Hydrogels for Immobilization of Trypsine Based on Poly-N-vinylpyrrolidone and Arabinogalactan Graft Copolymers

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Summary: By studying cross-linking of poly-N-vinylpyrrolidone [M_n=1×10^4 and 4×10^4] with N,N`-methylene-*bis*-acrylamide there were obtained water-swelling gel-forming polymers. Also was studied immobilization process of trypsin in polymer gels and Co(II), Cu(II), Ni(II) metal gel-complexes in polymeric gels. It was found that the amount and activity of immobilized trypsin gel carriers based on poly-N-vinylpyrrolidone less compared to the amount and the activity of immobilized trypsin gel carrier cross-linking copolymer of N-vinylpyrrolidone with arabinogalactan from cherry gum. The immobilization mechanism of trypsin into polymer gels investigated by FTIR and ^{1}H NMR spectroscopy.

Keywords: Poly-N-vinylpyrrolidone, Arabinogalactane, Hydrogel, Metal-polymer, Immobilization, Trypsin

Introduction

Immobilization of protein or enzyme into polymer spheres and capsules are of great scientific and practical interest. In recent years, immobilized enzymes are widely used in medicine and various enzymatic processes. This makes it possible to regulate the speed, the catalytic activity and the yield of the reaction product, and also continuously carry out the process [1-5].

One of the requirements to the polymeric systems used as carriers for biologically active compounds is to maintain activity of biologically active substances after the immobilization process, under the influence of the pH. There were synthesized copolymers of N-isopropylacrylamide, N-acryloyl phthalimide and polyacrylamide studied in the process of immobilizing them trypsin [6-8]. The authors found that in contrast to the free trypsin immobilized trypsin activity does not depend on temperature changes at temperatures above the lower critical solution temperature and regains its activity during cooling.

On the basis of poly-N-vinylpyrrolidone (PVPr), poly-N-vinylcaprolactam and tetramethoxysilane there were synthesized organic and inorganic hybrid hydrogels [9]. Arabinogalactane (AG) is a biologically active natural polysaccharide which has protective, membrane-conducting and immune activity properties. It is widely used as carrier of enzymes, drugs and essential minerals for the human body [10-14]. In that the natural polysaccharides as carriers of biologically active compounds which possess a large advantage over synthetic polymers.

It is known that among synthetic polymers PVPr has a high hydrophilic ability and the ability to form complexes with both organic and inorganic compounds. The high hydrolytic stability, non-toxicity and stability under sterilization in aqueous solution of polymer it has been used in synthesis of drugs [6, 9].

Despite of numerous studies conducted in the field of immobilization of biologically active compounds, this trend is relevant for today. Based on the mentioned above, this research has been conducted on trypsin immobilization polymer hydrogels obtained from the cross-linking of PVPr (molecular weight = 1×10^4 and 4×10^4) with N, N-methylene-*bis*-acrylamide (MBAD), their complexes with Cu(II), Co(II), Ni(II) metal ions and the hydrogel obtained by cross-linking copolymer of N-vinylpyrrolidone with MBAD which AG separated from cherry gum. Dependence of the activity of the immobilized enzyme from the pH medium, temperature, enzyme concentration and the initial time relatively and it was investigated with respect to the native trypsin.

Experimental

The used in PVPr with an average molecular weight = 1×10^4, 4×10^4 is a product of Belgium (Janssen Chimica), and trypsin Ukraine (Biofarma).

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Cross-linking reagent chemically pure MBAD (Sigma, Aldrich). AG was separated from cherry gum as described in [15, 16]. AG is a polysaccharide with branched structure, which contains 4.7% glucuronic acid and 0.4% of free -COOH groups, the aqueous solution has an acidic character pH=4.66. Cross-linking graft copolymers AG-VPr, PVPr and obtaining complexes of lower sorption capacity gels PVPr with copper, cobalt and nickel ions was performed as described in [13,14]. The structure of the polymer gels and their complexes with trypsin was determined by ^{1}H, ^{13}C-NMR (Bruker 300) and IR (Varian 3600 FTIR) spectroscopy.

