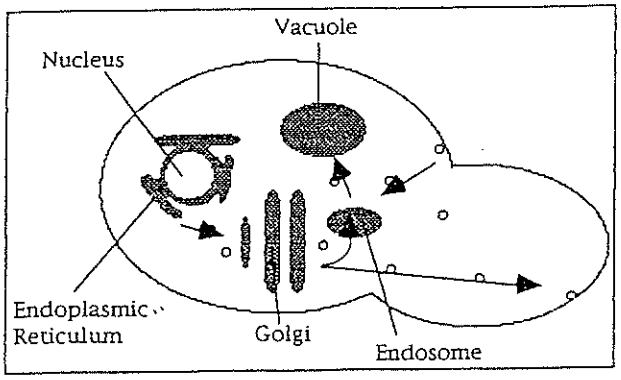

Figure 2: Overview of the yeast secretory pathway

The gene *PEP4* encodes a PrA precursor, an inactive preproenzyme of 405 aminoacids. A hydrophobic signal peptide at the N-terminus, a prepeptide, is cleaved during translocation into the lumen of the endoplasmic reticulum giving a proenzyme proPrA. The proenzyme is N-glycosylated in the endoplasmic reticulum, usually at two sites. The majority of the mature PrA molecules are glycosylated at two sites but a sub-population exists which contains only one oligosaccharidic chain. During the transit in the Golgi complex, the oligosaccharidic chains of the proenzyme undergo trimming and a limited extension. The propeptide is apparently required for proper folding and for targeting the protein to the vacuole. Finally a propeptide of 54 aminoacids is removed during or upon delivery to the vacuole, resulting in an active protease of 42 Kda. This removal requires the activity of Proteinase B (PrB) and is essential to obtain an active, mature PrA. Vacuolar activation of ProPrA can also take place in mutants lacking PrB activity. An active 43 Kda termed pseudoPrA is formed, probably by an autocatalytic process (Wolff *et al*, 1996).

Function and substrates of vacuolar proteolysis

The vacuole is a compartment in which non specific and relatively uncontrolled proteolysis takes place. The levels and the activity of proteases depend on the levels and type of nutrient in the extracellular media. PrA, PrB and CPY activities are low during exponential growth and increase strongly when entering stationary phase. Synthesis of the proteases appears to be repressed by glucose. Vacuolar proteolysis seems important under nutritional stress (Hansen *et al*, 1977). These vacuolar proteolytic enzymes are involved in the general breakdown of enzymes. Therefore it seems likely that these enzymes are important in the loss of viability and autolysis of brewing yeast cells in the post fermentation and storage phases. It should be noted, however, that cytoplasm contains specific inhibitors for each of the proteases. And also that the inhibitors themselves can be digested by the enzymes (Slaughter and Nomura, 1992). For example, PrA degrades the inhibitors for both PrB and CPY, whilst PrB can digest the inhibitor of PrA.

Proteinase A activity out of the vacuole

Vacuolar proteinases were supposed not to be released from living cells and their presence in the medium was thought to be exclusively due to autolysis. Nevertheless, Maddox and Hough (1970) and Dreyer and co-workers (1983) demonstrated a leakage of intracellular proteolytic enzymes through the cell wall of living yeast cells.

Maddox and Hough compared proteases from autolysing yeast, from protoplasts and extracellular secretion from whole living yeast cells (Maddox and Hough, 1970). They showed that proteolytic enzymes secreted by living yeast cells and from obtained lysed protoplasts are slightly different from those derived from autolysing cells (e.g. pH optima and temperature stability), suggesting different origins. Enzymes coming from autolysing cells were only released by digestion of the mother cell rather than produced for degrading the nutrients in the medium. Unfortunately, they did not determine the nature of the involved enzymes. In 1983, Dreyer and co-workers (Dreyer *et al*, 1983) using tritium labelled denaturated haemoglobin to measure the activity, demonstrated that proteolytic enzymes could escape from damaged cells.

