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Development of an immobilized brain glutamine synthetase liquid chromatographic stationary phase for on-line biochemical studies

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Abstract

Glutamine synthetase (GS) plays a key role in the regulation of glutamate availability to neurons. In the present study glutamine synthetase was immobilized on a silica-based immobilized artificial membrane liquid chromatographic stationary phase (IAM-SP) to create the GS-IAM. The stability of GS was improved by immobilization, but the enzyme's affinity for the substrates L-glutamate and D-glutamate was significantly decreased. In contrast, immobilization significantly increased GS sensitivity to inhibition by methionine sulfoximine. The GS-IAM was packed into a chromatography column to create an immobilized enzyme reactor (GS-IMER). On-line experiments with the GS-IMER demonstrated that the immobilized enzyme was comparable to the non-immobilized enzyme with regards to retention of activity and selectivity toward substrates and inhibitors and was reusable for several weeks.

Keywords: Immobilized proteins; Stationary phases, LC; Glutamine synthetase; Glutamate; Methionine sulfoximine

1. Introduction

Active biomolecules such as enzymes, receptors and transporters have been immobilized on chromatographic supports using a variety of processes [1–3]. In general, immobilization has produced stationary phases that retain the activity of the immobilized biopolymer with increased stability. The immobilized biomolecules could be reused multiple times and have been used in flow reactors.

Among the various supports used for the immobilization processes, Pidgeon et al. [4] have developed a silica-based immobilized artificial membrane stationary phase (IAM-SP), which in our hands has proven to be one of the most efficient and useful supports. The IAM-SP was produced though the covalent immobilization of 1-myristoyl-[(13-carboxyl)tridecanoyl]-*sn*-3-glycerophosphocholine to aminopropyl silica support through an ω-carboxyl group on the C2 fatty acid chain. In the resulting support, the phosphatidylcholine headgroups form the surface of the support and hydrocarbon side chains produce hydrophobic cavities which extend from the charged headgroup to the surface of the aminopropyl silica. This structure looks like the half of a plasma membrane, and is considered as a semi-artificial membrane. Indeed, IAM-SP was successfully used for immobilization of neuronal nicotinic

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acetylcholine receptors, a transmembrane ligated ion channel receptor [3].

Glutamate is one of the excitatory amino acids involved in neurotransmission. In order to avoid an over-excitation of glutamatergic neurons, the availability of glutamate to the brain is regulated by various mechanisms including the blood brain barrier and metabolism by glutamine synthetase (GS). This enzyme, therefore, plays an important role in neurotransmission in the central nervous system as it transforms glutamate into glutamine and it is responsible for ammonia scavenging [5,6].

GS is exclusively localized in glial cells, as astrocytes [7–9], and it is in these bodies that glutamate is transformed into glutamine. Indeed, astrocytes, which surround synapses, are endowed with significantly more powerful glutamate carriers than are neurons. Thus, released glutamate is very rapidly taken up by astrocytes and inactivated by GS through amidation to glutamine. The glutamine can be recycled to the neurons where it is reconverted to glutamate and γ-aminobutyric acid [10,11].

GS is specifically inhibited by methionine sulfoximine (MSO) which is a ''side-product'' of flour bleached by the agene process [12]. MSO induces seizures in various animal species [13–15] and this effect has been attributed to its inhibitory action on GS [16]. However, increased glutamate levels have not been found in rat brain after administration of epileptic doses of MSO [17]. In addition, genetically epilepsy-prone rats and seizure-prone gerbils have a significantly lower brain GS activity than controls [18,19].

Anti-epileptic drugs have been shown to have differing effects on GS activity. Valproate stimulates rat brain GS activity in vitro [20,21], while phenytoin and carbamazepine reduce mouse brain GS activity in vivo [22]. It is unlikely that the inhibition of GS activity is involved in the anti-epileptic action of these drugs, but rather may be a source of their toxicity. Thus, the effect on GS activity of marketed drugs and new drug candidates is one way to determine their potential central nervous system toxicity.

GS is a well-characterized cytosolic enzyme constituted of 12 identical subunits of M_r 51 000 each. The enzyme is enantioselective and displays greater affinity for L-glutamate than D-glutamate. GS activity is inhibited by various amino acids, AMP and MSO. Under standard experimental conditions, solubilized GS is unstable and can not be reused for multiple studies. The objectives of the present investigation were to increase GS stability through its immobilization on a silica-based liquid chromatographic support, to characterize this support and use it to create an on-line immobilized enzyme reactor, GS-IMER.

