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Effects of high pressure on amylases and starch in wheat and barley flours

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Application of high pressures (to 800 MPa) to 25% w/w slurries of barley and wheat flours indicated that pressures above 300 MPa caused an increase in the action of the α- and β-amylases due to starch gelatinisation, the apparent activity increasing with pressure to a maximum at 500–600 MPa. At higher pressures marked losses in action were observed. Pressure treatment caused inactivation of the isolated α- and β-amylases from malt barley and electrophoretic analysis suggested that disulphide bond-induced aggregation accompanied this loss in activity at pressures of 600 MPa and above. However, some loss of activity was seen at lower pressures, suggesting that aggregation was not the initial cause of inactivation. β-amylase from malt barley appears to be slightly more sensitive to pressure than α-amylase. Separate experiments showed that both pressure and heat gelatinised starch are more susceptible to amylase digestion than the intact granules. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The use of high pressure (100–1000 MPa) to process foods is not a new concept and was investigated over 100 years ago by Hite and co-workers (see Ledward, 1995). However, in recent years there has been renewed interest in this technology and it has become a commercial reality in some parts of the world, especially Japan.

As well as the well-established effects of pressure on micro-organisms and proteins (Ledward, 1995) work has shown that high pressure will bring about gelatinisation of starch, although the pressure stability of different starches does not reflect their relative sensitivities to thermal gelatinisation (Ezaki and Rikimaru, 1992). In addition, high pressure is known to modify the activity of a whole range of food enzymes including the amylases (Hayashi and Hayashida, 1989) and polyphenoloxidases (Gomes and Ledward, 1996). Under certain circumstances elevated pressures may bring about increased action of the enzyme, presumably due to some modification of the food enabling the substrate and enzyme to work more effectively together. However, at sufficiently high pressures enzymes will invariably lose their activity as the active site is modified, possibly due to unfolding or partial unfolding of their structure (Gomes and Ledward, 1996) or oxidation of thiol group(s) (Gomes *et al.*, 1997).

It is well established that the action of the amylases in wheat flour will modify the characteristics of bread dough. In addition, enzymic degradation of starch is used in the production of glucose syrups, which are important ingredients in many confectionery products. If high pressure is to become a realistic technology for flour-containing foods it is desirable that information be available about the relative activities of the different amylases as a function of pressure. Such knowledge could be useful in the development of novel foods or ingredients from such flours.

MATERIALS AND METHODS

Materials

Wheat (containing 10.6% protein, 71.6% carbohydrate and 1.6% fat) and barley (containing 7.9% protein, 83.6% carbohydrate and 1.7% fat) flour were obtained from a local supermarket. The same samples were used throughout and were stored at ambient temperature.

Potato starch and α-amylase from malt barley (E.C. 3.2.1.1) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

The α-amylase from malt barley (E. C. 3.2.1.1, Sigma, product no a 2771) was purchased as a dry powder

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containing 2.1 units/mg of α-amylase and 2.9 units/mg of β-amylase. All other chemicals used were of analytical grade.

Preparation and pressurization of the samples

Wheat and barley flour

A slurry of 25% barley or wheat flour in distilled water was prepared and 20.0 g samples were sealed in cryovac bags (high O_2 barrier) and subjected to pressures of 100–800 MPa for 10 and 20 min at ambient temperature in a laboratory model Stansted high-pressure rig (Stansted Fluid Power Ltd, Stansted UK). After pressure treatment, the samples were heated in water baths at 50°C for 20 min to increase the action of any surviving enzymes. After cooling to room temperature, 3.0 g of the slurry were made up to 30.0 g with distilled water and centrifuged at 2224 × *g* for 10 min at 25°C. For the total soluble carbohydrate assay, 2.5 g of the supernatant were made up to 50.0 g with distilled water. For the reducing sugar the supernatant was not diluted after centrifugation since it was in the appropriate range for the assay.

Enzyme solutions (5 ml of 1 unit/ml in distilled water) and 5% suspensions (w/w) of potato starch were also subjected to pressures of 200–800 MPa at ambient temperature.

Enzyme assays

Wheat and barley flour

The activities of the amylases from untreated or pressure-treated (100–800 MPa for 10 or 20 min at ambient temperature) barley and wheat flour slurries were determined by measuring the concentrations of the end products.

Total soluble carbohydrate and reducing sugar contents were measured as described by Chaplin (1986) and were expressed as glucose concentrations by preparing standard curves for the two assays using appropriate concentrations of glucose. For total carbohydrate, solutions of 0.25–2.5 mM D-glucose in distilled water were used and, for reducing sugar, solutions of 2.5–30 mM D-glucose.

Pressure-treated amylase from malt barley using heat-gelatinised potato starch as substrate

Potato starch (1.0 g) (Sigma Prod. No. S-2630) was mixed with 100 ml of 20 mM sodium phosphate containing 6.7 mM sodium chloride, pH 6.9 or 16 mM sodium acetate buffer, pH 4.8. The mixture was boiled for 15 min, left to cool to room temperature and then brought to its original volume (100 ml) with distilled water.

The activities of the amylases from malt barley were determined spectrophotometrically according to the method of Bernfeld (1955).

The assay was performed by placing 0.50 ml of 1% (w/v) of gelatinised potato starch, pH 4.8 or 6.9, into a clean oven-dried test tube in a water bath at 20°C. When 20°C was reached, the enzyme solution (0.50 ml of 1 unit/ml) was added, swirled and incubated for exactly 3 min. No enzyme was added to the blank.