The proteinases leaking out of the living cell is now well accepted. Even if this phenomenon is due to nutrient stress, the way by which the 42 Kda protein leaves the cell through the cell wall is not yet understood. However, Western blot analysis of beer samples revealed that the major form of Proteinase A released is a 54 Kda protein, and that this form is subsequently converted into an active form. This supports the idea that excretion of Proteinase A out of the cell might be a translocation error of vacuolar transport vesicles (Kondo *et al*, 1995). This missorting could be prompted by nutrition starvation. The proPrA might be autocatalytically converted into active Proteinase A by self-processing. This hypothesis of Kondo could explain the increasing of Proteinase A activity during the 37°C storage of beer. Studies of Wolff and co-workers suggest that the mature 42 Kda PrA and the 43 Kda pseudoPrA found in the growth medium under conditions of *PEP4* overexpression are result of proteolytic processing of proPrA occurring in the medium. Addition of PrB and PrA inhibitors altered the processing of the extracellular PrA species but had any effect on the intracellular processing of PrA (Wolff *et al*, 1996)

3. PROTEINASE A LEVEL DURING FERMENTATION

Maddox and Hough (1969) demonstrated that, when yeast was grown on a synthetic medium using protein as the nitrogen source, proteolytic activity was detected in the surrounding medium. This release of protease was stopped in the absence of protein but also at the presence of amino acids (including proline).

While Maddox and Hough (1969) found that the greatest excretion of proteolytic activity occurred when glucose and mannose amounts in the medium were equal, Hansen noted that glucose inhibited the proteinase release (Hansen *et al*, 1977).

Proteinase A evolution during beer fermentation

It is not possible to give a clear picture of the evolution of Proteinase A activity during fermentation, and the results obtained by several groups are contradictory. The main reason for this is the fact that the Proteinase A excretion seems to be strain- and medium-dependent.
Kondo and co-workers found that Proteinase A activity gradually increased during fermentation at both commercial and laboratory scale. This activity dramatically increased when nutrients in the wort were exhausted (especially amino-acids and nitrogen) and the temperature lowered. Kondo considered that Proteinase A may leak not only from autolysing cells but also from living cells because no connection could be found between the proteolytic activity and the number of dead cells. The following figure (Figure 3) illustrates the increasing Proteinase A activity during fermentation in a commercial tank (Kondo *et al*, 1995).

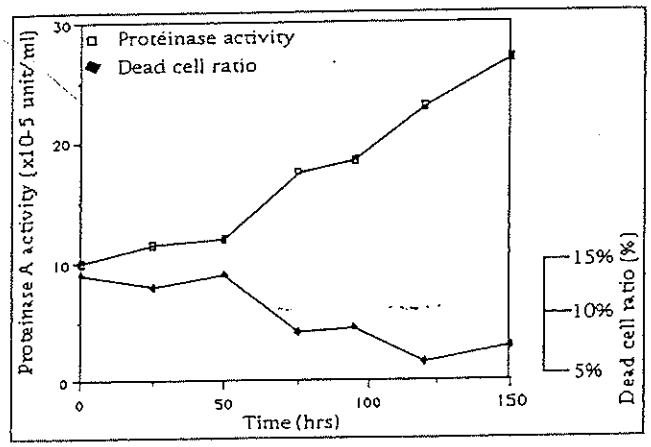

Figure 3: Proteinase A activity during fermentation (Kondo et al,1995)

The evolution of Proteinase A during the maturation seems to be clear for all searchers: there is an increase in the activity which is strain dependent and reliable to the cooling of beer in presence of yeast. Omrod and co-workers noted an important excretion of the acid protease during maturation steps (excretion did not seem significant, in this case during fermentation). A higher protease activity was observed in the rapidly cooled beer compared to that of the slowly cooled beer. But levels of Proteinase A activity were very similar in slow or rapid cooling fermentation if yeast is removed before cooling (Omrod *et al*, 1991). Muldbjerg also observed the increasing proteolytic activity during the maturation of a fermented beer (Muldbjerg *et al*, 1993).
Slaughter and Nomura reported the importance of inhibitor concentration for a correct determination of the proteolytic activity. The activity measured without an incubation time necessary for the complete inhibitor degradation is an underestimation of the total enzyme activity (Slaughter and Nomura, 1992). The authors suggest that the increase in specific activity measured for all three enzymes PrA, PrB and CPY as a function of the cell ageing, is an artefact which results more from a fall in the total protein content rather than a rise in the real activity of the enzymes. Proteinase A declines throughout the whole period of fermentation with no significant level of inhibitor. Proteinase B peaks before declining and Carboxypeptidase Y rises slowly during all the experiment. Figure 4 gives the evolution of PrA, PrB and CPY in the extracellular medium (free activity).

