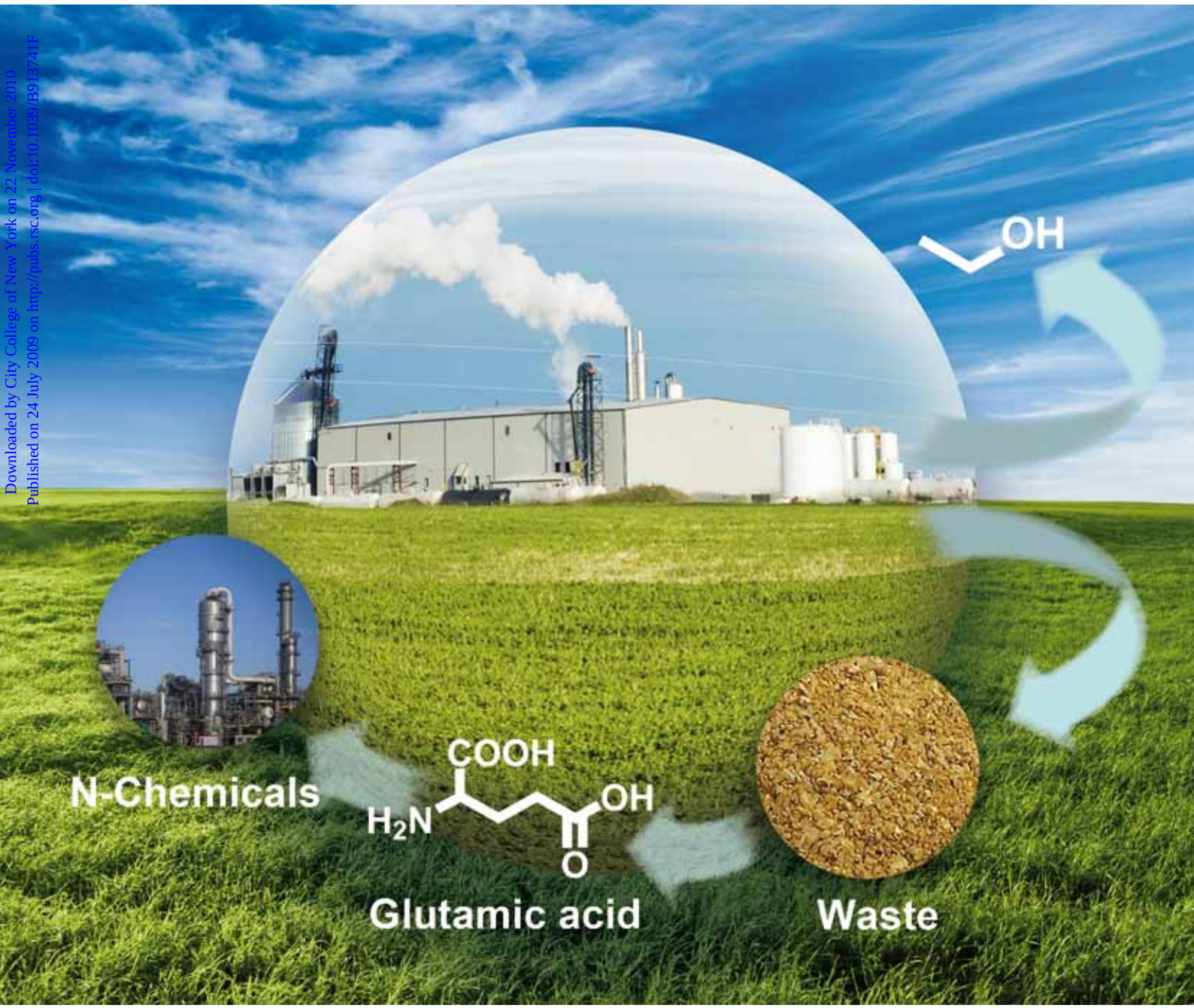
Green Chemistry

Cutting-edge research for a greener sustainable future

www.rsc.org/greenchem

Volume 11 | Number 10 | October 2009 | Pages 1485–1704



ISSN 1463-9262

RSC Publishing

The application of glutamic acid α-decarboxylase for the valorization of glutamic acid†

Tijs M. Lammens,[a] Daniela De Biase,[b] Maurice C. R. Franssen,*[c] Elinor L. Scott[a] and Johan P. M. Sanders[a]

Received 29th June 2009, Accepted 9th July 2009
First published as an Advance Article on the web 24th July 2009
DOI: 10.1039/b913741f

