PROTEIN HYDROLYSIS BY IMMOBILIZED AND STABILIZED TRYPSIN

Daniela Marques[1,4], Benavides C. Pessela[2], Lorena Betancor[3], Rubens Monti[4], Javier Rocha-Martin[1], Alfonso V. Carrascosa[2], Jose M. Guisán[1] and Gloria Fernandez-Lorente [2*]

1.- Instituto de Catálisis. CSIC. 28049 Madrid. Spain

2.- Instituto de Fermentaciones Industriales. 28006. Madrid. Spain

3.- IMDEA-Alimentos. 28049. Madrid. Spain

4.- Universidad Julio de Mesquita Filho - UNESP. Araraquara (SP, Brasil)

Corresponding authors

Gloria Fernandez-Lorente and Jose M. Guisan:

gflorente@ifi.csic.es

jmguisan@icp.csic.es

ABSTRACT

The preparation of novel immobilized and stabilized derivatives of trypsin is reported here. The new derivatives preserved 80% of the initial catalytic activity toward synthetic substrates (benzoyl-arginine *p*-nitroanilide) and were 50,000-fold more thermally stable than the diluted soluble enzyme in the absence of autolysis. Trypsin was supported on highly activated glyoxyl agarose following a two-step immobilization strategy: a) first, a multipoint covalent immobilization at pH 8.5 that only involves low pKa amino groups (e.g., those derived from the activation of trypsin from trypsinogen) is performed, and b) next, an additional alkaline incubation at pH 10 is performed to favor an intense, additional multipoint immobilization between the high concentration of proximate aldehyde groups on the support surface and the high pKa amino groups at the enzyme surface region that participated in the first immobilization step. Interestingly, the new, highly stable trypsin derivatives were also much more active in the proteolysis of high molecular weight proteins when compared with a non-stabilized derivative prepared on CNBr. In fact, all the proteins contained in a cheese whey extract had been completely proteolyzed after 6 hours at pH 9 and 50^{0}C, as judged by SDS-PAGE. Under these experimental conditions, the immobilized biocatalysts preserve more than 90% of their initial activity after 20 days. Analysis of the 3D structure of the best immobilized trypsin derivative showed a surface region containing two amino terminal groups and 5 lysine (Lys) residues that may be responsible for this novel and interesting immobilization and stabilization. Moreover, this region is relatively far from the active site of the enzyme, which could explain the good results obtained for the hydrolysis of high molecular weight proteins.

Keywords: cheese whey hydrolysis, two-step multipoint enzyme immobilization, multi-chain immobilization of trypsin

INTRODUCTION

The limited and selective proteolysis of low-cost proteins (e.g., casein, whey proteins, and denaturated proteins obtained as a by-product of oil extraction) is quite interesting. It is possible to obtain a pool of highly concentrated oligopeptides with interesting biological properties as a product of this kind of hydrolysis. Many studies have reported that the peptides obtained by the proteolysis of various protein sources possess bioactivities, including antihypertensive, antioxidant, anticancer, antimicrobial, and opioid activities, as well as immunomodulatory and cholesterol-lowering effects (1). For instance, the hydrolysis of milk proteins produces peptides with cardiovascular, digestive and endocrine activity (2). These bioactive peptides may be very useful as additives for functional foods (3). In fact, tryptic fragments of whey lactoglobulin exhibit an interesting inhibitory effect on angiotensin-converting enzyme (4). On the other hand, digestion of whey proteins with trypsin and chymotrypsin produces peptides with immunomodulatory effects (5). Interestingly, porcine blood proteins produced as by-products of agroindustries can be hydrolyzed by the simultaneous hydrolysis of trypsin, chymotrypsin and thermolysin, yielding interesting bioactive peptides (6).

A tailor–made, optimized protocol for the proteolysis of low-cost proteins to obtain highly valuable bioactive peptides should include the utilization of high temperatures to accelerate the reaction and should avoid microbial contamination and the use of highly selective and rather expensive endoproteases. The enzymatic hydrolysis would also benefit from the use of immobilized derivatives of selective proteases (e.g., trypsin), which enables the re-use of the catalyst and facilitates continuous protein hydrolysis.

