

HYDROLYSIS OF CASEIN AND β -LACTOGLOBULIN BY IMMOBILIZED PAPAIN AFTER PRE-TREATMENT WITH IMMOBILIZED TRYPSIN

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Protein hydrolysis using immobilized proteases may present problems, which are mainly caused by the diffusion of macromolecular substrate. Pre-hydrolysed substrate could be a viable alternative in this process. The aim of this work was to test the casein and β -lactoglobulin hydrolysis using immobilized papain on glyoxyl-agarose, following substrate pre-treatment with immobilized trypsin. Although immobilized papain showed difficulties to degrade the milk proteins, after 10 min of trypsin pre-hydrolysis, the immobilized papain was able to achieve the maximum degree of hydrolysis in shorter time. For pre-hydrolysate casein, the immobilized papain reached 98.9% at 60 min total reaction (including the 10 min pre-digestion). As for β -lactoglobulin, immobilized papain was capable of achieving maximum levels after just 60 min of reaction, where the free enzyme only achieved 60% of its maximum. Pre-hydrolysis with trypsin appears to have worked well as a pre-treatment method to increase the proteolytic action of immobilized papain.

Keywords: casein, β -lactoglobulin, papain, trypsin, enzyme immobilization, glyoxyl-agarose

Protein hydrolysates containing specific short-chain peptides have been highly desired in clinical diets or specific formulations (CLEMENTE, 2000). Enzymatic protein hydrolysis could release peptides, which provide health benefits beyond the basic nutritional effects, depending on protein amino acid composition and sequence. Milk proteins are well documented as “sources” of bioactive peptides (OTTE et al., 2007) that can have beneficial health effects on cardiovascular, digestive, immune, and nervous systems (SILVA & MALCATA, 2005). On the other hand, cow milk proteins constitute one of the most important food allergens in young children’s diet (SAMPSON, 1999). Caseins and β -lactoglobulin are considered major causes of allergic response to cow milk (EL-AGAMY, 2007; DUPONT et al., 2010). Since β -lactoglobulin is a major whey protein component in cow, buffalo, sheep, goat, mare, and donkey milk and it is absent in human milk, this protein could be resistant to infant gastric digestion (KLEBER et al., 2004). Therefore, cow milk hydrolysate proteins are also considered viable alternatives for infant formulas.

Although the appropriate choice of the enzymatic processing can provide specific and preserved peptides, enzymatic hydrolysis may present certain difficulties. For example, food samples may exhibit variable composition, being a rich culture medium. Hydrolysis at mild temperature conditions may provide microbiological growth, which calls for the use of higher processing temperatures. In this case, the thermal stability of the immobilized enzymes could

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be an attractive alternative. Multipoint enzyme immobilization can enhance their stability via the prevention of subunit dissociation and, moreover, the unfolding rate of protein chains, promoting “rigidification” of three-dimensional enzyme structures (ULBRICH-HOFMANN et al., 1999; MATEO et al., 2006). Different supports can be used. Glyoxyl-agarose support containing a large number of stable aldehyde groups can immobilize proteins via, at least, a two-point reaction involving the region/s of the protein surface with high densities of amino groups (MATEO et al., 2006). In addition to stabilization, the use of immobilized enzymes renders inactivation of the enzyme at the end of the process unnecessary, since the catalyst can be easily removed from the reaction medium. This avoids the usual thermal inactivation as the final step and its consequent protein/peptide aggregation, and could be especially important in limited hydrolysis, making it possible to obtain greater control of the desired hydrolysis degree (YUST et al., 2010).

Gastrointestinal digestion is the natural route to produce bioactive peptides through food protein hydrolysis, but other non-digestive enzymes have been studied for the release of different fragments with a wide range of actions (CLEMENTE, 2000; TAVANO, 2013). Papain, a member of the cysteine protease family, is an endopeptidase extracted from the latex of *Carica papaya*, which is widely studied for food process applications. Papain is a relatively heat stable and simple protein, presenting a single and small chain (YEOM et al., 1999), which makes it interesting for immobilization purposes. The aim of this work was to test hydrolytic response of casein and β -lactoglobulin using papain immobilized onto glyoxyl-agarose, considering trypsin pre-digestion.

