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Partial Purification and Immobilization/Stabilization on Highly Activated Glyoxyl-agarose Supports of Different Proteases from Flavourzyme

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The fractioning of some components and their immobilization of Flavourzyme, a commercial protease/aminopeptidase preparation, has been investigated to improve its specificity and stability. Adsorption of Flavourzyme on two ionic exchangers yielded two fractions with endoprotease activity and one fraction containing aminopeptidase activity. The use of an amine agarose gel has made it possible to purify a 43 kDa protein with only endoprotease activity. Immobilization of this endoprotease and the original Flavourzyme preparation onto glyoxyl-agarose provided derivatives that were more thermostable than their soluble counterparts. Tests using immobilized Flavourzyme and immobilized purified endoprotease for the hydrolysis of chickpea proteins showed that both preparations can be used for the production of protein hydrolysates and compare very favorably with the original crude Flavourzyme in terms of reducing the production of free amino acids. This was especially so in the case of immobilized endoprotease, which produced only 0.2% free amino acids. Keeping free amino acids content low is very important in protein hydrolysates for nutritional use to avoid excessive osmotic pressure.

KEYWORDS: Flavourzyme; immobilization; glyoxyl; protein hydrolysates

INTRODUCTION

Commercially available crude proteinase preparations are used extensively in the food industry to prepare protein hydrolysates with better nutritional or functional properties than intact protein (*1*, *2*). Manipulation of the conditions under which hydrolysis is carried out can be used, to some extent, to define the characteristics of the final hydrolysates. However, the specificities of the enzymes that are used determine the type of peptides that are produced and, therefore, the properties of the resulting food protein hydrolysates (*3*).

Flavourzyme, commercialized by Novo Nordisk, is a fungal protease/aminopeptidase mixture produced by fermentation of a selected strain of *Aspergillus oryzae* that is used for the hydrolysis of proteins under neutral or slightly acidic conditions. The optimal working conditions for Flavourzyme are reported to be pH 5.0−7.0 and 50 °C. The use of Flavourzyme facilitates the production of protein hydrolysates without the otherwise characteristic bitter taste (*4*).

Some of the enzymatic preparations that are used in food processing are not highly purified and contain undesirable enzymatic activities. Thus, the development of simple and rapid protocols to purify or at least fractionate the different components in these preparations is of great interest. Adsorption of proteins on ionic exchangers, requiring the interaction between active groups in the support and the protein (*5*), is one of the simplest and most used techniques for protein purification (*6*, *7*).

To use proteases or other enzymes at industrial scale, they usually need to be modified so that they can be recycled and reused. Although this can be accomplished using very different techniques, most efforts have focused on immobilization following different approaches (*8*). A second limiting factor for the implementation of enzymes as industrial biocatalysts is stability (*9*, *10*). Although several procedures have been employed to achieve stabilization of enzymes (e.g., genetic and protein engineering techniques) (*11*, *12*), coupling immobilization to stabilization has the advantage of facilitating both recycling and stabilization requirements. The immobilization/stabilization of enzymes can be achieved by multipoint attachment to porous solid supports, which increases the rigidity of the immobilized enzyme molecules and may result in a higher resistance to small conformational changes caused by heat, organic solvents, denaturating agents, etc. (*13*−*15*). However, the immobilization system and the reaction conditions have to

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be carefully chosen and optimized to succesfully induce intense covalent multipoint attachment (*16*−*18*).

Glyoxyl supports constitute a very good substrate for the immobilization/stabilization of proteins via multipoint covalent attachment (*18*−*20*). Many proteolytic enzymes have been stabilized using this technique, including trypsin, quimotrypsin, carboxypeptidase A, and Alcalase (*18*). The active glyoxyl groups found in these supports are aldehydes moderately separated from the support surface that form reversible and relatively weak Schiff bases with amine groups in enzymes (*18*, *21*). Because this initial link is weak, attachment of the enzyme to the matrix requires the formation of at least two simultaneous bonds (*19*, *20*). The driving force of the immobilization is not the reactivity of a single residue but the density of reactive groups on the protein surface. The Schiff bases can be later on stabilized by reduction to amine bonds (*18*). Pedroche et al. (*22*) showed that there is a direct correlation between the number of enzyme-support links and thermal stability and that some variables such as thickness of the agarose fiber and reaction time increase the number of enzyme-support bonds and stability.