Immobilized derivatives for use in biocatalysis should meet a number of conditions:

63 a. High stabilization to facilitate its reuse in many reaction cycles in moderately drastic reaction
64 conditions, which would result in a more cost-effective process even when using costly biocatalysts.

65 b. Proper orientation of the immobilized enzyme so that the active site remains fully accessible
66 even for the high molecular weight proteins that have to be proteolyzed during the first steps of
67 the hydrolysis.

68 c. A simple immobilization protocol that grants the use of a wide variety of commercial supports
69 and an easy control for the enzyme loading per gram of support.

70 Some years ago, our group was able to prepare stable trypsin catalysts that were useful for the
71 hydrolysis of denatured proteins obtained as by-products of oil hydrolysis (4,5). These derivatives
72 were obtained by covalent multipoint immobilization (at pH 10) between the region richest in
73 lysines on the trypsin surface and the highly activated glyoxyl derivatives.

74 It is possible that the rigidification of other regions of the enzyme structure (even those with less
75 lysines) generates even more stable derivatives because it may affect areas of the enzyme structure
76 involved in conformational changes (e.g., those induced by temperature) (6). Moreover, the
77 rigidification of the enzyme structure through different regions may favor the preparation of stable
78 derivatives in which the enzyme is better oriented to hydrolyze any type of protein, including those
79 of high molecular weight.

80 In this work, we will study the preparation of new trypsin derivatives obtained through a novel two-
81 step immobilization strategy: a) first, a multipoint covalent immobilization at pH 8.5 that only
82 involves low pKa amino groups (e.g., those arising from the activation of trypsin from trypsinogen)
83 occurs, and b) next, a further alkaline incubation of the derivatives occurs to favor an intense

multipoint covalent immobilization between the enzyme surface area that participated in the first immobilization step and the proximate aldehyde groups from the support.

These new derivatives will be compared to the previously prepared derivatives (on glyoxyl agarose at alkaline pH 10.0) (4) in terms of:

i. The simplicity of immobilization and the ability to immobilize high concentrations of trypsin.

ii. Their activity toward low molecular weight synthetic substrates.

iii. The thermal stability.

iv. The evaluation of the trypsin subunits that participate in the immobilization process.

v. Their activity toward crude extracts from *E. coli*.

vi. Their activity toward whey proteins.

The proteolysis will be analyzed by SDS-PAGE to evaluate whether the prepared derivatives are able to hydrolyze a number of proteins, including some as large as immunoglobulins (140 kDa), from cheese whey.

MATERIALS AND METHODS

Materials

The agarose CL- 4B gel, CNBr-activated Sepharose 4B support and the Low Molecular Weight (LMW) markers for electrophoresis were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Trypsin (E.C. 3.4.21.4) from bovine pancreas, BAPNA (benzoil- arginina ρ-nitroanilida), benzamidine, glycidol, benzamidine and sodium borohydride were purchased from Sigma Chemical Company (St. Louis, MO). Trypsin was a type III Sigma preparation that was dialyzed and lyophilized. Other reagents and solvents used were of analytical or HPLC grade.

Activation of Sepharose gels

Glyoxyl-agarose 4B-CL gel supports were prepared as previously described (7). Supports containing up to 25 μEquivalents of glyoxyl groups per ml were prepared. Commercial CNBr-Sepharose gels were treated before enzyme immobilization according to instructions from the commercial source.

