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The role of fimbriae in the flocculation of brewer's yeast

Graham, Lynne Theresè

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6.3.2 WALL CHARGE

The results presented show a distinct difference between the negative charge of log and stationary phase cells of the flocculent culture. They are interpreted as indicating a reduction in the intensity of the surface charge as cells proceed from the log to the stationary phase. The few log phase cells which showed labelling similar to that of the stationary phase cells were presumably either the original inoculum (comprising stationary phase cells) from which the log culture grew or cells which were just entering the stationary phase.

Surface charge has long been considered to be an important factor in the dispersion and subsequent agglutination of brewer's yeast and the results for this flocculent culture are in agreement with those previously documented (Geilenkotten and Nyns, 1971). As the fimbriae of the flocculated sample were not extensively labelled with the cationized ferritin they may, when exposed for flocculation, contribute to the overall reduction in surface charge. Alternatively , they may to some extent mask the negative charge and, not being strongly charged themselves, may bridge the gap between cells allowing for initial cell to cell contact.

If it were assumed that a low surface charge is specifically related to the aquisition of flocculence then the trend observed for the non-flocculent control culture would refute this. It did not display the same distinct charge differences between log and stationary phases that were observed for the flocculent culture although a slight decline in charge seemed to have occurred. While a greater negative charge may have been expected, labelling indicated a generally slightly lower overall charge than the flocculent culture. As the technique was not quantitative this result could not be verified, but it was consistent for all three experiments performed. A similar trend was noted by Harteng (1953) where the charge of a powdery yeast did decline as the fermentation proceeded, but more slowly than that of the flocculent yeast. Apparantly inconsistent results for changes in charge density due to phosphodiester groups of flocculent and non-flocculent cells were noted by Beavan et al. (1979).

Nonetheless, it is not impossible that changes in surface charge may be more pertinent to cultures which have the intrinsic ability to flocculate than to cultures which lack the biochemical machinery necessary to promote flocculation. Alternatively, the relatively low negative charge of the non-flocculent culture may have been related to its ability to acquire the potential to flocculate (see Chapter 2).

CHAPTER 7

DISCUSSION AND CONCLUSIONS

The aim of this study was to determine whether or not fimbriae play a role in the flocculation of brewer's yeast. Further, if they were found to be involved, the natures of this involvement and of the fimbriae themselves were to be investigated.

The study has produced evidence in support of a role for fimbriae, but due to the lack of success in isolating undamaged fimbriae, this evidence is correlative and therefore equivocal. It may be debatable whether or not these results are proof enough to ascribe a definite role in flocculation to fimbriae.

Fimbriae appear to be fairly widely distributed amongst yeasts, but their roles in the life cycles of these yeasts have not been extensively investigated. The one common observation was that the presence of fimbriae was associated with the increased agglutinability of the fimbriate cells in comparison with non-fimbriate cells.

The fimbriae of the smut fungus Ustilago violaceae are

thought to be involved in sexual 'agglutination' (Day and Poone 1975). Despite the differences between the fimbriae of this fungus and brewer's yeast a common antigen does exist since antiserum raised against pure smut fimbriae was also able to agglutinate fimbriate Saccharomyces cerevisiae.

Wild yeasts attached to the inside surfaces of beer dispense pipes (Casson,1984) appeared to be attached by means of fimbriae. These fimbriae were also associated with cell to cell binding.

A mutant of Schizosaccharomyces pombe which lacked the inactive form of the enzyme acid phosphatase was found to be more 'clumpy' than the wild type (Schweingruber et al.1984). As well as this, the surface hairs, or fimbriae, were longer than those of the wild type strain. The authors suggested that the inactive enzyme was involved in a structural capacity in morphogenesis, regulation of growth rate and cell agglutination. It was thought that a cell could alter its 'hair' structure depending upon the growth medium and could then control the nature of its contact with other cells. This study intimately linked fimbriae, enzymes and cell agglutination and gives credence to a potential role for fimbriae in flocculation of brewer's yeast.