The goal of the present work was to purify as much as possible the different enzymatic components in Flavourzyme by adsorption on ionic supports and to explore the possibility of immobilizing the original Flavourzyme preparation and the purified components on glyoxyl-agarose. The performance of the immobilized preparations has been tested for the hydrolysis of a chickpea protein isolate.

MATERIALS AND METHODS

Materials. Flavourzyme 1000 L was a kind gift from Novo Nordisk (Bagsvaerd, Denmark). Cross-linked 4% agarose beads (4 BCL) were purchased from Iberagar S.A. (Coina, Portugal). Boc-L-alanine-4-nitrophenyl ester (boc-L-ala-ONp) was from Bachem S.A. (Budendorf, Switzerland). L-Leucine-*p*-nitroanilide (L-leu-*p*Na), sodium borohydride, and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxymethyl (CM) Sepharose and low molecular weight markers were from Amersham Biosciences (Uppsala, Sweden). Glyoxyl-agarose beads (4 BCL) activated with 40 μmol of aldehyde groups/g of support were prepared as previously described (*21*). Monoaminoethyl-*N*-aminoethyl (MANAE) agarose gel was prepared from glyoxyl according to the method of Fernandez-Lafuente et al. (*23*). Chickpea protein isolate (CPI) was prepared as described by Sánchez-Vioque et al. (*24*). All other reagents were of analytical grade.

Methods. All results represent mean values of at least three experiments. In all cases, experimental error was lower than 5%.

Adsorption of Flavourzyme on CM Sepharose. The support (5 g) was suspended in 50 mL of 25 mM sodium acetate buffer, pH 5, and stirred for 30 min at room temperature. pH was kept at 5 throughout the procedure by the addition of 0.1 M NaOH as necessary. Aliquots were withdrawn at different times after the addition of 100 μL of Flavourzyme for the determination of enzymatic activities in the suspensions and supernatants. The derivatives obtained were washed with 25 mM sodium acetate buffer, pH 5. A control run, identical to the immobilization suspension but containing inert agarose beads, was also incubated under the same conditions.

Adsorption of Flavourzyme on MANAE Agarose. Adsorption on MANAE agarose was carried out following the procedure described above for immobilization on CM agarose, but using 25 mM sodium phosphate buffer at pH 7 instead of pH 5.

Immobilization of Flavourzyme on Glyoxyl-agarose. Flavourzyme (100 μL) was mixed with 50 mL of 100 mM sodium bicarbonate buffer, pH 10.05, and added to 9 g of glyoxyl-agarose gel. The resulting suspension was gently stirred for different times. The progress of the immobilization was monitored by measuring enzymatic activities in the supernatant and in the whole suspension at different time intervals. Additionally, a control consisting of soluble enzyme and no support was used to determine the possible enzyme-inactivating effect of pH, temperature, and dilution during the incubation period. The resulting derivatives were reduced with sodium borohydride as described by Mateo et al. (*18*) and washed successively with 100 mM sodium phosphate buffer at pH 7 and abundant distilled water.

Enzymatic Activity Assays. Enzymatic activities in the soluble and immobilized Flavourzyme preparations were determined as followed:

Endoprotease activity was measured by following the increase of absorbance at 405 nm that accompanies hydrolysis of the synthetic substrate boc-L-ala-ONp (to 1.98 mL of 50 mM sodium phosphate buffer, pH 7, containing 20% ethanol were added 20 μL of soluble or suspended enzyme and 20 μL of 100 mM boc-L-ala-ONp in acetonitrile). One NPA unit was defined as the amount of enzyme that hydrolyzes 1 μmol of boc-L-ala-ONp/min under the conditions described. The specific activity of commercial Flavourzyme was 19 NPA units/mg of protein.

