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Enzymatic hydrolysis of wheat proteins Part I. Enzymatic kinetics and study of limited hydrolysis in a batch stirred reactor [1]

L. Nouri [a], J. Legrand [a,*], Y. Popineau [b], P. Belleville [a]

[a] *Laboratoire de Génie des Procédés, Université de Nantes, IUT de Saint Nazaire, CRTT-BP 406-44602, Saint-Nazaire Cedex, France*

[b] *Laboratoire de Biochimie et Technologie des Protéines, INRA-BP 71627-44316, Nantes Cedex 03, France*

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Abstract

Gliadin peptides obtained by limited enzymatic hydrolysis were produced for their original properties. Experimental determination of the kinetic constants, K_m and V_{max}, was carried out to characterize the affinity of pepsin for native gliadin and to allow the performance prediction of the bioreactors in which the reaction is performed. Limited hydrolysis of native gliadin by pepsin in a batch stirred reactor was realized to study the effect of substrate concentration and impeller speed on the concentration of the free amino-acid group ($-NH_2$) obtained, and consequently on the degree of hydrolysis (DH). On the other hand, the resulting reaction products were qualitatively analysed by electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC). © 1997 Elsevier Science S.A.

Keywords: Enzymatic hydrolysis; Kinetics; Batch stirred reactor; Wheat proteins

1. Introduction

Plant proteins are extracted from abundant and renewable biological resources. They present a large variety of technological and biological functionalities. In addition their prices are generally lower than those of animal proteins. Therefore, they constitute a very attractive alternative for food and non-food uses.

Furthermore, it is possible to adapt functional properties of the proteins of chemical or enzymatic modifications. Among the possible reactions enzymatic hydrolysis has been shown to produce interesting effects on their technological and biological properties [1,2]. Particularly, limited digestion yields polypeptides and peptides with high solubility, low viscosity in solution and high emulsifying and foaming properties [3]. Experiments carried out on a number of proteins have indicated that the hydrolysis must be performed under strictly controlled conditions to obtain desirable functional properties.

In the case of wheat proteins, it has been shown that limited hydrolysis by protease destroyed viscoelasticity but made gluten soluble over a large pH range. Hydrolysates with a low degree of hydrolysis (DH = 1–2%) exhibit good emulsifying properties [4]. The effects of some proteases were compared [4,5]. Other studies have been carried out on gluten fractions, gliadin and glutenin, and also on a purified gliadin. The main peptide fractions were separated and characterized partially [6,7].

The purpose of this work is to study experimentally the enzymatic hydrolysis kinetics and to perform the limited hydrolysis of native gliadin by pepsin in a baffled stirred reactor with single impeller agitation.

2. Experimental

2.1. Description of the batch stirred reactor

Fig. 1 shows a schematic diagram of the experimental apparatus. The stirred-tank reactor was made of glass and equipped with flat bottom. The diameter (D) and the height of the liquid (H) were 150 mm, which correspond to a working volume (V) of 2.65 l. The reactor has four vertical baffles,

* Corresponding author. Fax: +33 2 40 17 26 18; e-mail: legrand@iutsn.univ-nantes.fr.

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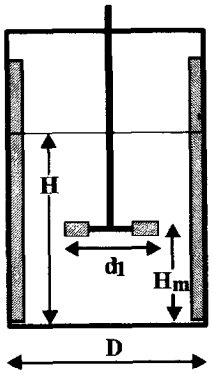

Fig. 1. Schematic diagram of the experimental apparatus.

of 280 mm height and 16 mm radial width, to prevent vortex formation at the free liquid surface.

The impeller was a four-blade paddle type (Rushton turbine) of 60 mm diameter (d_1), situated at $H_m = H/2$ from the tank bottom and was driven at speeds of 100 to 750 rpm, so that the impeller Reynolds number range ($Re_m = \rho N d_1^2 / \nu$) corresponded to 6000–45000.

2.2. Preparation of substrate

The chosen substrate is a native gliadin, obtained by extraction from industrial wheat gluten by a pilot process [8]. This native gliadin is composed of several components with a molecular weight between 30 and 40 kDa. They have a low percentage of basic amino acids and are poorly charged. For this study, the native gliadin was dispersed into 0.1M acetic acid and adjusted to pH 2 with 0.1M HCl.

The hydrolysis of native gliadin was performed by pepsin (Merck EC 3.4.23.1, 2000 U g^{-1}). This enzyme is characterized by a low isoelectric point (Pi < 1) and an optimal pH value towards wheat proteins [9] equal to 2. It has a broad spectrum action attributed to the high flexibility of its active site.