The results of the study demonstrate that GS can be immobilized on an IAM-SP to create the GS-IAM. The GS-IAM retained the enzymatic activity of GS with increased stability. While the enzyme kinetics of the immobilized GS were significantly altered as compared to those of native GS, the enzyme retained its substrate selectivity, including its enantioselectivity relative to L- and D-glutamate, and sensitivity to reversible competitive inhibitors. The results of chromatographic studies with the GS-IMER demonstrate that the GS-IMER can be used for the on-line determination of GS substates and inhibitors.

2. Experimental

2.1. Chemicals and materials

Sheep brain glutamine synthetase, L-glutamate, D-glutamate, L-alanine, L-aspartate, L-histidine, methionine sulfoximine and others chemicals (buffers, cofactors, etc.) were purchased from Sigma (St. Louis, MO, USA). The silica-based immobilized artificial membrane chromatographic support used in this study was the non-endcapped form of this support (the IAM-PC, 12 μm, 300 Å) obtained from Regis Technologies (Morton Grove, IL, USA). In this manuscript, this support will be identified as the IAM-SP.

2.2. Immobilization of glutamine synthetase (GS-IAM)

GS was reconstituted into imidazole buffer (100 m*M*, pH 7.2), containing 10% (v/v) glycerol, in order to obtain 50 U/ml, and the resulting solution was stored at −20°C. GS was immobilized onto the IAM-SP using a previously described experimental approach [1–3]. Briefly, 5 U of GS were mixed with

various amounts of IAM-SP that had been previously washed with imidazole buffer, the mixture was briefly shaken and then left standing at 4°C for 16 h. The GS-IAM was washed three times with 1 ml of imidazole buffer. The protein content of the GS-IAM was quantified directly using the BCA Protein Assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

2.3. Glutamine synthetase activity

The enzyme activity of GS was determined using the method of Wellner and Meister [23]. Known amounts of non-immobilized GS (expressed as μg protein) or of the immobilized GS (GS-IAM, expressed as μg protein/mg support) were incubated, for 30 min at 37°C, in a total volume of 600 μl of buffer A (20 m*M* ATP, 10 m*M* β-mercaptoethanol, 40 m*M* $MgCl_2$, 100 m*M* hydroxylamine and 100 m*M* imidazole; pH 7.2). The incubations were carried out in the presence of various concentrations of D- or L-glutamate alone or with different concentrations of agonists or inhibitors. After the incubation, the glutamylhydroxamate produced by the reaction was detected by addition of 900 μl $FeCl_3$ (0.37 *M* in a solution of 0.67 *M* HCl and 0.2 *M* trichloroacetic acid) either directly to the reaction mixture, or in the case of the GS-IAM, to the supernatant after centrifugation of the reaction mixture. The absorbance of the resulting solution was read at 535 nm and compared to the absorbances produced by standard solutions of authentic L-glutamylhydroxamate. The activity of GS was expressed as m*M* of L-glutamylhydroxamate/min per mg of protein.

2.4. Chromatographic experiments using the GS-IMER

The GS-IMER was created by packing the GS-IAM (0.2 g of support containing 1.5 μg of protein/mg of GS-IAM for a total of 300 μg of protein) into a glass column (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). The bed gel size of the resulting GS-IMER was 0.5×13.5 cm. The GS-IMER was placed in a chromatographic system consisting of a P1000 isocratic HPLC pump (Thermo Separations, San Jose, CA, USA) and a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 200-μl loop. The eluent from the GS-IMER was directed into a T-connector. A second P1000 isocratic HPLC pump (Thermo Separations) was connected to one of the other two ports of the T-connector. The third port was connected to a post-column reaction coil composed of Teczel tubing (25 cm×0.03 cm I.D., Alltech, Deerfield, IL, USA) and the coil was then connected to a Spectra 100 variable-wavelength UV detector (Thermo Separations). The complete chromatographic system is presented in Fig. 1.