After incubation, 0.50 ml of colour reagent (4.38×10^{-3} M 3,5 dinitrosalicylic acid, 1.06 M sodium potassium tartrate and 0.4 M sodium hydroxide) was added to the tubes. Enzyme solution was added to the blank after the colour reagent. The test and the blank samples were boiled for 15 min and then held in a water bath at 20°C for 30 min. Finally, 4.50 ml of distilled water were added and the absorbance at 540 nm was determined.

Amylase activity from malt barley using pressure-treated potato starch as substrate

One gramme of 5% suspensions (w/v) in distilled water of untreated and pressure treated potato starch were made up to 5.0 g with 16 mM sodium acetate buffer, pH 4.8 or 20 mM sodium phosphate containing 6.7 mM sodium chloride buffer, pH 6.9. The mixture was incubated in a water bath at 20°C for 30 min. After incubation, 5.0 ml of 1 unit/ml of enzyme solution in distilled water were added and the mixture incubated for 30 min at 20°C. After incubation the mixture was centrifuged at 2224 × *g* for 15 min at 20°C.

To 0.50 ml of the supernatant (starch and enzyme solution), 0.50 ml of 16 mM sodium acetate buffer, pH 4.8 or 20 mM sodium phosphate containing 6.7 mM sodium chloride buffer, pH 6.9 and 0.50 ml of colour reagent was added and the activities assayed as for the pressure-treated enzyme and gelatinised starch.

Heat treatment

α-Amylase from malt barley (E.C. 3.2.1.1) and potato starch

The enzyme solution (5 ml of 1 unit/ml in distilled water) or 5% suspension of wheat or potato starch in distilled water were heated in water baths at 20, 40, 50, 60 and 70°C for 30 min and analysed after cooling.

All experiments were repeated at least three times.

Thin-layer chromatography (TLC) analysis

Untreated and pressure-treated (100–800 MPa for 10 or 20 min at ambient temperature) 25% slurries of barley or wheat flour were centrifuged at 2224 × *g* for 10 min at 25°C and the supernatants applied to TLC plates (silica gel 60 on aluminium sheets). The standards used were glucose, maltose and raffinose.

The plates were eluted for 50 min in a mixture of 18.0 ml of ethyl acetate, 4.5 ml methanol, 4.5 ml acetic acid and 3.0 ml distilled water. After elution the plates were sprayed with a mixture of naphthoresorcinol/ethanol/sulfuric acid (24.0 ml of 0.4 g of naphthoresorcinol and 0.8 g of diphenylamine in 200 ml of 95% ethanol was mixed with 1.0 ml of sulfuric acid just prior to use). The plates were developed at 105°C for 7 min.

Microscopic analysis

Untreated and pressure-treated (100–800 MPa for 10 or 20 min at ambient temperature) slurries of 25% barley or wheat flour or 5% suspension (w/w) of potato starch were smeared onto a slide, dyed with 0.2% of trypan blue and viewed at 20 × magnification using cross polars (Flint, 1994). Trypan blue has the advantage of colouring damaged starch granules but leaving intact granules unstained so their birefringence is not masked by the stain (Flint, 1994).

Extraction of soluble proteins

Portions (4.0 g) of untreated and pressure-treated (200–800 MPa for 20 min at ambient temperature) slurries of barley or wheat flour (25% in distilled water) were made up to 30.0 g with 0.5 M sodium chloride. The mixtures were stirred for 1 h at 4°C and centrifuged at 2224 × *g* for 15 min at 4°C. The supernatants were collected and the precipitates stirred with 20.0 g of distilled water for 1 h at 4°C, after centrifuging at 2224 × *g* for 15 min at 4°C the combined sodium chloride and water supernatants were dialysed overnight against distilled water at 4°C. After dialysis, the globulins and albumins were separated by centrifugation at 2224 × *g* for 15 min at 4°C. The albumins, present in the supernatant, were freeze-dried.

Electrophoretic analysis

Wheat and barley flour

Native and SDS–polyacrylamide gel electrophoresis SDS–PAGE) was carried out in mixed gels (top half 10% acrylamide and the lower half 16.5% acrylamide) as described by Laemmli (1970).

The soluble proteins (5 mg) were dissolved in 1.0 ml buffer containing 10% glycerol, 0.002% bromophenol blue and 0.124 M Tris. For SDS–PAGE, the samples were mixed with buffer containing 2% SDS or 2% SDS plus 2% mercaptoethanol. In the presence of mercaptoethanol the samples were heated at 100°C for 3 min just prior to analysis. Barley samples were centrifuged before use to remove insoluble material. Samples (10 μl) were applied to each well. Electrophoresis at 150 V and 60 mA was carried out for 2.5 h.

Amylase from malt barley

Native PAGE (pH 4.75) was carried out in gels containing 7.5% acrylamide as described by MacGregor and Meredith (1971). SDS–PAGE was performed in gels containing 10% acrylamide as described by Laemmli (1970). Electrophoresis at 150 V and 30 mA was carried out for 2 h for SDS–PAGE and at 150 V and 60 mA for 2 h for Native PAGE (pH 4.75).

All gels were silver-stained as described by Nesterenko *et al.* (1994).