1. Materials and methods

1.1. Reagents

Agarose 4BCL was from GE HealthCare Life Science, papain from papaya latex (P-4762), trypsin from porcine pancreas (T-0303, type IX-S), casein (C-7078) and β -lactoglobulin (L-3908) from bovine milk were from Sigma Chemical Co.

1.2. Methods

All experiments were performed at least in triplicate and the results are presented as their mean values. Experimental error never exceeded 5%.

1.2.1. Enzyme immobilization. Glyoxyl-agarose support was prepared with the highest activation degree, as previously described by GUIÁN (1988). To 73 g of this agarose 50 ml of distilled water, 34 ml of 1.7 N NaOH containing 0.95 g of sodium borohydride, and 6.7 ml of glycidol (2,3-epoxy-propanol) were added. After 18 h of agitation, the support was washed with abundant distilled water and vacuum filtered. Glyoxyl-agarose was prepared with 825 ml of water and 175 ml of 0.1 M sodium periodate. The consumption of periodate (directly related to the aldehyde residue generated in the support) was checked by adding 0.2 ml of the supernatant of the oxidizing suspension to a mixture of 1.5 ml of 10 g/100 ml KI and 1.5 ml of saturated sodium bicarbonate. The absorption was read at 419 nm, considering the initial sodium periodate solution as 100% (0% aldehyde production). Enzyme derivatives were prepared using this highly activated glyoxyl-agarose support (71 micromoles of glyoxyl groups/g). This procedure enables the enzymes to interact with support through multiple

points. Increasing protein:support ratios were tested for each enzyme (trypsin: 0.2–3.0 mg g⁻¹ support; papain: 0.5–10.0 mg g⁻¹ support), with the highest possible protein loads being achieved using 2.0 mg g⁻¹ for trypsin and 8.0 mg g⁻¹ support for papain. The prepared support was suspended in an enzyme solution (10 g gel: 100 ml enzyme solutions in 100 mM sodium bicarbonate buffer pH 10.2) and gently stirred at 5 °C for 24 h. Prepared biocatalysts (derivatives) were reduced by adding sodium borohydride to reach 1 mg ml⁻¹ (MATEO et al., 2006).

1.2.2. Protein determination. Protein concentration was determined by the BRADFORD (1976) method using bovine serum albumin (BSA) as a standard.

1.2.3. Enzymatic activity. Trypsin activities were measured using BAPNA (benzoyl-DL-arginine-*p*-nitroanilide) as substrate (40 mg of BAPNA in 1.0 ml dimethyl sulfoxide in a total volume of 100 ml using 0.02 M Tris buffer, pH 8.2, as described by KAKADE and co-workers (1974); and papain activities were also measured using BAPNA in 0.02 M Tris buffer containing 1 mM EDTA and 5 mM cysteine, pH 7.0, as described by YEOM and co-workers (1999) with some modifications. Briefly, to 500 µl of enzyme (or 0.1 g of derivatives) 2.0 ml of substrate solution was added and the reaction mixture was incubated at 50 °C for 10 min. The reaction was stopped by adding 250 µl of 30% acetic acid and the Abs was measured at 410 nm. One unit of activity is arbitrarily defined as an increase of 0.01 Abs/min.

1.2.4. Influence of temperature and pH on the activities of immobilized enzymes. The effect of pH on immobilized enzymes was determined in 0.02 M TRIS-HCl buffer (containing 1 mM EDTA and 5 mM cysteine when papain was used) for pH 6.0–8.0 range, and 0.01 M glycine-NaOH buffer for pH 9.0, at 50 °C. The effect of temperature on the activity of enzymes was determined from 37 °C to 80 °C, at pH 7.0.