Immobilization of trypsin into hydrogels inclusions is conducted according to the method [14]. The concentration of trypsin in the liquid phase is determined by absorbance at 280 nm (molar absorbance coefficient trypsin $E^{1\%}_{1sm}$ = 14). The concentration of the enzyme in the cell is C = 1.1×10^{-6} M.

Degree of immobilization R, is determined according to the expression (1) [15, 16].

$$R = \frac{A_0 - A}{A_0}\times 100 \qquad (1)$$

where, the A_0-is absorption value of initial trypsin solution at 280 nm and A – is absorption value of trypsin solution after immobilization.

Determination of the Activity Based Immobilization Yield

The initial rate of hydrolysis of the substrate, N-*α*-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) was used for the determination of trypsin activity. Fresh, free trypsin solution of approximately 4 mg/mL and residual free trypsin solution (after immobilization) from each of the immobilization preparation were diluted (n= 4) with 0.1 M Na_3PO_4/HCl pH 7.2 buffer while 6.0 mg BAPNA were dissolved into 6.0 mL of 50% aqueous ethanol. The reactant solutions were separately thermally equilibrated at 40 °C for about 25 minutes prior to reactions in thermally controlled cuvettes set at 40 °C for 3 min. The reaction mixture in the cuvette contained 0.28 mL BAPNA solution, 0.52 mL of 0.1 M Na_3PO_4/HCl pH 7.2 buffer and 0.2 mL of the diluted trypsin solution. Two blank solutions comprising of only the BAPNA solution and the 0.1 M Na3PO4/HCl buffer were also prepared. The absorbances were recorded at 400 nm using a Shimadzu UV-1650 PC spectrophotometer. Activity based immobilization yield (IY) was determined as the percentage of the activity units of the actually immobilized trypsin (AI) to the activity units for the fresh free trypsin utilised for the immobilization (TI), *i.e.*,

$$IY = \frac{AI}{TI}\times 100 = \frac{TI - FI}{TI}\times 100\%$$

where FI is the activity units of the residual free trypsin.

Kinetic parameters K_M and V_{max}. Constant amounts of free and immobilized trypsin were subjected to reaction of BAPNA hydrolysis in solutions of increasing BAPNA concentration. For each experiment, the initial rate of reaction was measured, using a discontinuous assay.

Results and Discussion

It is known that the amount of immobilized enzyme and its stability and activity depends on the nature of the polymer support and the interaction between the enzyme and a macromolecule polymer. It was studied the immobilization of trypsin into synthesized natural and synthetic polymer-basis gels. Table-1 shows the dependence of the amount activity and stability of immobilized trypsin from the molecular weight of PVPr, the nature of the metal ion and the mass (%) of PVPr in graft copolymer composition which is obtained by reacting VPr with AG from the cherry gum.

Table-1 Influence of the molecular weight of PVPr and mass (%) VPr grafted on AG and the nature of the metal ions the activity and the amount of immobilized trypsin.

Carrying hydrogels	Amount of trypsin (mg/gr)	Degree of immobilization (%)	$C_{activ.}$ (U/gr)	S (%)	Relative activity (%)
PVPr $\bar{M}_n$ =1×10⁴	0.20	10.00	38	5.24	9.44
PVPr $\bar{M}_n$ =4×10⁴	0.45	22.50	51	7.42	11.68
PVPr4×10⁴ Cu (II)	3.60	72.35	92	12.8	20.60
PVPr4×10⁴ Co (II)	3.30	66.70	76	9.40	15.36
PVPr4×10⁴ Ni (II)	3.44	68.80	85	10.60	17.58
AQ-VPr (5%)	12.00	20.00	126	34.56	42.80
AQ-VPr(10%)	8.75	14.50	102	22.80	34.75
AQ-VPr(15%)	7.00	12.50	98	18.24	29.20
AQ-VPr(20%)	3.50	5.75	88	12.75	21.46