2.3. Determination of the degree of hydrolysis

The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved during the enzymatic reaction [10]. It is expressed by the following relation:

$$DH(\%) = \frac{h}{h_0} \times 100 \quad (1)$$

where h is the number of peptide bonds cleaved during an hydrolysis process, h is called the hydrolysis equivalence and is expressed as equivalents per kg protein; h_0 is the total number of peptide bonds in a given protein per kg protein. h_0 was obtained by hydrolysis of 60 μl of 10 g l^{-1} native gliadin under vacuum in 6M HCl for 24 h at 110 °C in sealed tubes.

The number of free amino groups was determined by reaction with trinitrobenzenesulfonic acid (TNBS). This method can be used for all protein products and protein hydrolysates.

The TNBS reaction was carried out as follows: 0.25 ml of the protein hydrolysate was mixed with 2 ml of 0.2125M sodium phosphate buffer (pH 8) in the presence of 1% SDS and 2 ml of 0.1% trinitrobenzenesulfonic acid, followed by incubation in the dark for 60 min at 50 °C. The reaction is quenched by adding 4 ml of 0.1M HCl and the absorbance is read at 340 nm. Six L-leucine solutions of 0.15–1.5mM were used as standard [10].

2.4. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)

SDS-PAGE was performed on polyacrylamide gradient slabs (13–24% of acrylamide) as described previously [6]. Gels were stained with Coomassie G brilliant blue. Molecular weights for calibration standards were phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000), lactalbumin (14000), and fragments of myoglobin (17200, 14600, 8240, 6380 and 2560 Da). Samples were analysed without and after reduction of the disulfide bond by 2-mercapto-ethanol.

2.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

Analytical separations were carried out on a Nucleosil, C18 column (particle size 5 μm, pore diameter ≤ 300 Å, internal diameter 4.6 mm, and column length 250 mm). Elution was achieved at 1 ml min^{-1} by gradients formed from solvent A (0.1 vol% TFA in distilled water) and B (99.1 vol% acetonitrile, 0.09 vol% TFA). The spectrophotometric detection of peptides was performed at 224 nm. An elution gradient was realized as follows: 15% B during 4 min, 15–45% B during 60 min, 45–80% B during 5 min, 80% B during 2 min and 80–15% B during 4 min.

3. Results

3.1. Kinetic study

The catalysis obtained by the major class of proteases occurs as three consecutive reactions, (1) formation of the Michaelis–Menten complex (ES) between the original peptide chain (substrate) and the enzyme; (2) cleavage of the peptide bond to liberate one of the two resulting peptides; (3) a nucleophilic attack on the remains of the complex to split off the other peptide and to reconstitute the free enzyme.

The enzymatic reaction studied in this work was:

native gliadin + pepsin → peptides

The model of the transient phase equation of the pepsin reaction is represented by the following expression:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_2} H\text{–}P' + E\text{–}P \xrightarrow[H_2O]{K_3} E + P\text{–}OH$$

where K_1, K_{-1}, K_2 and K_3 are the rate constants.

In the aforementioned model, the water activity has an important effect in the catalytic activity. Some studies [13] have shown that the water molecules play a determining role on affinity and specificity. Pepsin hydrolysis of gliadin was realized with different glycerol concentrations in the reaction medium [13]. The results obtained show that the presence of glycerol leads to an important perturbation effect on enzyme activity, due to the high solubility of the polyol. A non-Michaelian behaviour was obtained for glycerol concentrations higher than 1M.

The Michaelis–Menten equation which describes the reaction is expressed by:

$$V_i = V_{max}\frac{[S]}{[S]+K_m} \quad (2)$$

where K_m is the apparent kinetic constant,

$$K_m = \frac{K_{-1}+K_2}{K_1}\frac{K_3}{K_2+K_3}$$

and V_{max} the maximum reaction rate.

Practice trial experiments were carried out to determine the enzyme and substrate concentrations to be used. The determination of the initial reaction rate was obtained from a series of native gliadin concentrations ($0.286 < [S] < 4$mM). The hydrolysis was carried out during 10 min at 25 °C and the reaction was stopped by heating at 100 °C during 5 min after addition of sodium phosphate buffer (pH 8.2) in the presence of 1% SDS to prevent precipitation. The kinetics of native gliadin hydrolysis by pepsin was obtained from the determination of free amino-acid groups ($-NH_2$) by the TNBS method.

The determination of pepsin concentration (2 and 4 mg l^{-1}) was obtained from a preliminary study of the influence of the pepsin concentration on the initial reaction rate (Fig. 2). From the same plot (Fig. 2), the pepsin specific activity towards native gliadin was determined and was found to be equal to 2.5 U g^{-1}, where one unit (U) of proteolytic activity would be one micromole peptide bond cleaved per minute.