Glutamic acid is an important constituent of waste streams from biofuels production. It is an interesting starting material for the synthesis of nitrogen containing bulk chemicals, thereby decreasing the dependency on fossil fuels. On the pathway from glutamic acid to a range of molecules, the decarboxylation of glutamic acid to γ-aminobutyric acid (GABA) is an important reaction. This reaction, catalyzed by the enzyme glutamic acid α-decarboxylase (GAD) was studied on a gram scale. In this study, GAD was immobilized on Eupergit and in calcium alginate and its operational stability was determined in a buffer free system, using various reactor configurations. Immobilization was shown to increase the GAD stability. The conditions for the highest GABA production per gram of enzyme were determined by extrapolation of enzyme stability data. At 30 °C in a fed batch process this results in an average volumetric productivity of 35 kg m^{-3} hr^{-1}. The cost of using GAD immobilized in calcium alginate was estimated as €5 per metric ton of product. Furthermore it was shown that the cofactor pyridoxal-5′-phosphate (PLP) could be regenerated by the addition of a small amount of α-ketoglutaric acid to the reactor. In conclusion the application of immobilized GAD in a fed batch reactor was shown to be a scalable process for the industrial production of GABA from glutamic acid.

Introduction

Ever increasing oil prices, environmental concerns and the insecurity of oil supply force the need to look for alternatives to fossil fuels. This is not only the case for transportation fuels, but also for raw materials for the chemical industry. Most polymers still depend on fossil resources for their building blocks. In the case of nitrogen containing molecules, production usually involves reactions of molecules such as propylene with ammonia in very energy-intensive processes.[1]

A major challenge is to create new, economically feasible routes from plant materials to industrial chemicals. Amino acids could play an important role in the making of nitrogen-containing bulk chemicals, because amino acids already contain nitrogen. That makes them a good starting point to by-pass the use of ammonia and thus to save a lot of energy and investment costs.[2] With the current worldwide increase in biofuels production, it may be expected that in the near future it will be possible to cost-effectively isolate amino acids from protein rest streams. One can, for example, think of isolating L-glutamic acid from dried distiller's grains with solubles (DDGS), a cheap co-product of bioethanol production from maize or wheat.[3] DDGS from maize contains on average 25 to 30 wt% crude protein, which typically consists of 20% L-glutamic acid.[4–7] This means that there is a very high potential for using L-glutamic acid as a raw material for chemicals. Moreover, the structure of glutamic acid with its amine and carboxylate functionalities and the small carbon backbone resembles many industrial intermediates, so a variety of chemicals could be made from it in a relatively small amount of steps.

In a potential pathway from L-glutamic acid to industrial chemicals, γ-aminobutyric acid (GABA) could be an intermediate from which various other chemicals can be made. An example of a next step could be the simple lactamization of GABA to 2-pyrrolidone,[8] which is an industrial solvent and also the most important precursor for the monomer N-vinylpyrrolidone.[9] In this way, the use of biomass for the production of chemicals can be integrated in the current industrial infrastructure.

The reaction of L-glutamic acid to GABA can be done enzymatically, with the enzyme glutamic acid α-decarboxylase (GAD), a pyridoxal 5′-phosphate (PLP)-dependent enzyme widely distributed among living organisms.[10] Recently a study of this reaction using immobilized whole cells of *Lactobacillus brevis* was published.[11] However, using whole cells for this reaction has disadvantages such as the decomposition of product by GABA transaminase in the presence of pyruvate or α-ketoglutarate,[12] or the possible decrease in activity due to lack of nutrients for the cells.[11] Until now, to the best of our knowledge, it has not been shown that this process could be profitable

[a]*Valorisation of Plant Production Chains, Wageningen University and Research Centre, Wageningen, The Netherlands. E-mail: tijs.lammens@wur.nl*
[b]*Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Sapienza Università di Roma, Roma, Italy*
[c]*Laboratory of Organic Chemistry, Wageningen University & Research Centre, Dreijenplein 8, 6703 HB, Wageningen, The Netherlands. E-mail: maurice.franssen@wur.nl; Fax: +31 317 484914; Tel: +31 317 482976*
† Electronic supplementary information (ESI) available: Fed batch mass balance. See DOI: 10.1039/b913741f

for the manufacture of other than food or pharmaceutical grade products.

In this paper we develop an enzyme-based process for the scalable production of GABA from L-glutamic acid, applicable in the chemical industry. Purified GAD from *E. coli* was immobilized in two different manners (covalent binding to Eupergit and gel entrapment in calcium alginate) and the performance of the immobilized enzyme was studied in a buffer-free reaction system, to eliminate problems such as incompatibility with the co-enzyme PLP (the case with phosphate) or inhibition (the case with acetate).[13] Other advantages of a buffer-free system would be lowering process operation costs by using fewer chemicals and simplifying product recovery.

