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Modulation of protease specificity by a change in the enzyme microenvironment

Selectivity modification on a model substrate, purified soluble proteins and gluten

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Subtilisin BPN' activity on a synthetic substrate is found to decrease with the concentration of soluble additives such as sugars and polyols, the catalytic efficiency of the enzyme being related to the water activity in the reaction medium. Limited hydrolysis of ß chain of insulin is followed and the cleavage priority determined. When carried out in glycerol-containing medium, both enzyme catalytic behaviour and specificity are perturbed: a different cleavage order and a selectivity restriction are observed. The experiments were generalised to purified proteins and to an insoluble protein complex. The hydrolysis kinetics of purified gliadins by pepsin and of gluten by a Bacillus neutral protease are modulated in presence of water activity depressors. Glycerol is able to increase both pepsin efficiency and gluten protein solubility. The hydrolysis order is affected by water-structuring molecules in the enzyme microenvironment and new peptides appear whatever the size and initial solubility of the substrate.

Protease specificity; Insulin; Gliadin; Gluten; Selectivity; Water activity

1. INTRODUCTION

Molecular interactions between an enzyme and its substrate result from a complex interrelation between hydrogen bonding, van der Waal's and electrostatic forces, steric and hydrophobic interactions; the relative importance of these factors, combining through a still unclear way, determines substrate binding specificity [1]. While enzymes generally exhibit a narrow specificity – sometimes limited to a single substrate as for aspartate ammonialyase – some are able to react with a wide variety of molecules owing to a common structural character; proteases belong to this last class. Serine proteases are the most widely distributed proteolytic enzymes of both microbial and animal origin and microbial alkaline proteases are among the enzymes which exhibit the broader specificity [2]. Enzyme–substrate interactions have been modified by protein engineering of subtilisin showing the effect of steric and hydrophobic interactions [3] and of plasticity [4] on the protease selectivity. Engineering surface charge also induced rational modification of subtilisin catalysis [5].

Data from classical enzymology have been obtained in homogeneous conditions where kinetics are only depending on time and concentrations while actual applications of enzymes, either in vivo or in industry, occur in heterogeneous media where kinetics are also depending on space conditions [6]. In these last cases, modifications may be observed on activity, stability, nature of the reaction catalysed and selectivity of the concerned enzyme.

To mimic biological media [7] and try to understand the related mechanisms, aqueous media with restricted water activity have been used; under these conditions, modulations in the initial activity rate [8] and in the kind of reaction catalysed by enzymes have been observed [9]. The role of water on enzyme stability [10], and on the expression of catalytic activity [8] has been studied. But little has been done about the influence of water on enzyme specificity when the biocatalyst reacts in an aqueous medium thought it has been recently shown that bound water molecules play a determining role on affinity and specificity [11]. On the other hand, unusual reactions have been obtained when using proteases in the absence of water, in organic solvents [12–13].

In the present paper we have studied the interaction between water in the reaction medium and the activity and specifity of several proteases. We have evidenced a modulation in selectivity of these enzymes, induced by a controlled modification of water activity in the enzyme microenvironment, either on model substrates or on proteins.

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2. MATERIALS AND METHODS

2.1. *Insulin hydrolysis*

Bacillus subtilis subtilisin BPN' (Nagarse from Sigma) was chosen as this extracellular alkaline protease exhibits a broad substrate specificity [14] and as subtilisins have already been engineered [3-5]. As this enzyme may hydrolyze numerous synthetic substrates, we have chosen tosyl arginine methyl ester (TAME), a water-soluble compound, used at concentrations ranging from 10 to 300 nM. The reaction kinetics were followed by a pH-stat method, in 0.05 M Tris-HCl buffer pH 8.0 at 35°C with an enzyme concentration corresponding to 0.5 U/ml, the activity being expressed as μmol NaOH added$\cdot$min^{-1}. The NaOH concentration was varied so that the added volume was always less than 10% of the reaction volume.

To measure peptide bond cleavage specificity, oxidised insulin B chain (Sigma) has been used at substrate/enzyme ratio ranging from 1000 to 5000. The reaction was carried at 35°C in a batch reactor; the obtained peptides were analysed by a reverse-phase HPLC method. A Si C_{18} μBondapak column (internal diameter 3.9 mm, column length 25 cm and phase diameter 10 μm) was used with a 1 ml$\cdot$min^{-1} flow rate. The spectrophotometric measurements were performed at 215 nm. An elution gradient was realised as follows: 0–2 min: 100% A; 2–30 min: 75% A + 25% B; 30–90 min: 60% A + 40% B with A: TFA 0.05% in water and B: TFA 0.05% in acetonitrile. Previous purification of insulin was realised using a Si C_{18} μBondapak column (internal diameter 7.8 mm, column length 30 cm) with a 4 ml$\cdot$min^{-1} flow-rate. The obtained substrate and some peptides were collected and kindly characterised with a 477 A protein-sequencer coupled to a 120 A HPLC from Applied Systems by the team of Dr B. Ribadeau-Dumas (I.N.R.A., Jouy-en-Josas, France).

