Trypsin immobilisation on zeolites

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Abstract

This work compares different types of zeolites (NaA, NaX and NaY) as potential carriers for trypsin immobilisation. Silica was also used as a traditional carrier. 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 the three supports were studied. Optimum value of operation temperature was determined for trypsin immobilised on the best zeolite. Trypsin was most successfully immobilised on NaY by covalent attachment using glutaraldehyde. Though the best efficiencies were still obtained with silica, promising results were achieved with zeolite NaY. The ratio of immobilised protein achieved was very high, especially with silica, NaX and NaY. However there was a significant loss of enzyme activity with the immobilisation of trypsin. Optimal temperature obtained with immobilised enzyme was ca. 60 ºC, higher than the correspondent temperature for free enzyme (50 ºC).

1 Introduction

Enzymatic hydrolysis of food proteins is widely used to improve functional properties of foods. It is also interesting from a nutritional point of view e.g. to reduce allergenicity or to produce bioactive peptides (compounds with a health promoting potential). In fact, pancreatic enzymes - preferably trypsin – as well as other enzymes including Alcalase® and pepsin have been used for production of many known bioactive peptides (Gill *et al.*,1996; Mullaby *et al.*, 1997; Pihlanto-Leppälä *et al.*, 1997).
The immobilisation 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 porosity (2–10 Å) and particle size 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 can be generated (Gonçalves *et al.*, 1996).

This work compares different types of zeolites (NaA, NaX and NaY) as potential carriers for trypsin immobilisation. Silica was also used as a traditional carrier. 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 the three supports were studied. Optimum value of operation temperature was determined for trypsin immobilised on the best zeolite.

2 Materials and methods

All reagents used were of analytical grade and supplied by Sigma, Co. Trypsin from porcine pancreas

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with an activity of 1800 BAEE units/mg was also obtained from Sigma Chemical Co (one BAEE unit will produce a ΔA253 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). Four carriers were tested: porous silica with 30-45 mesh and 375 Å of pore diameter (ref. 27706, Fluka, Switzerland), and commercial zeolites NaY, NaA and NaX from Sigma (EUA).

2.1 Trypsin Immobilisation

Adhesion to the carriers 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). All immobilising tests were performed in duplicate.
For each carrier, trypsin (80 mg) was incubated with 0.5 g of carrier and 10 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. 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. The washing procedure was repeated four times.

2.2 Measurement of Trypsin Activity

Trypsin activity of immobilised 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 immobilised 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. 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 et al., 1996).

2.3 Storage Stability and Reusability

Storage stability was determined incubating the immobilised 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 immobilised trypsin was studied by measuring the residual activity after three operational cycles. Each time, immobilised trypsin was washed and centrifuged four times with TRIS buffer.

2.4 Enzymatic hydrolysis of whey protein concentrate

Hydrolyses of whey protein concentrate 70% (2.5 g in 50 mL) with 0.5 g of immobilised enzyme were carried out at several temperatures in a 0.05 L stirred batch reactor equipped with pH and temperature control. The pH was kept constant at 8.0 using 0.25 $mol.L^{-1}$ NaOH and and the temperature was kept at constant values ranging from 37 to 65 °C, depending on the experiment. The degree of hydrolysis (ratio between the amount of peptide bonds cleaved and the total amount of peptide bonds, DH) was monitored by the pH-stat method (Adler-Nissen, 1986).

3 Results and Discussion

Immobilisation 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 immobilise 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 immobilisation of 29% of total protein.

Sears et al. (1993) achieved 38 mg of immobilised 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.

Table 1. Immobilisation efficiency

Carrier	Immobilised protein (%)	Immobilised protein (mg/g carrier)
Silica	21.2	17.2
Silica with glutaraldehyde	62.7	39.1
Zeolite X	14.4	10.1
Zeolite X with glutaraldehyde	61.0	37.7
Zeolite A	49.9	11.1
Zeolite A with glutaraldehyde	62.8	36.8
Zeolite Y	14.2	11.5
Zeolite Y with glutaraldehyde	60.6	36.4

Although the amount of immobilised 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 zeolites without glutaraldehyde. The pore size of zeolites are too small (< 20 Å) and the inclusion of trypsin (38 Å) in the microporous structure is impossible (Diaz, 1996). This means that the area available for immobilisation 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
Zeolite X	69.2	6.86
Zeolite X with glutaraldehyde	23.8	0.628
Zeolite A with glutaraldehyde	27.9	0,757
Zeolite Y	67.8	5.91
Zeolite Y with glutaraldehyde	84.0	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	-
Zeolite X	69.2	34.6	33.8	51.1
Zeolite X with glutaraldehyde	23.8	7.36	7.44	68.7
Zeolite A with glutaraldehyde	27.9	14.4	5.34	80.8
Zeolite Y	67.8	35.1	15.7	76.8
Zeolite Y 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 immobilised enzyme with glutaraldehyde is much higher for silica, but not for zeolites. However, if we analyze only the loss of activity from the 2nd to the 3rd cycle, that loss is technically null (except for zeolite A). 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
Zeolite X	33.8	20.5	39.4
Zeolite X with glutaraldehyde	7.44	3.79	49.0
Zeolite A with glutaraldehyde	5.34	6.26	-
Zeolite Y	15.7	4.55	71.0
Zeolite Y 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 and zeolite X (Table 4). However the results shown are an average of two assays and in the second one activity loss of immobilised enzyme in silica with glutaraldehyde was also zero.

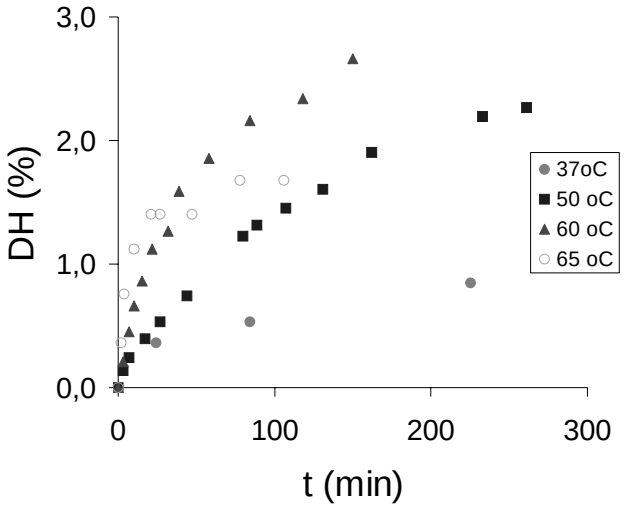

Figure 1. Degree of hydrolysis (DH) of whey protein concentrate achieved with immobilised trypsin on zeolite Y with glutaraldehyde at pH=8 and several temperatures

Figure 1 shows the degree of hydrolysis (DH) determined by the pH-stat method for whey protein concentrate hydrolysed at several temperatures with immobilized trypsin on zeolite Y. The activity of the enzyme increased with temperature and a maximum was found for the experiment performed at 60 °C for zeolite Y, slightly higher than the literature data for free trypsin: 50 °C (Godfrey, 1996).

4 Conclusions

Trypsin was most successfully immobilised on the supports by covalent attachment using glutaraldehyde, although with a small retention of activity. Though the best efficiencies were still obtained with silica, promising results were achieved with zeolite Y, particularly relating enzyme stability (to storage and to re-use).

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