1.2.5. Casein and β-lactoglobulin hydrolysis. Proteins were dissolved in 0.02 M TRIS-HCl buffer, pH 7.0, at a concentration of 2.4 g/100 ml and 0.6 g/100 ml, in order to simulate the same concentration of these proteins in normal cow milk. Digestions were carried out using 1:100 enzyme to protein ratio, for 120 min, at 60 °C. At different times aliquots of the supernatant were withdrawn. The reaction was stopped by boiling for 5 min. The extent of hydrolysis was measured by the increase of free amino groups, using OPA (*o*-phthalaldehyde) reagent as describe by CHURCH and co-workers (1983) with some modifications. Briefly, to 10 to 100 µl of hydrolysate 1.0 ml of OPA reagent was added, the solution was mixed briefly, and the absorbance at 340 nm was measured after exactly 2 min. A standard curve of L-leucine was constructed. The ratio used to the calculation of the percent hydrolysis (% H) was:

$$\% H = (AAs - AAba) / AAtm$$

where: AAs=moles of amino acids determined in the sample supernatant, AAba=moles of amino acids determined in the sample supernatant without hydrolysis, and AAtm=the calculated total moles of amino acids in the sample considering the mean molecular weight of the amino acids (MW=113).

2. Results and discussion

Using the glyoxyl-agarose support, it was possible to achieve high percentages of immobilization for both enzymes, as described in Table 1.

Table 1. Immobilization parameters of papain and trypsin on glyoxyl-agarose support

Enzyme	Initial protein charge (per mg support)	Immobilized protein/g derivative	Immobilization (%)*	Activity recovered** (%)
Trypsin	2.0	1.94 mg	96.7%	32.80
Papain	8.0	6.8 mg	84.9%	47.33

*: (Initial protein concentration – final protein concentration in supernatant solution/initial protein concentration) × 100

** : Considering (total activity of derivative/total initial activity of enzyme) × 100

Both enzyme suspensions kept 100% of the initial activity throughout the exposition time at pH 10.2 buffer during the immobilization procedure. pH stability of enzymes is an important factor to ensure the success of this procedure, since the immobilization on glyoxyl support is typically performed at high pH, where the reactivity of the terminal amino groups and ϵ -amino groups of the lysine residues of protein surface may be significant and multipoint interactions can be (MATEO et al., 2006). As previously mentioned, papain is particularly interesting for this immobilization procedure, since it presents a single peptide chain of 211 amino acid residues out of which 11 are lysine residues, but none of these residues is in its active site (YEOM et al., 1999; ROY et al., 2005). After the reduction of derivatives with sodium borohydride, their recovery activities were determined (Table 1). The observed loss of activity is probably related to diffusional problems. Diffusional limitations are usually considered to be a problem that reduces the immobilized enzyme activity when the substrate diffusion inside the support particle is slow. In this case, the enzymes in the core of the derivative particle will not reach the substrate as the surface enzymes would (RODRIGUES et al., 2013).

The goal of this work was to use immobilized trypsin as a pre-treatment possibility to improve papain performance. Thus, it was necessary to establish the best conditions for the immobilized papain action, where trypsin derivative also presents very good performance. The effects of pH and temperature on immobilized enzymes have been determined (Figs 1 and 2).

Papain derivative, as well as its free form, had better results when pH 7.0 was applied (Fig. 1A). By increasing the pH value to 7.5, activities of papain preparations were decreased by about 50%. Trypsin preparations presented optimum value at pH 8.5 (Fig. 1B), but at pH 7.0 the residual activity was over 75% to immobilized trypsin. Considering these results, pH 7.0 was chosen as the suitable environment to maintain both enzymes under the necessary conditions for proteins hydrolysis. When the optimum temperature was established, the activities of papain preparations were increased by increasing the temperature from 37 °C to 60 °C (Fig. 2). Above the optimum temperature (60 °C for both papain samples) a clear reduction in its activities was observed. The immobilized trypsin presented about 90% of activity at 60 °C (maximum activity at 65 °C). Thus, 60 °C and pH 7.0 were the choices to meet the best papain conditions, where trypsin derivative also presents very good activity.

