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Rhodanese-BSA polymers entrapped in insolubilized gelatin gels: Properties and behavior in continuous systems

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Bovine liver rhodanese (thiosulfate: cyanide sulfurtransferase, E.C. 2.8.1.1) was reticulated with bovine serum albumin (BSA) and the resulting rhodanese-BSA copolymers were entrapped in gelatin gels insolubilized with formaldehyde. Coreticulation prevented enzyme release from the gel matrix. Entrapment in the insolubilized gelatin gel allowed the capture of immobilizates having the geometrical configuration of membranes. Activity yields, pH optimum, storage, and thermal stability of both the soluble enzyme-albumin copolymers (SIE) and the gel-entrapped enzyme (GEE) were investigated and compared with those of free enzyme. Entrapment significantly affected activity yields due to partial denaturation during immobilization whereas enzyme stability was enhanced. GEE completely retained initial activity after 30 days of storage at 4°C or a 30 min incubation at 52°C. Hysteretic behavior of SIE was noted. Further, inactivation during the catalytic cycle of free enzyme and the two immobilized species was investigated and attributed to oxidation phenomena as demonstrated by the possibility of regenerating GEE with a reducing agent (sodium salt of the thioglycolic acid, TGA). TGA also enhanced storage stability of the three enzymatic preparations. The behavior of GEE in a plug-flow reactor (PFR) was examined and compared to that obtained in a continuously stirred tank reactor (CSTR).

Keywords: Rhodanese; thiosulfate:cyanide sulfurtransferase; immobilization

Introduction

Enzyme immobilization can be carried out with the goal of preparing biocatalysts, with improved stability suitable for specific industrial processes (which can be easily reused) and/or for studying the effects induced by immobilization and the existence of heterogeneous environments on both the enzyme properties and the reaction yield.

Bovine liver rhodanese (thiosulfate:cyanide sulfurtransferase, E.C. 2.8.1.1) catalyzes the *in vitro* conversion of the cyanide ion (CN^-) into the less toxic thiocyanate (SCN^-) according to the reaction scheme:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-} \quad (1)$$

The catalytic action of rhodanese follows a double displacement mechanism. Two distinct forms of the enzyme are present: the sulfur-free enzyme, *E*, and the covalent intermediate, *ES*.[1] A persulfide linkage is formed between the sulfur atoms and the sulfhydryl group of the active-site residue Cys 247.[2] During the last decade, rhodanese (a single 33 Da polypeptide chain) was extensively used either as model for the study of protein folding [3,4] or for its potential biotechnological applications.[5,6]

It is estimated that roughly 3 million tons of cyanide compounds per year are used worldwide in the production of chemical intermediates, and consequently, the problem of wastewater detoxification is very serious. In connection with the solution of this large environmental problem, rhodanese was immobilized on activated porous glass beads for use as an enzyme thermistor in environmental control[5] and on Sepharose in order to develop detoxification processes that continuously operate.[6]

This paper deals with a simple method for immobilizing

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rhodanese and its use in continuous systems. At first, the enzyme was reticulated with bovine serum albumin (BSA) in the presence of glutaraldehyde; then the soluble enzyme-albumin copolymers were entrapped in a gelatin matrix hardened with formaldehyde. Both the rhodanese-BSA polymers (soluble-immobilized enzyme, SIE) and the insoluble immobilized enzyme (gelatin-entrapped copolymers, GEE) were tested for optimal pH, storage, and thermal stability. The results were compared with those obtained using the native free enzyme. Thin membranes of the gelatin-immobilized biocatalyst were prepared and the enzyme behavior was investigated in a plug-flow reactor (PFR) (suitably conceived) and a continuous-stirred tank reactor (CSTR). The experiments were set up to monitor the intrinsic enzyme kinetics and verify the possibility of a long storage and reuse of GEE. Finally, the presence of intra- and interphase mass transfer resistances induced by the adopted immobilization procedure and the employed reactor configurations were also tested.

Materials and methods

Rhodanese from bovine liver (Type I, 30 U mg^{-1} solid) and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.). Fine gelatin leaf was a gift of Deutsche Gelatine-Fabrick Stoess (Eberbach, Germany), thioglycolic acid (sodium salt, TGA) was from Merck (Darmstadt, Germany). All other chemicals and reagents were analytical grade products.