2.2. *Gliadin hydrolysis*

The chosen substrate is either a pure γ gliadin or a γ-β gliadin preparation extracted from wheat (60% of wheat prolamins). These globular proteins have a low molecular weight (30 to 40 kDa), present a low percentage in basic amino acids and are poorly charged. They are made up of a single polypeptidic chain whose structure is stabilised by disulphide bonds and by hydrogen bonds due to numerous glutamic acid residues. Dr Popineau (INRA, Nantes) kindly gave us a well defined wheat source and proteins were extracted and electrophoretically analysed as previously described [15-17]. They have been sequenced and constitute a well characterised and renewable protein source. Experiments were also directly carried out on wheat flour.

For gliadin experiments, we used porcine pepsin (Merck) as the well-characterised substrate is only soluble in organic solvents and acid solutions. The enzyme exhibits hydrolytic activity only on peptide bonds and is characterised by its low P_i and optimal pH value ($P_i < 1$, pH = 2). It has a broad specificity attributed to the high flexibility of its active site [18]. 100 mg gliadin preparation have been solubilised for 2 h at room temperature in 10 ml of a 0.1 M CH_3COOH solution adjusted to pH 2 with HCl. Then 0.1 mg of 2500 FIP-U/g pepsin/ml was introduced and the reaction carried out at 20°C (1 FIP-u will produce a ΔA_{280} of 0.001 per min at pH 1.6 at 25°C measured as trichloracetic acid-soluble products using hemoglobin as substrate). Aliquots were regularly sampled and the reaction stopped by pH jump (addition of 0.05 M borate buffer, pH 9.5, first component of the Fields colorimetric reaction).

Protein hydrolysis was quantitatively followed by measuring the appearance of peptides using a colorimetric method [19] modified as follows: a 50 μl sample was mixed with 950 μl 0.05 M sodium borate buffer, pH 9.5; 20 μl of 1.8 M trinitobenzene sulphomic acid (TNBS) were then added and the obtained solution shaken for 5 min. Then 2 ml of a reaction stop solution consisting of 98.5 ml of a 0.1 M NaH_2PO_4 buffer + 1.5 ml of 0.1 M Na_2SO_3 were added. The mixture was diluted twice with 50% ethanol and the optical density followed at 420 nm. Activity is expressed in μmol Ile NH_2 eq$\cdot$l$^{-1}\cdot$min^{-1} by comparison with an isoleucine calibration curve. Qualitative analysis of gliadin hydrolysis products was realised using a HPLC method as previously described for insulin products.

2.3. *Flour hydrolysis*

For experiments with flour, the neutral protease from *Bacillus subtilis* (Gist Brocades, B 500 protease) has been used as this enzyme of industrial quality is one of the authorised biocatalysts for dough preparations. The enzymatic preparation only contains 4% protein (>95% being lactose), of which 35% are neutral protease, the preparation also contains an α-amylase, a β-glucosidase and an alkaline protease. To 90 ml 45°C water, 25 g T55 type wheat flour were added. 0.18 g B 500 protease are solubilised in 10 ml 45°C water and added to the substrate preparation. The reaction was stopped in 5 ml samples by addition of 25 ml of a solution containing 2×10^{-3} M EDTA in 0.02 M CH_3COOH and rapid cooling. Flour hydrolysis soluble products were extracted by continuous shaking in the acid solution for 16 h and then centrifuged at 12 000 × *g* for 20 min at 20°C. The supernatant was analysed using an FPLC (Waters 650 + Waters 481) method with two exclusion chromatography columns in series - Protein Pack Glass 200 W + Protein Pack Glass 300 W (Waters). Elution buffer is a 0.1 M phosphate buffer pH 7 containing 0.1 M sodium sulphide. Detection was realised at 280 nm.

Using the chromatographic methods, the activity was followed through the peak surface evolutions and the results were found to be reproducible with a relative error lower than 5%.

2.4. *Water activity*

Thermodynamical water activity (a_w) was measured using a Novasina cell consisting in an electric hygrometer [20]. Water activity was depressed by using glucose, sucrose, fructose, glycerol and sorbitol at various concentrations.

3. RESULTS

3.1. *Hydrolysis of model substrates*

When measured on TAME, the enzyme behaves following a Michaelis–Menten mechanism. The kinetic constants have been determined at K_m = 60 mM and V_m = 18.6 μmol NaOH$\cdot$min^{-1}. The effect of water activity depressors on enzyme catalysis was then measured with a TAME concentration of 200 mM. The relative activity of Nagarse was expressed as a function of water activity depressor concentration (Table I).

Obviously, glycerol presents the most important perturbating effect on enzyme activity. Due to the high solubility of the polyol, its concentration was increased up to 9 M and the influence on the catalytic efficiency of the enzyme measured (Fig. 1). This decrease in enzyme activity was correlated to the decrease in water activity (Fig. 2).