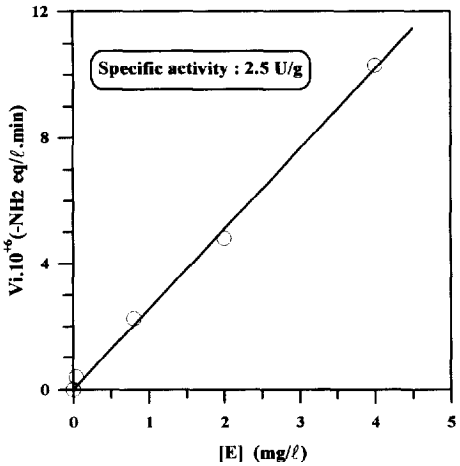

Fig. 2. Effect of pepsin concentration on initial reaction rate.

Fig. 3 shows the variation of the initial reaction rate, V_i, against native gliadin concentration, for two pepsin concentrations, 2 and 4 mg l^{-1}. The initial reaction rate is proportional to the enzyme concentration as predicted by the Michaelis–Menten equation. Then, the maximum reaction rate, V_{max}, is approximately proportional to the pepsin concentration. The determination of K_m and V_{max} is obtained by a direct linear transformation (Hanes' equation) of the Michaelis–Menten relationship (Fig. 4). This representation was recommended by Cornish-Bowden [11] to obtain K_m and V_{max} with good accuracy. The kinetic parameters are given in Table 1. The difference of K_m and V_{max} values observed for the two pepsin concentrations was attributed to experimental uncertainties.

In practice the determination of the kinetic constant parameters requires an acceptable range of substrate concentrations included between $K_m/4$ and $8K_m$ [11]. This condition was approximately respected in this study where the range of native gliadin concentrations used was comprised between

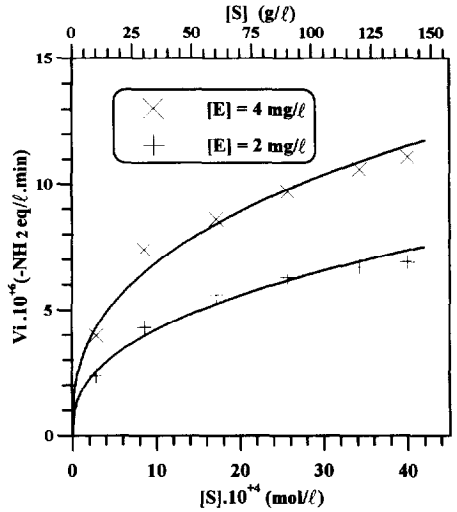

Fig. 3. Effect of native gliadin concentration on initial reaction rate.

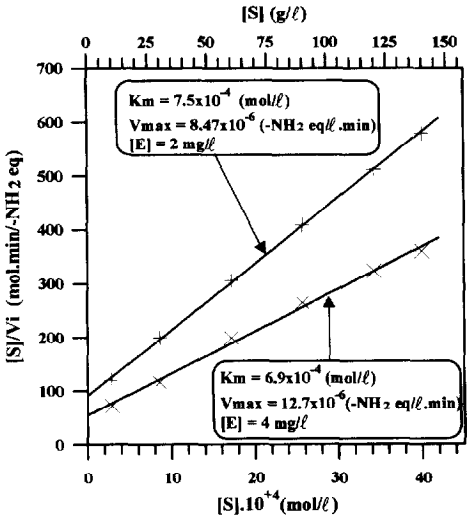

Fig. 4. Determination of kinetic parameters, K_m and V_{max}, by Hanes' representation.

Table 1
Michaelis–Menten constants, K_m and V_{max}, for hydrolysis of native gliadin by pepsin

Pepsin concentration ($mg\ l^{-1}$)	K_m ($mol\ l^{-1}$)	V_{max} ($eq\ -NH_2\ l^{-1}\ min^{-1}\ g^{-1}$)
2	7.5×10^{-4}	4.2×10^{-3}
4	6.9×10^{-4}	3.2×10^{-3}

$0.38K_m$ and $5.3K_m$. These kinetic parameters are used in part II of the article for modelling the enzymatic hydrolysis of native gliadin by pepsin in a torus reactor.

To characterize the affinity between the native gliadin and the pepsin, the Michaelis–Menten constant, K_m, is compared to other values obtained for the hydrolytic kinetics of some synthetic substrates by pepsin [12]. The apparent pepsin affinity is similar for native gliadin and for synthetic substrates (Table 2). The apparent pepsin affinity does not take into account the potential number of peptide bonds, which could be cleaved in the different substrates. On the other hand, the experimental value of K_m is also compared to the one obtained by Hertmanni et al. [13] from hydrolysis kinetics of a mixture of gamma and beta-gliadins by pepsin (Table 2). This difference in K_m values may be attributed to the narrower range of substrate concentrations (3–15 mg ml^{-1}) used by Hertmanni [14].