Rhodanese concentration is given as mg solid ml^{-1} unless otherwise specified. Protein determined with a Bio-Rad protein assay kit using bovine serum albumin (BSA) as standard accounted for 50% (w/w) of the total solid in the stock.

Immobilization technique: Copolymerization and entrapment

The soluble rhodanese-BSA copolymers (SIE) were prepared according to the method of Cantarella *et al.*,[7] but the procedure was modified as follows. Sodium phosphate buffer solutions (20 mM, pH 6.8) were prepared with the addition of 40 mg ml^{-1} BSA 1 mM sodium thiosulfate, and 0.2 mg ml^{-1} rhodanese in the presence of 0.6% (w/w) glutaraldehyde. The mixture was then allowed to react at 4°C for 10 h. Polymerization was stopped by addition of excess glycine, and dialysis of the solution against phosphate buffer was successively performed.

Rhodanese-BSA copolymers were entrapped in gelatin according to the procedure of Dhulster *et al.*[8] but modified as follows. A stirred vessel was filled with the liquid mixture containing the copolymers (rhodanese concentration, 0.2 mg ml^{-1} of final gel), gelatin (5% w/w), and 1 mM sodium thiosulfate and the hardening solution (20% formaldehyde, 40% water, and 40% ethanol, v/v) up to a final HCHO/gelatin weight ratio equal to 0.1. Immediately after the addition of formaldehyde, the mixture was rapidly poured on a glass surface and allowed to gel at –20°C. Film formation was completed in roughly 4 h; then the system was progressively heated to room temperature. The gel was gently removed from the glass support and rinsed several times with 0.9% (w/v) NaCl. This solution was also used for the storage of GEE rhodanese at 4°C. The film thickness varied between 0.05 and 0.14 cm depending on the mixture volume poured onto the glass support. Two films different in size were prepared (1.5 × 6.5 cm and 9.1 × 11.8 cm).

Film reactor

A photograph of the film reactor is reported in *Figure 1*. The gel film (9.1 × 11.8 × 0.14 cm) filled with the enzyme is set between the two plexiglass plates (held together with screws and nuts) and held in place by means of a *o*–ring. The reactor was continuously fed with a peristaltic pump which forced the reaction mixture to lap the upper surface of gel film. The lower reactor plate is completely in contact with the film while the geometry of the upper plate was prepared in order to form a continuous channel over the membrane. Because of the contact surface between the upper plate and the gel film, only 60% of the latter is directly wetted by the reaction stream. In this reactor configuration, the net flux of liquid across the film is not allowed. Reactants and products can only forward and backward diffuse into the gel. Under the experimental condition adopted, the hypothesis of a plug-flow reactor is found.

Enzyme assay

Rhodanese activity was assayed at 30°C. The colorimetric method based on the absorption at 460 nm of the ferric-thiocyanate complex was used to determine the formation of thiocyanate.[9] The standard reaction medium contained 50 mM potassium cyanide, 50 mM sodium thiosulfate, 40 mM potassium dihydrogen phosphate, and 200 mM glycine, pH 8.6.

In the batch experiments (40 ml), the reaction was started with the addition of free rhodanese (0.2 mg) or an equal enzyme amount of SIE. The reaction medium was confined in a stirred-batch reactor equipped with a water jacket for temperature control. In the case of runs with GEE, the film (1.5 × 6.5 × 0.14, filled with 0.273 mg of rhodanese) was cut into four pieces and suspended in the same reactor volume. At fixed time-intervals, aliquots of the reaction medium were taken, added to 1 ml of 15% formaldehyde to stop the reaction, and assayed for thiocyanate. Specific activity of free rhodanese, expressed as μeq of thiocyanate min^{-1} mg^{-1} of solid, was 55.4.

In PFR experiments, the film reactor was fed with the reaction medium at different flow rates from 0.1–2 ml min^{-1}. Because of the small reactor volume, the steady-state condition should be rapidly attained. On the other hand, the reactor volume and the enzymatic gel film used in CSTR experiments were the same as the batch system. The continuous-feed flow across the reactor was 1 ml min^{-1}. At steady-state conditions, rhodanese activity is given by Q × S where Q is the flow rate and S is the concentration of thiocyanate in the effluent.

pH optimum determination

The pH dependence of the free enzyme, SIE, and GEE was determined in reaction media prepared with 60 mM sodium phosphate buffer and bulk pH ranging from 6.0–8.0.