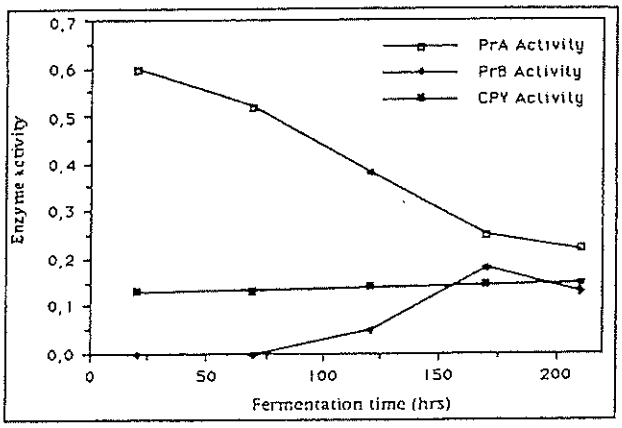

Figure 4: Proteinase activity during fermentation (Slaughter and Nomura, 1992)

Reicheneder and Narziss (1989) also noted an increase in Proteinase activity during fermentation after too many generations, dependent on temperature, time and media. The results obtained by Slaughter and Nomura (1992) have made evident the necessity of a perfect knowledge of the biochemistry of Proteinase A and its inhibitor.

Proteinase A level is strain dependent

If there is some controversy about the evolution of proteinase activity during fermentation, all researchers agree with the strain-dependency of the Proteinase A activity (Omrod *et al*, 1991; Muldjberg *et al*, 1993; Kondo *et al*, 1995). Figure 5 shows the activity as a function of the cell count. Obviously, each strain releases different amounts of the proteases. The discrepancies observed in figure 5 support the hypothesis that autolysis alone cannot explain the differences in the Proteinase A activities released from yeast cells.

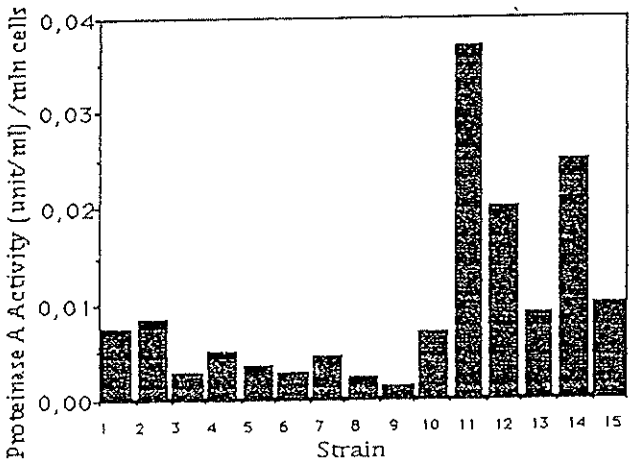

Figure 5a: Proteinase A activity from 15 different lager brewers yeast (per million cells) (Muldbjerg et al, 1993)

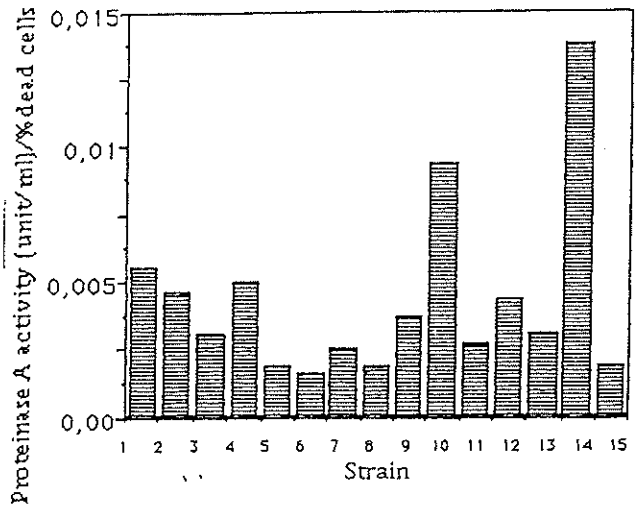

Figure 5b: Proteinase A activity from 15 different lager brewers yeast (per % dead cells) (Muldbjerg et al, 1993)