Materials and methods

Materials

Sodium alginate and Eupergit 250 C were both obtained from Sigma. All other chemicals were obtained from Sigma or Fluka. Their quality was analytical grade or higher and they were used as received.

The purified GAD isoform GadB was isolated from *E. coli* and purified as published before.[14]

Analytical methods

For HPLC analysis, glutamic acid and GABA were derivatized with phenylisothiocyanate (PITC).[15] A 50 µl sample of reaction mixture or of a standard solution of glutamic acid or GABA was dried under vacuum. The residue was dissolved in 20 µl ethanol-water-triethylamine (2:2:1 by volume) and dried under vacuum. Then the residue was re-dissolved in 30 µl ethanol-water-triethylamine-PITC (7:1:1:1 by volume), allowed to react for 20 minutes and subsequently left overnight to dry under vacuum at room temperature. The remaining dry residue was dissolved in 0.8 ml of mobile phase, consisting of an aqueous solution of 6.57 g sodium acetate, 0.4 ml triethylamine, 0.6 ml acetic acid and 125 ml acetonitrile, made up to 1000 ml with water.[16] The final pH was 5.9.

Isocratic HPLC separation was performed on a Waters apparatus with a model 600 controller, a model 717 plus autosampler with 100 µl injection loop, a model 2487 dual wavelength absorbance detector and a temperature control module. The column used was a HP Hypersil BDS C-18 (250 × 4.0 mm, 5 µm particle size). UV detection was done at 254 nm. The flow rate was 0.6 ml/min at 30 °C. Retention times of PITC-glutamic acid and PITC-GABA were 5.2 and 11.6 minutes, respectively.

GAD immobilization in calcium alginate

In a typical procedure, 1 mg/ml GAD solution was added to a 2% (w/v) solution of sodium alginate in sodium acetate buffer (0.1 M, pH 4.6), to give 25 µg/ml GAD. This solution was added dropwise with a syringe with a 0.4 mm (ID) needle to a continuously stirred aqueous solution of calcium chloride (0.2 M), resulting in the formation of off-white calcium alginate beads containing 0.05 mg GAD per 1 g of wet beads (GAD:alginate = 1:800 by weight). The beads were allowed to harden overnight at 4 °C, after which they were filtrated and stored as such at 4 °C. The filtrate was tested for GAD presence with an activity assay.

GAD immobilization on Eupergit 250 C

In a typical procedure, dry Eupergit beads (500 mg, 0.25 mm bead diameter, 100 nm pore diameter) were suspended in potassium phosphate buffer (5 ml, 1 M at pH 7.0) containing 0.1 mg/ml GAD and left overnight at room temperature on an overhead rotating disc. The suspension was filtered and the beads washed with sodium acetate (0.01 M, pH 4.6), re-suspended in sodium acetate (7 ml, 0.01 M, pH 4.6) and left for another 6 hours on the rotating disc at 4 °C, to remove any non-covalently bound enzyme. Then the beads were filtered again and stored as such at 4 °C, giving 0.24 mg GAD per 1 g of wet beads (theoretical value). Both filtrates were tested for GAD presence with an activity assay and a Bradford protein assay.

GAD activity assay

The enzyme activity assay was performed in water without any additional buffer present. The setup was a Metrohm 718 stat titrino with a titration vessel equipped with a thermostatic jacket. Titration was performed with an aqueous solution of HCl (0.1 M).

In a typical experiment, 10 ml of an aqueous solution of L-glutamic acid (0.08 M, 0.118 g) and PLP (0.5 mM, 1.33 mg) in water was brought to pH 4.6 with NaOH at 40 °C. Then 0.02 mg GAD (or an equivalent of 0.02 mg GAD in the case of immobilized GAD) was added and the titration curve recorded. GAD specific activity was determined as a function of the slope of the titration curve over 10 minutes time and is defined as U mg^{-1}, equal to µmol H^{+}_{added} min^{-1} mg^{-1}.

GAD stability assay

The enzyme stability was determined by measuring the activity for a prolonged period of time under steady-state conditions, with a continuously operated stirred tank reactor (CSTR) setup. For this a Gilson Minipuls 2 pump with 0.25 mm (ID) PVC tubing was connected to the reaction vessel, continuously pumping a solution of L-glutamic acid and PLP (0.1 M and 0.5 mM, respectively, pH 4.6) in at 0.06 ml/min and pumping reaction mixture out at the same rate. A Biozym polyethylene filter with 16 µm pore diameter was used to keep the Eupergit beads in the reactor. Titration was done with an aqueous solution of HCl (0.1 M).