Addition of substrates to phosphate buffer determined a pH

Figure 1 Photograph of the flow reactor with the two plexiglass plates and the gelatin film on the right side

shift towards alkaline values; therefore, the enzyme activity was measured in the pH range from 8.46–10.15. The pH of the system was measured at 30°C before the addition of enzyme, and because of the differential conditions adopted, it can be assumed that the pH did not change during the incubation time.

Storage stability and GEE reuse

Storage stability of the free enzyme and SIE was monitored at 4°C in two different media: phosphate/glycine solution, pH 5.0 and the same buffer but enriched with 100 mM thioglycolic acid-sodium salt (TGA) and 1 mM $Na_2S_2O_3$. The film with gel-entrapped enzyme (1.5 x 6.5 x 0.14 cm) was always stored in 0.9% (w/v) NaCl solutions unless otherwise specified.

The reuse of film-entrapped rhodanese at the end of each run either in batch or continuous reactors required a regeneration step. Therefore, the gel was soaked in sodium phosphate buffer (100 mM, pH 6.8), TGA (100 mM), and $Na_2S_2O_3$ (1 mM) for 12 h at 4°C.

Thermal stability

Thermal stability of the free enzyme, SIE, and GEE was measured in the phosphate/glycine solution at 52°C for the sake of comparison with other results reported in the literature.[10] In the case of soluble enzyme preparations, samples at fixed enzyme concentration (0.2 mg ml^{-1}) were incubated and at regular time-intervals were rapidly cooled in an ice-bath to stop the inactivation process. In the case of GEE, each sample was prepared by suspending a 1.5 x 6.5 x 0.14 cm membrane in 40 ml of storage medium. Residual activity was measured according to that adopted standard method.

Results

Time course of reaction in batch reactors for free enzyme, SIE, and GEE

The specific product formation (μeq mg^{-1} biocatalyst) for the reaction catalyzed by the free enzyme, SIE, and GEE in batch reactors is reported in *Figure 2* versus the process time. The highest conversion reached during two h incubation was roughly 16%. It can be observed that the reaction rate tends to zero independently of the rhodanese preparations. The different asymptotic values of SCN^- formation were attained in experiments with the three enzymatic samples.

The average activity yield (ratio between the specific activity of the immobilized enzyme species and that of the free one) was calculated from the final values of product concentration which is obviously related to the values of the instantaneous reaction rate during the course of the process. In soluble copolymers, rhodanese retains 71% of free enzyme activity while that saved in the gelatin gels is 33% of the free enzyme.

The behavior of the enzyme in the three samples is quite different at very short process times (less than 5 min, *Figure* 2 inset). In the case of GEE, the enzyme activity apparently increases because of the time needed to reach inside the matrix of the gel-film, initially filled with pure buffer, a substrate concentration equal to that in the bulk phase. In the case of SIE because of the absence of any void volume, the difference with the free enzyme might be attributed to an hysteretic behavior which is always noted when the fast-phase velocity of reaction exists. However, the amplitude of

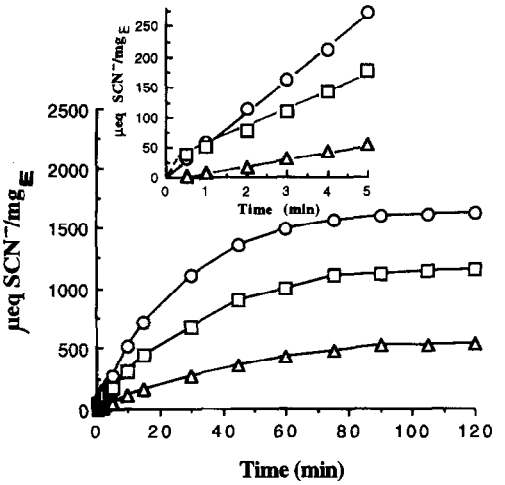

Figure 2 Time course of specific product formation for the rhodanese-catalyzed reactions in the case of free enzyme (○); SIE (□); and GEE (△). Thickness of GEE was 0.14 cm. The inset shows the progress reaction curves for the three enzyme preparations during the initial five min

the phenomenon appears to be limited both in length of time and excursion of values.

pH optimum of SIE and GEE

Activity vs. pH profiles for the free enzyme, SIE, and GEE are reported in *Figure 3.* All the values were calculated as the percentage of the highest observed reaction rate.