3.2. Enzymatic hydrolysis of native gliadin in a batch stirred reactor

The enzymatic hydrolysis of native gliadin in a batch stirred reactor was performed with an enzyme/substrate ratio of 1/100. The substrate was dispersed during 4 h at room temperature in 2650 ml of 0.1M acetic acid adjusted to pH 2 with HCl. The hydrolysis was carried out during 420 min at 25 °C. The reaction was stopped by heating at 100 °C, during 5 min, after addition of a sodium phosphate buffer (pH 8.2).

The evolution of the degree of hydrolysis (DH) was quantitatively determined by the trinitrobenzenesulfonic (TNBS) method. The hydrolysate composition obtained from the hydrolysis was followed by SDS-PAGE and RP-HPLC.

Table 2
Different values of the Michaelis–Menten constant, K_m, for the hydrolytic kinetics of some synthetic substrates by pepsin

Substrate	A	B	C	D	E
K_m (10^{-4}M)	6.9	7.7	5.4	130	7.5

A: Z–Phe(NO_2)–Phe–APM* [12].
B: Z–Phe(NO_2)–Val–APM* [12].
C: Z–Ala–Phe(NO_2)–APM* [12].
D: γ-gliadin [13].
E: Native gliadin (this work).
APM*: $NH(CH_2)_3NC_4H_8O$.

3.2.1. Effect of substrate concentration on the enzymatic hydrolysis

For different native gliadin concentrations, 10, 20, 40, 60, 100, and 200 g l^{-1}, the reaction in the batch stirred reactor was performed at low stirring speed ($N = 250$ rpm) to prevent any formation of foam at high substrate concentration. Fig. 5 shows that the variation of the concentration of free amino-acid group ($-NH_2$), obtained by hydrolysis, was proportional to the native gliadin concentration. On the other hand, the corresponding degree of hydrolysis (DH) obtained after 420 min remained approximately constant and is independent of native gliadin concentration. Furthermore, the action of the pepsin during the reaction on the viscosity of the gliadin solution is shown in Fig. 6. The decrease in viscosity is very rapid and corresponds to the breakage of a very small number of peptide bonds. Therefore it does not seem that the initial viscosity of the protein solution would have a limiting effect on the reaction rate.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) patterns show a number of distinct bands. They appear in chronological order and are distinguished by lower apparent molecular weights than

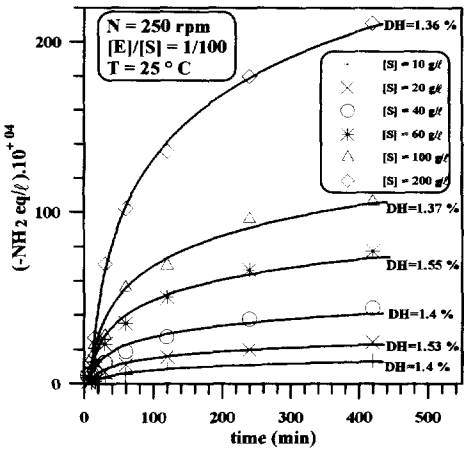

Fig. 5. Influence of native gliadin concentration on the enzymatic reaction in a batch stirred reactor.

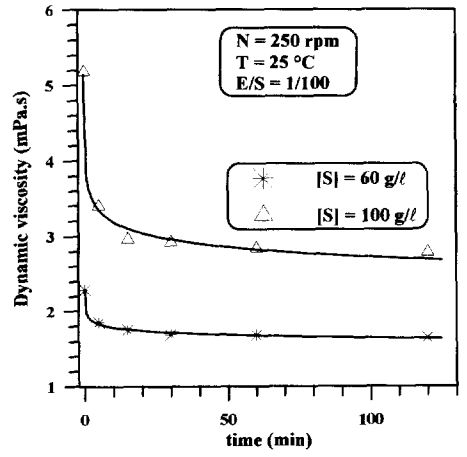

Fig. 6. Variation of the dynamic viscosity during the enzymatic reaction of native gliadin.