Preparation of immobilized trypsin derivatives

All immobilizations were performed in the presence of 3 mM benzamidine (a competitive inhibitor of trypsin) to prevent autolysis. The Tryp-glyoxyl-8.5/10 derivative was prepared according to the following procedure: 100 ml of 0.1 M bicarbonate buffer at pH 8.5 containing 40 mg of trypsin was added to 10 ml (7 wet grams) of 4%-glyoxyl agarose. The immobilization was carried out for 2 hours

119 at 25^{0}C. After the complete immobilization of the enzyme, the immobilized derivative was filtered
120 and incubated in 100 ml of 100 mM bicarbonate buffer, pH 10.0, for 22 hours at 25^{0}C. Finally, the
121 immobilization process was finished by reducing the derivative through the addition of 1 mg of
122 solid sodium borohydride per ml of suspension. The Tryp-glyoxyl-8.5 derivative was prepared under
123 the same conditions but was reduced without the additional incubation under alkaline conditions.
124 The Tryp-glyoxyl-10 derivative was also prepared under the same conditions but using bicarbonate
125 buffer at pH 10, and an additional long incubation (for 24 hours) was performed between the
126 immobilized enzyme and the activated support before borohydride reduction (8). The Tryp-CNBr
127 derivative was prepared with 25 mM phosphate buffer at pH 7.0 and a ratio of enzyme/support as
128 described above. The immobilization reaction was carried out for only 15 minutes at 4^{0}C to
129 minimize the possibilities of multipoint covalent immobilization. Then the derivative was filtered,
130 and the remaining active groups on the support were blocked by incubation of the derivative in 1 M
131 ethanolamine at pH 8.0 for 2 hours. The derivative was then washed and filtered for further
132 utilization in the hydrolysis reactions.

133 All immobilization processes were followed by testing the catalytic activity of the whole suspension
134 and the supernatant in BAPNA hydrolysis. A blank of soluble trypsin in the presence of benzamidine
135 preserved full activity under the different experimental conditions.

136

137 **Enzymatic assays**

138 The activity of the derivatives was measured by following the increase of absorbance at 405 nm
139 that accompanies the hydrolysis of 0.2 mM BAPNA prepared in a 50 mM phosphate buffer, pH 7.0,

containing 30% ethanol (9). Assays were performed in a 1-cm path-length cell with magnetic stirring.

Stability of the derivatives

The thermal stability of different derivatives was studied by incubation under different experimental conditions (pH 7.0 or 9.0 and temperatures between 50 and 75^{0}C). Usually, a ratio of 1/10 between the derivative and the whole suspension was used. At different times, aliquots of the suspensions of the derivatives (under stirring) were withdrawn and assayed for BAPNA hydrolysis at pH 7.0 and 25^{0}C.

Cosolvent stability was studied by incubation of the derivatives in 10 mM TRIS buffer, pH 7.0, containing 50% dioxane. The suspension (equal to the one described for thermal inactivation) was incubated at 40 °C, and the stability of the derivatives was analyzed as described above.

SDS-PAGE of the immobilized derivatives

A total of 0.7 grams of each derivative was added to 1 ml of dissociation buffer containing SDS and mercaptoethanol (10), and the suspension was boiled for 5 minutes. In this way, all trypsin chains that are not covalently attached to the support should become desorbed away from the support to the supernatant. The supernatant is then analyzed by SDS-PAGE using 15% polyacrylamide gels. A LMW marker set of proteins was used as a reference.

Hydrolysis of proteins by trypsin derivatives

Solutions containing 2-6 mg/ml of protein in different buffers were used as substrates for the hydrolysis using trypsin derivatives. Crude extracts of *E. coli* (2 mg/ml) or cheese whey (6 mg/ml) were used as the protein substrates for immobilized trypsin. A total of 1 ml (0.7 wet grams) of derivative was added to 10 ml of protein solution and incubated at different temperatures. At different times, aliquots of the supernatant (100 µl) were withdrawn. The different aliquots were mixed with 100 µl of dissociation buffer (10) and boiled for 5 minutes. Then the boiled aliquots were analyzed by SDS-PAGE using 12% polyacrylamide gels.

Evaluation of difussional problems.

A total of 1 ml of trypsin derivative was suspended in reaction buffer and strongly stirred with a magnetic bar inside an ice bath. In this way, the particle size of the derivatives was dramatically reduced (more than tenfold). Hydrolysis catalyzed with broken derivatives was compared with hydrolysis catalyzed by intact derivatives. Observed differences would be due to the existence of difussional limitations during hydrolysis with intact derivatives.