The specific activities of free enzyme, SIE, and GEE measured at optimum pH in phosphate buffer were in good agreement with those obtained using the standard reaction medium (pH 8.6).

Activity of free rhodanese was strongly dependent on pH. One unit variation caused, in comparison with the value at optimum pH, 47 and 26% reduction in activity, respectively, in the lower and upper range. The gel-entrapped enzyme, GEE, has a behavior similar to the free enzyme with the exception of a higher preferential range of pH (less drastic activity reduction above optimum pH). The SIE exhibited a much broader range for optimum pH. Activity remained almost unchanged between 9.25 and 10.15 while in comparison with the free enzyme and GEE, it is less curtailed between 8.46 and 9.25.

TGA effect on storage stability and GEE regeneration

The percentage of residual activity in the enzymatic preparations after 30 days incubation at 4°C are reported in the bar plot of *Figure 4.* The values referred to the activities measured at the beginning of the storage period. Storage stability of both the free enzyme and SIE was poor in phosphate/glycine buffer and was largely improved by the presence of 100 mM TGA and 1 mM $Na_2S_2O_3$ in the storage medium. In these latter media, rhodanese retained 100% of its initial activity.

On the other hand, storage stability of GEE was very good also in phosphate/glycine since rhodanese activity remained unchanged. These results elucidate that coreticulation with BSA is not sufficient to preserve enzyme activity

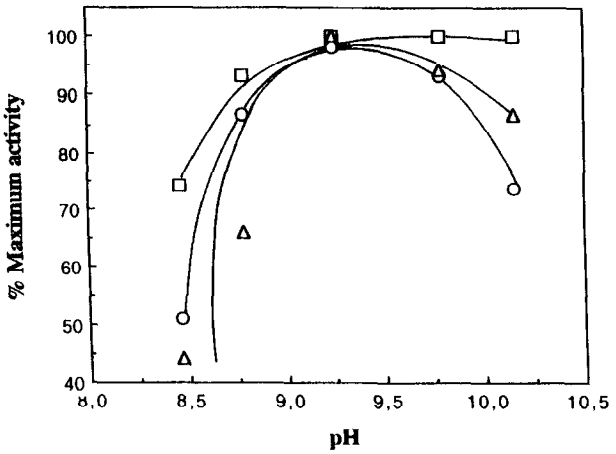

Figure 3 Rhodanese pH-activity profiles: free enzyme (○); SIE (□); and GEE (△). pH values are those of solution bulk. Thickness of GEE was 0.14 cm

during storage in pure buffer and the gel entrapment is necessary to meet this end. Data not reported confirmed that SIE was partially labile even in the presence of TGA and $Na_2S_2O_3$ since, after 70 days of storage, 70% of initial activity was loss.

It is also interesting to observe that during 30 days of storage in 100 mM TGA and 1 mM $Na_2S_2O_3$, activity of GEE increased up to 150% of the initial value and was higher (200%) after 70 days of storage. This increase in activity could be determined by a progressive regeneration of active sites of the enzyme not irreversibly denatured during the gel entrapment. According to this hypothesis, the results would be apparent because the true initial activity is masked.

A second behavior was typical of GEE. Inaccurate rinsings of the gel film after the incubation run determined complete loss of activity if the enzyme was stored overnight in the phosphate/glycine solution at pH 5.0. However, 40 and 100% of initial activity can be recovered after soaking the gel in 100 mM TGA and solutions prepared with 100 mM TGA and 1 mM thiosulfate, respectively.