RESULTS AND DISCUSSION

Effect of pressure and time on the activity of the amylases

Wheat and barley flour

Pressure treatment led to large increases in total soluble carbohydrate and reducing sugar content when barley and wheat flour slurries were subjected to 400–600 MPa for 10 or 20 min at ambient temperature (Figs 1 and 2). The level of total soluble carbohydrate was higher when the samples were pressurised at 600 MPa for 10 min compared with 20 min (Fig. 1(a) and (b)). At pressures of 100–300 MPa the levels of total carbohydrate and reducing sugars were similar to those observed in the untreated samples. At 700–800 MPa the levels of carbohydrate and reducing sugars were similar or less than that found in the untreated samples. Thus, treatment at 400–600 MPa leads to a massive increase in activity of the starch-degrading enzymes.

It should be noted that in both the untreated and treated flours the ratio of reducing sugars to total carbohydrate is approximately 2 suggesting the major

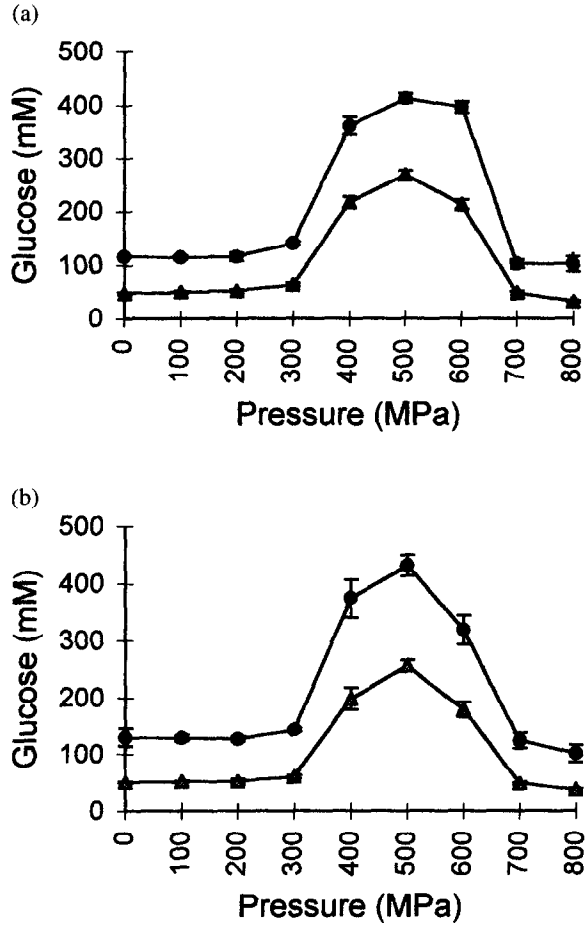

Fig. 1. Effect of high pressure treatment (100–800 MPa) for (a) 10 and (b) 20 min at ambient temperature on amylase activities in barley flour. Values are the means ± SD of three determinations. ●, total soluble carbohydrate; ▲, reducing sugars.

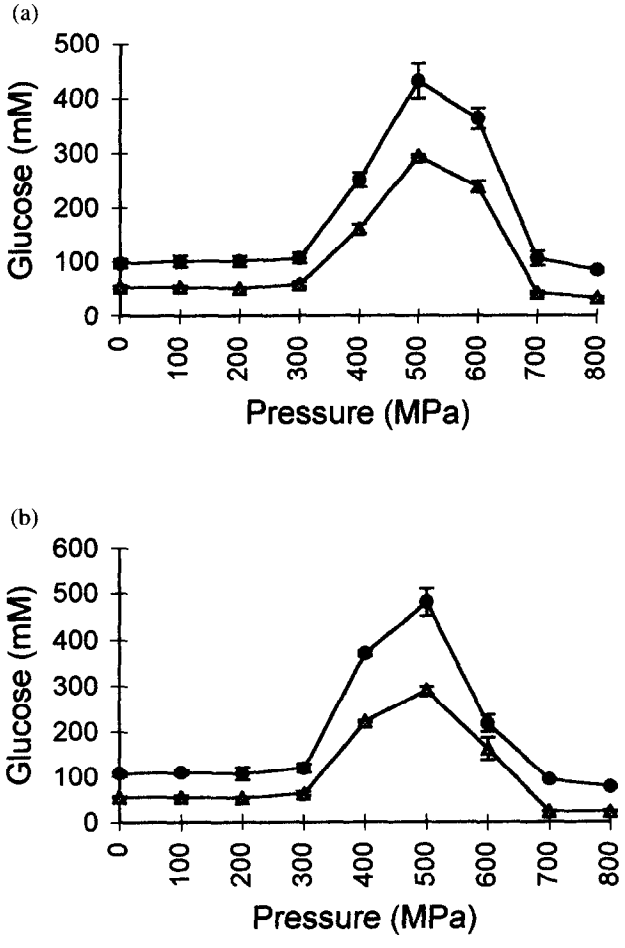

Fig. 2. Effect of high pressure treatment (100–800 MPa) for (a) 10 and (b) 20 min at ambient temperature on amylase activities in wheat flour. Values are the means ± SD of three determinations. ●, total soluble carbohydrate; ▲, reducing sugars.

product is a disaccharide, i.e. maltose. Thin-layer chromatography carried out on the soluble extracts of both flours confirmed these findings, since the major detectable sugar in both samples was maltose. Thus it would appear that enzyme action is increased with pressure treatments from 400 to 600 MPa and the increased activity is presumably due to the α- and β-amylases since maltose is the main product.