RESULTS

Multipoint covalent immobilization of trypsin.

Different trypsin derivatives were prepared using highly activated glyoxyl-agarose and the two-step strategy described in the Introduction and Materials and Methods sections. The best results in terms of the immobilization rate and the activity and stability of the biocatalysts were obtained with the following derivatives: a) an initial immobilization on glyoxyl-agarose at pH 10.0; the enzyme become immobilized through a first covalent multipoint immobilization *via* the area richest in Lys residues, and a further intense multipoint covalent attachment is achieved by a long incubation at pH 10.0 (tryp-glyoxyl-10) and b) immobilization on glyoxyl at pH 8.5; the first multipoint covalent immobilization at pH 8.5 only involves low pKa amino groups (e.g., those derived from the activation of trypsin from trypsinogen and by a very rapid autolysis phenomenon) (15,16). An additional alkaline incubation (pH 10) favors an intense multipoint immobilization between the high pKa amino groups from the enzyme surface that participated in the first immobilization step and the proximate aldehyde groups from the support (tryp-glyoxyl-8.5/10).

For each of these strategies, the enzyme became immobilized in less than three hours, and the intrinsic activity remained above 70% after immobilization (including the alkaline incubation) when measured with a synthetic small substrate such as benzoyl-arginine *p*-nitroanilide (Table 1).

As a reference to compare the activity and stability of the new derivatives, the enzyme was immobilized on CNBr-Sepharose (for 15 minutes at 4°C) to promote a very mild interaction

between the enzyme and the support (tryp-CNBr). Under these conditions, the enzyme generally becomes attached to the support only by its amino terminal moiety. The enzyme immobilized on CNBr-Sepharose behaves the same way as the diluted soluble enzyme in terms of activity and stability in the presence of inhibitors (e.g., 3 mM benzamidine; data not shown). For this reason, this one-point attachment strategy avoids the common problems of soluble enzymes such as autolysis, aggregation and interactions with hydrophobic interfaces, and thus, it is more useful for further comparative studies with other immobilized derivatives even in the absence of inhibitors of autolysis.

Stability of the different immobilized preparations.

A comparative analysis of the thermal inactivation of the different trypsin preparations at 75^{0}C and pH 7.0 showed that higher stabilization was achieved by immobilization at pH 8.5 and further incubation at pH 10.0 (tryp-glyoxyl-8.5/10) (Figure 1). This derivative preserved more than 80% of its initial activity after 24 hours of incubation and resulted in a 5-fold more stable derivative than the glyoxyl derivative prepared and incubated at pH 10.0 (Tryp-Glyoxyl-10.0, the most stable trypsin immobilized described thus far). Under the same conditions, Tryp-Glyoxyl-8.5/10 was 500-fold more stable when compared to the derivative with no alkaline incubation (Tryp-glyoxyl-8.5). It seems that incubation under alkaline conditions is a key step to achieve a very intense multipoint covalent immobilization and subsequent improvement of the stabilization of the immobilized enzyme.

On the other hand, the less stable derivative (Tryp-glyoxyl-pH 8.5) was 100-fold more stable than the Tryp-CNBr derivative (Figure 2). Thus, it is possible to estimate a global stabilization factor for

Tryp-glyoxyl-pH 8.5 of approximately 50,000-fold over the Tryp-CNBr derivative, which has the same stability as the diluted soluble enzyme in the presence of benzamidine. To our knowledge, there is no previous report of such a high stabilization factor for trypsin by immobilization strategies.

The stabilization gained with the Tryp-glyoxyl-8.5/10 preparation might be mostly a consequence of a 3D rigidification-stabilization because we compared the best derivative and a non-stabilized derivative (tryp-CNBr). To confirm this 3D rigidification, we also studied the inactivation of the different derivatives in the presence of organic cosolvents (Figure 3). Again, the results in terms of stability were similar to those in the thermal stability experiment. The 3D rigidification attained by multipoint immobilization seems to prevent the distortions promoted by any denaturing agent.