S.cerevisiae cells which had been dehydrated and then rehydrated were found to have become fimbriate (Rapoport et al.1983). These fimbriae were considered to have arisen as a result of the disruption of the structural organization of the surface mannoprotein. Due to the cleavage of some intermolecular linkage the glycoproteins may have acquired free ends which resembled fimbriae. The absence of fimbriae from intact cells suggests that, as appears to be the case with Saccharomyces uvarum 2036 they were an integral part of the cell wall network and probably not true fimbriae. Rapoport et al. 1983, noted that the fimbriate cells formed aggregates which did not separate easily, implying that the fimbriae may have been responsible for the aggregation.

These studies on fimbriate yeasts all indicate that fimbriae are involved in intercellular agglutination. Since, however, the results from the current study are not positive proof for the role of fimbriae in flocculation, the question must be asked: If fimbriae do not function in flocculation, what alternative purpose could they serve? Firstly, they may be a result rather than a cause of flocculation. If this is the case then the production of fimbriae may be one of the surface rearrangments which occurs as a result of, or during, the binding of the walls of adjacent cells. Secondly, they could be related to another stationary phase function such as the secretion or

uptake of protein (Day et al.1975). Even if their primary function is not cell attachment it is still feasible that they have a secondary role in flocculation. If one considers flocculation to be only a minor stationary phase function (albeit one for which brewers have selected) then it is very likely that even if fimbriae are able to effect or participate in flocculation, they probably do have another function which is more pertinent to the survival of the cell.

When asking why the fimbriae are present, it should perhaps also be asked why some yeasts flocculate. One possible explanation may be that it is a 'remnant' of the yeast mating system which has survived as a protection mechanism against adverse environmental conditions. Studies on mating cells of Hansenula wingei have shown that cell to cell recognition and agglutination is mediated by mannoproteins which act as agglutinin and receptor (Crandall and Brock, 1968). H.wingei cells are also fimbriate and it has been suggested that these fimbriae may be the sex-specific agglutination factors (Day et al.1975). Another possible link between the three features, flocculation, fimbriae and sexual agglutination is provided by S.pombe. This is a fimbriate yeast (Schweingruber et al. 1982) which flocculates prior to sexual agglutination (Calleja and Johnson, 1971). Although these facts arose from two separate studies it is likely that

further study may reveal an association between fimbriae and mating. Both sexual agglutination and the acquisition of flocculation appear to involve changes in the cell wall and in both cases it appears to be the mannoprotein fraction which is involved. The mannan structure and the gross cell wall organization of S.cerevisiae a cells which had been exposed to α-factor were found to be altered in comparison with control cells (Lipke et al.1976). The outer cell wall also became diffuse and, from electron micrographs presented, was not unlike the fimbriae of the wild yeasts isolated by Casson (1984). Haploid mating types of S.cerevisiae did produce sparse fimbriae, but the association with mating was not investigated (Day et al. 1975).

Because flocculation involves direct wall to wall contact of adjacent cells the presence of a dense mat of fimbriae radiating out from the cells cannot be ignored. If they are present before intercellular binding occurs and if they are not involved in this binding they could form a barrier to direct cell to cell contact. Therefore, they must either cause something to happen, or something must happen to them. It is certainly less difficult to visualise fimbriae playing an active role in flocculation than to speculate what would happen to them if they do not participate. In proposing an active role for them it is not presumed that they necessarily cause flocculation, but

merely that they do participate in an active capacity.

Some of the findings from this study do not necessarily support the involvement of fimbriae in flocculation and these will be discussed first.

That fimbriae are present on flocculated cells is irrefutable. They are not however, present on all flocculated cells and have been found on some actively growing (log phase) cells. As it was found that the number of fimbriate cells present in log phase populations could have been contributed by the original stationary phase inoculum of flocculated cells (Chapter 2) it is concluded that fimbriae are structures which appear to be peculiar to the stationary phase of growth. Since flocculation is also a stationary phase phenomenon there is a potential link between the two.

The presence of fimbriae on some cells of the non-flocculent strain, S.cerevisiae Y746 indicates that they are not flocculation-specific. On the other hand, this culture did become flocculent, which means that it does have the physical potential to flocculate. Its cell wall organization may therefore be suitable to allow for intercellular binding although under normal conditions, binding may not occur. The extensive variability and instability of flocculence, as well as the phenomenon of

co-flocculation indicate that the dividing line between flocculence and non-flocculence is very indistinct. S.cerevisiae Y746 probably falls into the grey area between flocculence and non-flocculence.

