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SPENT-GRAINS AND ZEOLITES AS POTENTIAL CARRIERS FOR TRYPSIN IMMOBILISATION

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Abstract. Trypsin is a widely used enzyme for protein hydrolysis and can be used to improve functional and nutritional properties of foods.
The immobilization of enzymes on solid supports can offer several advantages over free enzymes including easy handling, recovery from the reaction medium, reuse and operation in continuous reactors. Traditional carriers include porous silica, porous glass and cellulose derivatives. Zeolites are porous alumino-silicates available in a wide range of particle size and porosity and can also be used as carriers. Spent grains are a brewing by-product with a high content in cellulose and can also be interesting as carriers for enzyme immobilization because, besides having the necessary conditions (as stability, rigidity, low mass transfer limitations, for instance), they are cheap and food grade.
This work proposes the use of spent grains and zeolites as alternative carriers for trypsin immobilization and compares them with a traditional support (silica). Physical adsorption, ionic attachment and covalent attachment to the supports were tested. The efficiency of immobilization and activity, as well as the operation and storage stability of free and immobilized enzyme on the three supports were studied.
Trypsin was most successfully immobilized on the supports by covalent attachment using glutaraldehyde. Though the best efficiencies were still obtained with silica, promising results were achieved with both spent grains and zeolite.

Keywords: Trypsin, Immobilization, Zeolites, Spent grains, Silica and Protein Hydrolysis.

1. Introduction

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

The immobilization of enzymes on solid supports can offer several advantages over free enzymes including easy handling, recovery from the reaction medium, reuse and operation in continuous reactors. Traditional carriers include porous silica, porous glass and cellulose derivatives.

Zeolites are porous alumino-silicates available in a wide range of porosities (2–10 Å) and particle sizes and can also be used as carriers. They are built from a framework of silica and alumina tetrahedra with water and cations occupying the pores. By changing the Al/Si ratio carriers with different hydrophobic/ hydrophilic characters are generated (Gonçalves *et al.*, 1996).

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Spent grains are a brewing by-product with a high content in cellulose and can also be interesting as carriers for enzyme immobilization because, besides having the necessary conditions (as stability, rigidity, low mass transfer limitations, for instance), they are cheap and food grade.

This work proposes the use of spent grains and zeolites as alternative carriers for trypsin immobilization and compares them with a traditional support (silica). Physical adsorption, ionic attachment and covalent attachment to the supports were tested. The efficiency of immobilization and activity, operation and storage stability of free and immobilized enzyme on the three 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 was also obtained from Sigma Chemical Co (one BAEE unit will produce a ΔA_{253} of 0.001 per min at pH 7.6 at 25 C using BAEE as substrate; in a reaction volume of 3.2 ml and 1 cm light path). Three carriers were tested: porous silica with 30-45 mesh and 375 Å of pore diameter (ref. 27706, Fluka, Switzerland), spent grains (kindly supplied by UNICER, Porto, Portugal) and a commercial zeolite NaY from Davison Chemical Division of W.R. Grace (EUA).

2.1. Trypsin Immobilization

Dry spent grains were prepared as described by Branyik et al. (2001).

Adhesion to the three supports was tested without chemical modification of the carrier surface (by physical adsorption) and with activation using glutaraldehyde (both silica and zeolite were previously derivatized with 3-aminopropyltriethoxysilane). Diethylaminoethyl-modified spent grains (DEAE-cellulose) were also prepared according to the method described by Branyik et al. (2001), and ionic attachment to the carrier was tested. All immobilizing tests were performed in duplicate.

For each carrier, trypsin (50 mg) was incubated with 1 g of carrier and 30 ml of 0.05 M TRIS/HCl buffer, pH 8.0 with 0.02 M $CaCl_2$ (to reduce enzyme auto-digestion) overnight at 4 ºC. The supernatant was separated from the particles by centrifugation, in the case of zeolite and silica, and by vacuum filtration in the assays with spent grains. Samples were taken and the Bradford method was used for protein determination in the supernatant. The carrier was then washed with TRIS buffer and centrifuged/filtered. The washing procedure was repeated four times.

2.2. 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 buffer with 0.02M $CaCl_2$ at pH 8.0.

Hydrolyses of 1 mM BAPNA in TRIS 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 absorption of supernatant at 410 nm. The extinction coefficient used was 8.8 L/mmol.cm^{-1} (Huckel *et al*., 1996).

2.3. Storage Stability and Reusability

Storage stability was determined incubating the immobilized enzyme in TRIS-buffer with 0.020 % (w/v) sodium azide at room temperature for 8 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 buffer.

3. Results and Discussion

Immobilization efficiency in carriers with chemical modification of the surface with glutaraldehyde was better than in the same carriers with simple adsorption, with efficiencies around 60 % (Table 1). These results were expected, as chemical bonds are stronger, and are in accordance with those referred in literature. For instance, Kumar et al. (1998) were able to immobilize 75 to 86 % of protein by chemical bond to Eudragit S-100 and 30 % by physical adsorption to the same polymer. Huckel et al. (1996) obtained 16 mg/g carrier in porous silica, corresponding to the immobilization of 29% of total protein.