Trypsin is immobilized with different orientations

Trypsin becomes active as a consequence of autolysis of trypsinogen (11). In addition, trypsin normally becomes proteolyzed due to auto-digestion in at least 3 different sites (11). In this way, commercial trypsin has several amino terminal residues and is composed of several chains bonded by disulfide bridges. A good indication of the differences in the orientation of the immobilized trypsin could be obtained by SDS-PAGE analysis upon boiling of the different derivatives with SDS and mercaptoethanol. The electrophoretic pattern resulted in unique patterns for the two different derivatives analyzed (Figure 4). Tryp-Glyoxyl-8.5/10, the most stable trypsin derivative, did not release any large polypeptides, which correlates with the proposed immobilization mechanism: low pKa amino groups from different chains are involved in the first covalent attachment on the

support. On the contrary, the least stable derivative (Tryp-CNBr) liberates a number of trypsin chains, and this may correspond with a one-point immobilization mechanism.

Hydrolysis of protein extracts using immobilized and stabilized trypsin.

Crude protein extracts from *E. coli*.

The most and least stable derivatives (Tryp-Glyoxyl-8.5/10 and Tryp-CNBr, respectively) were tested in the hydrolysis of an *E. coli* extract. Under the conditions used in the experiment, both derivatives proved efficient in the hydrolysis of large and most likely multimeric proteins present in the extract (Figure 5). Interestingly, the most stable derivative was a better catalyst for protein hydrolysis, as shown by a "cleaner" electrophoretic lane upon catalysis of the extract. In fact, large molecular weight proteins disappeared faster using the Tryp-glyoxyl-8.5/10 derivative compared to the reference Tryp-CNBr derivative.

By analyzing the structure of partially autolyzed trypsin, it is possible to identify a region with several low pK terminal amino groups that might be responsible for the enzyme orientation in Tryp-Glyoxyl-8.5/10 (Scheme 1) (15, 16). Moreover, this region of the trypsin surface also shows several neighboring lysine residues that may promote an intense multipoint covalent immobilization upon incubation at pH 10.0. It is also possible to predict that, in this particular orientation, the active site lays exposed to the medium, leaving it easily accessible even for the proteolysis of high molecular weight proteins. On the contrary, there is an amino terminal group exposed on the enzyme surface proximal to the active site that may be involved in the one-point

265 immobilization on CNBr-sepharose. Therefore, this orientation results in less efficient hydrolysis of
266 the high molecular weight proteins.

267

268

269 **Hydrolysis of cheese whey**

270 The most stable and efficient derivative in the hydrolysis of high molecular weight proteins,
271 Tryp-Glyoxyl-8.5/10, was selected for the hydrolysis of whey proteins. At pH 7.0, the hydrolysis was
272 quite slow, but an increase in the pH to 9.0 increased the rate of hydrolysis, and the intensity of all
273 the bands was notably reduced after 60 min as analyzed by electrophoresis (Figure 6). After 6
274 hours of reaction, all the proteins from the extract had been fully hydrolyzed. The absence of steric
275 hindrances during proteolysis could also be attained for trypsin immobilized on agarose gels with
276 long spacer arms (e.g., hydrophilic residues and dextrans (17)) (data not shown). However, no
277 trypsin stabilization was observed in this case.

278 The stability of the derivatives was further studied under the optimal conditions for the
279 hydrolysis (pH 9.0 and 50°C) (Figure 7). Tryp-glyoxyl-8.5/10 preserved 100% of its initial activity
280 after 20 days incubation. On the contrary, the less stable derivative (Tryp-CNBr) had a half life of 3
281 days under the same conditions.

282 Considering the stabilization factors obtained for the inactivation at 70°C and pH 7.0
283 (50,000-fold advantage of the tryp-glyoxyl-8.5/10 derivative over the tryp-CNBr derivative), a
284 conservative calculation indicates a half-life of over one year for the Tryp-Glyoxyl-8.5/10.

