Slovak Society of Chemical Engineering
Institute of Chemical and Environmental Engineering
Slovak University of Technology in Bratislava

PROCEEDINGS

34th International Conference of Slovak Society of Chemical Engineering

Hotel Hutník
Tatranské Matliare, Slovakia
May 21 – 25, 2007

Editors: J. Markoš and V. Štefuca

ISBN 978-80-227-2640-5

Spent grain as a new carrier for trypsin immobilisation

Cristina Rocha[1,2], Victor M. Balcão[1,4], Maria Pilar Gonçalves[3], José Teixeira[1]

[1] IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal
[2] Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Viana do Castelo, Apartado 574, 4900-348 Viana do Castelo, Portugal; Tel. +351 258 819700, e-mail: crocha@deb.uminho.pt.
[3] REQUIMTE, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[4] Centro de Bioengenharia e FarmacoClínica (CBFC), Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Praça 9 de Abril nº 349, 4249-004 Porto, Portugal

Keywords: Trypsin, immobilisation, spent grains, whey protein isolate, BAPNA

Abstract

Trypsin is a widely used enzyme for protein hydrolysis and can be used to improve functional and nutritional properties of foods. This work compares different carriers obtained from spent grains as potential candidates for trypsin immobilisation. Covalent attachment to the carriers was tested using glutaraldehyde. The efficiency of immobilisation and activity, operation and storage stability of free and immobilised enzyme on supports were determined. The amount of immobilised protein achieved was high (up to 70 %) but there was a significant loss of enzyme activity with the immobilisation of trypsin. However, when a more purified enzyme from bovine pancreas was used, activity retention was almost 50 %. Trypsin was most successfully immobilised on the supports by covalent attachment using glyoxyl-derivatives. The stability of these immobilized enzymes was close to 100%.

1. Introduction

Trypsin is a widely used enzyme for protein hydrolysis and can be used to improve functional and nutritional properties of foods.

The conversion of enzymes into water-insoluble products possessing specific catalytic activity is of interest since such 'water-insoluble enzymes' may readily be removed from the reaction mixture, thus reducing downstream processing cost and allowing its reuse. If stable, they may be employed repeatedly to induce specific chemical changes in relatively large amounts of substrate (Bareli and Katchalski, 1960); this feature is especially useful for expensive enzymes. Other advantages include operation in continuous reactors (such as packed bed columns with enzymic activity), easy handling, less waste and enhanced stability towards temperature, pH or organic solvents (important when substrates or products have poor water solubility). However, during the immobilization procedure severe losses of activity often occur. Furthermore, additional costs of carriers and other immobilization reagents are involved and there may be mass transfer limitations during reactor operation.

Enzyme immobilization has been widely studied since the early 1960's. Trypsin, as well as pepsin, urease and invertase were often used as "model" enzymes and were immobilized in many different kinds of supports (Bareli and Katchalski, 1960; Levin and others, 1964; Glazer, 1967; Habeeb, 1967; Gabel and others, 1970; Goldstei and others, 1970; Goldstei, 1973; Stoner and others, 1975). More recent works with immobilised trypsin were presented by Kulik and others (1993), Sears and Clark (1993), Diaz and Balkus (1996), Huckel and others (1996), Huang and others (1997), Bryjak and Kolarz (1998), Kumar and Gupta (1998), Nouaimi and others (2001), Pedroche and others (2004), Goradia and others (2005), Amaral and others (2006).

Traditional carriers include porous silica, porous glass and cellulose derivatives. Spent grains are a brewing by-product with a high content in cellulose and can also be interesting as carrier for enzyme immobilization because, besides having the necessary conditions (as stability, rigidity, low mass transfer limitations), they are cheap and food grade (Branyik and others, 2001). Glyoxyl or glutaraldehyde activated supports have proven to be quite efficient in increasing the tertiary enzyme stability via multipoint covalent attachment (Lopez-Gallego and others, 2007).

Most of the reactive groups used for immobilizing proteins (glutaraldehyde, cyanogen bromide, etc.) are able to yield very stable enzyme-support bonds under mild immobilization conditions (e.g., neutral pH values). However, glyoxyl-derivatives immobilize proteins best at alkaline pH values.