As seen from the Table-1 when changing the molecular weight of PVP of 10.000 to 40.000 increases amount (from 0.2 to 0.45 mg/g) and activity (from 38 to 51 U/g) of immobilized enzyme in gels. This is due to the fact that increasing the sorption amount and swelling degree of gel obtained by cross-linking high molecular PVPr is more easily to

introduce the protein in macromolecules hydrogels. As seen from the Table-1 the amount of a trypsin-immobilized carriers in gels obtained by cross-linking various proportions different % AG, 10 % MBAD graft copolymer decreases with increasing amount of the graft copolymer AG-VPr. This is based on reducing the amount of acid in the glucuronic AG, which is the cause of immobilization trypsin [17-20]. We consider that the process of immobilization due to the formation of a hydrogen bond between -COOH group of the macromolecule and on the free -NH_2 groups and -CO-NH- group of macromolecule trypsin, and main as reduction glucuronic acid in the AG has a negative effect on immobilization of enzymes [21].

By ^{1}H NMR spectroscopy it was studied the chemical interaction between the macromolecule of trypsin and the carrier. This purpose is to use the PVPr+trypsin, PVPr-Cu(II)+trypsin model systems with presence of (D_2O) solvent.

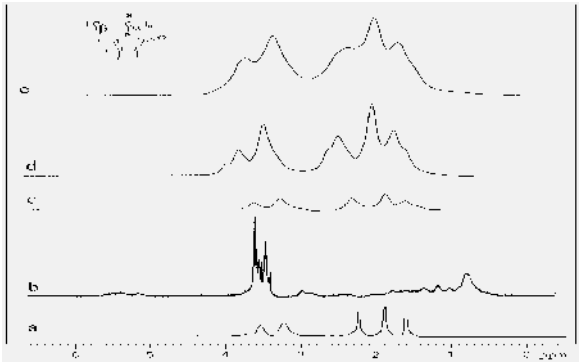

Fig. 1: Comparison ^{1}H NMR spectra PVPr (a), trypsin (b) PVPr-trypsin (c), PVPr-Cu(II) (d) and a complex of PVPr-Cu(II)-trypsin (e) in D_2O medium.

Interaction of trypsin with PVPr confirms the data by NMR spectroscopy. In the ^{1}H NMR spectrum registered in D_2O mixing solutions of trypsin and PVPr. Pyrrolidone ring proton signals are broadened and shifted toward to more weak fields (Fig. 1). At the same time, a ^{1}H NMR spectrum transformation occurs and some signals. C_5 proton signal at 3.23 ppm to the chemical shift comes in the form of widely signal with chemical shift 3.30 ppm. Also changes the signal of the protons at 2.22 ppm C_3, it comes with a chemical shift at 2.35 ppm. C_αH methine proton of the polymer backbone with chemical shift 3.52 ppm comes in the form of a broadened signal with chemical shift 3.65 ppm [22, 23]. Thus, in the NMR spectra of PVPr importantly change signals of two protons in the pyrrolidone circle C_5-H_2, C_3-H_2 and C_α-H backbone. These changes indicate that the immobilizing trypsin into carrier gel synthesized based on PVPr interacts with carbonyl group in pyrrolidone ring.