285 **Difussional problems.**

We did not detect difussional limitations when studying the activity of the derivatives using synthetic substrates (Table 1). In addition, the protein hydrolysis also exhibited very few difussional problems (Figure 8). In fact, upon mechanical breaking of the derivatives to reduce the particle size, the derivatives did not show a dramatic increase in activity.

Loading capacity of different supports.

A study of the loading capacity of different glyoxyl-agarose gels is shown in Table 2. As our main aim was the hydrolysis of high molecular weight proteins, we used 4% agarose and 4 mg of protein per mL of catalyst throughout the study to avoid difussional problems and steric hindrances during the hydrolysis of large proteins. However, for other applications, such as hydrolysis of low molecular weight proteins, it was possible to prepare derivatives at 100 mg/mL, which may be 16-fold more active in proteolytic reactions.

CONCLUSIONS

We have prepared different trypsin derivatives that favor covalent, multipoint attachment through different regions of the enzyme structure onto glyoxyl agarose. To do that, we have designed the immobilization strategy involved in two steps: a) first, an adsorption or covalent immobilization through diverse areas of the enzyme surface, and b) second, an additional alkaline incubation of each derivative to favor an intense multipoint covalent attachment between the glyoxyl groups from the support and the amino groups from the enzyme surface that it is near or in contact with.

Using a synthetic substrate, we have studied the activity and thermal stability of the different preparations. The most stable derivative was 50,000-fold more stable than the trypsin that was mildly immobilized onto CNBr-Sepharose. Tryp-glyoxyl-8.5/10 was first immobilized at pH 8.5, and immobilization probably involved the amino terminal residues released after the trypsinogen processing.

We studied the orientation of the immobilized derivatives and proved that in each of them, the trypsin had been attached through different peptide chains. The most stable derivative had two large peptide chains involved in the immobilization process, which seems to explain the immobilization mechanism and its high degree of stabilization.

Tryp-Glyoxyl-8.5/10 was not only stabilized structurally but also functionally and exhibited an interesting proteolytic activity in *E. coli* extracts and cheese whey. The derivative was efficient both in the lysis of large and small proteins, even at enzyme loadings as low as 4 mg/ml, and did not show severe difussional problems.

At 50°C, the proteolytic activity was higher at pH 9.0 than pH 7.0. Under these conditions, all of the proteins from the milk whey disappeared after 6 hours as judged by SDS-PAGE. Incubated in these conditions, the optimal derivative preserved more than 90% of activity after 20 days.

ACKNOWLEDGMENTS

The authors gratefully recognize the support from the Spanish R&D Program (Project No. AGL-2009-07625). We also gratefully recognize Spanish MICINN for “Ramon y Cajal contracts” to

328 Lorena Betancor and Gloria Fernandez-Lorente. A CAPES (Brazil) fellowship to Daniela Marques is
329 also recognized.

330

331

332

333

334

335 REFERENCES

336

337 1.- Shahidi, F.; Zhong, Y. Bioactive peptides. *J. AOAC. Int.* **2008**, *91*, 914-31.

338

339 2.- Meisel, H. Multifunctional peptides encrypted in milk proteins. *Biofactors*. **2004**, *21* ,55-61.

340

341 3.- Kitts, D.D. Bioactive substances in food: Identification and potential uses. *Can. J. Physiol. Pharm.*
342 **1994**, *72*, 423-434.

343

344 4.- Mullally, M.M.; Meisel, H.; Fitzgerald, R.J. Identification of novel angiotension-I- convering
345 enzyme inhibitory peptides corresponding to a tryptic fragment of bovine β-lactoglobuline. *FEBS*
346 *Letters*. **1997**, *402*, 99-101.

5.- Mercier, A.; Gauthier, S.F.; Fliss, I. Immunomodulating effects of whey proteins and their enzymatic digests. *Int. Dairy J*. **2004**, *14*, 175-183.

6.- Wel, J.T.; Chiang, B.H. Bioactive peptide production by hydrolysis of porcine blood proteins in a continuous enzymatic membrane reactor. *J. Sci. Food Agr.* **2009***, 89* 372-378.