Microscopic analysis of the untreated wheat and barley slurries revealed that the starch granules were mostly undamaged and birefrigent in the untreated samples and in those samples treated at 200–300 MPa for 10 or 20 min. However, after treatment at 400 MPa for 10 or 20 min at ambient temperature, both the barley and wheat starch granules had lost birefringence and appeared damaged. These results suggest that gelatinisation took place at about 400 MPa (Fig. 3). Douzals *et al.* (1996), found that at room temperature, wheat starch gelatinisation starts to occur at 300 MPa although potato starch was not altered under 600 MPa.

The enzyme assays (Figs 1 and 2) and microscopic analysis (Fig. 3) therefore strongly suggest that treatment at 400–600 MPa for 10 or 20 min at ambient temperature causes the wheat and barley starch granules to gelatinise which permits more aggressive attack by the amylases. However, treatment at 700–800 MPa significantly reduced the yield of sugar suggesting that, in this pressure range (700–800 MPa), the amylases are themselves modified and lose activity. It is worth noting that after treatment at 600 MPa less activity is seen after 20 min treatment compared with 10 min suggesting that some time-dependent modification of the enzymes is taking place at this pressure.

The decrease in activity, seen at 600 MPa and higher, is presumably due to modification of the active site, due to partial or total unfolding of the enzyme. That this decrease is time dependent, although gelatinisation is complete, suggests that the loss of activity of the amylases is both time- and pressure-dependent. Such time/pressure-dependence has been observed for other food enzymes (Gomes and Ledward, 1996) and thus work on the isolated enzyme from malt barley was carried out.

Amylase from malt barley

Pressure treatment caused a reduction in amylase activity when the enzyme solutions were subjected to 200–600 MPa for 10 min at ambient temperature (Fig. 4). At both pH 4.8 and pH 6.9 the effects were similar, although the loss in activity measured at 4.8 is greater than at 6.9. This suggests that β-amylase is more sensitive than α-amylase to pressure, since pH 4.8 is the optimum for β-amylase and pH 6.9 for α-amylase.

Heat treatment of amylase at temperatures up to 40°C had no significant effect on the activity when assayed at 20°C at both pHs but heating above 40°C caused irreversible loss of activity (Fig. 5). At 80°C all activity was lost and there was no significant difference between the loss in activities at pH 4.8 and 6.9 suggesting that both amylases are equally sensitive to heat.

Electrophoretic analysis

Wheat and barley flour

Most of the soluble proteins in wheat and barley are enzymes and Native PAGE showed that the soluble proteins in both barley and wheat were affected by pressure treatment at 400 MPa and above, since there was a marked loss of many proteins bands at these pressures (results not shown). Thus, at pressures of 400 MPa and above, denaturation and subsequent aggregation of many of the soluble proteins occurred.

SDS–PAGE under non-reducing conditions confirmed these observations since it showed that pressure treatment caused the loss of several SDS-soluble polypeptides; it is noteworthy that, not unexpectedly these were those of high molecular weight but, additionally, a polypeptide of molecular weigh about 7500, in both barley and wheat (Figs 6 and 7) was lost (denatured).

SDS–PAGE of wheat flour under reducing conditions (Fig. 7B) suggests that pressure caused aggregation