Sears et al. (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.

The results obtained with spent grains are very interesting as they immobilized almost as much protein as the supports with glutaraldehyde, only by physical adsorption.

Table 1. Immobilization efficiency

Carrier	Immobilized protein (%)	Immobilized protein (mg/g carrier)
Silica	21.2	17.2
Silica with glutaraldehyde	62.7	39.1
Spent grains	54.4	45.3
Spent grains with glutaraldehyde	62.9	60.9
Spent grains with DEAE	36.8	29.5
Zeolite	14.2	11.5
Zeolite with glutaraldehyde	60.6	36.4

Although the amount of immobilized protein was higher when chemical bonds were involved, the specific activity was lower (for the same carrier), indicating stronger 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. Besides, the active sites may not be as accessible to the substrate by partial obstruction or their conformation may be altered. Even so, silica with glutaraldehyde still showed higher specific activity than spent grains and

zeolite without glutaraldehyde. Although the structure of zeolite Y is one of the most open of all the zeolites, the pore size is still too small (< 20 Å) and the inclusion of trypsin (38 Å) in the pores of the zeolite (microporous structure) is impossible (Diaz, 1996). This means that the area available for immobilization is only the external surface area. Silica pore diameter is higher (375 Å) and, thus, the available area is also higher. This may be the reason for the better activity results achieved with silica.

Table 2. Activity retention

Carrier	Activity ($U/g_{carrier}$)	Specific activity ($U/mg_{protein}$)
Silica	347	20.2
Silica with glutaraldehyde	330	10.6
Spent grains	74.6	2.19
Spent grains with glutaraldehyde	59.7	1.00
Spent grains with DEAE	38.6	2.78
Zeolite	67.8	5.91
Zeolite with glutaraldehyde	83.9	2.38

In order to be economically interesting, this kind of systems must be re-usable. Thus, operational stability was tested (Table 3).

Table 3. Operational stability

Carrier	1st cycle activity ($U/g_{carrier}$)	2nd cycle activity ($U/g_{carrier}$)	3rd cycle activity ($U/g_{carrier}$)	Activity loss (%)
Silica	347	258	132	62.0
Silica with glutaraldehyde	330	325	335	-
Spent grains	74.6	34.9	36.2	51.5
Spent grains with glutaraldehyde	59.7	44.2	48.0	19.6
Spent grains with DEAE	38.6	19.1	13.0	66.3
Zeolite	67.8	35.1	15.7	76.8
Zeolite with glutaraldehyde	84.0	61.8	64.8	22.9

The 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, although losses of 10 or 20 % are still significant. However, if we analyze only the loss of activity from the 2nd to the 3rd cycle, that loss is technically null. This may indicate that there was still some enzyme weakly bonded to the support that leached during washings between the first two cycles.

Table 4. Storage stability

Carrier	Activity ($U/g_{carrier}$)	Activity after 8 days ($U/g_{carrier}$)	Activity loss (%)
Silica	132	51.8	60.8
Silica with glutaraldehyde	335	256	23.6
Spent grains	36.2	18.3	49.4
Spent grains with glutaraldehyde	48.0	53.0	-
Spent grains with DEAE	13.0	19.9	-
Zeolite	15.7	4.55	71.0
Zeolite with glutaraldehyde	64.8	79.1	-

Carriers with glutaraldehyde are able to retain all activity during 8 days of storage at room temperature, except silica (Table 4). However the results shown are an average of two assays and in the second one activity loss of immobilized enzyme in silica with glutaraldehyde was also zero.

4. Conclusions

Trypsin was most successfully immobilized on the supports by covalent attachment using glutaraldehyde. Though the best efficiencies were still obtained with silica, promising results were achieved with both spent grains and zeolite, particularly relating enzyme stability (to storage and to re-use).

References

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

Diaz, J. F., Kenneth, J. B. (1996). Enzyme immobilization in MCM-4 1 molecular sieve. *Journal of Molecular Catalysis B: Enzymatic*, 2, 115-126.

Gonçalves, A.P.V., Lopes, J.M., Lemos, F., Ramôa Ribeiro, F., Prazeres, D.M.F., Cabral, J.M.S., Aires-Barros, M.R. (1996). Zeolites as supports for enzymatic hydrolysis reactions. Comparative study of several zeolites. *Journal of Molecular Catalysis B: Enzymatic*, 1, 53-60.

Huckel, M., Wirth, H-J., Eran, M. (1996). Porous zirconia: a new support material for enzyme immobilization. *Journal of Biochemical and Biophysical Methods*, 31, 165-179.

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

Sears, P., Clark, D. (1993). Comparison of soluble and immobilized trypsin kinetics: implications for peptide synthesis. *Biotechnology and Bioengineering*, 42, 118-124.

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