Biochimica et Biophysica Acta, 484 (1977) 109—114

BBA 68216

PREPARATION AND PROPERTIES OF TRYPSIN AND CHYMOTRYPSIN COUPLED COVALENTLY TO POLY(*N*-VINYLPYRROLIDONE)

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(Received February 25th, 1977)

Summary

Water-soluble poly(*N*-vinylpyrrolidone) of molecular weight 10 000 was modified by hydrolysis of 5% of the γ-lactam rings, and formation of the *N*-hydroxysuccinimidester. This activated polymer was bound covalently to trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) to complexes of a molecular weight of about 150 000. The bound enzymes showed an increase in stability towards autolysis. Towards small molecular weight substrates the trypsin-poly(*N*-vinylpyrrolidone) conjugate showed an increase in specific activity of 1 : 1.6, whereas the activity towards larger substrate was found to be only 20%. Chymotrypsin-poly(*N*-vinylpyrrolidone) showed decreased activity against small (50%) and large (7%) molecular weight substrates.

Introduction

Protein modification by binding to soluble polymers is of special scientific and medical interest because by this method a variety of parameters of the bound protein molecule may be changed. By several groups it was shown that the immunogenic properties can be manipulated, e.g. antigenic determinants may become more immunogenic [1]. By changing the molecular weight the filtration rate through the kidneys may be minimized and the circulation time in the body prolonged [2]. The attack of proteolytic enzymes on the bound proteins was shown to be inhibited either through blocking of potential cleavage points or by steric hindrance [3]. A variety of polymers were used until now for binding of proteins. Dextran was used for the binding of hemoglobin [2] and trypsin (EC 3.4.21.4) [4]. Chymotrypsin (EC 3.4.21.1) was bound to carboxymethyl-cellulose [5]. But also polyamino acids like the D-Glu-Lys, a copolymer of glutamic acid and lysine [6], or polyethylenimine were used as carrier. The present work describes the partial modification of poly(*N*-vinylpyrrolidone), which is an accepted plasma additive in humans, to

enable covalent binding of proteins and properties of poly(*N*-vinylpyrrolidone)-bound chymotrypsin and trypsin. Part of this work was already presented in short [7].

Materials and Methods

Activation of poly(N-vinylpyrrolidone) and coupling to trypsin and chymotrypsinogen A. Poly(*N*-vinylpyrrolidone) K 15, (Fluka, Switzerland) with an average molecular weight of 10 000 was activated according to a modified procedure, which was described in short previously [7]. 5 g poly(*N*-vinylpyrrolidone) and 3 g NaOH were dissolved in 200 ml H_2O. The mixture was kept in an autoclave for 36 h at 120° C under a nitrogen pressure of 150 atm. The pH was then adjusted to 9 with boric acid and 2.86 ml of formaldehyde solution (35%) was added, followed by addition of 1.5 g of sodium tetrahydroborate. The solution is stirred for 45 min at room temperature, then the pH is adjusted to 2.5. The occasional occurrence of precipitate is removed by filtration.

The solution is then dialysed, concentrated, dried by azeotropic removal of water with alcohol and chloroform. The dry residual is dissolved in 100 ml chloroform and added dropwise to 500 ml of stirred diethyl ether. The white precipitate is collected on a glass filter, washed with ether and dried in a desiccator over P_2O_5 at high vacuum. The amount of ring opening at that stage was determined by titration in dimethylformamide with sodium methylate and thymol blue as indicator.

The dried polymer was dissolved in 100 ml dry dimethylformamide, then 1.035 g *N*-hydroxysuccinimide dissolved in dimethylformamide was added and 1.85 dicyclohexcylcarbodiimide in dimethylformamide was added dropwise. The solution was left to react overnight under stirring, cooled to 4° C and the formed precipitate was removed by filtration. The solution was added dropwise to 1.000 ml of stirred dry diethyl ether and the precipitate collected by filtration and washed with dry ether. If an oil is obtained, decantation and precipitation from chloroform ether yields a white powder. After removal of the ether in a desiccator about 3 g of activated poly(*N*-vinylpyrrolidone) is obtained which can be stored in a desiccator over P_2O_5 for several months at room temperature.