Glycerol inducing a more important perturbation on the kinetics of subtilisin BPN' than other water activity depressors, we have measured its influence on enzymic specificity. The specificity of subtilisin BPN' is broad

SO3H (on Cys) SO3H (on Cys)

Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

n°1 n°2 n°3 n°4 n°5

Table I

Relative activity of subtilisin BPN' expressed on 200 mM tosyl arginine methyl ester in the presence of different water activity depressors at various concentrations. Reference activity is 14 μmol NaOH · min^{-1}

Relative activity (%) [a_w depressor] (M)	0.5	1	1.5	2	2.5	3	3.5
Fructose	100	94	85	84	83	80	78
Sucrose	99	92	88	83	75	–	–
Sorbitol	97	90	88	86	82	76	66
Glucose	96	90	86	84	82	78	76
Glycerol	90	76	64	56	50	44	40

in complete hydrolysis, but restricted to 5 cleavage bonds on oxidised B chain of insulin in limited proteolysis [2].

First, several substrate/enzyme ratios were compared to determine the S/E value allowing to measure a sufficient but limited proteolysis. Hydrolysis was carried out for 30 min, stopped by quick freezing and the obtained peptides were analysed. An S/E ratio of 4000 was then chosen for the further experiments as a restrained number of peptides has been observed (Fig. 3.1). One cleavage bond was obviously hydrolysed first (no. 4).

Using this S/E ratio, B chain insulin proteolysis was carried out in buffer and the results observed as a function of time (Figs 3.1 and 3.2). After 1 h, insulin was completely hydrolysed. After 2 h, the obtained peptides were cleaved. Bond no. 5 was not hydrolysed.

The same experiments were realised with different glycerol concentrations in the reaction medium. The results are expressed for a 10 M glycerol concentration on Fig. 3.3. Hydrolysis was very slow, only 38% of insulin reacted after 8 h, 80% after 36 h. After 1 h, only two peptide bonds, sites no. 3 and no. 5, as shown by the analysis of the obtained products, were hydrolysed. A 25 amino acid peptide, more hydrophobic than insulin (retention time higher than that of the substrate) was observed when site no. 5 was cleaved. The evolution of hydrolysis products was compared in buffer and in the presence of 10 M glycerol on Table II. Peptides corresponding to the cleavage of bond no. 5 accumulated for 18 h before being hydrolysed.

3.2. *Gliadin hydrolysis*

The kinetics of gliadin hydrolysis by pepsin in buffer was followed during 400 min with a colorimetric method, the enzyme exhibited an important initial activity, a plateau was reached after 1 h (Fig. 4). The enzyme follows the Michaelis-Menten model and the apparent kinetic constants were determined at: $K'_m = 13$ mM and $V'_m = 1.2 \cdot 10^{-2}$ μmol Ile NH_2 eq · l^{-1} · min^{-1}. When adding glycerol at concentrations ranging from 0.1 to 10 M in the reaction medium, several unusual behaviours were observed:

(i) the initial activity was increased by up to 60% as compared to buffer for glycerol concentrations lower than 0.5 M. This phenomenon is reproducible.

(ii) contrary to what was observed with insulin, the enzyme expressed its activity even at high polyol concentrations (80% of reference activity in 10 M glycerol).

(iii) a non-michaelian behaviour was obtained for glycerol concentrations higher than 1 M, in this case, the Eadie-Hofstee linearisation showed that diffusion was controlling the reaction.

(iv) initial activity rate was not related to water activity.

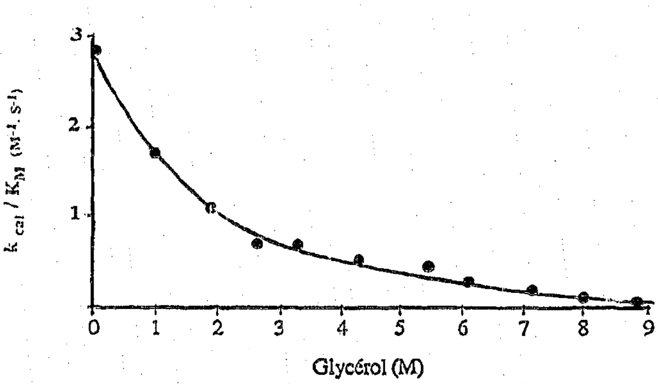

Fig. 1. Catalytic efficiency of subtilisin BPN' expressed on 200 mM tosyl arginine methyl ester as a function of glycerol concentration in the reaction medium. $(k_{cat}K_m^{-1})_{ref} = 2.8$ M^{-1} · s^{-1}.