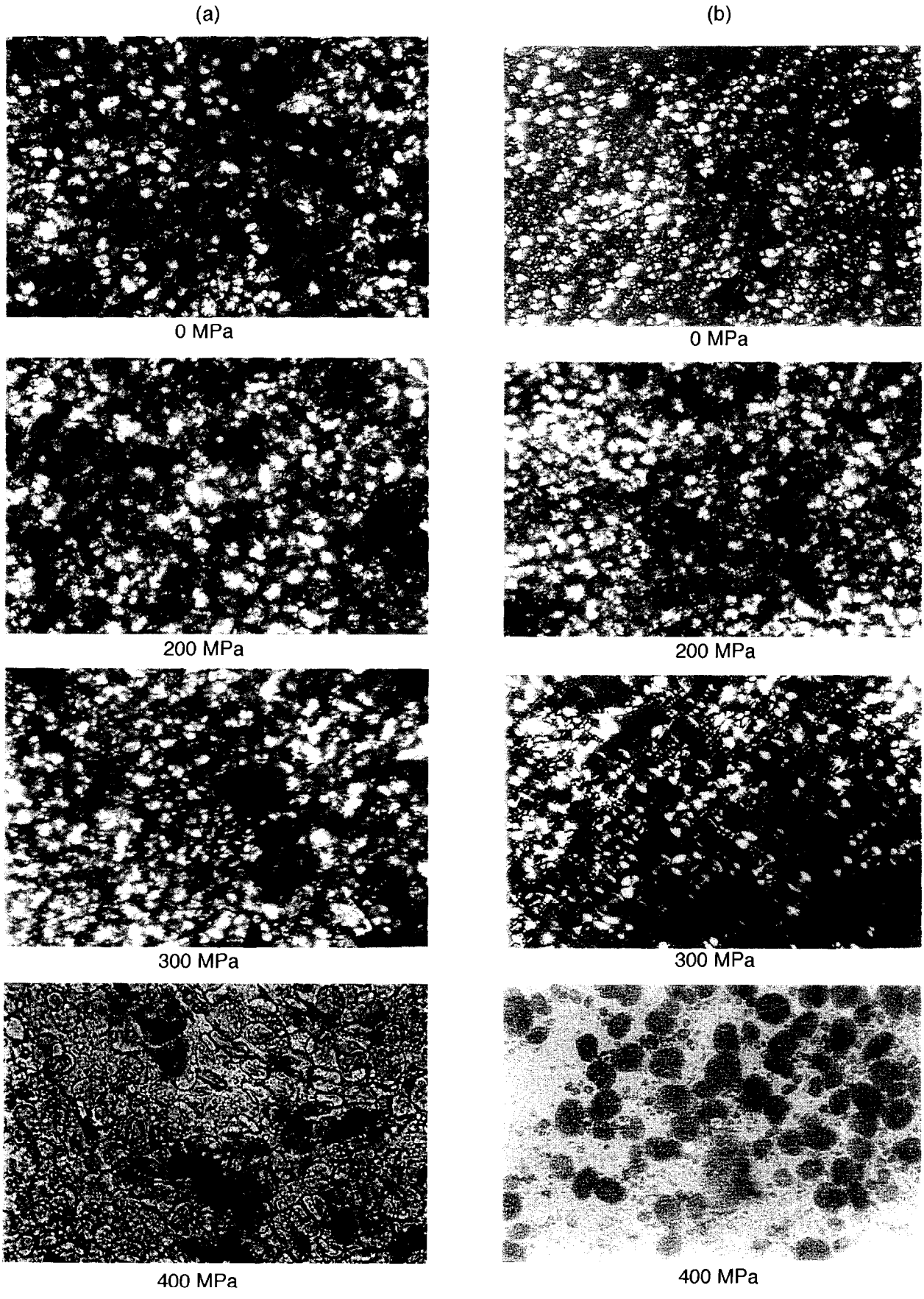

Fig. 3. Photomicrographs of 25% (a) barley and (b) wheat flour slurries after treatment at 200–400 MPa for 20 min at ambient temperature. Starch viewed at × 20 magnification using cross polars.

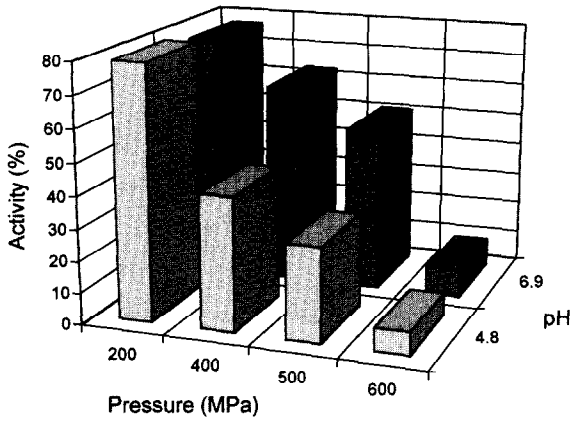

Fig. 4. Inactivation of amylases from malt barley as a function of pressure (200–600 MPa) for 10 min at ambient temperature and pH (16 mM sodium acetate buffer, pH 4.8 or 20 mM sodium phosphate buffer containing 6.7 mM sodium chrolide, pH 6.9). Analysis of variance indicated that the β-amylase (pH 4.8) was more labile to pressure than the α-amylase (pH 6.9); $P < 0.001$.

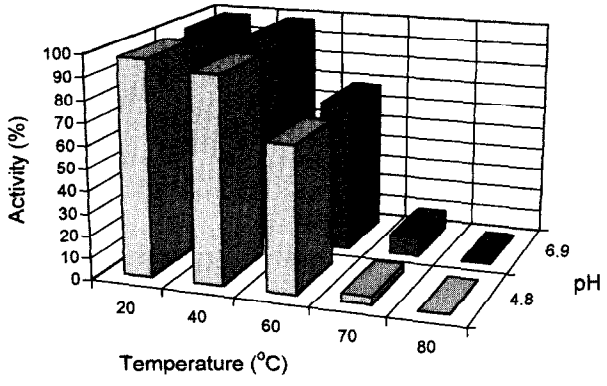

Fig. 5. Inactivation of amylases from malt barley as a function temperature and pH (16 mM sodium acetate buffer, pH 4.8 or 20 mM sodium phosphate buffer containing 6.7 mM sodium chloride, pH 6.9). The samples were heated at the temperature shown for 30 min and assayed at 20°C. Analysis of variance indicated no significant difference between the loss of activity at the two pHs. ($P > 0.1$).

involving disulfide bonds in wheat treated at 400–800 MPa since bands appeared under these conditions which were absent in SDS–PAGE gels prepared from these samples under nonreducing conditions (Fig. 7a). This observation supports the view that intermolecular disulphide bond formation is encouraged by pressure treatment (Galazka *et al.*, 1996).

A SDS–PAGE result, of relevance to this study, is the loss, in both non-reducing and reducing conditions, of a band corresponding to a molecular weight of about 60 000 following pressure-treatment of both wheat and barley flour (see arrow on Figs 6 and 7). This band may be β-amylase since 60 kDa falls within the range of 54–61 kDa reported for β-amylase from barley and 64 kDa for β-amylase from wheat (Shewry and Miflin, 1985; Fincher and Stone, 1993; Wong, 1995). In both samples it was still visible after treatment at 500 MPa, but not