Aminopeptidase activity was assayed by following the increase of absorbance at 410 nm, which accompanies hydrolysis of the synthetic substrate L-leu-*p*Na (to 2 mL of 50 mM sodium phosphate, pH 7, containing 20% ethanol were added 20−100 μL of soluble or suspended enzyme and 20 μL of 100 mM L-leu-*p*Na in acetonitrile). One LAP unit was defined as the amount of enzyme that hydrolyzes 1 μmol of L-leu-*p*Na/min in the described conditions. The specific aminopeptidase activity of commercial Flavourzyme was 2.1 LAP units/mg of protein.

Assays were performed in spectrophotometric glass cuvettes at 25 °C with magnetic stirring. The gels in suspension did not interfere with spectrophotometric measurements.

Thermal Stability. Samples of soluble and immobilized enzymes were suspended in 0.1 M sodium phosphate, pH 7, and incubated at 55 °C. Aliquots were withdrawn periodically, and enzyme activities were measured at pH 7 and 25 °C as described above. The thermal inactivation kinetics of the immobilized enzymes were complex and could not be described by a simple first-order model. Pseudo-half-life times, $pt_{1/2}$ (time necessary to reach 50% of residual activity), were taken directly from experimental time courses of inactivation for each sample. These values were representative of the stability of the derivatives and were used for comparing the stability of different derivatives.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (*25*). Gels were stained using Coomassie Brilliant Blue G-250.

Hydrolysis of CPI. The hydrolysis of CPI by soluble or immobilized enzymes was performed in a reactor with stirring and controlled pH and temperature. CPI was suspended in distilled water (5% w/v), and the pH was adjusted to 7 and the temperature to 50 °C. CPI was hydrolyzed using a relation E/S ratio of 2 mg of protein (soluble or immobilized) per gram of chickpea protein, and the pH was kept constant by the addition of 1 N NaOH as required. The biocatalyst was removed 2 h later by filtration (immobilized enzymes) or was inactivated by heating at 85 °C for 10 min (soluble Flavourzyme).

Degree of Hydrolysis (DH). The DH, defined as the percentage of peptide bonds that are cleaved during hydrolysis, was measured using the TNBS method according to the method of Adler-Nissen (*26*). The total number of amino groups was determined in a sample that had been 100% hydrolyzed at 110 °C for 24 h in 6 N HCl.

Determination of Free Amino Acids. Free amino acids were analyzed by high-pressure liquid chromatography (HPLC) of the derivatives obtained by precolumn derivatization with diethyl ethoxymethylenmalonate as described elsewhere (*27*).

RESULTS AND DISCUSSION

Characterization of Flavourzyme 1000 L. Commercial enzymatic extracts usually contain additives that stabilize enzyme molecules into a liquid medium. The time course of the inactivation of crude and dialyzed Flavourzyme at 50 °C and pH 7 was investigated to check whether commercial

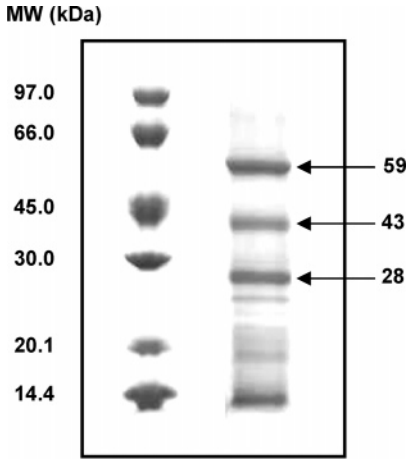

Figure 1. SDS-PAGE (15% polyacrylamide, 0.1% SDS) of commercial Flavourzyme: (lane 1) molecular weight standards; (lane 2) Flavourzyme.

Table 1. Adsorption Yields and Expressed Activity of Different Flavourzyme Derivatives

	adsorption yield (%)		expressed activity (%)	
support	NPA	LAP	NPA	LAP
CM Sepharose, 1 h, pH 5	48.9	0	11.5	
MANAE agarose, 1 h, pH 7	51.5	100	41.2	87.6

Flavourzyme contains such additives. The half-lives of endoprotease activity for undialyzed and dialyzed enzyme were 0.14 and 0.06 h, respectively, indicating that the crude preparation probably contains stabilizers that increased 2.3-fold the half-life time. Aminopeptidase activity was only 1.5-fold higher than that corresponding to dialyzed Flavourzyme, indicating that the effect of stabilizers on aminopeptidase activity was lower.