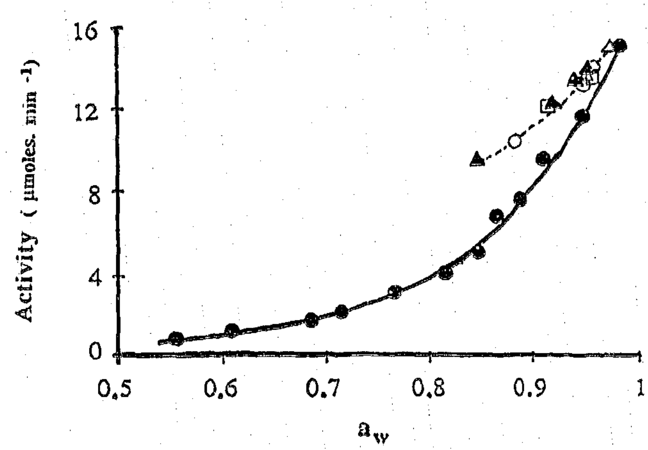

Fig. 2. Subtilisin BPN' activity as a function of the thermodynamical water activity in the reaction medium obtained by various glycerol concentrations (●) and with other additives (○: sucrose; □: glucose; ▲: sorbitol; △: fructose).

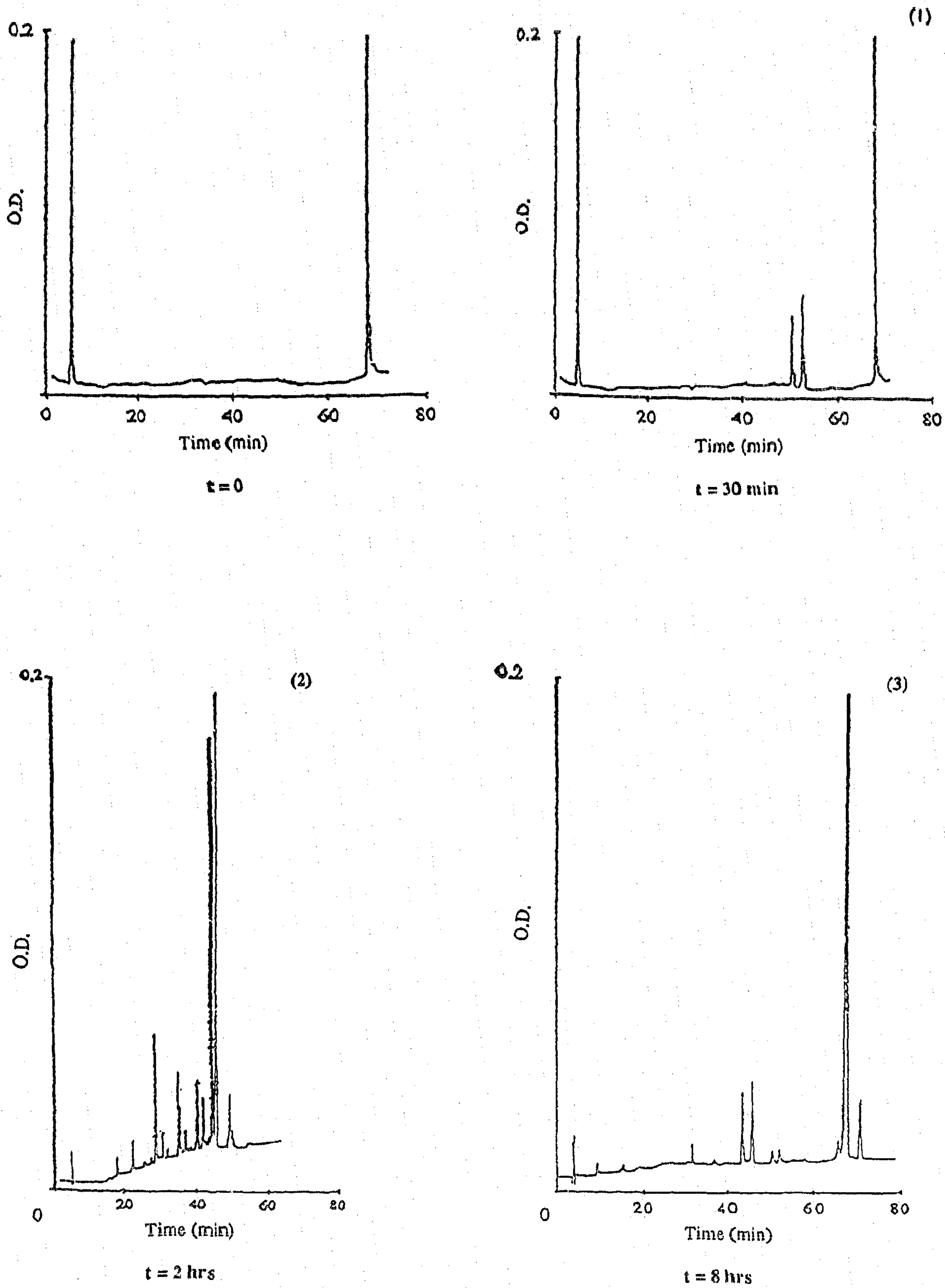

Fig. 3. Reversed-phase HPLC analysis of insulin (chain B) hydrolysis products for a 4000 S/E ratio and a reaction carried out in buffer for 30 min (1), for 2 h (2), or in a medium containing 10 M glycerol for 8 h (3).