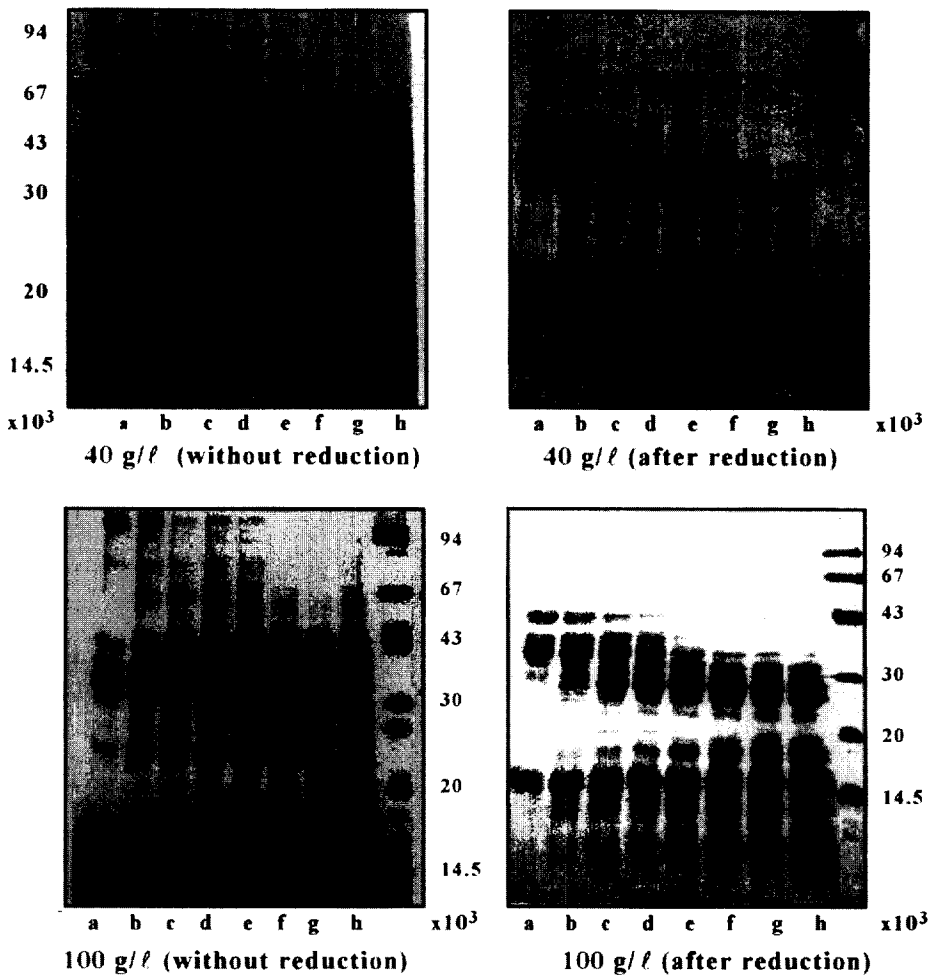

Fig. 7. SDS electrophoresis patterns of native gliadin hydrolyzed by pepsin for different native gliadin concentrations: incubation times, a: 0 min; b: 5 min; c: 15 min; d: 30 min; e: 60 min; f: 120 min; g: 240 min; h: 420 min.

the native gliadin bands (Fig. 7). Without reduction, patterns show that the pepsin action on the native gliadin results in the formation of a limited number of peptides, suggesting that intermolecular disulfide bonds limit the release of the peptide fragments. For different native gliadin concentrations, 40 and 100 g l^{-1}, no difference was observed on the appearance kinetics of the bands and their apparent molecular weights. The concentration of the major bands corresponding to the molecular weight of the native gliadins (35000–41000) decreased progressively and tended to disappear for the longest hydrolysis times. On the other hand, the peptides resulting from hydrolysis had apparent molecular weights of 30000, 28000, 26000, 18000, 17000, and 15000. The reduction of disulfide bonds by 2-mercapto-ethanol resulted in the formation of even smaller peptides with apparent molecular weights lower than 14000. Their concentration tended to increase with longer hydrolysis times. The change in pattern after reduction confirms that the same peptides are linked together by disulfide bridges.

Reversed-phase high performance liquid chromatography (RP-HPLC) emphasizes the complexity of the native gliadin sample and that of the hydrolysates (Fig. 8). However, the effect of enzymatic cleavage of the peptide bonds can be

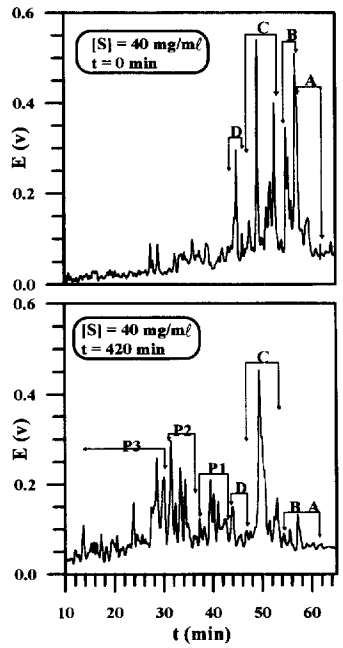

Fig. 8. RP-HPLC of native gliadin hydrolyzed by pepsin for various incubation times and for different native gliadin concentrations.