In the chromatographic experiments, the substrate (L-glutamate) alone or concomitantly with an inhibitor (1 m*M* MSO) was injected onto the column using buffer A as the mobile phase. The flow-rate was 0.1 ml/min and the experiments were conducted at room temperature. The eluent from the GS-IMER was mixed in the T-connector with a $FeCl_3$ solution (0.37 *M* in 0.67 *M* HCl and 0.2 *M* trichloroacetic acid) pumped into the T-connector at a rate of 0.1 ml/min. The output was monitored at 535 nm.

2.5. Sodium dodecylsulphate–polyacrylamide gel electrophoresis

GS and GS-IAM-SP were analyzed by gel electrophoresis according to the method of Laemmli [24] using a 10% acrylamide gel. The prestained molecular mass standard proteins were from Bio-Rad (Hercules, CA, USA).

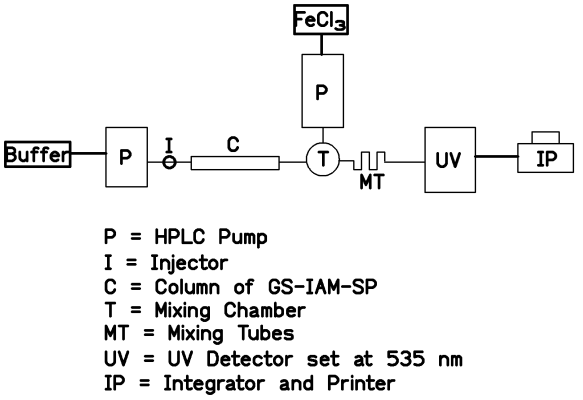

Fig. 1. Scheme of the chromatographic system used with the GS-IMER, where: C, GS-IMER; I, injector; IP, integrator and printer; MT, post-column mixing chamber; P, HPLC pump; T, T-connector; and UV, UV detector.

2.6. Statistics

The values were expressed as mean±S.E.M., and the significance was assessed using unpaired Student's *t*-test.

3. Results and discussion

The immobilization of GS on the IAM-SP was optimized relative to the amount of protein that was bound to IAM-SP and the activity of the immobilized enzyme. In these experiments, the amount of GS was held constant (5 U, 36 μg of protein) and the amount of IAM-SP was varied from 3 to 100 mg. The optimum yield of immobilization enzyme was obtained with 12 mg of IAM-SP, corresponding to the immobilization of 1.5 μg protein/mg of IAM-SP (Fig. 2A). This ratio also yielded the highest retention of GS activity, 60% of enzyme activity as compared to the activity of GS, which was processed as if for immobilization, but without IAM-SP (Fig. 2A). This ratio was used for the other immobilization experiments.

The experiments reported in Fig. 2A were carried out using 100 m*M* imidazole buffer (pH 7.2). The use of incubation buffer A (20 m*M* ATP, 10 m*M* β-mercaptoethanol, 40 m*M* $MgCl_2$, 100 m*M* hydroxylamine and 100 m*M* imidazole; pH 7.2) in place of imidazole buffer alone, did not improve the yield of the immobilization or the activity of the immobilized GS (data not shown). This suggests that the conformation of GS (with or without substrate) does not effect the immobilization of GS to the IAM-SP.

The immobilizations described above were carried out by adding the GS–imidazole solution to the IAM-SP, shaking the mixture for a brief period and then letting the mixture stand for 16 h at 4°C. If the GS–imidazole/IAM-SP mixture was stirred instead of allowed to stand, no GS activity was detected in the resulting GS-IAM. Indeed, stirring the GS–imidazole solution (Control) alone or the GS–imidazole solution in the presence of the IAM-SP (Experimental) for 4 h at 4°C also resulted in undetectable GS activities in both the Control and Experimental experiments. These results suggest that GS lacks mechanical stability which is not surprising since it is a rather large enzyme containing 12 identical subunits of M_r 51 000 each.