Another method for monitoring the GAD activity at a high substrate concentration for a long period of time was by titration with 0.08 M L-glutamic acid in water. In this case, GAD specific activity (U mg^{-1}) was determined as µmol L-Glu min^{-1} mg^{-1} added. When performing the reaction with GAD in calcium alginate, $CaCl_2$ (0.04 M) was added to the reaction mixture in both cases to keep the beads from dissolving in the course of time.

Computational fitting

Fitting of experimental data to model equations was performed with a Kevin Raner Software package called WinCurveFit, version 1.1.8, 2002.

Results and discussion

GAD immobilization

Two different ways to immobilize GAD were chosen, namely gel entrapment in calcium alginate and covalent binding to Eupergit 250C epoxide beads, in order to determine whether immobilization has an influence on the enzyme performance.

GAD entrapment in calcium alginate yielded beads of approximately 1.5 mm diameter. The filtrate showed no activity for conversion of glutamic acid into GABA, so apparently the immobilization yield was 100%.

GAD binding to Eupergit was performed at pH 7. Bradford analysis of the supernatants showed no residual protein after 18 hrs of reaction and there was no activity for the conversion of L-glutamic acid (at pH 4.6), suggesting the immobilization yield was 100%.

Enzyme activity assay and kinetics

In many micro-organisms, the GAD system is assumed to control the acidity of the cytosol, *via* the decarboxylation of glutamic acid to GABA, thereby incorporating a proton in the molecule.[17] Thus when performing this reaction without added buffer, the pH of the solution will rise in the course of the reaction. It was found that when comparing the titration curve with the GABA formation as measured by HPLC, these two graphs match (data not shown), indicating that both methods are equally suitable for determining the enzyme activity. Titration is the preferred method, because it provides a simple (online) activity assay.

With this assay Michaelis-Menten kinetics of the native and immobilized enzyme were studied. For this, the initial enzyme activity was measured as a function of the glutamic acid concentration and the data points were fitted to the Michaelis-Menten equation. The obtained values for the apparent K_m in the case of native GAD, GAD in calcium alginate and GAD on Eupergit were 2.0, 6.3 and 1.6 mM, respectively. So immobilization of GAD in calcium alginate increases the apparent K_m value. An explanation for this difference could be diffusion limitation. Since the alginate beads are much larger in size, glutamic acid needs to diffuse further to reach the GAD located in the center of the beads, a process which is favored by a higher concentration of glutamic acid. The obtained apparent K_m value of native GAD in water is a little higher than the K_m found by Shukuya in pyridine buffer (0.8 mM)[13] and lower than the K_m found in acetate buffer (6.7 mM).[18] The reason for a decrease in K_m compared to the one found in acetate buffer is thought to be the partial displacement of substrate from the enzyme surface by acetic acid.[13]

Enzyme stability

The GAD activity as a function of time was determined under semi steady-state conditions, keeping the substrate concentration well above K_m. This was done in order to eliminate any concentration effects from the measured enzyme activity and thus to determine the stability in time under turnover conditions. Two different ways to do this were chosen. The first was a CSTR setup, which is a convenient way to compare the stability of GAD on the two different carriers. However, it is unsuitable to determine the stability of native enzyme, because the enzyme will not be retained by the polyethylene filter. The activity of GAD on Eupergit and alginate was measured for 24 hours continuously. HPLC analysis of reaction samples showed that in the CSTR setup at 40 °C and pH 4.6, a steady state with a constant concentration level of both glutamic acid and GABA was reached after four hours. The activity measured as a function of time was for both GAD in calcium alginate and GAD on Eupergit the same as determined with a fed batch setup, which was chosen as the preferred method to assess the stability of the native GAD and compare it with immobilized GAD. In this case, titration was done with 80 mM glutamic acid in water instead of with hydrochloric acid, in order to replenish the substrate and in doing so keeping the substrate concentration well above K_m. A comparison of the enzyme stability of native GAD, GAD in calcium alginate and GAD on Eupergit during 24 hours of turnover conditions is shown in figure 1. This shows that immobilizing GAD significantly improves the enzyme stability. Similar curves were obtained at 40 °C and 50 °C (data not shown).