Given a catalytic amount of metal ions in enzymatic reactions in the test systems the amount of Cu(II), Co(II) and Ni(II) ions compiled 0.56 mmol/gr. As seen from Table-1 the amount of activity and stability of immobilized trypsin gel-based carrier with a molecular weight PVPr 4×10^4 are of less value than immobilization trypsin to Cu(II), Co(II) and Ni(II) complexes a PVPr gel, which were obtained after the sorption of metal ions. Formation of complexes with trypsin PVPr hydrogels with the participation of Me(II) ions during immobilization affirmed the results of ^{1}H NMR spectroscopy. In the ^{1}H NMR spectrum of PVPr in the presence of Cu(II) it was observed the following chemical shifts: C_5 3.32 ppm, 2.32 ppm C_3, C_4 and 1.88 ppm 3.65 ppm C_α, C_β 1.58 ppm, which correspond to the signal of the protons pyrrolidone rings and protons of the polymer chain. When the system is added, the trypsin character of the spectrum changes, the area broadened, signals are shifted toward the high-field chemical shifts: C_5 3.21 ppm, C_3 2.21 ppm, C_4, 1.85 ppm and 3.58 ppm C_α, 1.55 ppm C_β, respectively. This may explain the process involving metal ions coordinated to PVPr gels trypsin immobilization process. Based on literature data [24, 25], immobilization of enzymes into polymer macromolecule proceeds effectively involving the metal ions. Metal ions play the role of a bridge between the polymer macromolecule and the enzyme and stored in a stronger electrostatic interaction with the enzyme. From the FTIR spectra in immobilized metal-gel trypsin complex it is seen that there is a maximum of the absorption at 1655 cm^{-1} which is characteristic to the pyrrolidone ring in PVPr macromolecule. PVPr complexes with metal ions $[MeX_2\times2L]_n$, give the shift in at 1670 cm^{-1} due to the carbonyl group in the pyrrolidone ring. Trypsin, consisting of an amino acid functional groups is immobilized in the metal-gel through hydrogen bond formation between carboxyl, an amino group macromolecule of peptide and the carrier. A small part of the coordination of immobilization is formed by the electrostatic interaction between the excess charge generated on the local area of macromolecules of trypsin and metal ions [26].

As a result it is increased interaction of metal ions with the enzyme. It was ascertained in the study of the FTIR spectra of the complex formation with PVPr-Cu(II)-trypsin.

The FTIR spectra of PVPr gels there were detected absorption bands characterized by the following frequencies: 3415, 2955, 2130, 1655, 1291, 931, 648, 576, cm^{-1}. In its complexes with Cu(II) there were detected bands with frequencies 3419, 2132, 1659, 941, 658 cm^{-1}. After immobilization of trypsin there were absorption bands with frequencies of 3410, 2137, 1655, 1228, 1020, 651, 573 cm^{-1} corresponding to the triple complexes. Investigation of the structure and gel complex hydrogels before and after immobilization by FTIR spectroscopy (Fig. 2) indicates that the immobilization proceeds is an evidence of hydrogen bonding that the process is in chemical way. In the FTIR spectrum of hypertension observed absorption bands with characteristic intensity, which refers to the -COOH group. Absorption bands were identified gels obtained by cross-linking MBAD grafted copolymers and 5% AG-VPr in the following frequencies: 3436, 1650, 1074, 609 and 540 cm^{-1}. After immobilization the trypsin into gel complex absorption bands are 3414, 1076, 636, 586 cm^{-1} frequencies. These displacements in the AG based gels show that the immobilization of trypsin occurs due to the formation of a hydrogen bond between the free -COOH and -OH groups of macromolecules AG and -CO-NH- and $-NH_2$ groups of macromolecules trypsin and it is completely similar to the mechanism of enzyme immobilization in gels basis polysaccharide [27].

Table-2 shows the results of the effect of pH, time and concentration of enzyme immobilization on activity, the degree of preservation and the relative activity of trypsin activity immobilized synthesized in 5% AG-VPr.

As seen from Table-2 during the immobilization by varying trypsin concentration in a buffer solution pH 9.2, an increase (with concentration) in specific activity was observed for trypsin solutions of concentrations below 0.8 mg/mL and the opposite behavior was observed for solutions of higher concentrations. We assume that, for higher concentrations, there were protein-protein interactions (autolysis of the trypsin) and/or distortion of the protein molecules, which resulted in its denaturation. Thus, a 0.8 mg/mL trypsin solution was chosen to study the volume effect.