Table II

Evolution of hydrolysis ratio and of hydrolysis products as a function of time for an ordinary buffer medium and for a medium containing 10 M glycerol 'hydrolysis (%)' expresses the ratio:insulin surface/total product surface. 'peak no. x (%)' is the ratio between the surface of the two peaks corresponding to the cleavage of bond no. x and the total surface of hydrolysis products

	Time	30 min	45 min	1 h	2 h	4 h	8 h
Buffer	hydrolysis (%)	31	97	100	100	–	–
	peak no. 4 (%)	99	70	68	58	–	–
	peak no. 3 (%)	–	7	6	8	–	–
10 M glycerol	hydrolysis (%)	–	–	8	13	19	38
	peak no. 3 (%)	–	–	78	72	68	63
	peak no. 5 (%)	–	–	22	23	23	23
	peak no. 4 (%)	–	–	0	4	9	8

Pepsin hydrolysis of gliadins was followed by HPLC peptide analysis (Fig. 5). The chromatograph shows 4 peaks for gliadins (named A, B, C and D for increasing elution times - Fig. 5.1) and 9 peaks for hydrolysates (named from 1 to 9 for increasing elution times - Fig. 5.2). The evolution of total surfaces for hydrolysis products is well correlated to results obtained with the colorimetric method (Fig. 4). The enzymatic reaction did not affect gliadin hydrophobicity as the retention times were not modified in buffer. It is important to note, that all hydrolysate peaks increased as a function of reaction time, with a major increase for peaks no. 5 and 6. The hydrolysis of gliadins appeared to be ordered. During the first 10 minutes, only peak D corresponding to γ_{44} gliadin was hydrolysed, then peak C, then peaks A and B: the most hydrophobic peaks were hydrolysed first.

When glycerol was added to the hydrolysis reaction medium, the hydrolysate surface was decreased as compared to the buffer reference medium, except for short hydrolysis time and glycerol concentrations lower than 0.5 M (Fig. 6). This result is in good agreement with colorimetric analysis. The ratio product surface/substrate surface (*P/S*) is decreasing with glycerol concentration.

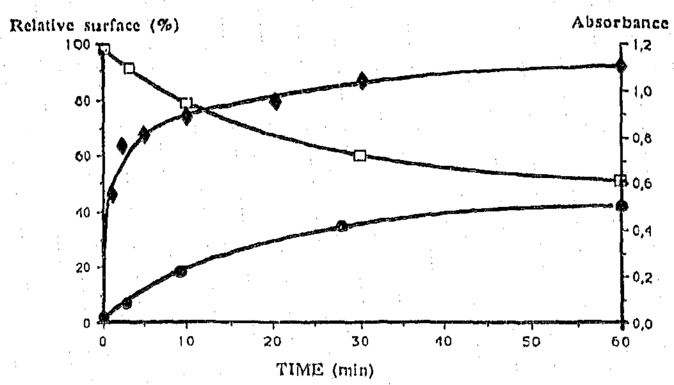

Fig. 4. Pepsin hydrolysis of γ-β gliadins in buffer. The enzyme kinetics were measured by a colorimetric method (◆), and with a reverse-phase HPLC method through the disappearance of gliadins (□) and the evolution of hydrolysates (●).

In glycerol containing media, gliadin hydrolysis followed an order different from that observed in buffer (Table III). Fraction D, hydrolysed first in buffer, did not react while fraction A was more hydrolysed than in reference medium. On the other hand, the retention times of gliadin peaks were increased in glycerol indicating an increase in hydrophobicity (Fig. 5.3).

For the less hydrophobic hydrolysates (peaks 1–5), no difference in retention time due to the presence of glycerol was observed while their surface increased as a function of reaction time. For more hydrophobic peptides the behaviour was different:

(i) peak 6 corresponding to the site hydrolysed in priority in buffer revealed an activation in enzyme reaction for a glycerol concentration of 0.1 M and an inhibition for concentrations higher than 1 M. It was partly hydrolysed at 10 M glycerol (peaks 6 and 6′ on Fig. 5.3).

(ii) a new peak (between peaks 8 and 9), never observed in buffer, was revealed for glycerol concentrations higher than 0.5 M after 10 min of hydrolysis. Its retention time was not varying with glycerol concentrations. It was hydrolysed after 30 min of enzymatic reaction.

(iii) peak 9 exhibited a retention time varying with the glycerol concentration, indicating a modification in peptide hydrophobicity. As previously described for other peptides, it revealed an activation for 0.1 M glycerol and decreased at 10 M glycerol.

3.2. *Wheat flour hydrolysis*

Wheat flour hydrolysis was followed by the FPLC method and the evolution of peak surfaces as measured (Table IV). The total surface evolution showed that the enzymatic reaction was fast even on the insoluble substrate and that a plateau was reached after 45 min of hydrolysis. The surfaces of high molecular mass products (>36 kDa) relatively decreased during the first hour, indicating that hydrolysis did not induce an important solubilisation of the gluten proteins. The enzyme was mainly inducing an increase of low molecular mass peptides.