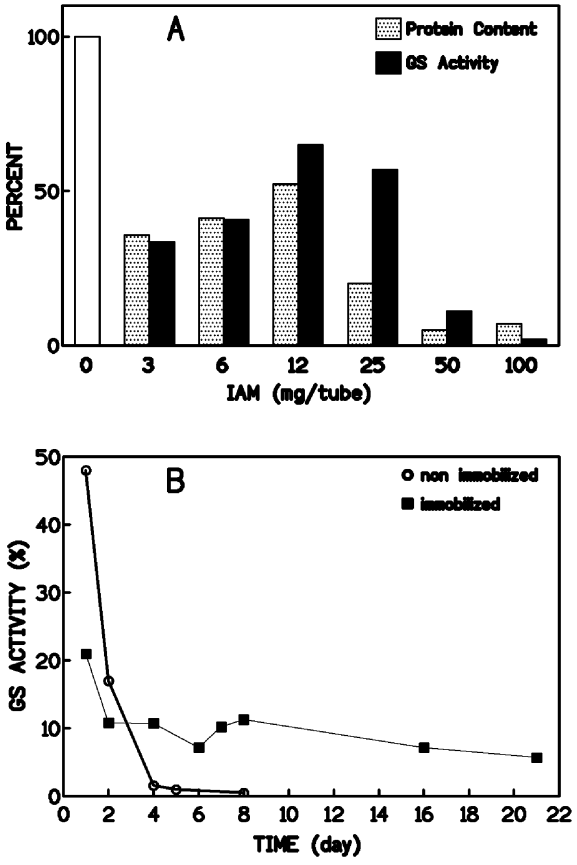

Fig. 2. Immobilization of glutamine synthetase (GS) on IAM-SP as a function of amount of IAM-SP. The IAM-SP was washed three times with imidazole (100 m*M*, pH 7.2), mixed with 5 U (36 μg of proteins) for 15 s, and incubated at 4°C for 16 h. (A) Yields of immobilization in terms of protein bound to IAM-SP and GS activity recovered. The 100% GS activity corresponded to the control experiment performed with the same amount of GS without IAM-SP. Values represent the mean of two independent immobilizations. (B) Stability of GS activity of both GS and GS-IAM-SP conserved at 4°C. Activity was compared to the activity of GS-stock before the immobilization. Values represent the mean of two independent immobilizations.

The immobilization of GS on the IAM-SP improved the overall stability of the enzyme including its mechanical stability. The GS–imidazole solution and the GS-IAM (with imidazole buffer; 100 m*M*, pH 7.2) were stored at 4°C for 21 days and the enzymatic activity was periodically assessed (Fig. 2B). In each experiment, the GS–imidazole solution

and the GS-IAM were processed in the same manner. At the initiation of the experiments, day 1, a 50% reduction in the observed GS activity of the GS–imidazole solution (as compared to the GS stock solution) was observed (Fig. 1B). By day 2, the activity of the GS–imidazole solution had decreased to less than 20% of the expected value and by day 4, no significant enzymatic activity was detected (Fig. 2B).

With respect to the GS-IAM, the activity of the immobilized enzyme on day 1 was only 20% of the GS stock solution (Fig. 2B). On day 2, this activity decreased to ~10% of the control activity. However, in contract to the GS–imidazole solution, the enzymatic activity of the immobilized GS stabilized at day 2 and no significant reduction in activity was observed throughout the remainder of the 21-day experiment (Fig. 2B).

The effect of immobilization on the molecular composition of GS was investigated using SDS–gel electrophoresis. The observed protein patterns from the GS–imidazole solution and the GS-IAM demonstrate that the protein composition is very similar in both samples (Fig. 3). These data suggest that the immobilization procedure did not alter the protein composition of the enzyme, since additional bands of degraded or aggregated proteins were not observed.

The effect of immobilization on the biochemical properties of GS were assessed through the determination of the Michaelis–Menten constants K_M (substrate affinity for the enzyme) and V_{MAX} (maximum enzyme velocity) for the GS–imidazole solution and GS-IAM. The values were calculated as a function of the concentrations of the substrates L-glutamate and D-glutamate. Both the non-immobilized and immobilized enzymes demonstrated Michaelis–Menten kinetics, and L-glutamate was a better substrate for both enzymes than D-glutamate (Fig. 4). The data indicate that the immobilization of GS did not result in a qualitative change in the biochemical properties of the enzyme, as reflected by its enantioselectivity.

The K_M values for L-glutamate determined for the non-immobilized GS and the GS-IAM were 4.89±0.32 m*M* (n=3) and 12.12±0.66 m*M* (n=3), respectively, and the K_M values for D-glutamate determined for the non-immobilized GS and the GS-IAM were 7.75 m*M* (n=2) and 19.67 m*M* (n=2), respectively (Fig. 4). The affinities for both

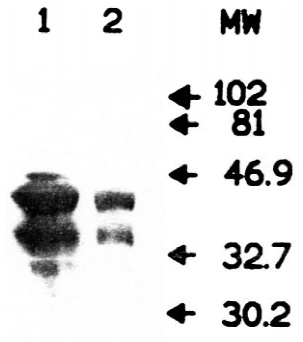

Fig. 3. Gel electrophoresis on 10% polyacrylamide gel of the GS-stock (lane 1) and of GS-IAM-SP (lane 2). MW indicates the molecular mass ($\times 10^{-3}$) of standard proteins.