7.- Pedroche, J.; del Mar Yust, M.; Mateo, C.; Fernández-Lafuente, R.; Girón-Calle, J.; Alaiz, M.; Vioque, J.; Guisan, J.M.; Millan, F. Effect of the suport and the experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme-support linkages and termal stability. *Enzyme Microb.Tech.***2007***, 40*, 1160-1166.

8.- Pedroche, J.; del Mar Yust, M.; Lqari, H.; Megias, C.; Girón-Calle, J.; Alaiz, M.; Vioque, J.; Millan, F. Production od Brassica carinata Protein hydrolyzates with a high Fischer´s ratio using immobilized proteases. *J. Agr. Food. Chem*. **2006**, *54*, 7621-7627.

9.- Bolivar, J.M., López-Gallego, F., Godoy, C., Rodrigues, D.S., Rodrigues, R.C., Batalla, P., Rocha-Martín, J., Mateo, C., Giordano, R.L.C., Guisán, J.M. The presence of thiolated compounds allows the immobilization of enzymes on glyoxyl agarose at mild pH values: New strategies of stabilization

by multipoint covalent attachment. *Enzyme Microb. Tech*. **2009**, *45*, 477-483.

10.- Guisán, J.M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzyme. Microb. Tech*. **1988**, *10*, 375-382.

11.- Guisan, J.M.; Alvaro, G.; Fernandez-Lafuente, R.; Rosell, C.M.; Garcia, J.L.; Tagliani, A. Stabilization of heterodimeric enzyme by multipoint covalent immobilization: Penicillin G acylase from Kluyvera citrophila. *Biotech. Bioeng*. **1993**, *42*, 455-464.

12.- Díaz, J.F.; Balkus, Jr. K.J. Enzyme immobilization in MCM-41 molecular sieve. *J. Mol. Catal B: Enzymatic*. **1996**, *2*, 115-126.

13.- Schägger, H. SDS electrophoresis techniques. Membrane Protein Purification and Crystallization. **2003**, 85-103.

14.- Muhlia-Almazán, A.; Sanchez-Paz, A.; García-Carreño, F.L. Invertebrate trypsins: a review. J*. Comp. Physiol. B.* **2008**, *178*, 655-672.

15.- Walter, J.; Steigemann, W.; Singh, T.P.; Bartunik, H.; Bode, W.; Huber, R. On the Disordered Activation Domain in Trypsinogen. Chemical Labelling and Low-Temperature Crystallography . *Acta Crystallogr. B*. **1982**, *38***,** 1462-1472.

16.- Varallyay, E.; Gabor, P.; Patthy, A.; Szilagyi, L.; Graf, L. Two mutations in Rat trypsin confer resitance against autolysis. *Biochem. Bioph. Res. Co*. **1998**, *243*, 56-60.

17.- Penzol, G.; Armisen, P.; Fernandez-Lafuente, R.; Rodes, L.; Guisan, J.M. Use of dextrans as long and hydrophilic spacer arms to improve the performance of immobilized proteins acting on macromolecules. *Biotech. Bioeng*. **1998**, *60*, 518-523.

FIGURE LEGENDS

Scheme 1.- An image of the surface of a partially autolyzed trypsin molecule including the active site, several terminal amino groups and several lysine residues. The image was obtained with the PyMOL 0.99rc6 program from the structure of intact trypsin (pdb file **2 ptn**) (Ref 15) and includes the two main autolysis positions (Ref 16).

Figure 1. Thermostability of different immobilized trypsin derivatives. Inactivation was performed at 75˚C in 5 mM sodium phosphate at pH 7.0. Tryp-Glyoxyl-10 (◆); Tryp-Glyoxyl-8.5 / 10 (■); Tryp-Glyoxyl-8.5 (▲).

Figure 2. Thermostability of different immobilized trypsin derivatives. Inactivation was performed at 70˚C in 5 mM sodium phosphate buffer at pH 7.0. Tryp-CNBr (◆); Tryp-Glyoxyl-8.5(▲).