Coupling of the activated polymer to bovine pancreatic trypsin EC 2.4.4.4 (Sigma Nr. T-8253) and chymotrypsinogen A from bovine (Marck, GRF) was performed by dissolving 10 mg of enzyme in 5 ml of borate buffer, pH 8.5, and adding 100 mg of dry activated poly(*N*-vinylpyrrolidone) while stirring. The pH is kept constant by addition of sodium tetraborate. After 30 min the reaction mixture is transferred to 4° C, stirred overnight, then centrifuged to remove insoluble material and concentrated by ultrafiltration in an Amicon cell with an XM 50 membrane to a sample volume of 1 ml. The sample is then diluted 10 times and concentrated again in order to remove most of the unbound poly(*N*-vinylpyrrolidone). Finally the sample is concentrated to about 0.5 ml on an Amicon B 15 apparatus and then passed through a Sephadex G-150 column (1 × 90 cm) at a rate of 5 ml/h. The elution was performed with 0.001 M HCl containing 0.9% NaCl for trypsin and 0.05 M

phosphate, pH 7.1, for chymotrypsinogen. The first peak is collected. Protein was determined by quantitative amino acid analysis according to ref. 8 after acid hydrolysis. Polymer-bound and free enzyme samples of similar activity were both hydrolysed in 6 M HCl for 24 h at 110°C in vacuo in seeled vessels. The amino acid analysis of the hydrolysate was performed with a Beckman multichrome liquid column amino acid analyser. The protein content was calculated from the recovery of glutamic acid, valine and glycine.

The amount of poly(*N*-vinylpyrrolidone) in the conjugate was determined with the iodine reagent [9]. To 1 ml of sample dilution 60 µl of iodine reagent (0.08 M KJ, 0.01 M J_2) is added and the absorbance read at 490 nm. The amount of poly(*N*-vinylpyrrolidone) is read from a standard curve between 10 and 100 µg poly(*N*-vinylpyrrolidone) per ml.

Activity determinations. The activity determinations of polymer-coupled and unbound trypsin were measured according to ref. 10. As substrate were used benzoyl-arginine-*p*-nitroanilide (Bz-Arg-$NPhNO_2$) (Sigma), and azocasein (Sigma). For the Bz-Arg-$NPhNO_2$ measurement the enzyme concentrations used for a 3 ml assay were approx. 3.3 µg/ml trypsin or trypsin coupled to poly(*N*-vinylpyrrolidone). The weight of polymer-protein complexes is always refering to the weight of protein. The substrate concentration was 0.77 mM in 110 mM Tris buffer, pH 8.1.

The assay conditions for azocasein determinations were between 3 and 7 Bz-Arg-$NPhNO_2$ munits trypsin or polymer-bound trypsin per assay mixture, 13 mg azocasein/ml in 30 mM phosphate buffer, pH 7.6 [10].

Inhibition of trypsin and trypsin bound to poly(*N*-vinylpyrrolidone) by soybean inhibitor (Sigma) and bovine pancreatic trypsin inhibitor (Kunitz) (Bayer FRG) was determined using Bz-Arg-$NphNO_2$ according to ref. 10. 7.3 m-units trypsin or 6.73 m-units polymer-bound trypsin were incubated with various amounts of inhibitor for 5 min at 25°C and then the activity determined using Bz-Arg-$NPhNO_2$ as substrate.

Chymotrypsinogen A and chymotrypsinogen A bound to poly(*N*-vinylpyrrolidone) were activated by incubation with trypsin 1 : 30 at 5°C for 24 h in 0.1 M phosphate buffer, pH 7.8. Activation was followed by taking samples at various intervals which were assayed towards activity. Activation was stopped, when no more increase of activity could be observed, by dialysis against 0.001 M HCl.

Activity of chymotrypsin A and polymer-bound chymotrypsin A was determined using *N*-glutaryl-L-phenylalanine-4-nitroanilide (Glt-Phe-$NPhNO_2$) as substrate [11]. The incubation mixture (3 ml) contained 40—50 µg enzyme in 67 mM phosphate buffer, pH 8.0, and 12 mM Glt-Phe-$NPhNO_2$. Activity of free and polymer-bound chymotrypsin was also determined with azocasein [10]. The incubation mixture (3 ml) contained 0.1 M phosphate buffer, pH 7.6, 13 mg/ml azocasein and 5—50 µg of enzyme. 1 unit of trypsin or chymotrypsin corresponds to an extinction change ΔE_{405} of 3.32 per min in a 3 ml assay mixture with Bz-Arg-$NPhNO_2$ or Glt-Phe-$NPhNO_2$.