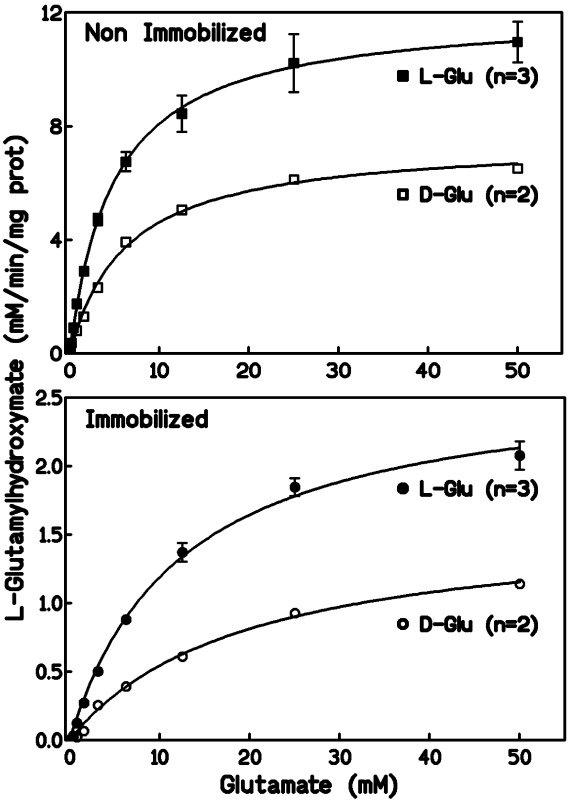

Fig. 4. Glutamine synthetase activity as a function of substrate concentrations. As described in the Experimental section, 1 U of GS was incubated for 30 min at 37°C with various concentrations of either L-glutamate or D-glutamate. Each point represents the mean±S.E.M. of two or three (as indicated) independent immobilizations.

substrates were significantly lower ($P<0.001$) for the GS-IAM than for the non-immobilized GS. The same trend was observed for the V_{MAX} values. With the non-immobilized enzyme the calculated V_{MAX} of L-glutamate and D-glutamate were 12.07±1.05 m*M*/min per mg protein ($n=3$) and 6.28 m*M*/min per mg protein ($n=2$), respectively. The same parameters on the GS-IAM were significantly lower ($P<0.001$), 2.66±0.14 m*M*/min per mg protein ($n=3$) and 1.61 m*M*/min per mg protein ($n=2$), respectively.

The inhibition of GS activity by MSO was also investigated through the determination of the inhibition constants (K_I) of MSO for non-immobilized GS and the GS-IAM. In this case, MSO was three times more efficient ($P<0.01$) as an inhibitor for GS-IAM than for non-immobilized GS, $K_I=1.77\pm0.20$ versus 0.53±0.04 m*M*, respectively (Fig. 5). The K_I values for three additional competitive inhibitors were determined for non-immobilized GS and the GS-IAM: L-alanine ($K_I=47$ m*M* (non-immobilized), 66 m*M* (GS-IAM)); L-aspartate ($K_I=66$ m*M*, 111 m*M*)); L-histidine ($K_I=72$ m*M*, 128 m*M*).

The relationship between the K_I values determined for the non-immobilized GS and the GS-IAM was investigated using regression analysis. In order to enhance the range of the correlated values, the K_M values determined for L-glutamate and D-glutamate were added to the data sets. A linear correlation ($r=0.9809$, $P=0.001$) was identified between the K_M and K_I values for GS-stock and GS-IAM-SP. Since both K_I and K_M values reflect the affinities of the substrate or inhibitors for the active site of the enzyme, the observed correlation indicates that immobilization produced quantitative, not qualitative changes in the enzyme.