SDS-PAGE analysis of crude Flavourzyme showed that this preparation contains three major proteins with apparent molecular masses of 59, 43, and 28 kDa (**Figure 1**). Minor electrophoretic bands at 21 and 14 kDa were also observed. This is consistent with this preparation not having a single enzymatic activity.

Partial Purification of Different Enzymes in Flavourzyme 1000 L. Considering that Flavourzyme presents aminopeptidase and endoprotease activity, the possibility of purifying the enzymes responsible for these activities using the ionic exchangers MANAE agarose and CM Sepharose was explored. Yields of adsorption were expressed as percentage of the initial activity that was adsorbed on the supports (**Table 1**).

When CM Sepharose was used, only 49% of NPA activity was bound to the support, whereas the other 51% and the whole LAP activity remained in the supernatant (**Table 1**). A longer time of contact between the enzyme and the support did not lead to a greater NPA activity adsorption. The same results were obtained when the experiment was carried out using lower amounts of enzyme, confirming that the adsorption process is not limited by the loading capacity of the support. These results indicate that Flavourzyme probably contains, at least, two enzymes with endoprotease activity. On the other hand, the enzymatic activity decreased after adsorption on the support to 25%. Thus, this support did not seem to be suitable for the purification of these enzymes.

Adsorption on MANAE agarose was higher. Thus, all LAP activity and 51% NPA activity were adsorbed on the support.

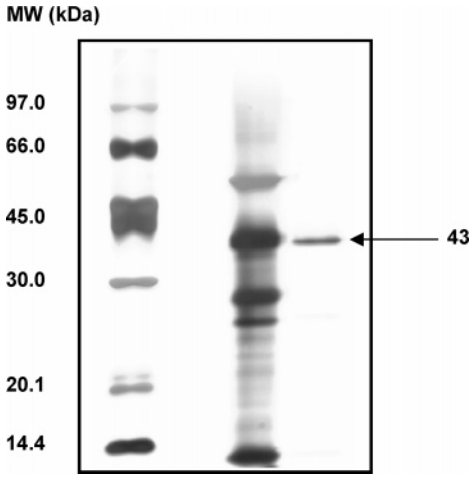

Figure 2. SDS-PAGE (15% polyacrylamide, 0.1% SDS) analysis of the supernatant resulting from adsorption of Flavourzyme on MANAE agarose: (lane 1) low molecular weight standards; (lane 2) commercial Flavourzyme; (lane 3) supernatant after 1 h.

Only a 43 kDa band appeared in the supernatant (**Figure 2**), indicating that this endoprotease activity can be isolated in a single, easy chromatographic step. This 43 kDa endoprotease presented a specific activity of 35 NPA units/mg of protein versus 19 NPA units/mg of protein in the crude preparation. The obtention of this endoprotease without contamination with aminopeptidase activity is especially important because it could be used for the production of protein hydrolysates enriched in small peptides and osmotically equilibrated that are of nutritional interest. The relatively high osmotic pressure that results from a high concentration of free amino acids may cause intestinal secretion and diarrhea (*28*), so that the presence of these amino acids in the form of peptides is a great advantage in this type of hydrolysates (*29*). Moreover, it has been reported that di- and tripeptides are more easily adsorbed than free amino acids (*30*) and that enteral diets containing peptides are more effective than diets containing free amino acids in the nutritional recovery of starved rats (*31*).

On the other hand, the proteins adsorbed on MANAE agarose preserved more than 80% of their initial activities at the end of the process. We tried to separate the endoprotease and aminopeptidase activities by desorption with increasing amounts of sodium chloride, but desorption was not very effective (only 50% desorption could be achieved using 1.7 M NaCl), and both activities were equally recovered.