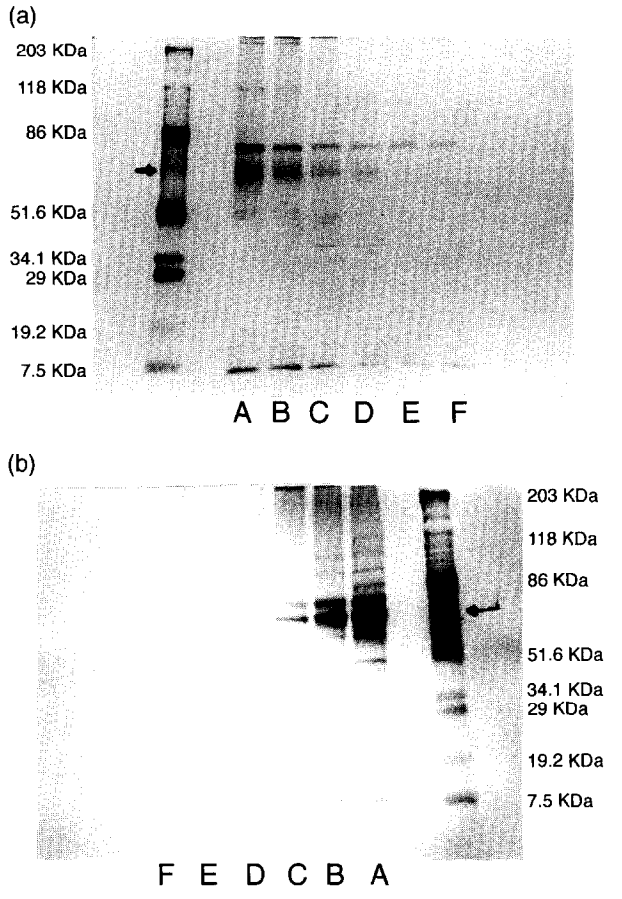

Fig. 6. SDS–PAGE of soluble proteins from barley flour (5 mg/ml) in the presence of 2% SDS (a) and in the presence of 2% SDS and 2% 2-mercaptoethanol (b) before and after treatment at 200–800 MPa at ambient temperature for 20 min. A, native; B, 200 MPa; C, 400 MPa; D, 500 MPa; E, 600 MPa; F, 800 MPa.

after treatment at 600 MPa which correlates well with the apparent activities, in the flours (Figs 1 and 2), i.e. the starch gelatinises at slightly lower pressures than those at which the enzyme is inactivated.

It has been reported that α-amylase from barley or wheat has a molecular weight in the range 42 000–54 000 (Kruger and Reed, 1988; Fincher and Stone, 1993, Wong, 1995). This protein band is not clearly seen in the electrophoretograms but bands in this molecular weight range also disappear at pressures above 400–500 MPa (Figs 6 and 7). Since the results with the slurries, although indicative, were not conclusive the isolated enzyme was studied.

α-Amylase from malt barley

Changes in α- and β-amylase were seen on Native and SDS–PAGE (Figs 8 and 9).

Native Page showed a large loss in intensity of the major band when the enzyme was treated at 600 MPa at ambient temperature for 10 min and total loss on treatment at 800 MPa (Fig. 8).

It has been reported that α-amylase is stable only in the presence of an excess of calcium ions (Greenwood

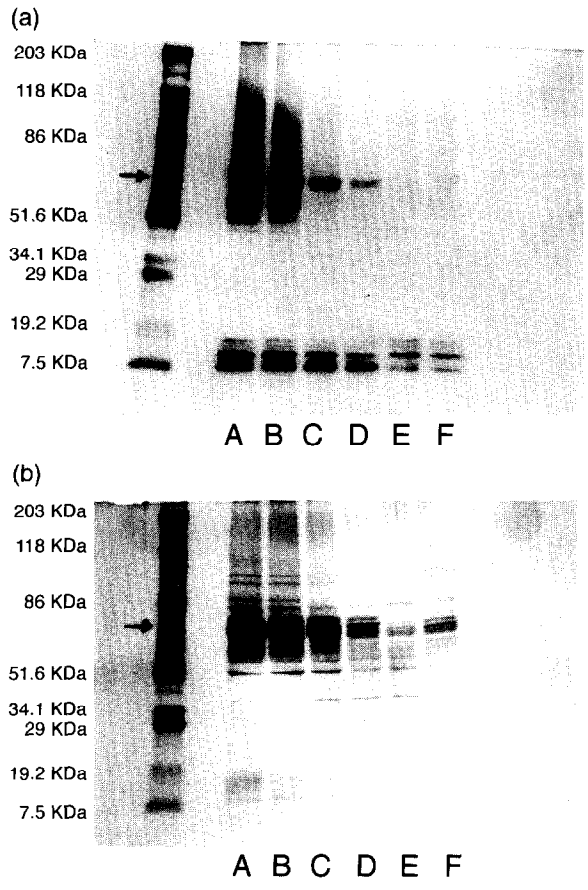

Fig. 7. SDS–PAGE of soluble proteins from wheat flour (5 mg/ml) in the presence of 2% SDS (a) and in the presence of 2% SDS and 2% 2-mercaptoethanol (b) before and after treatment at 200–800 MPa at ambient temperature for 20 min. A, native; B, 200 MPa; C, 400 MPa; D, 500 MPa; E, 600 MPa; F, 800 MPa.