According to the results of Kondo and co-workers (Kondo *et al*, 1995), top fermentation yeasts tend to release more Proteinase A than bottom fermentation yeasts. A comparison between Proteinase A activity of top- and bottom-fermentation yeasts is graphically presented in figure 6.

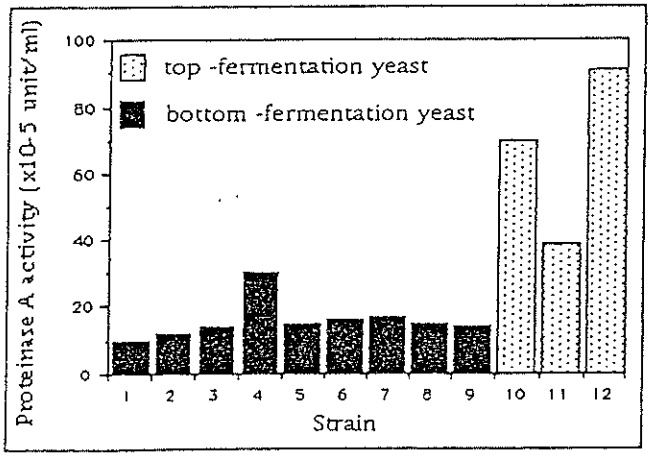

Figure 6: Proteinase A activity from top and bottom brewers yeast (Kondo et al, 1995)

It appears from these results that there is a difference in the cell wall permeability of the different yeast strains or in the amount of protease synthesised. Many reports on excretion of compounds into the medium are consistent with the finding that walls of growing cells of *Saccharomyces cerevisiae* are permeable to compounds of high molecular weight. Indeed *S.cerevisiae* has often been used as host for the production and secretion of proteins like cellobiohydrolase, haemaglutinine or α-glucosidase. Plasticity of the cell walls has been considered as a prerequisite for growth which is assured by insertion of new wall material. The growing part of the cell should have a more porous wall than the non-growing part.

In accordance with this, fast-growing cells have a higher wall porosity than slow-growing cells (De Nobel and Barnett, 1991).

4. EFFECT OF PROTEINASE A ACTIVITY ON FOAM STABILITY

Proteases excreted by the yeast during fermentation and/or maturation and storage are well known to influence substantially the foam. This phenomenon can be easily repeated by adding purified Proteinase A to a beer and monitoring the resulting foam stability (Dreyer *et al*, 1983, Steiner and Länzlinger, 1989).

For many constituents, the optimal concentrations and measurements to improve the foam stability have been described (Lebouille and Moonen, 1989; Maeda *et al*, 1991; Mohan *et al*, 1993). But concentration is not sufficient: the concentration of beer protein is not significantly changed by Proteinase A activity. Shimizu and co-workers have described quantitative and qualitative changes of beer components by proteolysis (Shimizu *et al*, 1995). Yokoi and co-workers also demonstrated the importance of hydrophobic proteins in foam stability (Yokoi *et al*, 1994).

The reason for a decrease in foam stability was supposed to be a decomposition of hydrophobic proteins. After a storage in the presence of Proteinase A, the total protein of a beer was fractionated and quantified. The results of protein analysis were correlated with the foam stability. Figure 7, based on the results of Shimizu, shows the evolution of proteins and of foam stability during storage. The amount of proteins in each fraction does not vary significantly while the foam stability decreases. On the other hand, the hydrophobocity properties of each fraction change during storage. This quality has been measured by the velocity of increase in surface viscosity (Shimizu *et al*, 1995).