This work compares different methods of immobilisation with spent grain and modified spent grain to evaluate its potential as carrier for trypsin. Adsorption, ionic binding, covalent attachment (with glutaraldehyde and with glyoxyl) and a combination of adsorption and covalent binding (physical adsorption of the protein onto the carrier and intermolecular crosslinking with glutaraldehyde as a bi-functional reagent) was tested. The efficiency of immobilisation and activity, operation and storage stability of free and immobilised enzyme on the supports were studied.

2. Materials and methods

All reagents used were of analytical grade and supplied by Sigma, Co. Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg (one BAEE unit will produce a ΔA_{253nm} of 0.001 per min at pH 7.6 at 25 ºC using BAEE as substrate; in a reaction volume of 3.2 ml and 1 cm light path) and trypsin from bovine pancreas with an activity of 9000 BAEE units/mg (chymotrypsin =0.2%) were also obtained from Sigma Chemical, Co. Spent grains were kindly supplied by UNICER, Porto, Portugal. Whey protein isolate (WPI) powder (Lacprodan DI-9212, batch R320215) was kindly supplied by Arla Foods Ingredients (Viby, Denmark). According to the suppliers, the WPI protein content was 91 % dry basis, moisture was 5.5 % (maximum), the ash content was 3 % and the ion content was: sodium, <0.1 %, phosphorus, 0.2 %, chloride, 2.2 %, potassium, <0.1 % and calcium, <0.1 %.

2.1. Spent grains

Dry spent grains were prepared as described by Branyik and others, 2001. Dry spent grains (100 g) were mixed in 1500 mL of 3% (v/v) HCl solution at 60 ºC for 2.5 hours in order to hydrolyse the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was cooled and washed with water. The remaining solids were partially delignified by shaking (120 rpm) in 500 mL of 2% (w/v) NaOH solution at 30 ºC for 24 hours. After being several times washed with water until neutral pH and dried, the carrier (ca. 10 g) was ready to be used. Diethylaminoethyl-modified spent grains (DEAE-cellulose) were also prepared according to the method described by Branyik and others (2001), and ionic attachment to the carrier was tested. Spent grains were activated using glutaraldehyde 1% (w/v) for 2 hours in 0,05 M pH 7 phosphate buffer at room temperature or glycidol (2,3-epoxypropanol) with subsequent oxidation with periodate as described by Guisan, 1988. Glyoxyl-spent grains were also further activated with ethylenediamine to an amine-spent grain.

2.3. Trypsin Immobilization

Adhesion to the carriers was tested without chemical modification of the carrier surface (by physical adsorption) and with activation using glutaraldehyde. All immobilizing tests were performed at least in duplicate.
For each carrier, 100 mg of trypsin were incubated with 200 mg of the appropriate form of spent grain and 0.05 M phosphate buffer, pH 7.0 overnight at 4 ºC, except for glyoxyl-spent grain which

was incubated in 0.05 M carbonate buffer, pH 10.0. In some cases, crosslinking was made at the end with 1% glutaraldehyde as bi-funtional reagent for 1 h more at pH 7.
The supernatant was separated from the particles by vacuum filtration. Samples were taken and the Bradford method was used for protein determination in the supernatant. The carrier was then washed first with phosphate and then with TRIS/HCl buffer and filtered. The washing procedure with TRIS/HCl buffer was repeated four times. Benzamidine (3mM as final concentration) was used in some cases as a reversible trypsin inhibitor to prevent auto-proteolysis that could promote enzyme inactivation.

2.4. Measurement of Trypsin Activity

Trypsin activity of immobilized and native enzyme preparations was monitored hydrolyzing N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) in 0.05 M TRIS/HCL buffer with 0.02M $CaCl_2$ at pH 8.0 (Erlanger and others, 1961).
Hydrolyses of 1 mM BAPNA in TRIS/HCl buffer (a dilution from a 25 mg/mL BAPNA solution in DMSO was freshly prepared) with immobilized enzyme were carried out at 25 ºC in a 0.05 L stirred, tank-type, batch reactor equipped with temperature control. Samples of 1 mL were collected and the reaction was stopped with 0.25 mL of acetic acid 30 % (v/v). The supernatant was once again centrifuged, in the case of zeolite and silica, and vacuum filtered in the case of spent grains. The rate of p-nitroaniline formation was determined by measuring absorvance of supernatant at 410 nm. The extinction coefficient used was 8.8 L/mmol.cm^{-1} (Huckel and others, 1996).