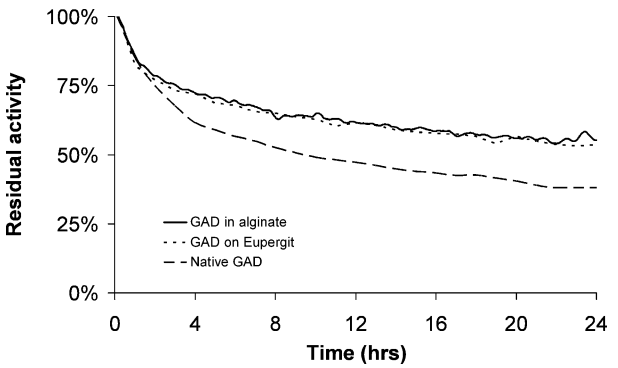


Fig. 1 The influence of immobilization on GAD stability in a fed batch setup. Individual titration curves with native GAD, GAD in calcium alginate and GAD on Eupergit were recorded for 24 hours at pH 4.6 and 30 °C. The residual activity is defined as the specific activity at a given time divided by the initial specific activity.

Reaction conditions

The effect of pH on the enzyme activity was determined for the immobilized as well as the native GAD. The native enzyme showed a strong decrease of the activity above pH 5, which is similar to data reported before.[13] The immobilized enzyme showed in both cases this profile too (data not shown), indicating that the immobilization had no influence on the pH dependency of the enzyme activity. The pH dependency of the enzyme activity is known to be associated to a protein conformational change,[19] so the immobilization had no effect on the conformational mobility of the enzyme. 24 Hour fed batch experiments were also performed at different pHs in the range of 3.6 to 4.6 (data not shown). No differences were found in the enzyme stability.

The influence of temperature on the enzyme performance (activity and stability) was also studied. A higher temperature gives a higher enzyme activity, but also a faster decrease of the activity, as can be seen from figure 2, which shows the specific

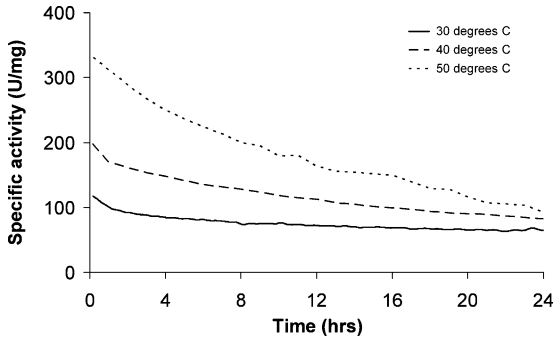


Fig. 2 The influence of temperature on the long-term GAD activity in a fed batch setup. Individual titration curves with GAD in calcium alginate were recorded at 30, 40 and 50 °C for 24 hours at pH 4.6.

enzyme activity as a function of time with GAD immobilized in calcium alginate. Similar curves were obtained with native GAD and GAD on Eupergit (data not shown).

GAD deactivation

The cause of the decrease of GAD activity with time is known to be related to a side reaction that takes place once every 300,000 turnovers, which is an abortive transamination reaction.[20,21] In this case, GAD replaces a carbonyl group of PLP with an amine group from a GABA molecule, thereby producing pyridoxamine-5-phosphate (PMP) and succinic semialdehyde. PMP dissociates from the active site, leaving GAD in its apo-form, which is conformationally less stable.[22] Meeley and Martin showed that the inactivation of GAD from hog brain is not first order, but is best described as the sum of two exponential decay processes.[23] The reason for this biphasic inactivation could be in the multiple PLP binding sites that GAD contains, which might inactivate at different rates. In the case of GAD from *E. coli* the measured deactivation curves of GAD show a good fit to this model, at different temperatures and for both the immobilized and the native GAD. The results for the fit of the curves in figure 2 are shown in table 1. The rate constant of the first term (a) shows a clear temperature dependency, with an increasing rate of deactivation at increasing temperature. The second rate constant (b) shows no clear temperature dependency; however, the errors in the determined values for b are high, making it difficult to draw any conclusions from the second term of deactivation.

Cofactor regeneration

It was shown before that the stability of GAD can be greatly increased by adding PLP to the reaction mixture.[18,21] Another approach can be the regeneration of PLP from PMP by GAD transaminase activity, which is known from human GAD.[24] This is shown in figure 3. When a small amount of α-ketoglutaric acid is added to the system, GAD catalyzes the transamination of α-ketoglutaric acid to glutamic acid, thereby converting PMP into PLP.