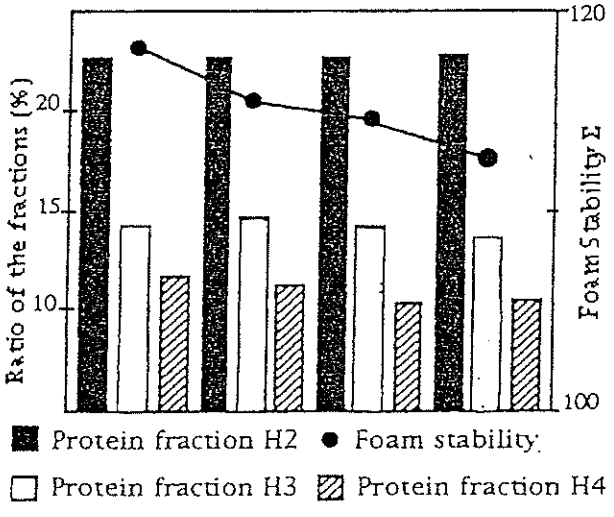

Figure 7a: Changes in ratio of the H2, H3 and H4 fractions in the collapsed foam after storage at 37°C (Shimizu et al, 1995)

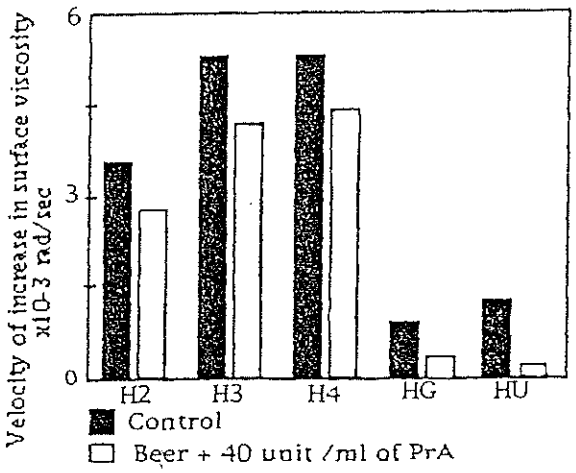

Figure 7b: Changes of velocity of increase in surface velocity of the H2, H3 and H4 fractions and fractions obtained by hydrophobic chromatography (HG and HU) (Shimizu et al, 1995)

The major consequence of Proteinase A activity seems to be a change in the quality of the hydrophobic proteins which act as a positive factor of foam.

5. PROTEINASE A ASSAY

Several methods have been developed to measure the activity of Proteinase A but their sensitivity are very slow. Since Proteinase contents in beer are very low, the lack of a suitable method makes this measurement in beer time consuming and of low accuracy.

Classical Anson assay and derived methods

The classical assay for Proteinase A has been proposed by Anson in 1939. Lanoë and Dunningan have proposed a modification of the Anson assay in 1977, still based on the original concept of Anson.
Anson measurements of the protease activity in acidic conditions are the following: an enzymatic preparation incubated with an excess of protein releases peptides of various sizes; the extent of proteolysis is evaluated by the amount of UV absorbing group soluble in trichloroacetic acid (TCA). The optimised method of Lanoë and Dunningan is given below:

* Activation of the zymogens for 20 minutes at 25°C;
* Incubation from 5 to 180 minutes at 37°C;
* Stop the reaction with TCA and precipitation of the undigested products at 30°C for 30 minutes increased to 48 hours according to Omrod (1991);
* Recovery of the digested products by centrifugation;
* Estimation of their concentration by measuring 280 nm absorbance;
* Conversion of A_{280} into proteolytic units.

The assessment of soluble peptides content of TCA supernatant has been modified several times due to the lack of sensibility of A_{280} measurement. Maddox and Hough (1970) used the Folin reagent for measuring peptide content of the supernatant. Dreyer used a method derived from Lanoë and Dunningan where absorbance measurement has been replaced by scintillation counting. The substrate he used was radioactively labelled ^{3}H-haemoglobin (Dreyer *et al*, 1983; Omrod *et al*, 1991). Another assay took advantage of the use of iode labelled proteins or synthetic substrates (Fukal *et al*, 1986).