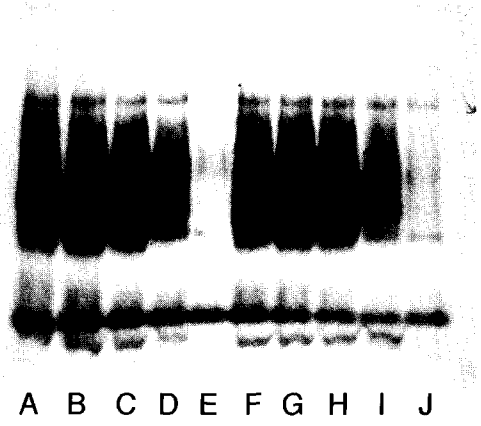

Fig. 8. Native PAGE (pH 4.75) of amylase from malt barley (20 mg/ml) before and after treatment at 200–800 MPa for 20 minutes at ambient temperature. A, F native; B, G 200 MPa; C, H 400 MPa; D, I 600 MPa; E, J 800 MPa; A–E The enzyme was prepared in 0.2 M acetate buffer pH 5.5, F–J The enzyme was prepared in 0.2 M acetate buffer pH 5.5 containing 0.0002 M calcium chloride.

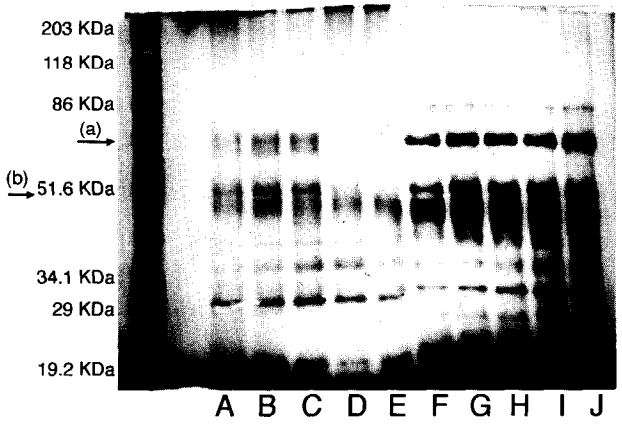

Fig. 9. SDS–PAGE of amylase from malt barley (20 mg/ml) in the presence of 2% SDS or 2% SDS and 2% 2-mercaptoethanol before and after treatment at 200–800 MPa for 20 min at ambient temperature. A, F native; B, G 200 MPa; C, H 400 MPa; D, I 600 MPa; E, J 800 MPa; A–E, in the presence of 2% SDS. F–J, in the presence of 2% SDS and 2% 2-mercaptoethanol.

Table 1. Effect of pressure-treatment (200–800 MPa for 20 min at ambient temperature) on potato starch digestibility by amylase. Values are the means ± SD of three determinations

	Maltose released (mg $\times 10^1$)	
Pressure	pH 4.8	pH 6.9
0	0	0
200	0	0
400	0	0
500	0	0
600	0.61 ± 0.04	0.47 ± 0.01
800	1.29 ± 0.09	1.13 ± 0.08

Table 2. Effect of heat-treatment (20–60°C for 30 min) on potato starch digestibility by amylase. Values are the means ± SD of three determinations. The samples were assayed at 20°C

	Maltose released (mg $\times 10^1$)	
Temperature (°C)	pH 4.8	pH 6.9
control	0	0
20	0	0
40	0	0
50	0	0
60	0.43 ± 0.02	0.32 ± 0.02

and Milne, 1968). No significant difference in electrophoretic patterns was observed when the samples were prepared in the presence or absence of calcium chloride (Fig. 8). It is likely that calcium ions were added during the purification of the enzyme and therefore the addition of more calcium ions during electrophoresis had no further effect on the enzyme.

In non-reducing conditions, SDS–PAGE revealed a decrease in intensity of a band corresponding to a molecular weight of about 60 000 (see arrow on Fig. 9)