In the non-flocculent state this culture had a similar proportion of densely fimbriate cells in both the log and stationary phases (about 25-30%). As the proportion of flocculent cells did not increase when the culture entered the stationary phase the correlation between the presence of fimbriae and the occurence of flocculation, which was observed for the flocculent culture, still holds; although the specificity of fimbriae for flocculation is doubtful. In other words, there is a possibility that fimbriae are a feature which is common to many yeasts, but which may have the capacity to participate in flocculation.

The inability of S.cerevisiae Y746 and of log phase S.uvarum 2036 with 'ether-induced' fimbriae to flocculate in the Helm medium could also be regarded as evidence against fimbrial involvement in flocculation. If, however, the flocculating ability of log phase cells of S.uvarum 2036 is considered there is no reason why fimbriate log phase cells should flocculate, even if fimbriae are involved in flocculation. For example, the inability of log phase S.uvarum 2036 to flocculate in the Helm medium gives no indication as to the potential flocculence of the

strain. It thus follows that even if fimbriae are involved, their appearance on cells which are physiologically not ready to flocculate should not necessarily induce floc formation. It does nonetheless confirm that their presence is alone insufficient to promote flocculation and that other factors must be involved.

Perhaps the strongest evidence in support of fimbriae being flocculation-related is provided by the non-flocculent culture, since the proportion of fimbriate stationary phase cells was about 30% when the culture was non-flocculent, but increased to about 75% when it became flocculent. A further correlation was evident when non-brewery yeast strains were examined. Those which did not agglutinate had only a few densely fimbriate cells and those which flocculated strongly had numerous fimbriate cells.

Removal of fimbriae was achieved by prolonged ultrasonication and by treatment of the cells with the enzyme, pronase. In each case the cells lost the ability to flocculate in both the Helm medium and in fermented wort. Further support for this was provided by the ability of pronase treated cells to multiply when repitched into fresh wort. While the pronase treatment removed the fimbriae and destroyed the ability to flocculate it did not damage the cells so severely that they could no longer bud.

The structures seen with the zymolyase treated cells may have been part of the fimbriae although the inability to reisolate them casts some doubt on this suggestion.

The striking feature of the ultrasonication experiment was the simultaneous loss of fimbriae from most cells and of the inability of these cells to regain their flocculation potential. After each period of treatment where densely fimbriate cells had still predominated over non-fibriate cells, the samples had been able to flocculate when Helm medium was added.

Both of the methods which removed the fimbriae doubtless also damaged the cell walls (even though this was not apparant from electron microscopy) and other wall components involved in flocculation may have been destroyed. It is acknowledged that the evidence is only correlative and as such is inadequate to prove conclusively the involvement of fimbriae in flocculation. Despite this, the fact that in both cases fimbriae and flocculation potential were lost at the same time, strongly suggests that the two events may be connected.

That the fimbriae are proteinaceous is in agreement with the results of Poone, et al. (1978) for S.cerevisiae fimbriae, and is significant in the light of other research on flocculation which has proved beyond doubt, the

importance of protein in flocculation. Apart from the requirement for continued cytoplasmic protein synthesis (Baker and Kirsop, 1972) proteins are also required in a structural capacity (Nishihara et al. 1977; Day et al.1975). While it could not be confirmed that the fimbriae (or parts of them) were the structural units necessary for flocculation, the fact that they are protein and that they are exposed when flocculation occurs, means that it is quite feasible that they are agents of flocculation.

The 48,000 dalton protein present in wall extracts of flocculated cells, but absent from log phase extracts appeared to be flocculation specific since it was also absent from stationary phase extracts of the non-flocculent strain. While it is possible that this is a fimbrial protein it would be necessary to separate fimbriae from the other wall components in order to prove that it is. It could not be a major fimbrial protein subunit because fimbriae are present (although not necessarily exposed) on both S.cerevisiae Y746 and log phase S.uvarum 2036 cells, and the major structural components would therefore be common to all extracts.

The flocculation specific 48,000 dalton protein was probably responsible, at least in part, for the different antigenicities of log and stationary phase flocculent cells

which were observed with fluorescent antibodies. It is possible that the additional antigen is associated with another stationary phase function, but as flocculation is a cell wall phenomenon and only occurs in the stationary phase it is very likely that the antigen is associated with flocculation.