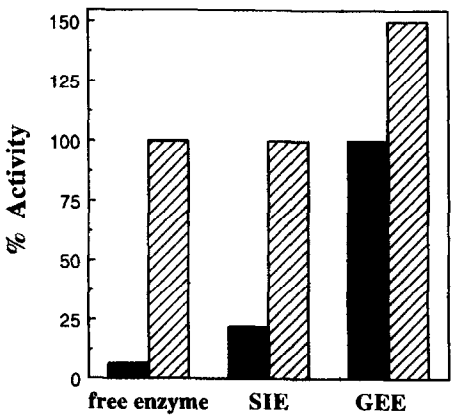

Figure 4 Storage stability after 30 days at 4°C of free enzyme, SIE, and GEE without (black bars) or with (grey bars) 100 mM TGA plus 1 mM thiosulfate in phosphate/glycine solution. Activities of the enzyme preparations are the values at zero time

The positive effect exerted by TGA was also evident at operational conditions. A slightly lower rate of activity decrease occurred in batch experiments carried out with GEE when 50 mM TGA was added to the reaction medium (*Figure 5*).

Thermal stability of free enzyme, SIE, and GEE

The inactivation kinetics of the free enzyme, SIE, and GEE at 52°C are illustrated in *Figure 6*. The linear decay in the semilog plot of A/A_0 vs. time (A and A_0 are the activities of partially inactivated and noninactivated enzyme at zero time) affirms that enzyme thermal inactivation obeys a first-order mechanism. The slope allows measurement of the rate constant of the enzyme inactivation reaction (k_d) at 52°C/. The different k_d values calculated for the free enzyme and SIE (0.234 and 0.083 min^{-1}, respectively) proved that enzyme coreticulation with BSA significantly enhanced thermal stability of rhodanese. GEE residual activity was still 100% after 30 min of incubation at 52°C and remained 80% of the initial value after 60 min of storage. Stabilization of the enzyme in gelatin gel was very satisfactory. This immobilization method seems to be more effective in comparison with the coupling of rhodanese to activated CH–Sepharose since, following this procedure, residual activity of immobilized enzyme was only 30% of the initial value after 15 min of incubation at 52°C.[10]

Continuous reactors

Further experiments studied the use of the gel-entrapped rhodanese in continuous reactors. Both continuous stirred tank reactors loaded with a portion of the GEE film or flow reactors (plug-flow condition) with the lower plate totally covered by the gelled film were utilized. *Figure 7* shows the behavior of GEE in the CSTR at 1 ml min^{-1} flow rate. Residence time in the reactor was 40 min and the reactor operated at strongly differential conditions. The initial portion of the curve (between 0–90 min) depicts the transient behavior due to product accumulation in the reactor. In the case of constant enzyme activity, the second portion of the curve should be a zero slope line since a steady state should be attained after 120–160 min from run start-up. Conse-

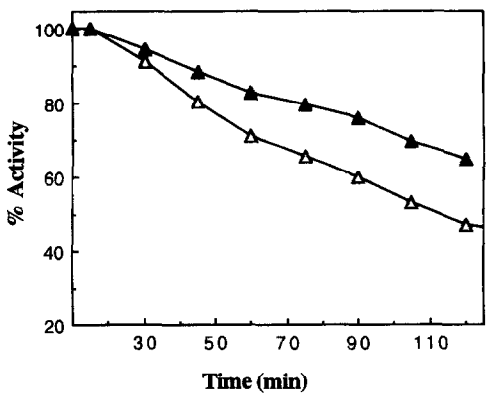

Figure 5 Ratio of instantaneous to initial activity of GEE versus process time in batch reactors: medium without (△) or with 50 mM TGA (▲)

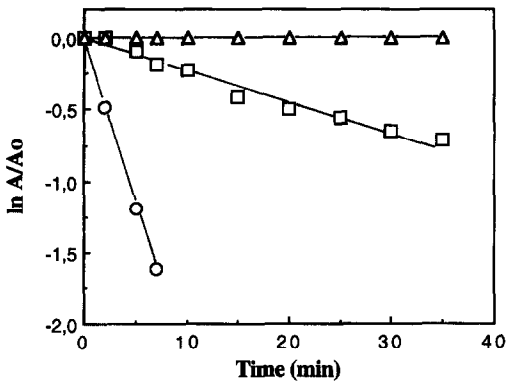

Figure 6 Thermal stability at 52°C of free enzyme (○), SIE (▲), and GEE (△)