2.5. Storage Stability and Reusability

Storage stability was determined incubating the immobilized enzyme in TRIS/HCL-buffer with 0.020 % (w/v) sodium azide at 4 °C for 60 days. The remaining enzyme activity was determined with BAPNA as above.
The reusability (or operational stability) of immobilized trypsin was studied by measuring the residual activity after three operational cycles. Each time, immobilized trypsin was washed and centrifuged/filtered four times with TRIS/HCl buffer.

2.6. Enzymatic hydrolysis of whey protein concentrate

Solutions of WPI (prepared suspending the desired amount of WPI in 50 mL of distilled water) were heated to the hydrolysis temperature and adjusted to pH 8.0 with concentrated NaOH. Enzymatic degradations were performed with free or immobilised bovine trypsin in a 0.05 L stirred batch reactor equipped with pH and temperature control. The pH was kept constant at 8.0 with a 0.25 mol L^{-1} NaOH solution and the temperature was kept at constant values ranging from 37 to 70 °C, depending on the experiment. The degree of hydrolysis (DH) achieved (ratio between the amount of peptide bounds cleaved and the total amount of peptide bounds) was measured by the pH-stat method (Adler-Nissen, 1985). Glyoxyl-spent grain was used as carrier for the bovine trypsin in the presence of benzamidine.

3. Results and discussion

Immobilization efficiency (expressed as the percentage of immobilized mass of protein referred to the initial mass of protein in solution) in carriers with chemical modification of the surface was better than with simple adsorption, with efficiencies around 60 % (Figure 1). As chemical bonds are stronger these results were expected and are in accordance with those referred in literature. For instance, Sears and Clark (1993) achieved 38 mg of immobilized protein per gram of carrier (porous size controlled glass), corresponding to 95 % of protein. However, only 4 mg of enzyme per gram of carrier were active. Huckel and others (1996) obtained 16 mg g^{-1} carrier in porous silica, corresponding to the immobilisation of 29 % of total protein. Kumar and Gupta (1998) were able to

immobilise 75 to 86 % of protein by chemical bond to Eudragit S-100 and 30 % by physical adsorption to the same polymer.
The best results concerning protein retention were obtained with glyoxyl and glyoxyEDA (amine) spent grain (almost 70 % in the former case and around 60% in the second one). The driving force for the immobilization with glyoxyl-carriers is the density of lysines in the enzyme (the reactive group). As the pKa of lysine is around 10, immobilisation always takes place at alkaline pH's. Trypsin may have rich lysine areas which could explain why this support has been successfully used. As the isoelectric point of trypsin is 10.5 (Diaz and Balkus, 1996), electrostatic repulsions are probably lower and binding more efficient.
In the case of DEAE-spent grains no improvements from simple adsorption were observed. Higher pH should be tested, as the isoelectric point of the enzyme is 10.5 and DEAE-cellulose is an anionic exchanger, though reversible bonds are involved and the optimum working pH of the free enzyme is 8. Transforming spent grains in a cationic exchanger (as a carboxymethyl-derivative) is probably a better alternative.