As cells are negatively charged they would repel one another and in order for flocculation to occur this repulsion must be overcome. Fimbriae could provide a means to accomplish this. Firstly, they do not appear, from labelling with cationized ferritin, to be negatively charged. They may even be responsible for the overall decrease in cell charge from log to stationary phase since their appearance coincided with this decrease in charge. Secondly, they radiate outwards from the cell by about 0,5um which would increase the effective cell diameter by 1um and would therefore increase the chance of contact with other cells. They could thus be responsible for the initial contact between cells, and firmer binding would follow after this.

The results from this study, although not direct proof, do point more towards the participation of fimbriae in flocculation than towards their not participating. In view of this the manner in which they could function should be considered.

Two mechanisms via which flocculation could occur have been proposed, namely calcium bridge formation and a specific biological recognition system, such as lectin type of reaction. Calcium bridge formation is the generally accepted mechanism, but the possibility of a specific biological recognition system is gaining increasing support. It is possible that fimbriae could function in either system. As conditions in the fermenting wort change and the cells enter the stationary phase, so a series of genetically controlled events are probably triggered and these would culminate in flocculation. If fimbriae are involved in flocculation these events would include those which would expose and modify them so that they could function in recognition and binding between cells.

That only about 60% of cells become fimbriate means either that they are only exposed on some cells, or alternatively (and which is more likely) that they are not exposed on all cells at the same time. It is uncertain in what form the fimbriae exist in the wall prior to being exposed. As the width of the wall is probably only about 0,1um this excludes the possibility of their radiating out from the inner wall components into a matrix which could subsequently be removed leaving the fimbriae extending into the fermenting medium. They therefore probably lie adpressed to the wall, forming a network which may or may

not be embedded in a mannan matrix.

The removal of mannan may be part of the exposure process and may only occur once the sugars in the wort have been utilised. The addition of any glucose containing sugar to the fermentation delays the onset of flocculation, whereas sugars such as arabinose and xylose do not (Axcell, pers. comm.) These sugars may competitively inhibit the dissolution of the mannan linkage which may be necessary for fimbrial exposure. Alternatively, the addition of these sugars may prevent the secretion of the enzymes necessary to expose the fimbriae by a negative feedback mechanism. One such enzyme may be α-mannosidase, the concentration of which has been found to increase during the transition from active growth to flocculation (Beavan, et al. 1979). The induction of flocculation in mid log phase cells (Chapter 3) by this enzyme also suggests that it has a role in flocculence.

Fimbriae which are artificially produced on cells fail to promote flocculation. Therefore if naturally produced fimbriae promote flocculation, either the 'artificial' fimbriae do not possess the functional groups necessary for intercellular binding, or these groups are masked. Under natural conditions therefore, they would need to be modified to enable them to recognise and bind to active sites on adjacent cells. This could involve the synthesis

and insertion of a glycoprotein (or protein), or the loss of a fimbrial component, or both. A precedent for modification of the fimbriae i.e. that the fimbriae of log and stationary cells are different, is provided by S.pombe since the hair structure of 'clumpy' and wild type cells was different (Schweingruber et.al.1982).

The next step in the process would be the actual binding of adjacent cells. That non-fimbriate cells are found in flocs means that either they are passively caught up in flocs as they form, or that they also participate in floc formation. In other words, fimbriate cells would either bind to other fimbriate cells or to smooth cells. In the event of calcium bridge formation 'end on' binding between fimbriae of adjacent cells could occur with calcium bridges forming between terminal carboxyl groups (assuming that the terminal groups are carboxyl). In the event of a lectin-type system operating, binding would occur between protein and carbohydrate groups of adjacent cells and in this case it is more likely that fimbriate cells would bind to non-fimbriate cells. Evidence is further weighted in favour of binding between fimbriate and smooth cells for two reasons. Firstly, the proportion of non-fimbriate cells in flocs (35-40%) seems to be too great to have arisen purely by chance, and secondly, there appears to be a precedent from co-flocculent strains. One strain of the co-flocculent pair examined (Day, et.al.1975) was densely fimbriate.