Figure 2 also indicates that the immobilized enzymes were actually more stable than the free enzymes. At 65 °C more than 80% of the original activity was lost for papain free enzyme, while the immobilized form presented about 65% of residual activity. This could be related to a higher stability of the immobilized form via rigidification of the enzyme structure. This effect was more pronounced in the trypsin assay (Fig 2). For free trypsin the optimum temperature was 60 °C, while the immobilized enzyme presented about 90% of activity at 60 °C and maximum activity at 65 °C, maintaining a high activity even up to 80 °C (about 85%). At this temperature the free trypsin was almost inactive.

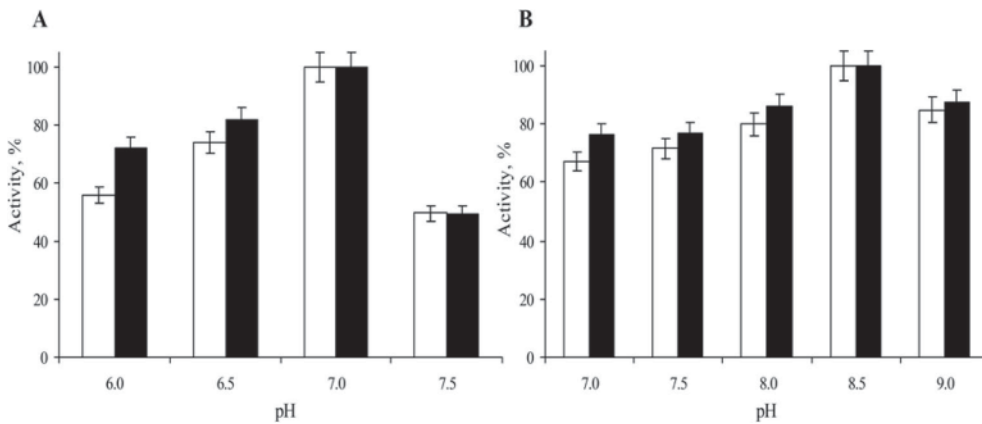


Fig. 1. Effect of pH on free and immobilized papain (A) and trypsin (B) activity, measured at 50 °C, using BAPNA substrate, as detailed in Methods. Error bars show standard deviation. Results are means of triplicate determinations. □: free; ■: immobilized

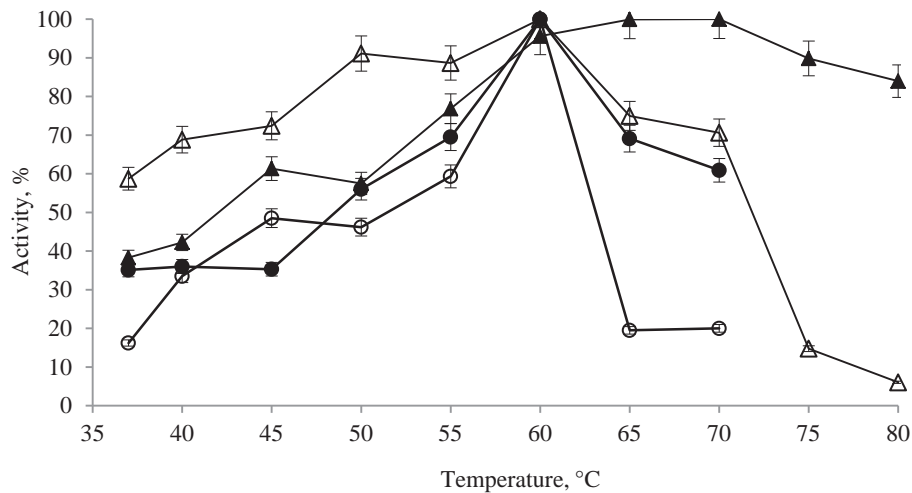


Fig. 2. Effect of temperature on activity of free and immobilized papain (○, ●) and trypsin (△, ▲), measured at pH 7.0, using BAPNA substrate, as detailed in Methods. Error bars show standard deviation. Results are means of triplicate determinations.