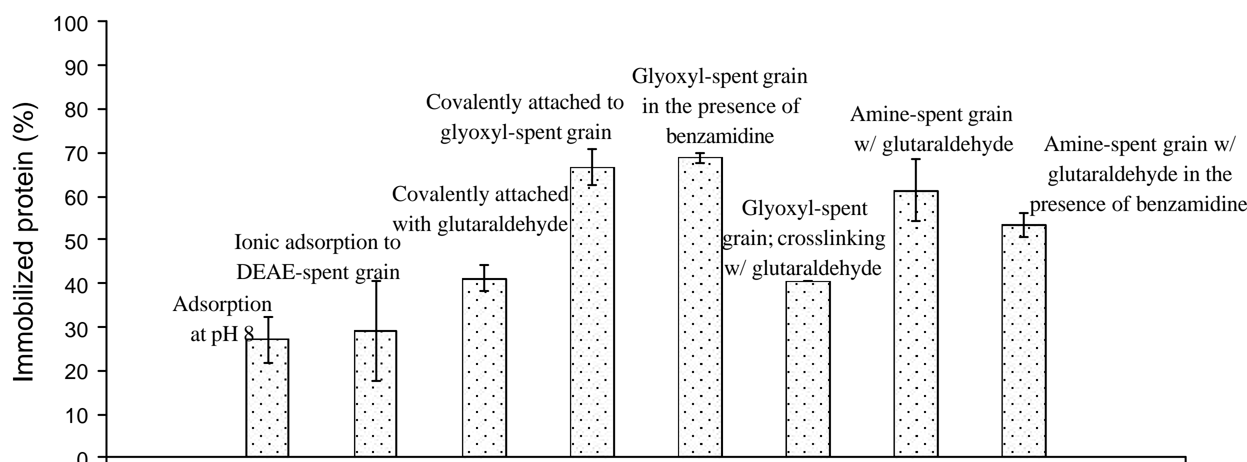

Figure 1 Immobilization efficiency

Although the amount of immobilised protein was higher when chemical bonds were involved, the specific activity was lower, indicating enzyme inactivation (Table 2). The necessary conditions to covalent attachment of an enzyme to a carrier are such that some loss of activity is inevitable.

Table 2. Activity retention

Immobilisation strategy	Activity (U/g carrier)	Specific activity (U/mg protein)	Activity retention (%)
Adsorption at pH 8	5.01±0.56	0.0428±0.013	15,2±10.1
Ionic adsorption to DEAE-spent grain	-	-	-
Covalently attached w/ glutaraldehyde	1.07±0.36	0.0064±0.001	3.06±0.78
Covalently attached to glyoxyl-spent grain	9.07±4.14	0.026±0.010	11.5±4.6
Covalently attached to glyoxyl-spent grain in the presence of benzamidine	8.25±0.96	0.024±0.001	10.5±0.5
Covalently attached to glyoxyl-spent grain and crosslinking with glutaraldehyde	2.15±0.19	0.0152±0.002	6.59±0.76
Covalently attached to glyoxylEDA-spent grain with glutaraldehyde	5.60±0.05	0.0196±0.002	8.51±0.96
Covalently attached to glyoxylEDA-spent grain with glutaraldehyde in the presence of benzamidine	5.06±0.45	0.0202±0.002	8.80±0.002

Besides, the active sites may not be as accessible to the substrate by partial obstruction or their conformation may be altered. Even so, spent grain with glutaraldehyde showed a much higher enzyme inactivation during immobilisation than the others (only 3% of the initial activity was retained). It should be noted than protein retentions above 95 % were achieved with glyoxyl- and glyoxylEDA-carriers using a slightly lower ratio quantity of trypsin/quantity of carrier (1:3) suggesting that the carrier's surface might be saturated and the enzyme might be adsorbing in multilayers (results not shown).

In order to be economically interesting, this kind of systems must be re-usable. Thus, operational stability was tested (Figure 2).

The operational activity loss is high (above 50%) when only (weak) physical bonds are involved, probably due to enzyme leaching during washings. Operational stability of immobilized enzyme with glutaraldehyde is much higher and with glyoxyl and EDA-glyoxyl is close to 100 %. Carriers with glyoxyl are also able stable during storage and they retained all activity during 60 days at 4 ºC (Figure 2).