quently, the calculated specific reaction rates ($Q \cdot C_p m_E$ where m_E is the enzyme weight) are apparent but the plot clearly indicates that enzyme underwent a progressive and significant loss of activity. On the contrary, data obtained in the PFR (*Figure 8*) seem to depict a different situation. Reaction rates attained different steady-state values at the end of a transient period, the length of which also depended on the flow rate. Because of the very small reactor volume and the plug-flow condition, the initial portion of the curve cannot be related to product accumulation in the reactor but more reasonably, to the saturation of gel void volume with substrate. The possibility of observing for a significant time a constant reaction rate depended on the larger amount of enzyme loaded in the reactor (3.0 mg) in comparison with that present in the CSTR (0.273 mg).

The enzyme reuse in the PFR was quite simple and did not require removal of the film from the reactor. At the end of each run, 200 ml of a 100 mM TGA plus 1 mM $Na_2S_2O_3$ solution were fed to the reactor at 1.5 ml min^{-1} flow rate. Repeated cycles of operations and film rinsing permitted the use of GEE for 15 days in the PFR without apparent loss of rhodanese activity.

Discussion

Although less popular than other commonly used gels, gelatin has proven to be a convenient matrix for entrapping

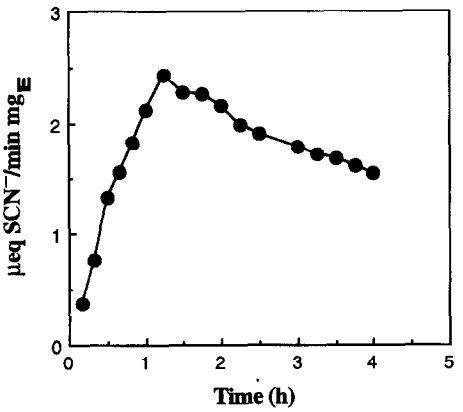

Figure 7 Specific rate of GEE-catalyzed reaction (1.5 × 6.5 × 0.14 cm film) in the CSTR system. Experiments were carried out at 30°C. Flow rate was 1 ml min^{-1}

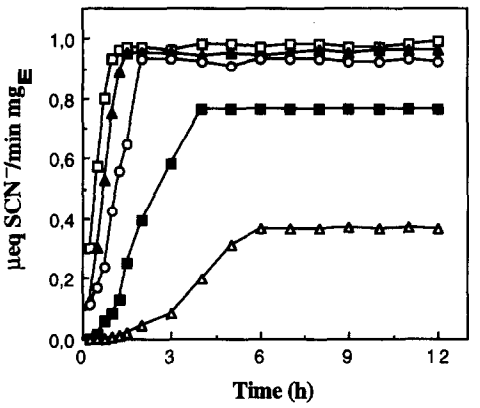

Figure 8 Specific rate of GEE-catalyzed reaction (9.1 × 11.8 × 0.14 cm film) in the PFR system at different flow rates: 0.1 (△); 0.25 (■); 0.5 (○); 1.0 (▲); and 2.0 (□) ml min^{-1}

either microbial cells or enzymes provided the solution/gel change is made irreversible by means of cross-linking agents such as formaldehyde[8] or polyaldehydes.[11,12] This technique has proven to be adequate also in the case of an enzyme of low molecular weight such as rhodanese on the condition that coreticulation with an inert protein (BSA) is carried out to provide an effective enlargement of the size of the enzyme. Activity yields were comparable with those reported for rhodanese immobilized with different techniques.[5,6]

The entrapment of SIE in gelatin gels insolubilized with formaldehyde resulted in physically stable films. In a previous study, these showed good mechanical properties and were characterized by a sponge-like structure which ensured a large surface available for interactions between substrate and biocatalyst.[13] This observation seems to be confirmed by the constancy of activity yield when the membrane thickness was increased from 0.05 to 0.14 cm (data not shown). However, the experiments in batch reactors showed that soluble-immobilized rhodanese (SIE) and gel-entrapped rhodanese (GEE) did not have the same activity and therefore, if internal diffusional resistances were not present, the conclusion must be reached that during gel entrapment, the activity of rhodanese is partially destroyed even though not irreversibly as suggested by the results of storage stability.