Because co-flocculation occurred when the ratio of either strain to the other was a maximum of 90:10 (Stewart and Garrison, 1972), it is probable (if fimbriae did play a role) that fimbriate cells bound to smooth cells and not to other fimbriate cells. The critical minimum density for each cell type would have been 10%. In other words, provided that there is a critical minimum proportion of both cell types present, the precise numbers are irrelevant. This is represented diagramatically in figure 51.

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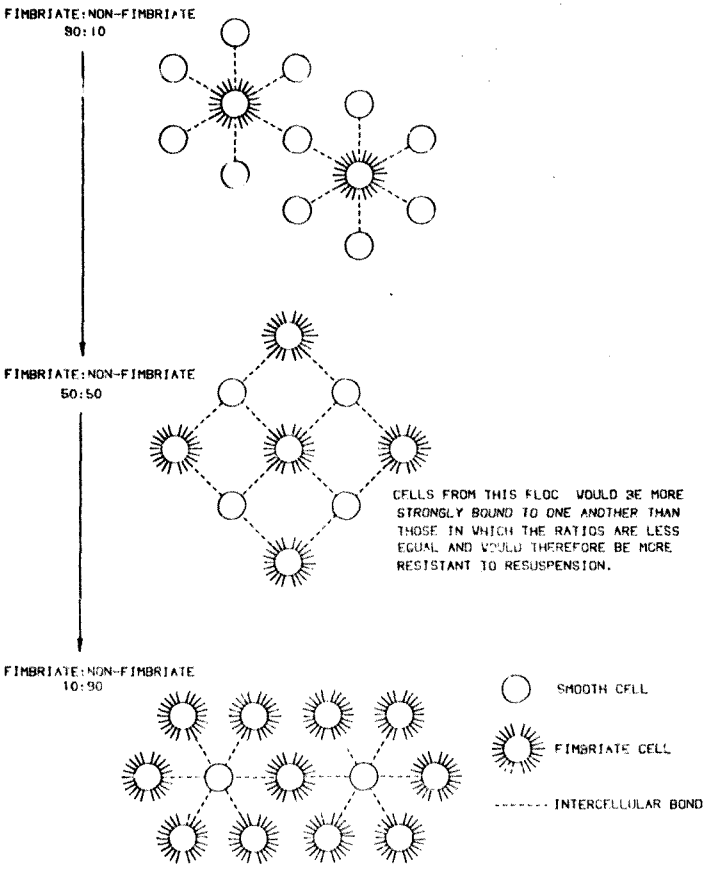


FIG. 51. DIAGRAMATIC REPRESENTATION OF A PROPOSED MECHANISM FOR FLOCCULATION IN WHICH BOTH FIMBRIATE AND SMOOTH CELLS ARE INVOLVED AND WHICH COULD ACCOUNT FOR VARIATIONS IN FLOCCULATION INTENSITY

Evidence exists for the involvement of both protein and mannan cell wall components as separate entities. If calcium bridge formation is the means by which cells attach to one another the mannan component of the wall would probably not be necessary except as part of the glycoprot[illegible]ose carboxyl groups were involved in bridge formati[illegible]annan is required specifically for flocculati[illegible] shown by the competition of non-flocculen cells for binding sites on flocculent cells whose proteins had been destroyed (Miki et al.1981).

If fimbriae are involved in a fashion similar to this it would be necessary, once initial cell to cell contact had been established, for the walls of the cells to be brought into direct contact. The fimbriae may therefore contract or intertwine in order to achieve this.

The ability of fimbriae to promote flocculation could possibly be determined by adding purified fimbriae under suitable conditions, back to the cells from which they had been removed. Their composition and charge could be accurately determined as well as any difference between fimbriae of log and stationary phase cells of a flocculent strain, and fimbriae of non-flocculent strains. It could also then be established whether or not the apparantly flocculation-associated 48000 dalton protein is a fimbrial

protein. The use of a number of flocculent strains would be necessary to determine whether it is a strain specific protein or one which is commonly associated with stationary phase flocculent cells. The use of genetically defined flocculent and non-flocculent strains would make direct comparisons more reliable and more meaningful.

A strong case therefore exists to support a role for fimbriae in flocculation. They are found predominantly on flocculated (as opposed to actively growing and non-flocculent) cells; their removal (with pronase) coincides with the loss of flocculence, but not with the inability to multiply; they appear to be comprised of mannan and protein, and do not have a strong negative charge. As it was not however possible to isolate intact fimbriae, unequivocal proof for their participation in flocculation was not obtained.