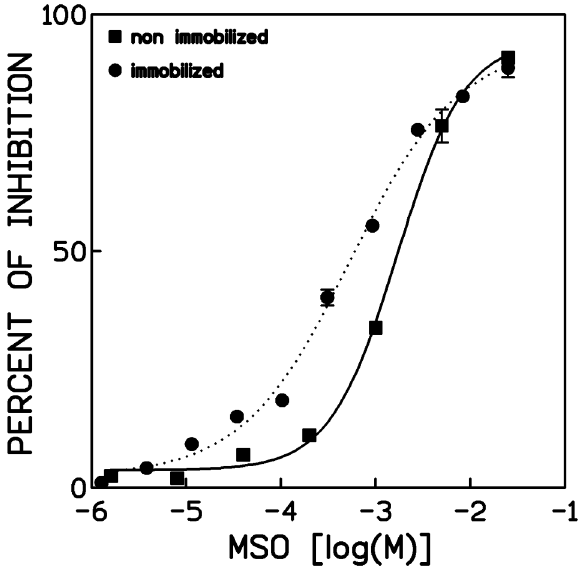

Fig. 5. Inhibition of glutamine synthetase activity of both GS and GS-IAM as a function of methionine sulfoximine. Each point represents the mean±S.E.M. of three independent immobilizations.

The data suggest that the source of these differences was a change in the accessibility of the GS active site produced by the immobilization. This is quite likely since GS is a large enzyme (M_r 600 000) constituted of 12 identical subunits, that exists as a cytosolic protein, i.e. it is not a membrane-bound enzyme. Thus, immobilization of GS on the IAM support could affect the configuration and the transconformational mobility of the enzyme. Nevertheless, the IAM immobilized enzyme retained the basic characteristics of the native GS including the capability to transform glutamate into glutamine and its sensitivity to inhibition by known competitive inhibitors.

Since the K_M and K_I values obtained on the GS-IAM are comparable to results obtained using the non-immobilized enzyme, the data suggest that the GS-IAM can be used to screen for GS agonists or antagonists. This is particularly attractive since the immobilization of GS on the IAM support increased the stability of the enzyme and the GS-IAM is reusable. Similar results have been reported for the immobilization of nicotinic receptors on the IAM-SP [1]. The IAM-immobilized receptors correctly determined the relative order of binding affinities for tested ligands, although the K_d values determined with the immobilized nicotinic receptors were consistently lower (up to five-fold) than the corresponding affinities determined using cell membrane homogenates.

In order to optimize the use of the GS-IAM in high-throughput screening, the stationary phase was packed into a column to create an immobilized enzyme reactor (the GS-IMER). Previous studies have demonstrated that IMERs can be used for on-line metabolic and inhibition studies (cf. Refs. [2,25–27]). The GS-IMER was used as a flow

reactor in the chromatographic system depicted in Fig. 1. The activity of the immobilized GS in the flow system was investigated using a series of L-glutamate concentrations (6.25–50 m*M*). The substrates were injected onto the GS-IMER and GS activity assessed by measurement of the L-glutamylhydroxamate produced in the post-column reactor. The areas of the L-glutamylhydroxamate peaks were proportional to the amount of substrate injected as demonstrated in Fig. 6A–D. The sensitivity of the GS-IMER to competitive inhibition was demonstrated by reduction in the observed L-glutamylhydroxamate peak produced by 50 m*M* L-glutamate after the addition of 1 m*M* MSO to the mobile phase (Fig. 6A) (50 m*M* L-glutamate) versus Fig. 6E (50 m*M* L-glutamate plus 1 m*M* MSO). The GS-IMER retained its enzymatic activity for 12 days when kept at room temperature.