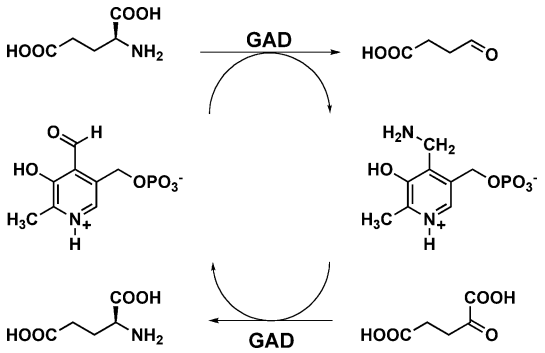


Fig. 3 The regeneration of PLP with α-ketoglutaric acid.

Figure 4 shows that if neither PLP nor α-ketoglutaric acid is added, that leads to a fast deactivation of GAD. If no PLP is added, it will only be present in a stoichiometric amount, for in active GAD from *E. coli* PLP is bound covalently to the active site. This stoichiometric amount of PLP will be transaminated over time, leading to a fast decrease of the enzyme activity. The addition of 5 mM α-ketoglutaric acid, however, has the same influence on the enzyme activity as the addition of extra PLP to the system, probably because the PLP is now regenerated. Addition of less (0.5 mM) and more (50 mM) α-ketoglutaric acid was also tested. The first showed no effect, which is likely due to unfavorable kinetics, and the second showed a stable but lower GAD activity, probably due to competitive inhibition of glutamic acid by α-ketoglutaric acid. The fact that replacing PLP with α-ketoglutaric acid is a possibility for this process is especially interesting from an economic point-of-view, because α-ketoglutaric acid is much less expensive than PLP. Moreover, the transamination reaction regenerates glutamic acid which can then be recycled.

Table 1 The determined coefficients and rate constants for a fit of the obtained data for GAD in calcium alginate with a double exponential decay function: residual activity $= A_0 e^{-at} + B_0 e^{-bt}$. Given errors are the deviations between two measurements

	Coefficient (% of initial rate)		Rate constant (hr^{-1})	
T	A_0	B_0	a	b
30 °C	71 ± 0.2	36 ± 6.5	0.014 ± 0.002	0.74 ± 0.19
40 °C	85 ± 3.8	19 ± 1.7	0.037 ± 0.008	1.13 ± 0.45
50 °C	84 ± 0.6	20 ± 1.2	0.058 ± 0.016	0.40 ± 0.01

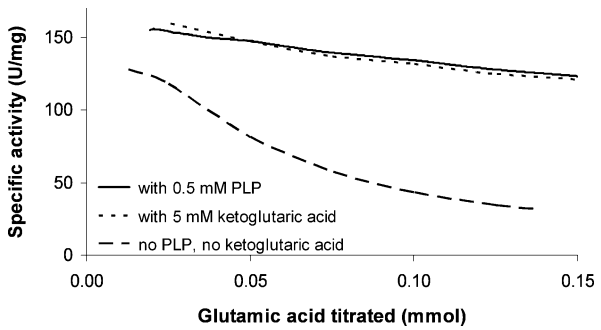


Fig. 4 The influence of α-ketoglutaric acid on the GAD stability. Individual titration curves were recorded with native GAD at pH 4.6 and 40 °C, in the presence of 0.5 mM PLP, in the presence of 5 mM α-ketoglutaric acid and in the presence of neither PLP, nor α-ketoglutaric acid.

Reactor design and productivity

The long-term experiments with immobilized GAD described in this article have been performed in two different ways of operation, in a CSTR and in a fed batch reactor. From the obtained data it is possible to derive what would be the best mode of operation and to calculate if this process would be economically viable in an industrial application. In order to do this, a mass balance of the reactor was made and from this the amount of GABA that can be produced per gram of GAD was calculated, as was the volumetric productivity of the reactor in that case.† GAD immobilized in calcium alginate was chosen as the model catalyst, as it has been shown to perform better than GAD immobilized on Eupergit. Alginate is also less expensive than Eupergit. The chosen operating conditions for these calculations were calcium alginate at pH 4.6 and 30 °C. These conditions were chosen because an extrapolation of the reactor productivity based on the derived inactivation data (Table 1) shows that at 30 degrees the total expected GABA yield per gram GAD is the highest, although it takes more time (figure 5).

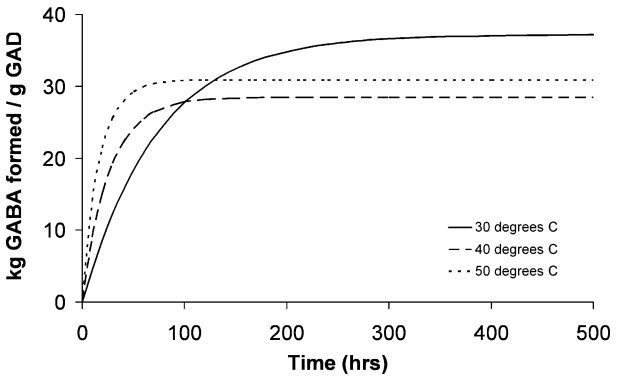


Fig. 5 The expected productivity of GAD in calcium alginate at different temperatures, based on the inactivation data shown in table 1.