Synthetic substrates and fluorometric assay

Yokosawa and co-workers first described a fluorometric assay using a synthetic substrate and a new protein: the dimethylcasein (Yokosawa *et al*, 1983).
The fluorogenic assay of Muldjberg was based on peptide substrates containing the anthraniloyl group (*Muldjberg et al*, 1993). In this assay, the anthraniloyl group fluorescence is quenched of a 3-nitrotyrosine residue located at or near the carboxyl terminus of the peptide as shown in figure 8. When the substrate is cleaved, the fluorescence, not longer quenched, increases to 30 fold and can be monitored.

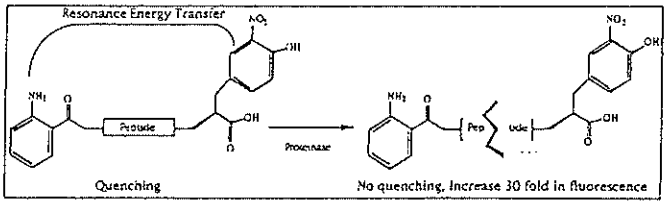

Figure 8: Principle of fluorogenic assay using anthraniloyl group (Muldbjerg et al, 1993)

Kondo and co-workers (1995) used another fluorogenic group: 4-methyl-coumaryl-7-amide (MCA) and optimised the polypeptide in order to gain in specificity for Proteinase A. A large number of peptides were designed to screen the most sensitive substrate and "Ala-Pro-Ala-Lys-Phe-(NO_2)Phe-Arg-Leu" was shown to have the highest kinetic parameters (Kondo *et al*, 1995). According to Kondo, this peptide coupled to MCA showed unique specificity for Proteinase A and the measurement can take only a few hours.
As we can see, evolution has been made in the measurement of Proteinase A activity: from the cleavage of a labelled protein (not easy to use and time consuming), to fluorogenic substrates with high specificity for the proteolytic enzyme studied.

6. CONCLUSION

By way of conclusion, we will try to summarise the available data on Proteinase A secretion by yeast and to give some practical clues in the hope of diminishing its disturbing activity. As we will see, the effects are better known than the fundamental processes.

What we know...

It is of clear evidence that Proteinase A acts as a negative factor on foam stability, probably by hydrophobic proteins of beer. Proteinase A may escape from autolysing cell but also may leak from living yeast cell. The enzyme

concentration clearly increases during maturation and storage, and even to a higher extent in the case of a rapid cooling at the presence of yeast. Medium factors seem to be of prime importance: extracellular protein containing material and the lack of aminoacids and nutrients may enhance the activity.

What we don't know...

The major discrepancy concerns the evolution of the proteolytic activity during fermentation. If recent results have showed an increasing activity, it seems that it is not a general rule. This evolution in fermentation is probably strain dependent.
Even if we know that proteolytic activities are strain dependent not only for the activity itself but also for the leaking out of yeast cell, a lack of explanations hampers the understanding of that phenomenon. For example, the leaking from living yeast cell and the permeability of the cell wall are key mechanisms that are not well understood. The role of Proteinase A inhibitors is unclear. Furthermore proteinase inhibitors have been found recently (Campbell *et al*, 1995).

What we may do to improve foam stability ...

Two ways may be explored to reduce the effect of Proteinase A. The first one is to limit the production and/or the excretion of the enzyme. This seems very difficult to achieve in view of our knowledge of the biochemistry and physiology of yeast proteases and their excretion. Several authors pointed out some critical factors which may control the enzyme excretion: number of generations, temperature, cooling with or without yeast, time of maturation, infections... Factors like the yeast strain or the nutrient content of the final beer are more difficult to deal with.
The second way consists of reducing the enzyme activity when secreted or freed by the yeast cell. This may be achieved by using inhibitors or by denaturing Proteinase A. At this time, no commercial Proteinase A inhibitor is available for the brewer. But pasteurisation was shown to denature enzyme: pasteurised beers contain no Proteinase A activity whereas than filtered beers do (Reicheneder and Narziss, 1989). Metal ions like zinc may also play a role in the enzyme inactivation.

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