MEDIA AND SOLUTIONS APPENDIX A

Wickerham Stock Culture Agar

0,3 % .....malt extract

0,3 % .....yeast extract

0,5 %.....peptone

1% .....glucose

2% .....agar

tap water

The medium was sterilized by autoclaving for 15 minutes. The pH of the agar was adjusted to pH 3,5 with 20% tartaric acid prior to pouring.

Synthetic medium

Composition was the same as the Wickerham Stock Culture Agar with the agar omitted.

Helm Sedimentation Test

Solution A (washing solution)

$CaSO_4 2H_2O$ .....0,645 g/l in distilled water.

Solution B

$CaSO_4 2H_2O$ ..... 0,86 g /l in distilled water.

Solution C

Sodium acetate $3H_2O$.....34g/l in distilled water.

Solution D **APPENDIX A**

Solution B .....375ml.

Solution C ..... 50ml.

Distilled water.....500ml.

Method:

The solutions were mixed and adjusted to pH 4,5 with 1N HCL.

Reagents for Protein Determination

Reagent C:

Reagent A.....50ml

Reagent B..... 1ml

Reagents A and B were mixed just before use.

Reagent A

Na_2CO_3 .....20g

Sodium potassium tartarate 0,2g

The chemicals were disolved in distilled water and made up to 1 l.

Reagent B:

$CuSO_4$ $5H_2O$.....50g

The copper sulphate was disolved in distilled water and made up to 1000ml.

Formvar Films **APPENDIX A**

0,35g formvar

100ml chloroform

Formvar was disolved in chloroform and used immediately.

Phosphotungstic acid (PTA)

0,2-0,3g of PTA was dissolved in 9ml of glass distilled water. The pH was adjusted to pH7,0 and the solution made upto 10ml with glass distilled water.

Ferritin Con A Labelling

Sodium phosphate buffer pH 6,8

Solution A: 0,2 M monobasic sodium phosphate (27,6g/l)

Solution B: 0,2 M dibasic sodium phosphate heptahydrate (53,65 g/l)

44 ml of solution A were added to 56 ml of solution B, the pH checked and the buffer made up to 200 ml with distilled water.

APPENDIX A

Glucose-6-Phosphate Dehydrogenase Test

Solution 1

NADP 12.2mg

ATP 28.8mg

NADP and ATP dissolved in 20ml of 0,1m triethanolamine/ chloride buffer pH 7,6.

Buffer:

Solution a: Triethanolamine hydrochloride.....0.372g in 20ml distilled water.

Solution b: $MgCl_2 6H_2O$.....0,2g in 10ml distilled water.

For 20ml of buffer 1,5ml of solution b. was added to 18,5ml of solution a.

TTC overlay technique

TTC agar

0,1g TTC

1,0g glucose

3,0g agar

333ml distilled water

The chemicals were combined and heated to boiling point but were not autoclaved.

The agar was attemperated to 45-50 degrees centigrade and poured slowly over the cultured wort agar plates. After 30 minutes the plates were examined for the presence of petite colonies. These colonies remained white while the respiratory-sufficient colonies turned pink.

ANTIBODY LABELLING APPENDIX B

(i) Conjugation of antiserum with HRP

Buffers:

0,1 M Sodium bicarbonate buffer pH 9,5

Solution A 0,1M $NaHCO_3$

Solution B 0,1M Na_2CO_3

Solution B was added to solution A to reach pH 9,5 (approximately three parts of solution A to two parts of solution B)

1M Sodium bicarbonate buffer pH9,5

Solution A.....1M $NaHCO_3$

Solution B .....1M Na_2CO_3

The carbonate was titrated against the bicarbonate solution until the pH reached 9,5.

(ii) Immunoelectrophoresis

Preparation of agar coated slides:

A glass microscope slide was thorougly cleaned and wiped with alcohol. The slide was precoated by dipping it in sloppy agar (0,2 %) at about 55 degrees centigrade and allowed to dry. The slide was then placed on a levelled surface and carefully coated with 3 ml of 3 Noble agar (Difco.Lab.,Ltd. Surrey, England) in barbitore buffer. A central well was punched in the agar, once this had set. The well was filled with 10-12 ul of conjugate. The slide was then placed in an electrophoresis tank, the buffer

chambers filled with barbitone buffer pH 8,2 and ionic strength 0,08 and the ends of the slides connected with filter paper wicks (Watman No 1) to the buffer chambers. A current of 8 milliamps was applied to the slide.