Reversible Immobilization of the 59 and 28 kDa Proteins. The results of adsorption on MANAE agarose indicated that although this support is not useful for the purification of the 59 and 28 kDa proteins, it may be suitable for their reversible immobilization because the retention of activity and the adsorption were very high. The thermal inactivation of the adsorbed enzymes (**Figure 3**) showed that the endoprotease activity was less stable than the aminopeptidase activity, suggesting that the activities could be associated with different enzymes. Because of its aminopeptidase activity and stability, the MANAE agarose immobilized derivative could be used as a biocatalyst for the removal of the bitter taste that is characteristically generated during the extensive hydrolysis of proteins. A procedure for recycling of the support could be incubation in 10 volumes of 10 mM HCl/1 M NaCl after enzyme inactivation, which resulted in the complete removal of the immobilized enzymes.

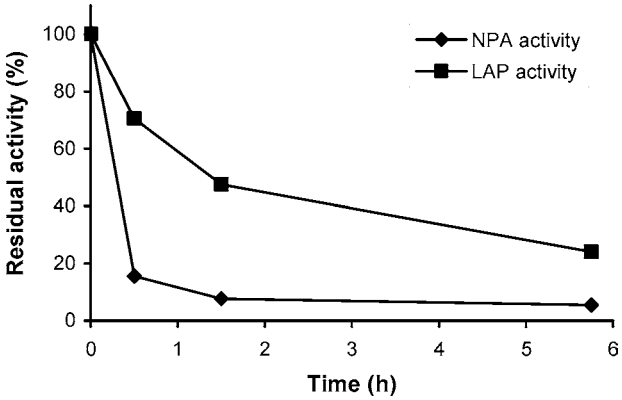

Figure 3. Thermal inactivation of the Flavourzyme–MANAE agarose derivative at pH 7 and 50 °C.

Table 2. Analysis of the Immobilization of Flavourzyme on Glyoxyl-agarose at Room Temperature and 4 °C

	immobilization yield (%)		expressed activity (%)	
	NPA	LAP	NPA	LAP
0.5 h, room temperature	87.5	75.2	48.8	20.42
0.5 h, 4 °C	73.5	71.1	95.3	58.2
2 h, room temperature	95.4	91.8	20.4	12.7
2 h, 4 °C	77.7	78.0	95.3	38.6

Immobilization of Crude Flavourzyme and the 43 kDa Endoprotease on Glyoxyl-agarose Supports. A high decrease in enzymatic activities was observed after immobilization of Flavourzyme at pH 10 and room temperature. Thus, the enzyme preserved 49% NPA activity and 20% LAP activity after only 30 min (**Table 2**) and less than 21% after 2 h. To avoid this fast decrease in catalytic activities, the immobilization process was carried out at 4 °C, which slows enzyme inactivation (*18*). At this temperature, autolysis should be minimized and the reaction between enzyme and support may be slower. As expected, the drop in NPA activity was very low at 4 °C, presenting the derivative more than 95% of initial activity after 2 h, although the effect on aminopeptidase activity was much smaller (**Table 2**). On the other hand, the rate of immobilization was lower at 4 °C than at room temperature, which is consistent with previous findings (*19*).

Considering these results, the immobilization of Flavourzyme and the 43 kDa endoprotease at 4 °C for 1 h followed by different periods of time at room temperature was studied as a possible optimized procedure for immobilization/stabilization. A 1 h immobilization time at 4 °C was chosen to minimize losses of activity, and the subsequent incubation was carried out at room temperature to increase immobilization yield and the number of linkages between enzyme and support. The time course of the immobilization on glyoxyl-agarose is shown in **Figure 4**.

The rates of immobilization were very similar for both enzymatic preparations, reaching more than 90% NPA activity immobilized after 2 h of contact between enyzme and support. However, longer incubation times were studied because this may have an effect on the stabilization of the enzymatic activity (*22*). It has been described that immobilization on this support depends on the formation of at least two points of attachment with the enzyme (*19*, *20*). The formation of additional points of attachment is more difficult than the initial immobilization (insolubilization process) and may require longer incubation times to allow the correct alignment of active groups in enzyme and support (*18*).