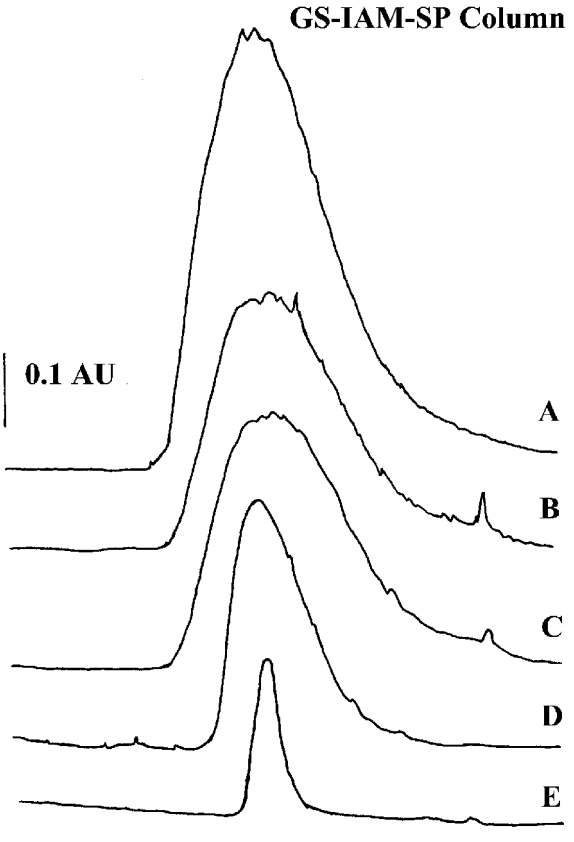

Fig. 6. Chromatograms obtained using GS-IMER as a flow reactor. Samples of 200 μl of various L-glutamate concentrations were injected onto the column. The mobile phase was buffer A. Chromatograms: (A) 50 m*M* L-Glu; (B) 25 m*M* L-Glu; (C) 12.5 m*M* L-Glu; (D) 6.25 m*M* L-Glu; (E) 50 m*M* L-Glu with 1 m*M* MSO in the mobile phase.

The immobilized GS retained its Michaelis–Menten properties in the on-line flow reactor. The amount of L-glutamylhydroxamate produced in the flow-system varied in a hyperbolic fashion as a function of increasing amounts of L-glutamate injected onto the GS-IMER (Fig. 7). Analysis of the data yielded a K_M value of 6.9±1.6 m*M* and a V_{MAX} of 0.20±0.01 m*M*/min per mg protein. These values were consistent with those found with the GS-IAM using batch-wise experiments where the calculated K_M value was 12.12±0.66 m*M* and the V_{MAX} was 2.66±0.14 m*M*/min per mg protein. The percentage of conversion of L-glutamate into L-glutamylhydroxamate is presented in Fig. 8. The enzyme (1 U of GS was used) in GS-IMER was less efficient than the non-immobilized enzyme. Nevertheless, the enzyme is more efficient in GS-IMER (25 U in the whole column, V_{total}=800 μl) than in the batch-wise procedure.

The data demonstrate that the immobilized GS was more efficient in the column than when used in the batch-wise procedure. This was probably due to the fact that after the injection of the substrate, the L-glutamate migrates through the GS-IMER column in a band that produces a higher concentration of substrate relative to immobilized enzyme than is achieved in the batch-wise procedure where the substrate is dispersed throughout the reaction solu-

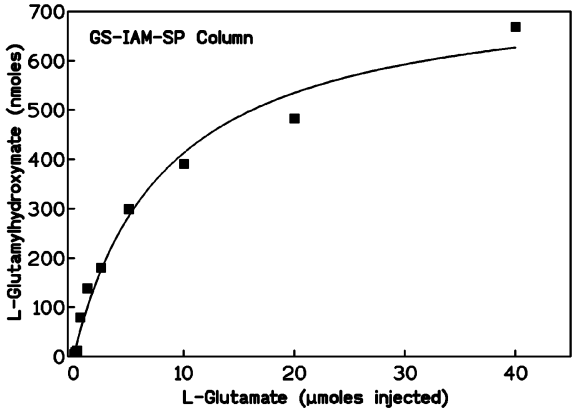

Fig. 7. GS activity of the GS-IMER as a function of L-glutamate injected onto the column.

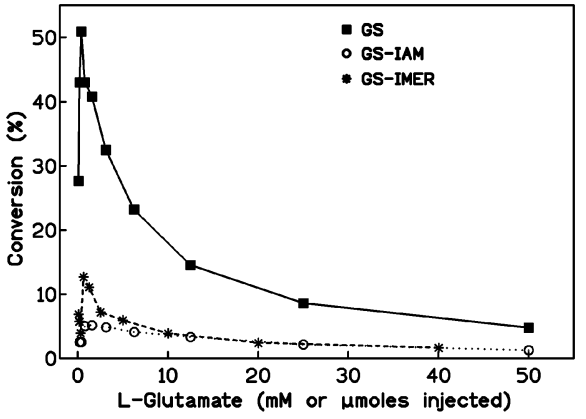

Fig. 8. Percentage of conversion of L-glutamate into L-glutamylhydroxamate as a function of L-glutamate by non-immobilized GS and immobilized GS in the form of the GS-IMER; where: 1 U of GS was used for both enzymes and 100 µl of substrate was injected onto the GS-IMER that contained 25 U of immobilized GS.