(iii) Fluorescent Antibody Labelling

Borate buffer pH 9,3

Solution A:

Boric acid .....1,55 g

KCl .....1,865 g

Chemicals were disolved and made up to 250 ml.

Solution B:

6 M NaOH

Solution A was adjusted to pH 9,3 with solution B and made up to 400 ml with distilled water.

APPENDIX C

POLYACRYLAMIDE GEL ELECTROPHORESIS

12% Gels:

The following solutions were mixed together and degased for five to ten minutes:

Acrylamide stock.....48,4 ml.

1M Tris HCI pH 8,8.....45,0 ml.

Glass distilled water..19,6 ml.

10 % SDS..... 1,2 ml.

To the degassed solution;

1,5% ammonium persulfate (6ml) and Temed (30 ul) were added rapidly and thoroughly mixed. This was poured into prepared casettes and left to polymerize for about 90 minutes.

Acrylamide stock

15g acrylamide

0,4g bis acrylamide

glass distilled water to make 50ml of solution.

1M Tris HCI buffer pH 8,8

6,06 g Tris

0,76ml of 11,6N HCl

Glass distilled water was added to make 50ml of buffer.

APPENDIX C

Bath buffer (10 times concentrate)

30,3g Tris 144,1g glycine

10,0g SDS

distilled water to make 1L of buffer.

Staining solution

0,25% coomassie brilliant blue (CBB)

45% methanol

77% glacial acetic acid

47,75% distilled water

Chemicals were mixed together and filtered.

Destaining solution

135ml methanol.

21ml glacial acetic acid

Chemicals were made up to 3 l with distilled water.

Splitting solution

2% SDS

5% mercaptoethanol

8M urea.

For a sample with a low protein concentration splitting solution was added at a ratio of 1:1 and for a sample with a high concentration at 5:1. Samples were covered and placed in a boiling water bath in an extraction hood for five

minutes.

Low molecular weight calibration markers.

(Pharmacia Fine Chemicals, New Jersey)

Protein	sub-unit molecular weight	Source
Phosphorylase b	94000	rabbit muscle
Albumin	67000	bovine serum
Ovalbumin	43000	egg white
Carbonic anhydrase	30000	bovine erythrocyte
Trypsin inhibitor	20000	soybean
l lactalbumin	14400	bovine milk

APPENDIX D : ENZYME BUFFERS APPENDIX D

α-amylase

0,2M Sodium phosphate buffer

0,006M NaCl

pH was adjusted to 6,9.

Cellulase, β-glucuronidase, zymolyase

0,05M Tris-HCl (pH 7,4) 0,5ml

1,2M KCl 3ml

0,02M $MgSO_4 7H_2O$ 3ml

Acid phosphatase, α-mannosidase

citrate buffer

1,05g citric acid

1,47g sodium citrate

The solution was made up to 100 ml with distilled water.

For acid phosphatase the buffer was adjusted to pH5,6.

For α-mannosidase the buffer was adjusted to pH4,5.

Pronase

0,1 M Tris-HCl

1,21g Tris was made up to 100 ml with distilled water.

The buffer was adjusted to pH6,8 with concentrated HCl.

APPENDIX D

Leucine aminopeptidase

The enzyme was activated at 37 degrees centigrade for two hours in 0,1ml of 0,025M Manganese chloride
0,1ml 0,5 M Tris HCl buffer, pH8,5.

Reaction buffer:
1ml 0,125M Magnesium chloride
1ml 0,5M Tris-HCl buffer, pH8,5
2ml glass distilled water

Lipase

2ml 3,0M NaCl
1ml 0,075M $CaCl_2$
2ml 0,5% albumin (BSA fraction V)
The buffer was adjusted to pH8,0.

APPENDIX E

NOTES ON ENZYMES USED

Acid phosphatase from potatoes EC 3.1.3.2 (Boehringer Mannheim)

This enzyme (which has an acid optimum) has a broad esterase activity and catalyses the reaction.