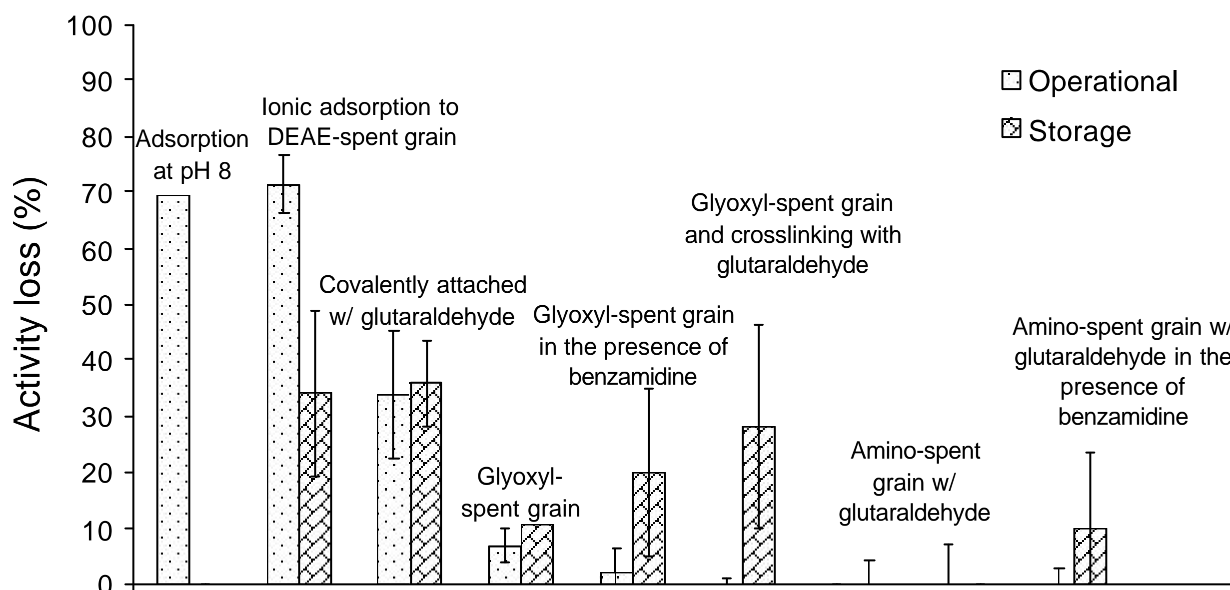

Figure 2 Operational stability after four cycles and storage stability after 60 days in TRIS/HCL buffer at 4 ºC

Figure 3 shows the degree of hydrolysis (DH) determined by the pH-stat method for whey protein isolate hydrolysed with free and immobilised trypsin on glyoxyl-spent grains.

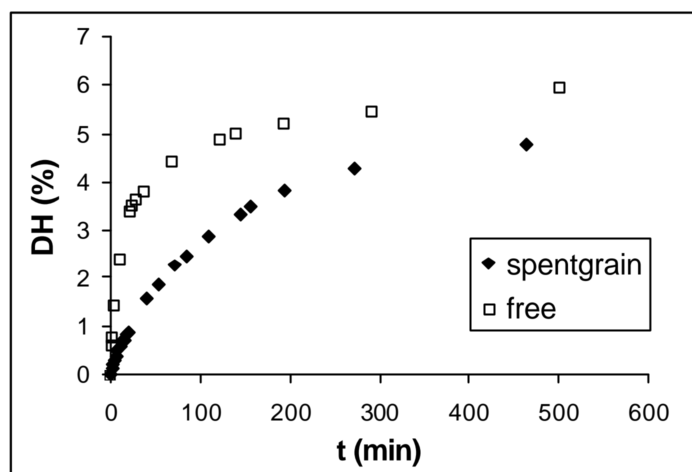

Figure 3 Degree of hydrolysis of whey protein concentrate with free and immobilized trypsin at 37 ºC and pH 8

The maximum degree of hydrolysis achieved was 5.9 % after 8 hours of hydrolysis with immobilised trypsin or 4.8 % with free trypsin. This result is slightly lower than those found in literature, but would probably be higher if a longer time, a higher temperature or a combination of several enzymes had been used. The immobilised enzyme had lower expressed activity and thus it is expected that the degree achieved is lower for the same time of hydrolysis. Perea and others (1993) mentioned a DH of 9.9 % for β-lactoglobulin and 7.4 % for α-lactalbumin with free pancreatin trypsin Novo 3.0S at 50 ºC and pH 8.0 and 12.4 % for β-lactoglobulin and 15.2 % for α-lactalbumin with a mixture of free trypsin and chymotrypsin. Mullally and others (1994) achieved a DH of 6.0 % for the hydrolysis of α-lactalbumin with trypsin at 50 ºC and pH 8.0. Mercier and others (2004) reported a DH of 12 to 17 % with a mixture of free trypsin and chymotrypsin.
In the experiments with WPI, a more purified enzyme (chymotripsin =0.2%) was used. As already found referred in literature, the immobilization activity retention was much better in this case and 46% were obtained (against 11 % with the unpurified enzyme). When the macrosubstrate (WPI) was used, this activity decreased to 18% (Table 3). As the whey proteins are much bigger molecules than BAPNA the binding of the active sites to the enzyme might be more difficult. The introduction of a spacer could improve the results. Activity with WPI was determined as the number of peptide bonds broken per min during the first three minutes of hydrolysis.