tion. In addition, since the immobilized GS in the GS-IMER was active and reusable for at least 12 days, the GS-IMER can be used for the on-line screening of compounds for GS antagonist and agonist properties. Thus, the GS-IMER could be useful a tool for screening of drugs for central nervous system toxicity since it has been reported that the toxicity of anti-epileptic drugs was due to their inhibition of GS [22].

Acknowledgements

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References

[1] Y. Khang, Y. Xiao, K.J. Kellar, I.W. Wainer, Anal. Biochem. 264 (1998) 22.
[2] V. Sotolongo, D.V. Johnson, D. Wahnon, I.W. Wainer, Chirality 11 (1999) 39.
[3] M.P.J. Kierstan, M.P. Coughan, in: R.F. Taylor (Ed.), Protein Immobilization. Fundamentals and Applications, Marcel Dekker, New York, 1991, p. 13.
[4] C. Pidgeon, C. Marcus, F. Alvarez, in: T.O. Baldwin, J.W. Kelly (Eds.), Applications of Enzyme Biotechnology, Plenum, New York, 1992, p. 201.
[5] W.W. Souba, in: R.G. Landes (Ed.), Glutamine – Physiology, Biochemistry, and Nutrition in Critical Illness, R.G. Landes Co., Austin, TX, 1992, p. 5.
[6] A. Meister, in: P.D. Boyer (Ed.), The Enzymes, Vol. 10, Academic Press, London, 1974, p. 699.
[7] A. Martinez-Hernandez, K.P. Bell, M.D. Noremberg, Science 195 (1977) 1356.
[8] M.D. Noremberg, J. Histochem. Cytochem. 27 (1979) 756.
[9] M.D. Noremberg, A. Martinez-Hernandez, Brain Res. 161 (1979) 303.
[10] R.A. Wanieski, D.L. Martin, J. Neurochem. 47 (1986) 304.
[11] R.A. Wanieski, J. Neurochem. 58 (1992) 167.
[12] P.N. Campbell, T.S. Work, E. Mellanby, Nature 165 (1950) 345.
[13] E.L. Peters, D.B. Tower, J. Neurochem. 5 (1959) 80.
[14] O.S. Sellinger, R.A. Schatz, R. Porta, T.E. Wilens, Ann. Neurol. 16 (1984) S115.
[15] R.A. Swanson, K. Shiraishi, M.T. Morton, F.R. Sharp, Stroke 21 (1990) 322.
[16] A. Meister, in: J. Mora, R. Palacios (Eds.), Glutamine: Metabolism, Enzymology and Regulation, Academic Press, London, 1980, p. 1.
[17] F. Fonnum, R.E. Paulsen, J. Neurochem. 54 (1990) 1253.
[18] G.F. Carl, L.K. Blackweel, F.C. Barnett, L.A. Thompson, C.J. Rissinger, K.L. Olin, J.W. Critchfield, C.L. Keen, B.B. Gallagher, Epilepsia 34 (1993) 441.
[19] P.R. Laming, S.L. Cosby, J.K. O'Neill, Comp. Biochem. Physiol. 94 (1989) 399.
[20] P.M. Nolan, P.P. Phelan, C.M. Regan, IRCS Med. Sci. 13 (1985) 873.
[21] P. Phelan, C.M. Regan, C. Kilty, A. Dunne, Neuropharmacology 24 (1985) 895.
[22] C.M. Fraser, G.J. Sills, G. Forrest, G.G. Thompson, M.J. Brodie, Br. J. Pharmacol. 126 (1999) 1634.
[23] V.P. Wellner, A. Meister, Biochemistry 5 (1966) 872.
[24] E.K. Laemmli, Nature 227 (1970) 680.
[25] P. Jadaud, I.W. Wainer, J. Chromatogr. 476 (1989) 165.
[26] W.K. Chui, I.W. Wainer, Anal. Biochem. 201 (1992) 237.
[27] Y. Zhang, F. Leonessa, R. Clarke, I.W. Wainer, J. Chromatogr. B 739 (2000) 33.