Casein and β -lactoglobulin were able to be degraded when subjected to both papain preparations (Fig. 3A and B). The slower hydrolysis rate using immobilized trypsin was expected, and appears to be more related to diffusional problems of substrates, as described previously, than environmental conditions.

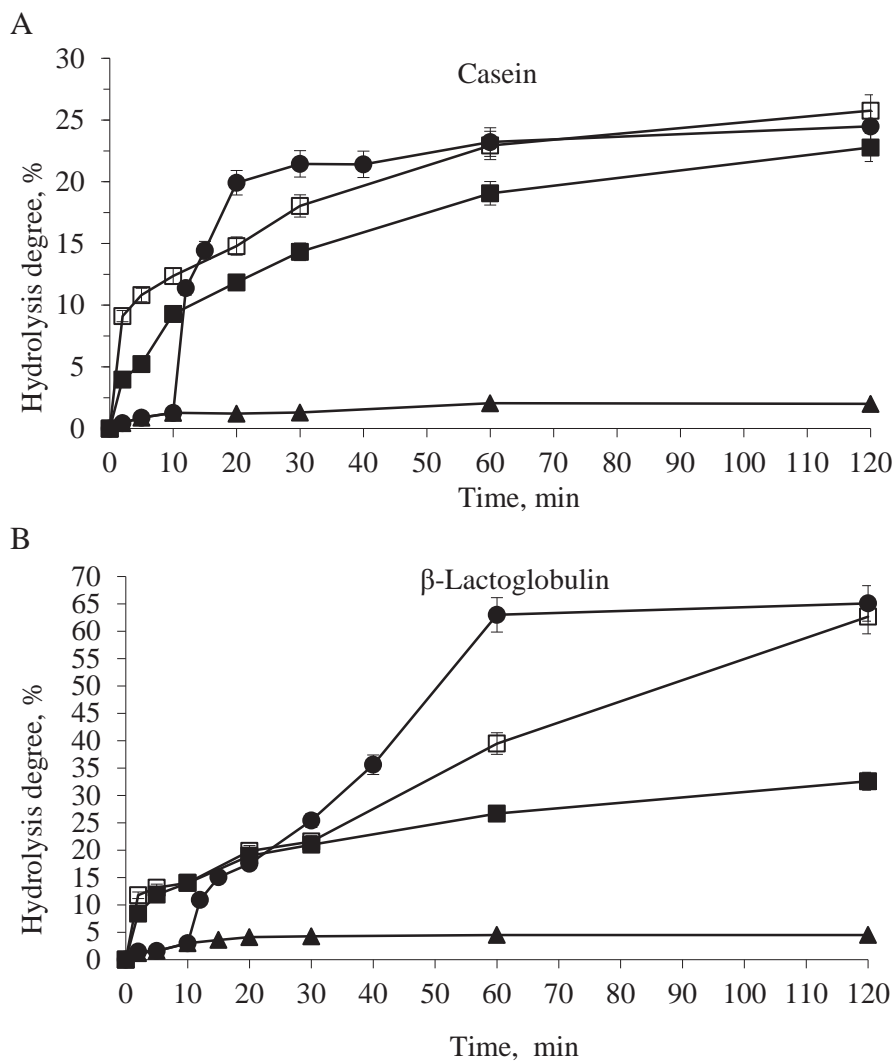


Fig. 3. Hydrolysis patterns for casein (A) and β -lactoglobulin (B) by free papain (\square), by immobilized papain (\blacksquare), immobilized trypsin (\blacktriangle), and by immobilized trypsin (10 min) combined with immobilized papain (\bullet), at 60 °C and pH 7.0, as detailed in Methods. Error bars show standard deviation. Results are means of triplicate determinations