Table 3. Comparison of activity retention (%) in the hydrolysis both substrates (BAPNA and WPI) with free and immobilised purified enzyme

BAPNA	WPI
46.0	17.6

A simple Michaelis-Menten model was considered to the kinetics analysis. The concentration of all cleavable sites was used as the substrate concentration. The kinetics analysis showed that the immobilized trypsin apparent K_m was about 2 times lower than that found for the soluble enzyme (Tabel 4). ν_{max} of the immobilised enzyme is around 15% of the ν_{max} from the free enzyme as expected from Table 3. There may be substantial multi-layers of trypsin on the flat surface area of spent grains, and the internal layers of enzyme are less accessible for the substrate molecules, specially the larger ones, and therefore, a significant amount of trypsin may not be readily available to the substrate.

Table 4. Kinetics of free enzyme and enzyme immobilised on spentgrains evaluated at 37 ºC and pH 8.

	K_m (mol_{lig}/L)	ν_{max} (mol_{lig}/min)
Free enzyme	0.933	$6,4\times10^{-4}$
Immobilised enzyme	0.570	$4,6\times10^{-3}$

4. Conclusions

Trypsin was most successfully immobilised on the supports by covalent attachment using glyoxyl-derivatives, although with a small retention of activity. However, when a more purified enzyme from bovine pancreas was used, the activity retention was of almost 50 %. The stability of these immobilized enzymes was very high. The immobilized enzyme had a much higher activity towards low molecular weight substrates.

Acknowledgments

This work was co-financed by Social European Fund (public call 1/5.3/PRODEP/2003, financial application 1012.012, of measure 5/action 5.3 – Post-graduate training for academic staff submitted

by Escola Superior de Tecnologia e Gestão do Instituto Politécnico de Viana do Castelo).

References

Adler-Nissen, J. Enzymic hydrolysis of food proteins. 12-19, 1985. London, Elsevier Applied Science.

Amaral, I.P.G., Carneiro-da-Cunha, M.G., Carvalho, L.B., and Bezerra, R.S. Fish trypsin immobilized on ferromagnetic Dacron. *Process Biochemistry,* 41(5), 1213-1216, 2006.

Bareli, A. and Katchalski, E. Water-Insoluble Trypsin Derivative and Its Use As A Trypsin Column. *Nature,* 188(4753), 856-857, 1960.

Branyik, T., Vicente, A.A., Machado Cruz, J.M., and Teixeira, J.A. Spent grains - a new support for brewing yeast immobilisation. *Biotechnology Letters,* 23(13), 1073-1078, 2001.

Bryjak, J. and Kolarz, B.N. Immobilisation of trypsin on acrylic copolymers. *Process Biochemistry,* 33(4), 409-417, 1998.

Diaz, J.F. and Balkus, K.J. Enzyme immobilization in MCM-41 molecular sieve. *Journal of Molecular Catalysis B-Enzymatic,* 2(2-3), 115-126, 1996.

Erlanger, B.F., Cohen, W., and Kokowsky, N. Preparation and Properties of 2 New Chromogenic Substrates of Trypsin. *Archives of Biochemistry and Biophysics,* 95(2), 271-&, 1961.

Gabel, D., Vretblad, P., Axen, R., and Porath, J. Insolubilized Trypsin with Activity in 8 M Urea. *Biochimica et Biophysica Acta,* 214(3), 561-&, 1970.

Glazer, A.N. Specific Binding of Thionine to Active Site of Trypsin. *Journal of Biological Chemistry,* 242(14), 3326-&, 1967.

Goldstei, L. New Polyamine Carrier for Immobilization of Proteins - Water-Insoluble Derivatives of Pepsin and Trypsin. *Biochimica et Biophysica Acta,* 327(1), 132-137, 1973.