When measured in the presence of sorbitol and

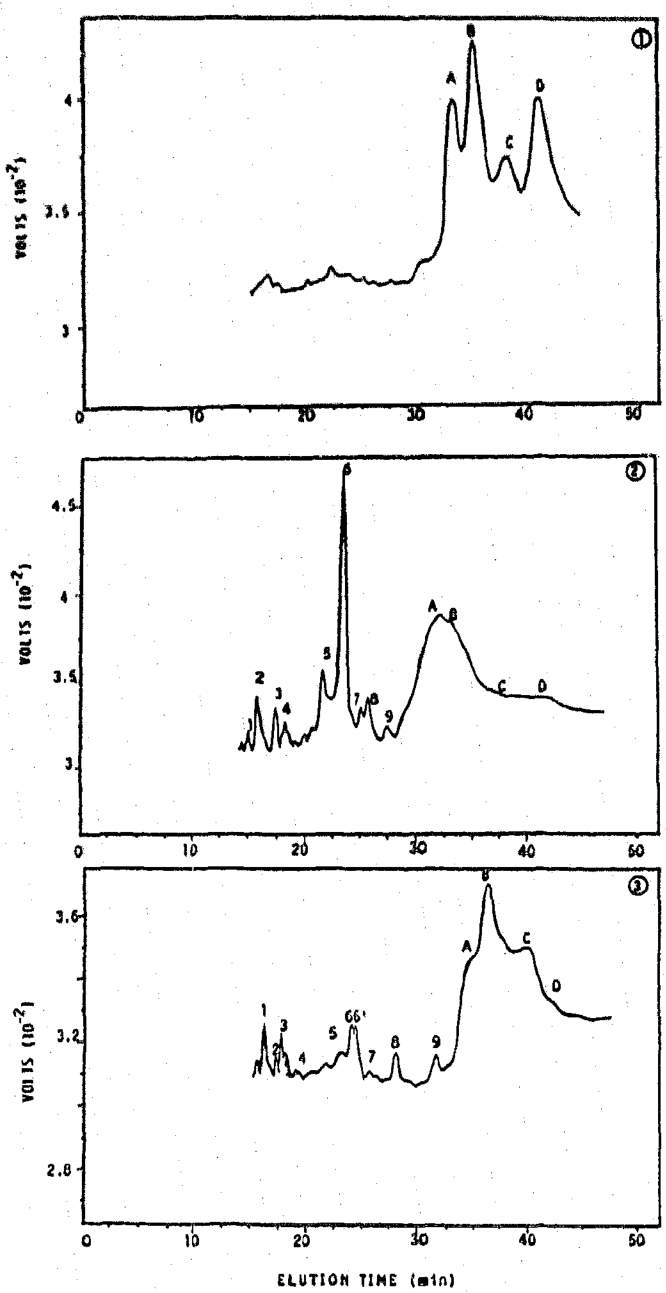

Fig. 5. HPLC elution profiles of γ-β gliadins before hydrolysis (1) and after 60 min hydrolysis in buffer (2) and in 10 M glycerol (3).

glycerol (0.5, 1, 2 and 2.5 M), hydrolysis was found to be influenced relatively to the polyol concentration. The surface before the enzymatic reaction was increased by 22% for 2.5 M glycerol when compared to the reference in buffer, indicating that the first effect of the additive was to solubilise more proteins than buffer. The results are described for 2.5 M glycerol (Fig. 7). The presence of additive did not diminish the expression of enzyme activity:

$$T_{60}\ \text{glycerol}/T_{0\ \text{glycerol}} \sim T_{60\ \text{reference}}/T_{0\ \text{reference}} = 1.6$$

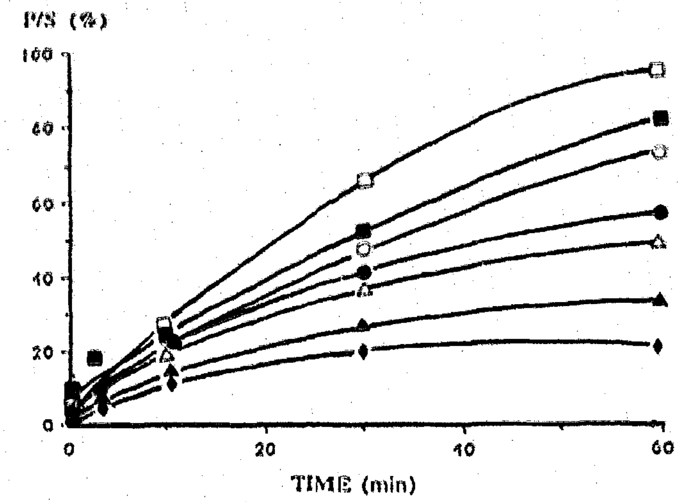

Fig. 6. Total hydrolysate surface/total gliadin surface measured as a function of hydrolysis time in media containing glycerol at various concentrations. (□ : 0 M; ■ : 0.1 M; ○ : 0.5 M; ● : 1 M; △ : 2 M; ▲ : 5 M; ◆ : 10 M).