Figure 3. Stability of different immobilized trypsin derivatives against cosolvents. Inactivation was performed at 40˚C and pH 7.0 in 50% dioxane. Tryp-Glyoxyl-8.5/10 (■); Tryp-Glyoxyl-10 (◆); Tryp-Glyoxyl-8.5(▲); Tryp-CNBr (●).

Figure 4. **SDS-PAGE** a**nalysis of the supernatants of different trypsin derivatives boiled under dissociating conditions.** *Lane 1.* Molecular weight marker; *lane 2.* Commercial trypsin preparation (5 mg/mL); *lane 3.*

Commercial trypsin preparation immobilized on a CNBr-activated Sepharose support; *lane 4.* Commercial trypsin preparation immobilized on glyoxyl-agarose at pH 8.5 and incubated at pH 10.0.

Figure 5. SDS-PAGE analysis of hydrolysates of crude protein extracts from *E. coli* obtained with different immobilized trypsin derivatives. *Lane 1.* Molecular weight marker; *lane 2. E. coli* extract preparation (2 mg/mL); *lane 3.* 24-hour *E. coli* extract hydrolysis by immobilized trypsin on a CNBr-activated Sepharose support; *lane 4.* 24-hour *E. coli* extract hydrolysis by immobilized trypsin on glyoxyl-agarose at pH 8.5/10.

Figure 6. SDS-PAGE analysis of hydrolysates of cheese whey protein obtained with most stable trypsin derivative (Tryp-glyoxyl-8.5/10). *Lane 1.* Molecular weight marker; *lane 2.* Bovine milk whey protein (6 mg/mL); *lane 3.* 30 minutes of hydrolysis at pH 7; *lane 4.* 60 minutes of hydrolysis at pH 7; *lane 5.* 30 minutes of hydrolysis at pH 9; *lane 6.* 60 minutes of hydrolysis at pH 9; *lane 7.* 6 hours of hydrolysis at pH 9.0. Hydrolysis was performed at 50°C.

Figure 7. Thermostability of immobilized trypsin preparations under alkaline conditions. Inactivation was performed at 50°C and pH 9.0 (sodium bicarbonate buffer 5 mM). Tryp-glyoxyl-8.5/10(■); Tryp-CNBr (▲).

Figure 8. **SDS-PAGE of hydrolysates of cheese whey proteins obtained by intact and broken glyoxyl-tryp-8.5/10. Experiments were performed as described in Methods.** Proteolysis was performed at pH 7.0 and 50°C. *Lane 1.* Molecular weight marker; *lane 2.* Bovine milk whey protein (6 mg/mL); *lane 3.* 30 minutes hydrolysis by the intact derivative; *lane 4.* 30 minutes hydrolysis by the broken derivative; *lane 5.* 60 minutes hydrolysis by the intact derivative; *lane 6.* 60 minutes hydrolysis by the broken derivative; lane *7.* 120 minutes hydrolysis by the intact derivative; *lane 8.* 120 minutes hydrolysis by the broken derivative.

449

450

Table 1. Yield and expressed activity of the immobilized preparations of trypsin using different supports.

Activated Support	Yield (%)	Expressed activity (%)
CNBr-activated sepharose	15	100
Glyoxyl-agarose pH 10.0	>95	75
Glyoxyl-agarose pH 8.5	>95	100
Glyoxyl-agarose pH 8.5/10	>95	80

Immobilizations were performed as described in the experimental section. Yield is defined as the percentage of soluble enzyme that becomes attached to the support. Expressed activity is defined as the ratio between the observed activity and the activity of soluble enzyme that has been immobilized.

451

452

453

454

455

456

457

458

459

460

Table 2.- Different trypsin-glyoxyl-agarose derivatives

Support	mg of trypsin/ ml of support *	Protein substrates , KDalton **
4% agarose	25	200-400
6% agarose	60	75-200
10 % agarose	100	15-75

* Maximum loading capacity

** approximate size of proteins that can be hydrolyzed by trypsin immobilized on different supports

461

462