This reaction can conveniently be performed in a fed batch process, as was shown above. In order to keep the volume of the reactor constant, the reaction could be performed by the addition of solid glutamic acid instead of an aqueous solution of glutamic acid. In this way, the substrate concentration would remain constant as the product concentration increases, resulting in an accumulation of product. Therefore, it is important that product inhibition of GAD by GABA does not take place. This was examined up to a GABA concentration of 5 mol/L. No decrease in enzyme activity was detected, as was expected because the presence of two free carboxylic acid groups in a molecule is required for effective inhibition of GAD.[25]

Figure 5 showed the expected productivity of the reactor with GAD in calcium alginate at 30 °C. After eight days only 5% of the initial GAD activity remains. After this time 1 gram of GAD has formed 34 kg GABA. For a production of 1000 kg GABA in these eight days, 29.4 gram GAD of high purity would be needed. The activity of the purified GAD is 5.2 times higher than that of the crude extract as reported by De Biase *et al.*[14] Should a crude extract of GAD be used (which is likely to be used in industry), 150 gram would be needed.

For the immobilization of an enzyme in calcium alginate, an enzyme to alginate weight ratio of 0.5 is achievable with lipase.[26] Assuming that would also be the case with GAD, 300 gram alginate is needed for the immobilization of 150 gram GAD. The alginate concentration is 2% (w/v), so 300 gram alginate is equal to 15 L of beads. If the catalyst loading is 10%, then the total reactor volume would be 0.15 m^3. The average volumetric productivity of this reactor over eight days based on the above assumptions would then be 35 kg GABA m^{-3} hr^{-1}.

The same calculation can be done for performing the process at 50 °C. Then the reaction time to 5% remaining enzyme activity is 50 hours and by then 1 gram GAD has formed 29 kg GABA. The average volumetric productivity would then become as high as 111 kg GABA m^{-3} hr^{-1}.

A cascade of three CSTR's which each 80% conversion (overall giving 99% conversion) showed to be less effective than a fed batch process. This is because of the low glutamic acid concentration in the second and third reactor (4 and 1 mM, respectively). These concentrations are below the determined apparent K_m of the enzyme in calcium alginate and therefore the reaction rates are relatively low.

Cost analysis

To estimate the price of the needed enzyme per metric ton of produced GABA, one can assume that a typical price for a crude enzyme applied in the chemical industry would be €100 per kg of pure enzyme equivalent (private communication from industry). Then the cost of enzyme would be €3 per ton GABA. Using crude enzyme and immobilizing that with alginate would cost 0.3 kg alginate at €6 per kg is €1.80 per ton GABA extra. The cost of using immobilized GAD in this process would therefore be around €5 euro per ton of produced GABA. That means the price will be in the same range as for example amylase (used for the hydrolysis of starch), which costs €2–3 per ton of product.[27]

Furthermore, because of the high volumetric productivity of this bioreactor (which is related to the high enzyme activity), the operational costs are not expected to form an obstacle. Also the costs of PLP and α-ketoglutaric acid are not expected to pose a problem, because the amounts used in the process will be very limited and most of it can be recycled.

The major obstacle for the application of this process is the current cost of glutamic acid, which is too high because it is still produced by a fermentative process.[28] The isolation of glutamic acid from protein rest streams is essential to make this process cost-effective, and is therefore a challenge that is under current investigation.

Conclusion

Here we have shown that it is possible to develop a practical and economically feasible process for the industrial bulk production of GABA, based on glutamic acid derived from plant materials. GABA can then be a precursor for different nitrogen-containing materials in such a way that it can be implemented in the current infrastructure of the chemical industry, thereby reducing the dependency on fossil fuels.

By way of immobilization, the enzyme (GAD) was made more stable and easily separable from the reaction mixture.

Furthermore, a reactor setup with a pH stat was tested on a gram scale with the immobilized enzyme, showing that it is possible to perform the reaction without any buffer present. The subsequent reactor design, based on the acquired results and assuming no major scale-up problems, showed that a fed batch process would be the preferred method. A major advantage of this is that the process can be run up to a high product concentration, making the product recovery easier. Finally, and important for an industrial application, it was shown that the enzyme cost will not be an obstacle for this process.