On the other hand, the NPA activity of both the purified endoprotease and Flavourzyme derivatives decreased in a time-dependent manner at longer incubation times. As small amounts of the derivatives were loaded (less than 1.5 mg of protein/g of support) and the maximum capacity of the support is about 20 mg protein/g of support, we can assume that mass transfer limitations were not masking the activity results. In addition, the possibility of autolysis taking place should be discarded because more than 90% of the activity was still bound to the support after 2 h. Therefore, the activity loss could be attributed at least partially to distortions in the enzyme structure promoted by the additional enzyme-support attachments, as has been reported for other enzymes (*32*). After 23 h, the endoprotease derivative maintained 50% of initial activity, whereas the Flavourzyme derivative maintained only 38%.

Concerning the LAP activity of Flavourzyme, the suspension maintained less than 20% of the initial activity after 2 h of incubation. This drop in aminopeptidase activity would have the positive effect of reducing the release of free amino acids, which otherwise would lead to an increase in osmotic pressure in the final resuspended hydrolysate.

Thermal Stability of Glyoxyl Derivatives. One of the main requirements for the application of enzymes as industrial biocatalysts is their stability under industrial reaction conditions, typically high temperature, for extended periods of time (*18*). In this sense, the thermal stability at 55 °C of the purified endoprotease and Flavourzyme glyoxyl derivatives obtained by immobilization of the two fractions was investigated. The stabilization of each fraction was calculated as the ratio between its $pt_{1/2}$ and the $pt_{1/2}$ corresponding to the soluble enzyme. The stabilizers in crude Flavourzyme are lost during the washing of glyoxyl-agarose derivatives. Nevertheless, the stability of the derivatives was compared with that of the soluble enzymes, containing stabilizers, in order to compare enzymatic preparations as they would actually be used in an industrial setting. The half-lives of purified endoprotease and Flavourzyme at 55 °C are 0.2 and 0.1, respectively.

As shown in **Figure 5**, immobilization on glyoxyl-agarose increased the thermal stability of all the enzymatic activities. This improvement may be attributed to several factors including prevention of autolysis due to restriction of intermolecular contacts and protection of enzymes from structural rearrangement due to multipoint attachment to the support (*33*). Moreover, the stabilization of the derivatives greatly increased with incubation time, which is characteristic of immobilization on glyoxyl-agarose (*18*, *22*, *32*). This effect could be correlated with an increasing number of lysine residues that react covalently with the support (*22*).

The stability of the purified 43 kDa endoprotease immobilized on glyoxyl-agarose was higher than that of crude Flavourzyme derivatives. The derivative obtained after 23 h was the most stable, presenting a half-life 226 times higher than that of soluble endoprotease. Likewise, the LAP activity of all Flavourzyme derivatives was also stabilized, and the stability increased with incubation time. However, this stabilization was lower than the stabilization of NPA activity, the 23 h derivative showing a stabilization factor of 85.

Hydrolysis of a CPI. The performances of crude Flavourzyme, immobilized Flavourzyme, and immobilized 43 kDa endoprotease were compared by carrying out hydrolysis of a CPI using the same E/S ratio. Considering the activity and stability of the derivatives obtained after different incubation

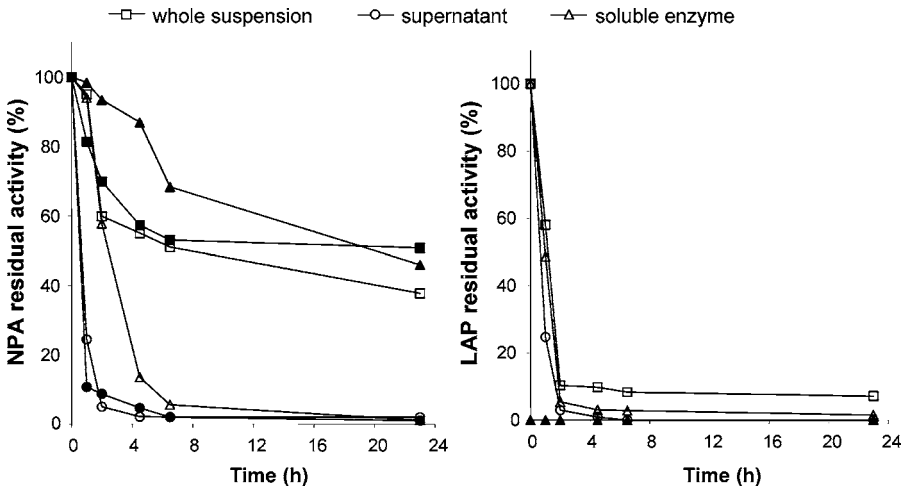

Figure 4. Time course of the immobilization of Flavourzyme (open symbols) and purified endoprotease (solid symbols) on glyoxyl-agarose (4 BCL) at 4 °C for an hour followed by incubation at room temperature for up to 22 h.