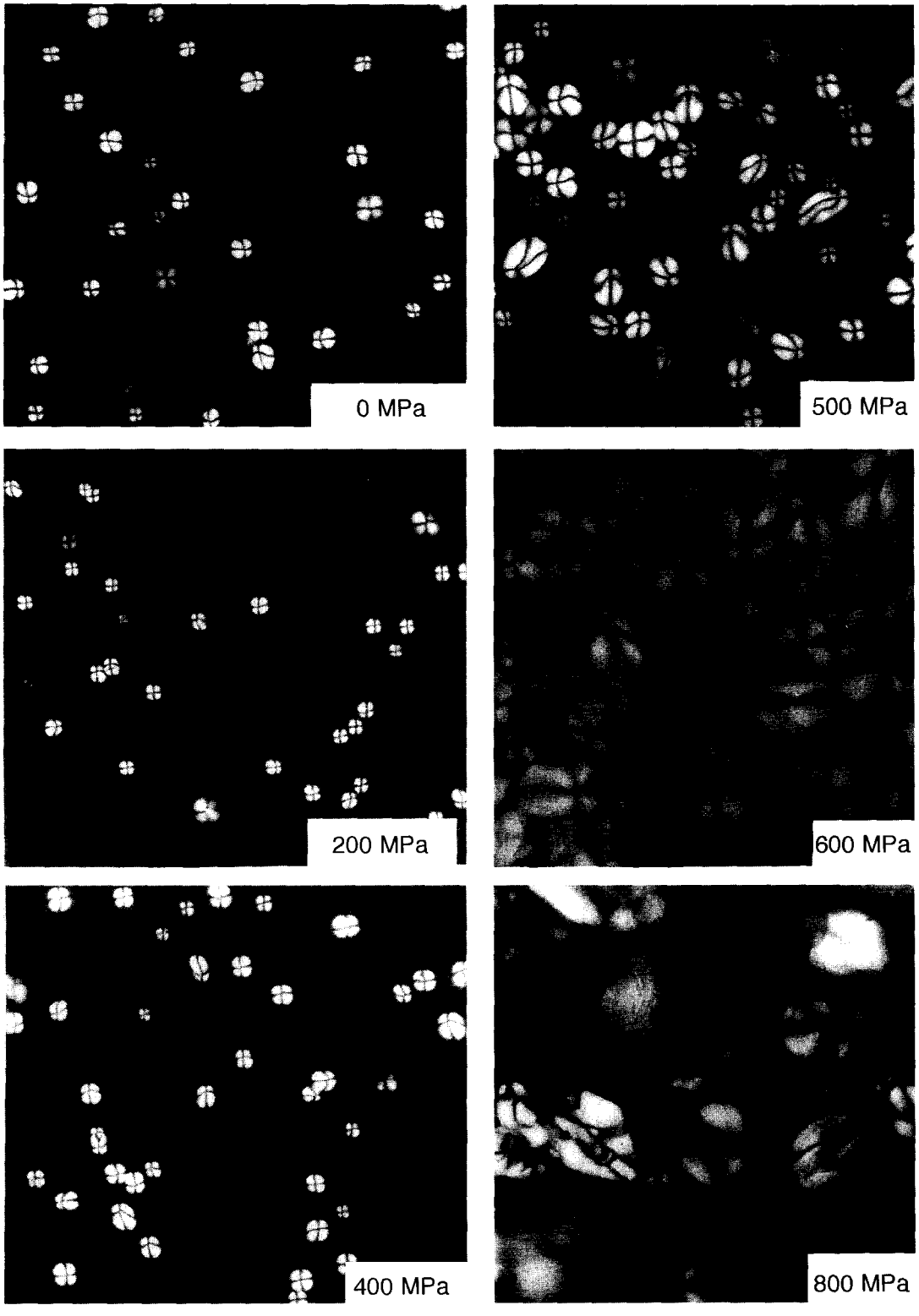

Fig. 10. Photomicrographs of 5% potato starch after treatment at 200–800 MPa for 20 min at ambient temperature. Starch viewed at × 20 magnification using cross polars.

when the enzyme solution was pressure treated at 600–800 MPa for 10 min at ambient temperature, which is probably β-amylase. In addition, a decrease in intensity of a band of molecular weight about 51 000 was observed at 600–800 MPa, which is probably α-amylase.

No significant difference between untreated and pressure-treated samples was observed in SDS–PAGE in reducing conditions (Fig. 9). Thus, it can be concluded that the decrease in activity of amylases from malt barley involves aggregation due to disulphide bond

formation since little difference is seen in SDS–PAGE carried out under reducing conditions. It is likely that the formation of the disulfide bonds is time/pressure-dependent and this may explain the relatively slow loss of activity under pressure, assuming that the formation of these bonds prevents the refolding of the native structure on release of pressure and reformation of the active site.

The work on the isolated enzymes does suggest that some activity is lost at pressures at which no aggregation is seen (200–500 MPa, Fig. 4). This might imply that some pressure-induced change affecting the activity occurs before aggregation takes place. In β-amylase there is some evidence that modification of a cysteine-residue near to the active site can decrease the activity of the enzyme (Wong, 1995); α-amylase possesses no such thiol group and it is possible, if oxidation of this group occurs under pressure, that this contributes to the greater sensitivity of β- compared with α-amylase at pressures below 600 MPa (Fig. 4).

Effect of pressure and temperature on potato starch

No attack by the amylases was observed on untreated and pressure-treated (200–500 MPa for 20 min at ambient temperature) potato starch since no maltose was detected. However, pressure treatment of the starch at 600 or 800 MPa led to an increase in the levels of maltose when assayed against amylase at both pH 4.8 and pH 6.9 (Table 1).

Similarly an increase in maltose concentration was observed when potato starch was heated at 60°C for 20 min and assayed at both pHs at 30°C (Table 2). No hydrolytic attack was observed in potato starch heat treated at 20–50°C for 30 min.

Microscopic analysis of the untreated and treated (200–500 MPa for 20 min at ambient temperature) potato starch revealed that the starch granules were mostly undamaged and birefrigent. However, after treatment at 600 MPa or 800 MPa for 20 min at ambient temperature, the potato starch granules had lost birefringence and appeared damaged (Fig. 10). Thus gelatinisation took place at about 600 MPa. Similarly heat-gelatinisation of starch took place at 60°C. Thus the results shown in Tables 1 and 2 suggest that both pressure- and heat-gelatinised starch are very susceptible to amylase digestion.

In agreement with the present studies it has been reported that susceptibility of pressure treated wheat, potato and corn starch (0.1–500 MPa at 40–50°C) to digestion by α-amylase from *Bacillus* species was enhanced but on treatment at these pressures and 25°C no susceptibility of the starch to amylase digestion was seen (Hayashi and Hayashida, 1989).

These results confirm the hypothesis discussed in the work on the flours that pressure-induced starch gelatinisation increases amylolysis. However, further increase in pressure limits the hydrolyis since the enzymes themselves become inactivated.

CONCLUSION

This work confirms that pressure gelatinises wheat and barley starch and suggests that this transition increases the efficiency of starch hydrolysis by α- and β-amylases in flour. Heat can also bring about a similar phenomenon and is utilised in the manufacture of glucose syrups but, given the different sensitivities of various starches and enzymes to heat and pressure, the potential of manipulating the properties of such systems, using both temperature and pressure, in food manufacture is of interest.

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