Goldstei, L., Pecht, M., Blumberg, S., Atlas, D., and Levin, Y. Water-Insoluble Enzymes - Synthesis of A New Carrier and Its Utilization for Preparation of Insoluble Derivatives of Papain, Trypsin, and Subtilopeptidase-A. *Biochemistry,* 9(11), 2322-&, 1970.

Goradia, D., Cooney, J., Hodnett, B.K., and Magner, E. The adsorption characteristics, activity and stability of trypsin onto mesoporous silicates. *Journal of Molecular Catalysis B-Enzymatic,* 32(5-6), 231-239, 2005.

Guisan, J.M. Aldehyde-Agarose Gels As Activated Supports for Immobilization-Stabilization of Enzymes. *Enzyme and Microbial Technology,* 10(6), 375-382, 1988.

Habeeb, A.F.S. Preparation of Enzymically Active Water-Insoluble Derivatives of Trypsin. *Archives of Biochemistry and Biophysics,* 119(1-3), 264-&, 1967.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. Comparison of the properties of trypsin immobilized on 2 Celite(TM) derivatives. *Journal of Biotechnology,* 53(1), 21-27, 1997.

Huckel, M., Wirth, H.J., and Hearn, M.T.W. Porous zirconia: A new support material for enzyme immobilization. *Journal of Biochemical and Biophysical Methods,* 31(3-4), 165-179, 1996.

Kulik, E.A., Kato, K., Ivanchenko, M.I., and Ikada, Y. Trypsin Immobilization on to Polymer Surface Through Grafted Layer and Its Reaction with Inhibitors. *Biomaterials,* 14(10), 763-769, 1993.

Kumar, A. and Gupta, M.N. Immobilization of trypsin on an enteric polymer Eudragit S-100 for the biocatalysis of macromolecular substrate. *Journal of Molecular Catalysis B-Enzymatic,* 5(1-4), 289-294, 1998.

Levin, Y., Pecht, M., GOLDSTEI.L, and KATCHALS.E. A Water-Insoluble Polyanionic Derivative of Trypsin .I. Preparation and Properties. *Biochemistry,* 3(12), 1905-&, 1964.

Lopez-Gallego, F., Betancor, L., Hidalgo, A., lamora-Ortiz, G., Mateo, C., Fernandez-Lafuente, R., and Guisan, J.M. Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. *Enzyme and Microbial Technology,* 40(2), 278-284, 2007.

Mercier, A., Gauthier, S.F., and Fliss, L. Immunomodulating effects of whey proteins and their enzymatic digests. *International Dairy Journal,* 14(3), 175-183, 2004.

Mullally, M.M., Ocallaghan, D.M., FitzGerald, R.J., Donnelly, W.J., and Dalton, J.P. Proteolytic and Peptidolytic Activities in Commercial Pancreatic Protease Preparations and Their Relationship to Some Whey-Protein Hydrolysate Characteristics. *Journal of Agricultural and Food Chemistry,* 42(12), 2973-2981, 1994.

Nouaimi, M., Moschel, K., and Bisswanger, H. Immobilization of trypsin on polyester fleece via different spacers. *Enzyme and Microbial Technology,* 29(8-9), 567-574, 2001.

Pedroche, J., Yust, M.M., Lqari, H., Giron-Calle, J., Vioque, J., Alaiz, M., and Millan, F. Production and characterization of casein hydrolysates with a high amino acid Fischer's ratio using immobilized proteases. *International Dairy Journal,* 14(6), 527-533, 2004.

Perea, A., Ugalde, U., Rodriguez, I., and Serra, J.L. Preparation and Characterization of Whey-Protein Hydrolysates - Applications in Industrial Whey Bioconversion Processes. *Enzyme and Microbial Technology,* 15(5), 418-423, 1993.

Sears, P.S. and Clark, D.S. Comparison of Soluble and Immobilized Trypsin Kinetics - Implications for Peptide-Synthesis. *Biotechnology and Bioengineering,* 42(1), 118-124, 1993.

Stoner, G.E., Gileadi, E., Ludlow, J.C., and Kirwan, D.J. Immobilization of Trypsin on Carbon. *Biotechnology and Bioengineering,* 17(3), 455-456, 1975.