As it is known, K_m value can increase due to restricted diffusion of substrate or the high structural rigidity of immobilized enzyme (RODRIGUES et al., 2013; TAVANO et al., 2013). HOMAEI and co-workers (2010) observed a slight increase in K_m value when papain was

immobilized in cyanogen bromide activated Sepharose. In immobilized protease applications, the macromolecular nature of the substrates may be a problem (RODRIGUES et al., 2013). This can be particularly important at the beginning of the proteins hydrolysis process, when whole proteins are present and the large size of their chains could restrict the access to the inside of the derivative. In the course of the reaction, surface immobilized enzymes begin to release smaller fragments, which make the substrate solution more susceptible to derivative hydrolysis, progressively overcoming the support barrier. The larger and more complex the protein chain is, the bigger difficulties in starting hydrolysis can appear. This can be clearly noted about casein profile, and especially in the β -lactoglobulin hydrolysis profile (both complex protein chains), when immobilized papain was used on comparison to the free enzyme (Fig. 3A and B).

As a means of speeding up the reaction, the purpose was the pre-hydrolysis of the milk substrates with immobilized trypsin before using papain immobilized for digestion. Casein and β -lactoglobulin hydrolysis profile using immobilized trypsin (Fig. 3A and B) indicated that 10 min of digestion process was enough for digestion by immobilized trypsin to reach the highest possible hydrolysis degree, and, on the other hand, to avoid a very time consuming step on the protocol. This procedure was enough to accelerate the papain derivative performance. In fact, when the pre-hydrolysis was used, the immobilized papain was able to achieve the maximum degree of hydrolysis (considering the level reached at the end of the free enzyme process) in shorter times. As shown in Figure 3, for pre-hydrolysate casein, the immobilized papain reached 19.9% of hydrolysis within the first 10 min of contact with this digest, which means after a total 20 min hydrolysis, and reaching 23.22% at 60 min total reaction (including the ten min pre-digestion). The highest casein hydrolysis degree was 24.49%. As for β -lactoglobulin (Fig. 3B), immobilized papain was capable of achieving maximum levels of hydrolysis after only 60 min of reaction (63.0%), where the free enzyme only achieved about 60% of its maximum activity (39.47%). These results suggest that in fact the macromolecular characteristic of substrates hinders the access to immobilized enzymes, since the same substrate, when offered as smaller fragments, facilitated the course of hydrolysis. Given these observations, immobilized trypsin could be applicable for this pre-hydrolysis step, partly due to its specificity, which cleaves Lys and Arg amino acid residues. The large generated fragments may well contain further sites for hydrolysis by surface or internal immobilized papain, which catalyses hydrolysis of peptide bonds of basic amino acids such as leucine or glycine (GANAPATHY et al., 2001).

These observations demonstrate that conducting hydrolysis with the use of immobilized enzymes can be highly feasible. While there may be problems, such as diffusional difficulties, and consequent reduction in the speed of reaction, these can be circumvented. Beside that, we have to consider the ease of handling of the catalyst and the possibility of interrupting the reaction, which may allow for greater control of the process and products generated.

3. Conclusions

Pre-hydrolysis with trypsin appears to work well as a pre-treatment method for the action of papain. The pre-hydrolysis reduced the time needed for immobilized papain to reach the hydrolysis levels of its free form when β -lactoglobulin and casein were tested. Protein hydrolysis using immobilized proteases can have disadvantages when compared to the free enzyme, such as reducing the release rate of the peptides. But if this problem is mainly

caused by the diffusion of macromolecular substrate, as suggested in this study, it can be circumvented by using a pre-hydrolysed substrate.

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