Orthophosphate monoester + H O enzyme ⟶ alcohol + phosphate

It hydrolyses phosphomonoesters and phosphoproteins, but does not hydrolyse phosphodiester bonds. (Boehringer Mannheim Biochemicals catalogue)

α-amylase *from porcine pancreas.* EC 3.2.1.1 (Boehringer Mannheim)

This enzyme is a 1,4-α-D-glucan glucanohydrolase with a pH optimum of 7 and molecular weight of 50000 daltons. It acts randomly on starch glycogen, polysaccharides and digosaccharides, and releases reducing groups in the α-configuration. It catalyses endohydrolysis of 1,4-α-D-glucosidic links in polysaccharides contain three or more 1,4-α-linked D-glucose units ids a mixture of maltose and glucose. (Boehringer Mannheim Biochemicals catalogue; Enzyme Nomenclature 1978)

APPENDIX E

Cellulase from Trichoderma viride. EC 3.2.1.4
(BDH Chemicals, England)
This enzyme is a 1,4-β-D-glucan glucanohydrolase which catalyses the endohydrolysis of 1,4 β-D-glucoside linkages of cellulose and cereals of an optimum pH of 4,2-5,2. Cellulase is a complex of enzymes which act in concert to hydrolyse cellulose. (Boehringer Mannheim GmbH Biochemicals catalogue 84/85; Enzyme Nomenclature, 1978).

β-D-glucuronidase from Helix pomatia Type H-2. EC 3.2.1.31
(Sigma Chemical Company, St.Louis, U.S.A.)
This enzyme is widely distributed in mammalian tissues and is thought to have a role in the catabolism of mucopolysaccharides. It is a glycosidase which can catalyse the hydrolysis of β-glucuronides (Levy and Marsh, 1960). Reaction: β-D glucuronidase catalyses the reaction between β-D-glucuronide and water to produce an alcohol plus D-glucuronate (Enzyme Nomenclature, 1978).

Lipase from Rhizopus arrhizus (Trihlycerol acylhydrolase) EC 3.1.1.3 (Boehringer Mannheim)
Lipase catalyses the reaction:
Triglyceride + H O → diglyceride + fatty acid at pH 3,5-pH 7,0. It also attacks phospholipids, digalactosyl diglycerides, sulfolipids and acyl groups attached to C-6 of hexoses. Only substrates not in true solution i.e. only

emulsified substrates are hydrolysed.

Leucine aminopeptidase from hog kidney
(α-aminoacyl-peptide hydrolase (cytosol) EC 3.4.11.1
(Boehring ⁄ Mannheim)
LAP is an exopeptidase with a molecular weight of 255ooo daltons and a pH optimum of 9,1. It releases amino acids from the N-terminal end of proteins and polypeptides and is most active on leucine residues.

α-mannosidase from Jack bean (Canavalin ensiformis)
α-D-mannosi[illegible]annohydrolase EC 3.2.1.24 (Boehringer Mannheim).
This enzyme hydrolyses terminal, non-reducing α-D-mannose in α-D-mannosides.
α-D-mannoside + H O $\xrightarrow{\text{α-mannasidase}}$ [illegible]cohol + D-mannose
(Enzyme Nomenclature, 1978; Boehri[illegible]r Mannheim Biochemicals catalogue.)

Pronase from Streptomyces griseu[illegible] 3.4.24.4 (Boehringer Mannheim)
Pronase is a neutral metalloprot[illegible]se which preferentially cleaves bonds adjacent to a hydrophobic amino acid residue (Enzyme Nomenclature, 1978). It has a wide range of side chain specificity being able to hydrolyse many dipeptides,

tripeptides, acyl amino acids, acyl peptides, amides and esters (Hagihara, B., 1960).

Zymolyase from Arthrobacter luteus

(Kirin Laboratories, Yokohama)

Zymolyase has a pH optimum of 6,5 and a temperature optimum of 45 degrees centigrade. It hydrolyses heat treated pachyman (whose predominant glucosidic bonds are β-1,3 and β-1,6). The main product liberated is laminaripentaose. It does not hydrolyse phosphomannan or cellulose, requires long sequences of β-1,3 linked glucose residues and can catalyse either end of exohydrolysis (Kitamura and Yamamoto, 1972).

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