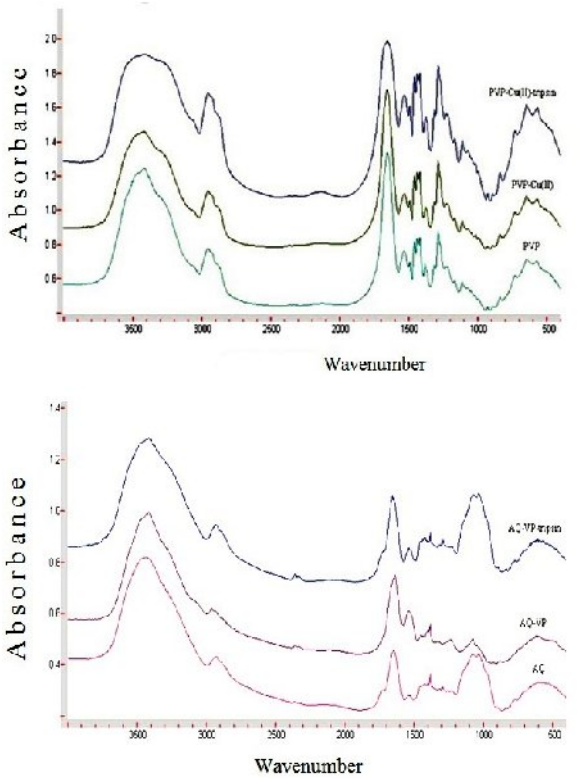

Fig. 2: Comparison of FTIR spectra of metal-polymer complexes and AG-VPr, which immobilized trypsin.

As a result of compaction of immobilized trypsin gel pores observed difficulties in effective interaction with the substrate, which leads to a lower degree of relative activity and activity maintenance of the immobilized enzyme. As a result, there are active only those immobilized trypsin with active sites that are on the surface of the gels [28]. Active sites of trypsin macromolecules, which act on the pore screened inactive parts of the macromolecule enzyme.With an increase in the period of immobilization for 1-12 hours, an increase in specific activity value of the enzyme activity and the degree of conservation of the relative potency values.

Table-2: Results of the effect of the initial concentration, the time of immobilization and pH on the enzyme activity and the relative activity of the immobilized trypsin in AG(5%) -VPr.

Concentration trypsine mg/ml	$C_{activ.}$ U/gr	S (%)	Relativity activity (%)	Time, hour	$C_{activ.}$ (U/gr)	S (%)	Relativity activity (%)	pH	$C_{activ.}$ (U/gr)	S (%)	Relativity activity (%)
1.00	**32**	**46.76**	**84.40**	**1**	**82**	**8.76**	**10.18**	**6.1**	**54.87**	**9.76**	**11.30**
2.00	**94**	**42.40**	**63.78**	**2**	**88**	**11.43**	**12.65**	**7.3**	**76.31**	**16.48**	**13.42**
3.00	**126**	**34.56**	**42.80**	**4**	**94**	**18.50**	**14.75**	**7.8**	**96.54**	**18.24**	**15.28**
5.00	**178**	**28.82**	**24.36**	**8**	**102**	**27.44**	**28.40**	**8.0**	**61.32**	**19.16**	**19.28**
6.00	**214**	**11.20**	**14.76**	**12**	**121**	**32.56**	**37.60**	**9.0**	**43.76**	**20.43**	**24.33**
8.00	**232**	**6.32**	**9.34**	**24**	**126**	**32.86**	**38.00**	**10.0**	**38.78**	**19.80**	**28.56**

Immobilization Conditions: mass gel 100 mg, 3 ml of the enzyme solution, enzyme concentration of 3 mg/ml, pH =7.2, immobilization time 24 hour, T =293 K, activity of the native trypsin 5.50 U/g

We studied the changes in specific activity, the relative activity and the degree of conservation in the activity depending on pH, and we found that the specific activity of the trypsin was immobilized in a 5% AG-VPr reaches its maximum at pH =7.83. The relative activity of the immobilized trypsin was estimated as the ratio between the specific activity of the enzyme and its maximal specific activity. Higher enzymatic activities were achieved with more alkaline solutions (pH between 9 and 11). This may result from the fact that a basic pH favoured the attachment of the enzyme in a more active conformation. A pH of 9.2 resulted in the highest activity, so it was chosen for the next assays.