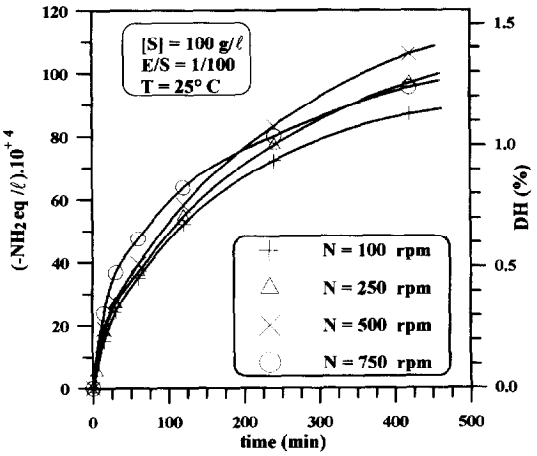

Fig. 9. Effect of impeller speed on the enzymatic reaction in a batch stirred reactor.

easily followed on the RP-HPLC patterns. They show that the peptides were eluted earlier than native protein due to a lower hydrophobicity and to their smaller size. But even after 420 min of hydrolysis a significant amount of peptides were eluted after 45 min, i.e. as native gliadins. This is not unexpected since SDS-PAGE shows that the same large peptides with apparent molecular weights 15000–30000 were still present in the hydrolysate after 420 min. This indicates that some peptides are resistant to pepsin action. This was described previously in studies of the hydrolysis of gamma-gliadin by pepsin and chymotrypsin [6,7]. It was also shown on gamma-gliadin hydrolysates that earlier eluting peptides and later eluting peptides arise from two different regions of the protein [6,7]. The results obtained here suggest that the same types of peptide are produced when whole gliadin is hydrolysed by pepsin. The same effect was observed when whole gluten was submitted to enzymatic hydrolysis [4]. Increasing the protein concentration in the 10–150 g l^{-1} range did not change the composition of hydrolysates for a given degree of hydrolysis.

3.2.2. *Effect of the impeller speed on the enzymatic hydrolysis*

The impeller rotational speed (N) in the batch stirred reactor was varied from 100 to 750 rpm. The native gliadin concentration and enzyme/substrate ratio were respectively 100 g l^{-1} and 1/100. The reaction was carried out during 420 min at 25 °C.

It can be seen in Fig. 9 that the degree of hydrolysis, and therefore the concentration of free amino-acid groups ($-NH_2$), is proportional to impeller speed for low rotational speed. At an impeller speed of 750 rpm the degree of hydrolysis was affected by the formation of foam generated by the high impeller rotational speed and favoured by foaming properties of the peptides obtained by enzymatic hydrolysis. In this case, the degree of hydrolysis decreased from 1.4% to 1.25% respectively for 500 and 750 rpm.

For two stirring speeds, 250 and 500 rpm, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) without reduction (Fig. 10) showed that the peptides obtained by hydrolysis are characterized by similar mobility and apparent molecular weight. Otherwise, the appearance kinetics of the bands corresponding to the different products is nearly independent of impeller rotational speed. On the other hand, reversed-phase high performance liquid chromatography (RP-HPLC) patterns (Fig. 11) showed that after 420 min of reaction the concentration of hydrophobic peptides obtained was clearly important at high stirring speed and consistent with the value of the degree of hydrolysis. Both electrophoresis and RP-HPLC patterns show that some peptides can withstand high stirring speeds.

3.2.3. *Effect of temperature on the enzymatic hydrolysis*

The temperature is a determining factor in enzyme activity and consequently of the number of peptide bonds cleaved during an hydrolysis process. In this last part, the enzymatic reaction in a batch stirred reactor has been carried out at three temperatures, 25, 37 and 50 °C. It can be seen in Fig. 12, that