Digestion of free and polymer-bound enzymes. Free and polymer-bound trypsin (3 mg/ml) were incubated in borate buffer, pH 8.5, at 25°C. The activity was monitored by taking samples at various intervals up to 24 h and measuring the activity with Bz-Arg-$MPhNO_2$. 1 mg/ml chymotrypsin and 1.5

mg/ml chymotrypsin coupled to poly(*N*-vinylpyrrolidone) were incubated for 6 days in 67 mM phosphate buffer, pH 7.6, at 25° C. Aliquots were taken and assayed with Glt-Phe-$NPhNO_2$.

Titration of the carboxyl groups of poly(N-vinylpyrrolidone). The amount of carboxyl groups which was present on the polymer after hydrolysis of the lactam rings and methylation of the formed secondary amino group was titrated with 0.05 M sodium methylate in dimethylformamide [12]. Care was taken to prevent uptake of CO_2 from the atmosphere. Thymol blue was used as indicator. Because of the yellow colour of the polymer solution it was titrated to a green endpoint.

Results

Activation of poly(N-vinylpyrrolidone) and binding ratios to trypsin and chymotrypsin. The titration of the carboxyl groups present at the polymer after blocking the γ-amino groups by methylation showed that an average of 4.8 carboxyl groups per M polymer was formed. This indicates that 5% of the γ-lactam rings were opened. When hydrolysis was performed for 24 h only the amount of carboxyls formed was reduced to 2.2 groups per M polymer.

All the protein which was put into reaction was converted into high molecular weight product. By calibration of the Sephadex G-150 column with mixtures of not activated poly(*N*-vinylpyrrolidone) and of trypsin or chymotrypsin and with a mixture of γ-globulin and albumin an average molecular weight of 150 000 was obtained for the poly(*N*-vinylpyrrolidone)-enzyme conjugates. However, the peak obtained is not sharp, which shows that different sizes of polymer are present. By this method it was also shown that under the elution conditions no absorption of protein to polymer took place. The molar ratio of polymer to chymotrypsinogen was found to be 12 : 1.

Activity of the modified enzymes against high and low molecular weight substrates. Trypsin conjugated covalently to poly(*N*-vinylpyrrolidone) showed an increase in specific activity to 19 units/mg compared to 11 units/mg of the original enzyme, when assayed with the low molecular weight substrate Bz-Arg-$NPhNO_2$. Addition of poly(*N*-vinylpyrrolidone) in equal concentrations to the reaction assay showed to have no effect on the activity of trypsin or chymotrypsin.

When similar Bz-Arg-$NPhNO_2$ units of trypsin or trypsin coupled to poly(*N*-vinylpyrrolidone) were assayed with azocasein it was observed that the modified trypsin liberated about 80% less trichloroacetic acid-soluble material from azocasein. The specific activity of trypsin towards azocasein was found to be 381.65 munits/mg compared to 68.64 munits/mg (18%) for trypsin coupled to poly(*N*-vinylpyrrolidone).

This difference in activity between high and low molecular weight substrates was also observed when inhibition studies on free and polymer-bound trypsin were performed. Fig. 1 shows the inhibition of the reaction with Bz-Arg-$NPhNO_2$ of trypsin and trypsin coupled to poly(*N*-vinylpyrrolidone) by soybean inhibitor. As can be seen, trypsin is totally inhibited by 5 μg inhibitor. Trypsin coupled to poly(*N*-vinylpyrrolidone), however, could only be inhibited up to 80%. Inhibition studies with bovine pancreas trypsin inhibitor, molecular