Acknowledgements

We wish to thank professor Kenji Soda (Kansai University, Japan) for his fruitful suggestions concerning the reactivation of apo-GAD and Kees van Kekem (WUR, The Netherlands) for providing HPLC apparatus and assistance. Further, we are grateful to NWO-Aspect for funding of this work.

Notes and References

1 F. Veatch, *Chem. Eng. Prog.*, 1960, **56**, 65–67.
2 E. Scott, F. Peter and J. Sanders, *Appl. Microbiol. Biotechnol.*, 2007, **75**, 751–762.
3 J. Sanders, E. Scott, R. Weusthuis and H. Mooibroek, *Macromol. Biosci.*, 2007, **7**, 105–117.
4 R.L. Belyea, K. D. Rausch and M.E. Tumbleson, *Bioresour. Technol.*, 2004, **94**, 293–298.
5 M. J. Spiehs, M. H. Whitney and G.C. Shurson, *J. Anim. Sci.*, 2002, **80**, 2639–2645.
6 Y. Kim, N. S. Mosier, R. Hendrickson, T. Ezeji, H. Blaschek, B. Dien, M. Cotta, B. Dale and M. R. Ladisch, *Bioresour. Technol.*, 2008, **99**, 5165–5176.
7 J. L. Wolfson and G. Shearer, *Agronomy J.*, 1981, **73**, 611–613.
8 A. Blade-Font, *Tetrahedron Lett.*, 1980, **21**, 2443.
9 A. L. Harreus, 2-Pyrrolidone, In: *Ullmann's Encyclopedia of Industrial Chemistry*, Online edn., 2000, Wiley InterScience.
10 H. Ueno, *J. Mol. Catal. B: Enzym.*, 2000, **10**, 67.
11 J. Huang, L. Mei, H. Wu and D. Lin, *World J. Microbiol. Biotechnol.*, 2007, **23**, 865–871.
12 B. J. Shelp, A. W. Bown and M. D. McLean, *Trends Plant Sci.*, 1999, **4**, 446–452.
13 R. Shukuya and G. W. Schwert, *J. Biol. Chem.*, 1960, **235**, 1649–1652.
14 D. De Biase, A. Tramonti, R. A. John and F. Bossa, *Protein Expression and Purif.*, 1996, **8**, 430.
15 S. Gunawan, N. Y. Walton and D. M. Treiman, *J. Chromatogr. A*, 1990, **503**, 177.
16 V. Rossetti and A. Lombard, *J. Chromatogr. B*, 1996, **681**, 63.
17 G. Capitani, D. De Biase, C. Aurizi, H. Gut, F. Bossa and M. G. Grütter, *EMBO J.*, 2003, **22**, 4027–4037.
18 A. Tramonti, D. De Biase, A. Giartosio, F. Bossa and R. A. John, *J. Biol. Chem.*, 1998, **273**, 1939–1945.
19 H. Gut, E. Pennacchietti, R. A. John, F. Bossa, G. Capitani, D. De Biase and M.G. Grütter, *EMBO J.*, 2006, **25**, 2643–2651.
20 T. G. Porter, D. C. Spink, S. B. Martin and D. L. Martin, *Biochem. J.*, 1985, **231**, 705–712.
21 V. P. Almazov, I. V. Morozov, F. A. Savin and B. S. Sukhareva, *J. Mol. Biol. U.S.S.R.*, 1985, **19**, 359–370.
22 P. L. Grant, J. M. Basford and R. A. John, *Biochem. J.*, 1987, **241**, 699–704.
23 M. P. Meeley and D. L. Martin, *Cell. Mol. Neurobiol.*, 1983, **3**, 39–54.
24 T. G. Porter, S. B. Martin and D. L. Martin, *J. Neurochem.*, 1986, **47**, 468–471.
25 M. L. Fonda, *Biochemistry*, 1972, **11**, 1304–1309.
26 K. Won, S. Kim, K. Kim, H. W. Park and S. Moon, *Process Biochem.*, 2005, **40**, 2149–2154.
27 P. S. J. Cheetham, in *Applied Biocatalysis*, ed. J. M. S. Cabral, D. Best, L. Boross and J. Tramper, Harwood Academic Publishers, Chur, 1st edn., 1994, ch. 11, pp. 431–469.
28 T. Kawakita1, C. Sano, S. Shioya, M. Takehara and S. Yamaguchi, Monosodium Glutamate, In: *Ullmann's Encyclopedia of Industrial Chemistry*, Online edn., 2000, Wiley InterScience.