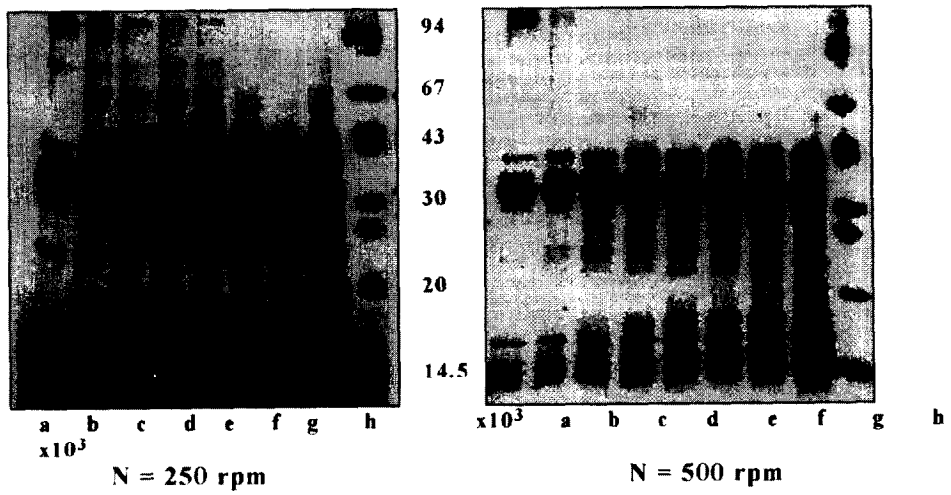

Fig. 10. Effect of impeller speed on the SDS electrophoresis patterns of native gliadin hydrolyzed by pepsin: incubation times, a: 0 min; b: 5 min; c: 15 min; d: 30 min; e: 60 min; f: 120 min; g: 240 min; h: 420 min.

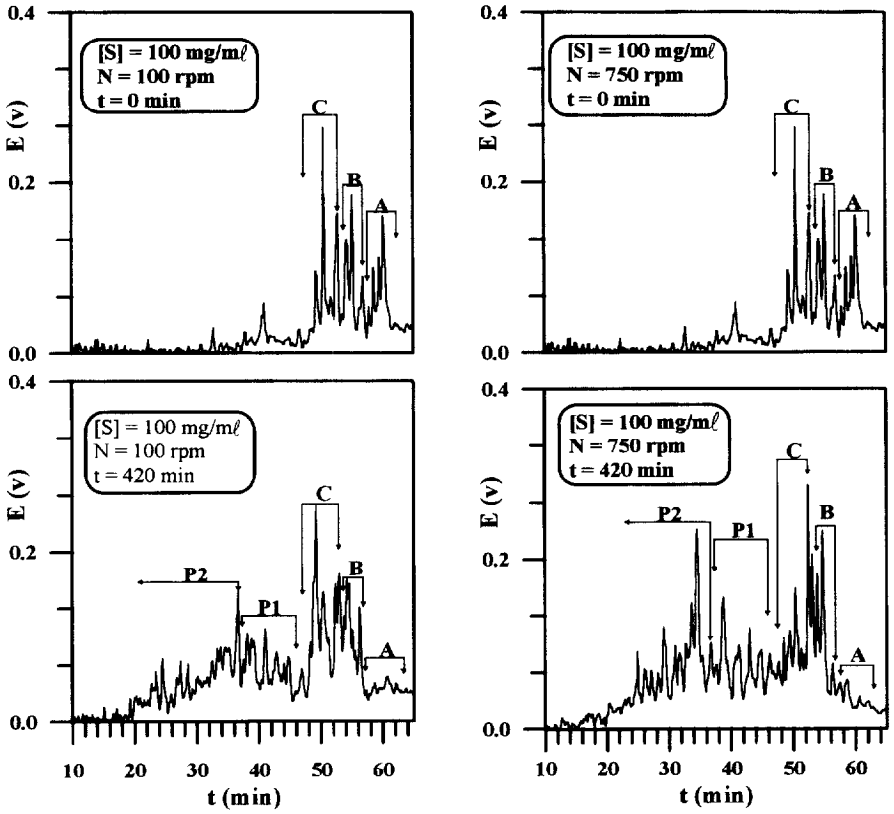

Fig. 11. RP-HPLC of native gliadin hydrolyzed by pepsin for various incubation times and for different impeller speeds.

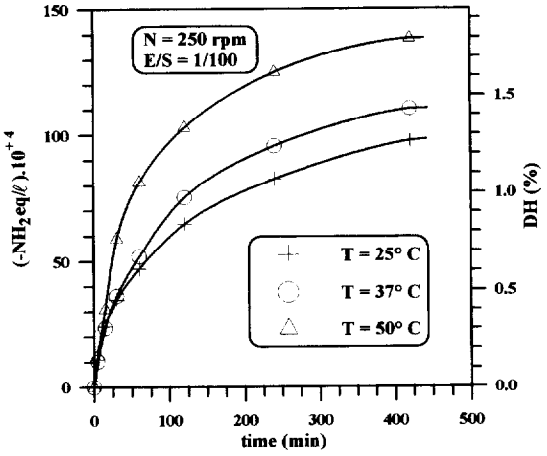

Fig. 12. Effect of the temperature on the enzymatic reaction in a batch stirred reactor.

the degree of hydrolysis after 420 min of reaction increased from 1.26% for 25 °C to 1.8% for 50 °C, an overall increase of 43%. Accordingly, SDS-PAGE without reduction (Fig. 13) shows that the disappearance kinetics of the major bands (35000–41000) corresponding to the native gliadin is more rapid at 50 °C than at 25 °C, and consequently, the concentration of peptides of lower apparent molecular weight (13000–15000) obtained is more important at the higher working temperature.