It is known that native trypsin at pH=9 is unstable, however, immobilized trypsin 5% AG-VPr, retain its catalytic activity at 20.43%. This allows to immobilization enzymes unlike native enzyme exhibiting catalytic activity over a wide pH range. It was found investigated that the effect of temperature is on the relative activity of native and immobilized trypsin. Found that the relative activity of immobilized trypsin is optimal at 308 K and for native trypsin at 310 K. This indicates that the temperature differently affects the relative activity of the native and immobilized trypsin. The decrease in the activity of native trypsin with an increase in temperature is the denaturation of protein macromolecules. A reduction in the activity of immobilized trypsin in an increase in temperature occurs due to the interaction of the free -COOH group AG functional groups in the active sites of the enzyme molecule. This fact coincides with the literature data [5, 29,].

The kinetic results obtained for the free and immobilized trypsin are presented in Fig. 3 as Lineweaver-Burk plots, $1/v_0$ versus 1/[S]. The linear nature of the Lineweaver-Burk plots proves that, in the ranges of trypsin concentration examined, both enzymes followed the Michaelis-Menten kinetics. K_M increased and V_{max} decreased, for immobilized trypsin, compared to its free counterpart, which indicates that the immobilized trypsin had less affinity for the substrate.

However, the K_M values were of the same order of magnitude. This means that the catalytic function of trypsin was not significantly impaired by the immobilization procedure. This increase in K_M might has been a consequence of a lower accessibility of the substrate to the active site of the immobilized enzyme, which was caused by several factors such as protein conformational changes induced by attachment to the support, steric hindrance and diffusion effects. This increase is not very significant when compared with others observed in the immobilization of proteases in solid supports. A quite significant decrease was observed in the V_{max} value after immobilization: $V_{max,imob} / V_{max,free} = 0.25$. This is not sufficient to draw conclusions about the intrinsic activity of the immobilized trypsin, though. In fact, the activity assay was conducted at pH 7.0 and 37 ºC, which are almost the optimal pH and temperature of the free trypsin. At these conditions, the catalytic power of the immobilized trypsin is less than half of its maximum. If the assays were carried out in conditions closer to the optimal conditions of the immobilized trypsin, results would have been more favourable to it. However, it is natural that some aminoacids, essential to its catalytic activity, were involved in the covalent bonding. This happens very frequently in covalent bonding of enzymes to supports, and it is one of its main disadvantages.

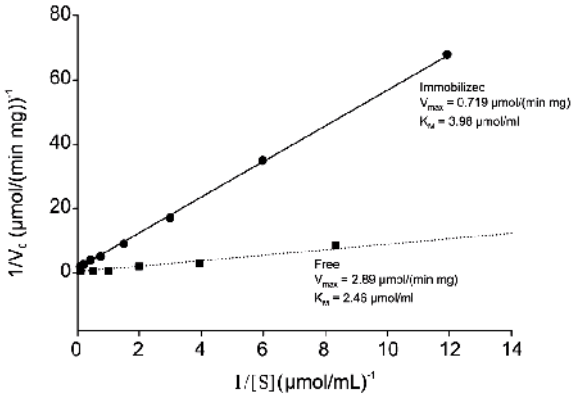

Fig. 3: Lineweaver-Burk plots for free (■) and immobilized (●) trypsin. Reactions were carried out in phosphate buffer pH 7.0 at 37 ºC.

Conclusions

In this study, trypsin was physically immobilized on poly-Ninylpyrrolidone graft copolymers basis on arabinogalactane and its metal derivatives. The immobilization procedure provided for both suitable coupling environment and good recovery of activity. Activity recovery of about 60 to 70% were obtained for bovine pancreatic trypsin immobilized onto the macroporous poly-Ninylpyrrolidone graft copolymers basis on arabinogalactane hydrogels. The physico-chemical properties of trypsin were changed after immobilization, but whilst the kinetic parameters were slightly less favourable than those of the free trypsin, the excellent storage stability and reusability demonstrate the potential of the trypsin-polymer

system for practical applications. Most importantly, any enzyme with free amino groups can be successfully attached to this support. Thus, poly-Ninylpyrrolidone graft copolymers basis on arabinogalactane is a promising biocatalytic support.

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