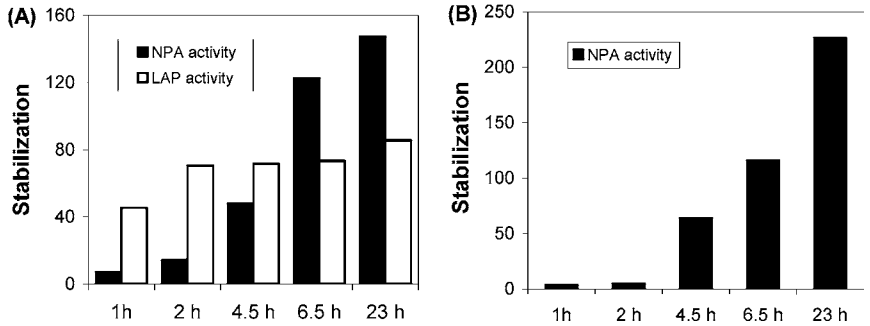

Figure 5. Thermal stabilization factors at 55 °C and pH 7 of Flavourzyme (**A**) and purified endoprotease-glyoxyl derivatives (**B**) obtained after different immobilization times. Stabilization was calculated as the ratio between the half-life time of immobilized enzyme and that corresponding to soluble enzyme.

Table 3. Degree of Hydrolysis (DH, Mean ± SD) and Amount of Free Amino Acids (Mean ± SD) of Chickpea Protein Hydrolysates Obtained by Treatment of Chickpea Protein Isolate with Different Enzymatic Preparations

catalyst	DH (%)	free amino acids (%)
Flavourzyme soluble	18.86 ± 0.19	11.63 ± 0.12
glyoxyl-Flavourzyme derivative	7.25 ± 0.14	7.06 ± 0.06
glyoxyl-endoprotease derivative	10.58 ± 0.26	0.20 ± 0.01

times (see above), the derivatives that were chosen for this study were the glyoxyl-endoprotease and the glyoxyl-Flavourzyme obtained by immobilization for 23 and 6.5 h, respectively. The highest DH was achieved using Flavourzyme in soluble form because immobilization led to a decrease in enzymatic activity (**Table 3**). Diffusional limitations caused by the higher molecular weight of chickpea proteins are probably why the glyoxyl derivative showed lower DH than may be expected bearing in mind the results of activity assays using smaller artificial substrates. Interestingly, immobilization resulted in a very significant reduction in free amino acid content. This reduction occurred in all cases, but was especially apparent in the endoprotease-glyoxyl derivative, which produced only 0.2% free amino acids, as compared to 12 and 7% in the case of crude Flavourzyme and immobilized aminopeptidase, respectively.

Therefore, the immobilized derivatives described in this paper have the advantages of being more stable than the original crude preparation and can be used for the production of osmotically equilibrated protein hydrolysates that are of interest for the food industry. In addition, immobilization facilitates recycling of the catalyst, which together with stability is of great interest for their application at an industrial scale. Immobilization also eliminates the need for an inactivation process because the derivatives can be easily removed by filtration, facilitating a better control of the hydrolytic process and avoiding contamination with the catalyst.

ABBREVIATIONS USED

boc-L-ala-ONp, Boc-L-alanine-4-nitrophenyl ester; CPI, chickpea protein isolate; CM, carboxymethyl; DH, degree of hydrolysis; L-leu-*p*Na, L-leucine-*p*-nitroanilide; MANAE, monoaminoethyl-*N*-aminoethyl; $pt_{1/2}$, pseudo-half-life time; TNBS, trinitrobenzenesulfonic acid.

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