Hydrolysis was more complete in glycerol than in buffer, as:

$\text{Surface}_{<20\ \text{kDa peptides}}$ in glycerol = 87% total surface
$\text{Surface}_{<20\ \text{kDa peptides}}$ in buffer = 52% total surface.

Selectivity was modified, peptides of 0.5, 10 and 18 kDa appearing in the presence of glycerol. When sorbitol was used instead of glycerol, peptides of 8 and 18 kDa were observed. Sorbitol, like glycerol, was found to be able to favour the extraction of low molecular mass molecules.

4. DISCUSSION

The presence of a_w depressors (sugars and polyols, particularly glycerol) makes subtilisin BPN′ activity decrease. This effect is exponentially related to water activity. The experimental results show that the catalytic activity is expressed whatever the water activity but with different efficiencies. Water availability which has been shown to be a key parameter for hydrolysis by several enzymes in aqueous media [9] and for non-conventional reactions in organic solvents [21] also seems to be a determining factor for the proteolytic reaction. The microenvironment modifiers used here may affect the thermodynamics of most processes in which the substrate may be involved, including enzymatic transformations. It is important to note that in the case of subtilisin BPN′, glycerol which is a very efficient water activity depressor does not behave like the other additives used. Such a difference has previously been observed with lysozyme whose activity, on the contrary, was less affected by glycerol than by other additives [8]. These results show that a_w partly controls the reaction but also that the enzymatic activity is

Table III

Residual γ and β gliadins after 3 min hydrolysis as a function of glycerol concentration in the reaction medium

	% of total gliadin surface				
Glycerol (M)	0	0.1	0.5	1	10
Peak A	32	25	26	12	12
Peak B	16	20	17	32	16
Peak C	23	24	24	7	12
Peak D	18	21	26	45	42

Table IV

Wheat flour hydrolysis by B 500 *Bacillus subtilis* protease as a function of reaction time in buffer. The hydrosoluble products were analysed by a reverse-phase HPLC method

Molecular mass of hydrolysis products (kDa)							
% Total surface	≥220	65	36	24	15	≤12	Total surface (arbitrary units)
Time (min)							
0	7	6	22	24	18	23	376
5	4	3	21	22	22	28	412
10	3	4	20	22	21	30	459
15	2	3	18	21	22	34	494
30	1	3	16	21	23	36	532
45	1	3	14	22	24	36	570
60	0.5	2	13.5	22	25	37	574

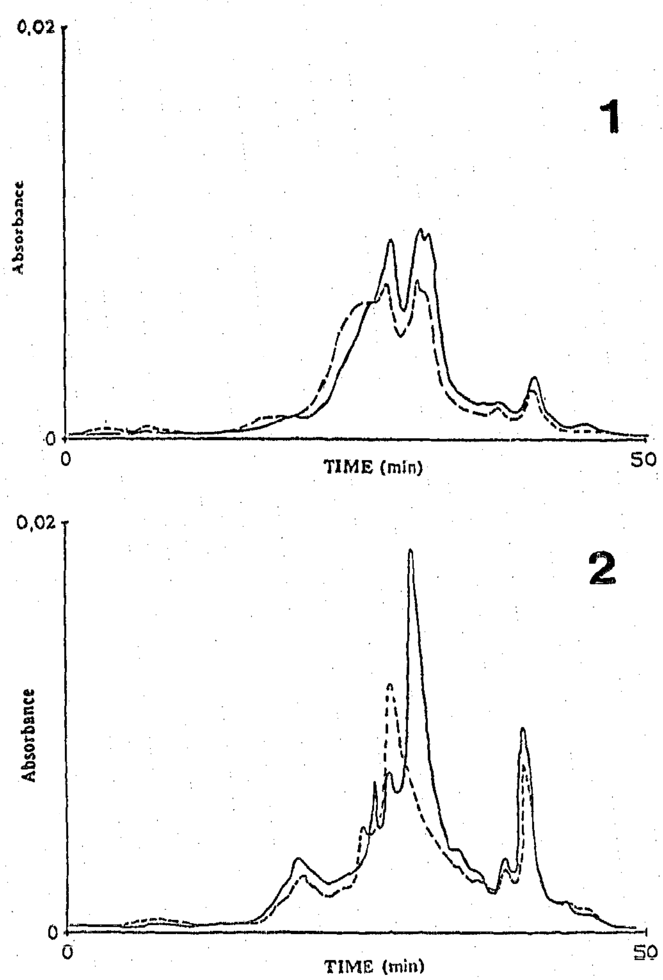

Fig. 7. Wheat flour hydrosoluble products measured before (----) and after 60 min (——) of hydrolysis with B 500 protease in buffer (1) and in 2.5 M glycerol medium (2).

dependent on the particular relation between the additive and the reaction medium.