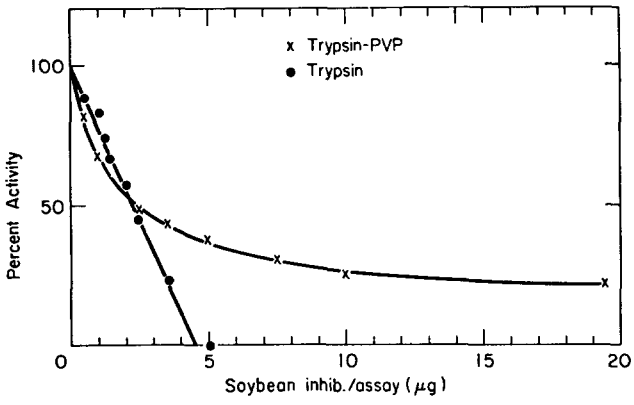

Fig. 1. Inhibition of trypsin and trypsin bound to poly(*N*-vinylpyrrolidone) (PVP) by soybean inhibitor. To similar activities of enzyme (6.77 munits trypsin bound to poly(*N*-vinylpyrrolidone) and 7.3 munits trypsin) increasing amounts of soybean inhibitor was added and the residual activity was determined with Bz-Arg-NPhNO$_2$. Activity is expressed in percent of the original activity without inhibitor.

weight 6300 showed that with the small molecular weight inhibitor trypsin coupled to poly(*N*-vinylpyrrolidone) could be inhibited up to 97%. When chymotrypsin coupled to poly(*N*-vinylpyrrolidone) was assayed against the synthetic low molecular weight substrate Glt-Phe-NPhNO$_2$ 23.87 munits/mg were measured which is about 60% of the activity of the unmodified enzyme (40 munits/mg).

Against the high molecular weight substrate azocasein for the modified chymotrypsin only 24 munits/mg (7%) were measured compared to 331 munits for the original enzyme.

Inhibition of autolysis. Autolysis of trypsin and trypsin coupled to poly(*N*-vinylpyrrolidone) in borate buffer, pH 8.5, at 35° C showed a marked degree of stabilization of the modified trypsin. After 24 h the activity of trypsin was reduced to 16%. Trypsin coupled to poly(*N*-vinylpyrrolidone), however, showed still 70% of its original activity.

When chymotrypsin or chymotrypsin coupled to poly(*N*-vinylpyrrolidone) were incubated in phosphate buffer, pH 8, at 75° C for 117 h a loss of 39% of its original activity was observed. Under the same conditions the modified chymotrypsin retained almost 100% of its activity.

Discussion

Hydrolysis of the γ-lactam rings of poly(*N*-vinylpyrrolidone) K 15, (molecular weight 10 000) and blocking of the formed γ-amino group with formaldehyde yields a polymer which carries about five carboxyl groups per 10 000 molecular weight. The fact that all the protein which was put to reaction with poly(*N*-vinylpyrrolidone) was converted into high molecular weight product shows that the coupling method is very efficient.

An important factor in coupling proteins to soluble carriers is to control the molecular weight of the product because high molecular weight conjugate tend to loose activity especially towards high molecular weight substrates, as

reported previously for the case of trypsin-dextran conjugates [4]. As seen from the data, even against high molecular weight substrates a considerably high activity is preserved. The difference in activity towards high and low molecular weight substrates indicates that the main changes which are caused by binding short chains of poly(*N*-vinylpyrrolidone) to the terminal amino groups of the enzymes are of steric origin. The fact that the activity of typsin towards Bz-Arg-NphNO$_2$ is increased by the binding to poly(*N*-vinylpyrrolidone) may be interpreted by increased local substrate concentration. Poly(*N*-vinylpyrrolidone) is known to bind small molecules as dyes and drugs. The increased activity is proven by two independent experiments: the titration with bovine pancreas trypsin inhibitor and the amino acid analysis, from which almost identical values were obtained. In the case of chymotrypsin the strong inactivation also against small molecular weight substrates indicates changes at the active side which causes part of the polymer-bound enzyme to be totally inactive.

The data presented show that proteins can be increased in molecular weight without becoming inactivated. This was confirmed in recent studies [3] where hexosaminidase A, the missing enzyme in Tay-Sachs disease, was coupled to poly(*N*-vinylpyrrolidone). In vivo studies in rabbits showed an increase of circulation time in terms of activity from 20 min for the free enzyme to 48 h for the polymer-bound enzyme. Immunological studies with albumin coupled to poly(*N*-vinylpyrrolidone) (von Specht, B.U. and Segal, S., unpublished results) show that the ability of albumin coupled to poly(*N*-vinylpyrrolidone) to recall memory in albumin immunized animals is drastically reduced. These facts indicate that the presented method of modifying poly(*N*-vinylpyrrolidone), which is known to be non-toxic and applicable to humans by its usage as a plasma expander for many years, so that binding of proteins is enabled, may serve as a powerful tool in enzyme replacement therapy and in cases where proteins have to be administered to humans.

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