Substrate partition phenomena exerted by the polyanionic gelatin matrix on CN^- and $S_2O_3^{2-}$ should also play a minor role if present. Of course, the partitioning phenomena cannot be responsible for the pH–activity profile shift toward more alkaline values observed using SIE.

The drop in activity observed during batch experiments with free enzyme and the two immobilized species (SIE and GEE with or without TGA) could be ascribed to a gradual oxidation of the enzyme promoted by both substrates and/or products. Rhodanese is oxidatively inactivated by a number of reagents (such as phenylglyoxal, dinitrobenzene, and its derivatives) either directly or indirectly since these reagents can produce reactive intermediates consequent to their own oxidation.[14] It was also demonstrated that cyanide and sulfite can oxidize rhodanese.[15,16] The oxidation determines the formation of an intraprotein disulfide; the sulfhydryl group of Cys 247 takes part in bridge formation, resulting in the loss of thiocyanate-forming activity.[17,18]

In this concern, the effect exerted by TGA and $Na_2S_2O_3$ during rhodanese activity regeneration in films as well as storage of the three different enzyme preparations can be explained as follows. TGA acts as a reducing agent toward disulfide bonds whereas $Na_2S_2O_3$ stabilizes the enzyme in the sulfur-donor form (ES). The latter has been reported to be more stable and resistant to oxidation;[19] therefore, the oxidative phenomena involving the sulfhydryl group of the active-site residue Cys 247 could be considered, to a large extent, responsible for the loss of the enzyme activity observed.

The different behavior exhibited by GEE when employed in PFR and CSTR reactors provided some additional information on the mechanism of enzyme inactivation and the combined kinetics and external mass transfer resistances. In the stirred CSTR, external mass transfer resistances are not present and because of the relative small amount of enzyme loaded in the reactor and the fast inactivation induced by oxidative agents, the true kinetics are observed and the effects of activity decay can be easily determined. In the PFR, external mass transfer resistances play an important role as confirmed by the different time periods required to reach a steady-state value of apparent reaction rate at the different driving force (mass transfer coefficient changes with flow velocity). On the other hand, because of the large amount of enzyme entrapped in the film, only a portion of the enzyme effectively works and enough rhodanese is present during the whole length of the experiment to mask enzyme inactivation. This is also confirmed by the value of the specific reaction rate in the CSTR which is unusually higher than that in the PFR. The difference in asymptotic reaction rates during PFR experiments could also be ascribed to product inhibition since it is also known from the literature that both substrates and products can form deadend complexes with both E and ES.[20] At high residence time (low fluid flow rate), substrate conversion increases and product inhibition tends, more significantly, to depress enzyme activity. However, the dependence of reaction rate on product concentration does not fit any mechanism for product-inhibited enzyme kinetics. Therefore, the conclusion was reached that overall kinetics is mainly controlled by external mass transfer resistances and product inhibition even if at the present, they cannot be determined by these experiments.

Rhodanese immobilization was found to be a useful tool to point out another peculiar property of the enzyme, i.e., its flexibility displayed by the hysteretic behavior of SIE. Hysteresis has to be attributed to the existence of several enzyme conformers with different kinetic properties, the transitions of which are sufficiently slow compared to the reaction rate measurement.[21,22] From the results of these experiments, it is possible to speculate about differences between free enzyme and SIE in conformational transitions because of constraints induced on the enzyme molecules by covalent bonds. These transitions are probably too fast to be monitored using free rhodanese while cross-linking with BSA allows this observation. Consequently, the reaction rate catalyzed by SIE showed a time increase during the first few minutes. The phenomenon cannot be observed in reactions catalyzed by GEE since the characteristic time of gel saturation with substrate and that of enzyme hysteresis should have the same order of magnitude.

In conclusion, this work reports on the preparation and characterization of immobilized enzyme obtained by entrapping rhodanese-BSA polymers in insolubilized gelatin gels. These preparations show good storage and thermal stability, can be easily regenerated, and are suitable for use in continuous systems. Although a constant conversion can be achieved in the PFR system, the GEE performance is not yet satisfactory. A study is in progress with the goal of improving conversion yield and reactor configuration, and to further optimize the immobilization procedure.

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