4. Conclusion

The experimental results reported in this paper can be summarized as follows.

In the first part, in spite of the complexity of the substrate, the enzymatic kinetics follows the Michaelis–Menten model. The kinetic constants, K_m and V_{max}, for pepsin hydrolysis of native gliadin were determined. The pepsin affinity towards native gliadin is similar to that towards synthetic substrates.

In the second part, pepsin hydrolysis of native gliadin was carried out in a batch stirred reactor. The results obtained from this study are the following.

- The concentration of the free amino-acid group ($-NH_2$) or the degree of hydrolysis is dependent on the substrate concentration. Moreover, the enzymatic hydrolysis is not inhibited by the substrate.
- No effect of substrate viscosity on the kinetics of hydrolysis was observed. This result is interesting for the industrial application of this reaction, because of the possibility of working at high substrate concentration.
- In a low range of stirring speed, the degree of hydrolysis of the reaction is proportional to the impeller rotational speed.
- For high stirring speeds, the pepsin hydrolysis reaction is affected by the formation of foam in the reaction medium.
- It is possible to modify the kinetics by controlling the temperature.

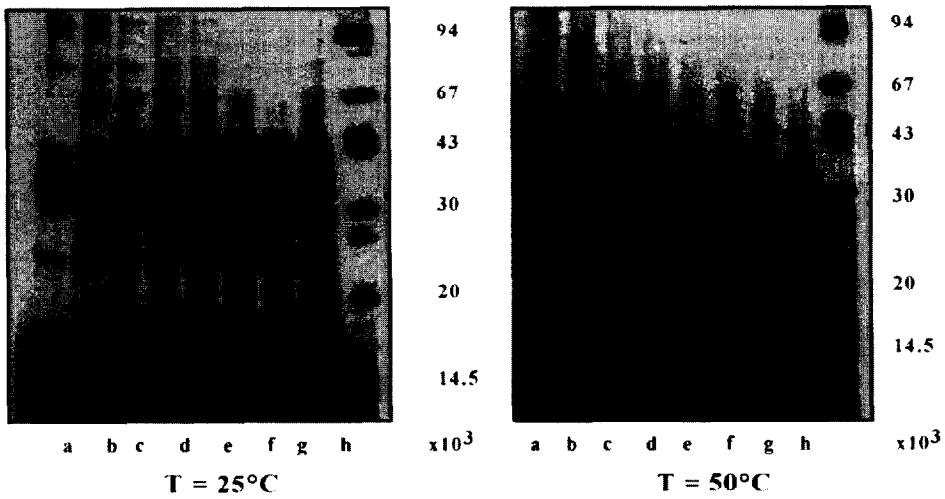

Fig. 13. Effect of the temperature for SDS electrophoresis patterns of native gliadin hydrolyzed by pepsin: incubation times, a: 0 min; b: 5 min; c: 15 min; d: 30 min; e: 60 min; f: 120 min; g: 240 min; h: 420 min.

- Qualitatively, for a given degree of hydrolysis, the products resulting from the reaction are independent of substrate concentration, stirring speed and working temperature.
- The results obtained showed a correlation between the degree of hydrolysis and the quality and the size of the resulting products.

Appendix A. Notation

D	Diameter of the reactor
d_1	Diameter of the impeller
DH	Degree of hydrolysis
H	Height of liquid in the reactor
h	Number of peptide bonds cleaved during an hydrolysis process per kg protein
h_0	Total number of peptide bonds in given protein per kg protein
K_m	Apparent kinetic constant
N	Impeller speed
Re_m	Impeller Reynolds number ($\rho N d_1^2/\nu$)
U	Unit of proteolytic activity (one micromole peptide bond cleaved per minute)
V	Volume of the reactor
V_{max}	Maximum reaction rate
[E]	Enzyme concentration
[S]	Substrate concentration
SDS-PAGE	Electrophoresis in presence of sodium dodecyl sulfate
RP-HPLC	Reversed-phase high performance liquid chromatography
TNBS	Trinitrobenzenesulfonic acid
TFA	Trifluoracetic acid

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