Under these conditions, the kinetic parameters of the enzyme are strongly modulated. The catalytic efficiency decreases with the glycerol concentration. The decrease in affinity may be related to the increase in medium viscosity due to the presence of glycerol. These results confirm those of Drapron [13] while Xu [22] observed with yeast alcohol dehydrogenase in the presence of sorbitol a decrease in both V_m and K_m indicating that, in this case, the affinity for the substrate was not diminished. This particular effect was attributed to a conformational modification of the enzyme.

On the other hand, the enzymatic cleavage priorities on a well defined substrate appeared modulable following the microenvironment conditions. Sobtilisin BPN' has 5 theoretical hydrolysis sites on the B chain of insulin. In limited proteolysis in buffer medium, only 4 are expressed with an obvious priority for one. This preferential orientation of hydrolysis has been observed on related enzymes: Novo and Carlsberg subtilisins [23].

The modification of enzyme microenvironment, due to high glycerol concentration, has allowed to direct hydrolysis towards a cleavage site never expressed in an aqueous medium. The obtained peptide has a higher hydrophobicity than the substrate from which it arises, so that it is eluted later. Diffusion cannot explain this modification of enzyme reaction.

We suppose that the hydrophobicity of the medium increased by glycerol leads to an increased presentation of hydrophobic parts of the substrate to the enzyme, so that the no. 5 cleavage site, inaccessible in buffer, is hydrolysed first. On the other hand, the preferential site in buffer (site no. 4) is cleaved only after sites no. 3 and 5. A threshold of 7 M glycerol was found necessary to obtain the hydrophobic peptide. Such a polyol concentration corresponds to a water activity of 0.74 and a water concentration of 27.4 M.

The presence of water activity depressors in the reaction medium modified the hydrolysis kinetics of both purifed and insoluble proteins. In contrast to what was obtained with insulin, the enzyme activity was always expressed with a high rate in water-restricted media.

On the other hand, the influence of glycerol (or sorbitol) on protein hydrophobicity seemed to be determining for the enzyme efficiency as an important increase in initial activity was obtained with gliadin hydrolysis medium containing low glycerol concentrations. On the other hand, the polyol was shown to increase the solubility of gluten proteins and did not affect the neutral protease efficiency.

As for the B chain of insulin, the protease selectivity was modified by the presence of glycerol. For gliadins, the order of hydrolysis was modulated, the most hydrophobic proteins, hydrolysed first in buffer did not react in polyol-containing media for concentrations higher than 0.5 M glycerol. The hydrophobicity of the proteic substrates was modified by itself, as their retention times were affected by the concentration of glycerol. A new peptide peak, corresponding to a hydrophobic product appeared. The evolution of hydrolysate products also revealed a modification in hydrolysis priority as compared to the behaviour in buffer (the cleavage bond most hydrolysed in buffer does not react well for high glycerol concentrations).

When gluten was used, the presence of glycerol leads to an increase in available substrate and to the obtaining of new products. The molecular weights of the products are dependent on the nature of the additive used as observed in a comparison between glycerol and sorbitol effects.

The physicochemical properties of the enzyme microenvironment, particularly viscosity, were modified by the presence of the additive. The effects of substrate diffusion, solution viscosity and water concentration on the enzymatic behaviour have been described [24], while relationships between the collisional activation mechanism and exclusion effects on the one hand and Michaelis constants on the other have been demonstrated [25]. The study of viscosity intensifier additives to modulate the activity of a hydrolase has shown that above a viscosity threshold, the medium conditions were determining for the enzymatic activity while below this value, the particular role of each additive was predominant [26]. In the results here presented, the enzyme seemed to be affected by the medium conditions in a similar way; moreover, it was shown that the medium mofification may participate in changing the enzyme selectivity.

Nasri [27] has shown a modification in specificity of restriction enzymes in the presence of glycerol. Under these conditions, the enzymes were able to recognise sites different from standard sites. This relaxation phenomenon was attributed to the loss of hydrogen bonds between endonucleases and each base pair recognised by the enzymes, so that the enzyme specificity diminished. In our case, numerous hydrogen bonds were created between polyol and water molecules and the polyol-containing medium presented an increased hydrophobicity as compared to the buffer medium. The complex interactions between the different forces regulating substrate binding specificity were changed, resulting in a modification of enzyme selectivity.

From our results, we may conclude that the selectivity of proteases is affected by water-structuring additives such as polyols, whatever the size of the substrate and its initial solubility.

With an enzyme of low specificity, an important selectivity restriction was observed. This result is of considerable importance as it could explain how enzymes may be so weakly specific in vitro. In the cell where proteases are synthesised, the water concentration is restricted [7] and we may assert that proteolytic activity cannot be expressed at the maximal rate, and that substrate specificity may be modulated in such a way that the proteins necessary for the cell life are not hydrolysed. In this case, the cell would synthesise aspecific proteases without being affected in its metabolism.

The search for extremely specific natural proteases did not produce the wanted genetically programmed enzymes, but rare exceptions [28], in contrast to what happened for DNA cleaving enzymes with the discovery of restriction enzymes. The in vivo selectivity of proteases could be phenotypic rather than genotypic and thus, partly controlled by the enzyme microenvironment. Our results are a contribution to this hypothesis.

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