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Development of an alliin-biosensor

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The quality of fresh garlic (*Allium sativum* L., Alliaceae) and garlic containing remedies made from garlic powder or dry extracts is defined by their content of the flavour precursor alliin. In the last decade, numerous chromatographic methods for the quality control of garlic preparations have been developed. Nearly all of them include an extensive sample treatment like pre-column derivatization. In order to simplify sample treatment and to reduce the time needed for analysis, we developed a novel analytical method based on an ammonium gas electrode combined with immobilized alliinase (EC 4.4.1.4). A direct coupling of an enzyme and a physical sensor like an electrode is named "biosensor". The electrode was fitted to a specially designed flow-through cell to achieve highest reproducibility of results. Working conditions of the ammonia gas electrode such as pH, temperature, and flow rates were optimized and adapted to the environmental requirements of the enzyme alliinase. Satisfactory results were obtained at pH 7.0, 35 °C, and a flow rate of 1.0 ml/min.

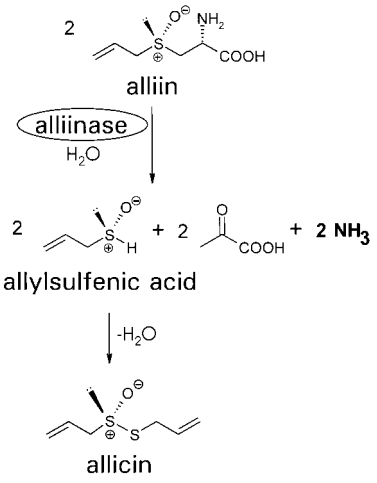
1. Introduction

Since ancient times, garlic (*Allium sativum* L., Alliaceae) has been used world-wide as a spice and as a remedy in folk medicine for the treatment of infectious diseases as well as for the prevention of stroke and arteriosclerosis [1, 2]. Garlic-containing remedies were usually standardized on their content of the flavour precursor alliin, (+)-*S*-allyl-L-cysteine sulfoxide, or the enzymatically formed allicin (2-propenyl-2-propenethiosulfinate). In recent years, numerous chromatographic methods for the analysis of the sulfur-containing constituents of garlic and related species have been developed [1, 3, 4]. All these techniques usually require a time-consuming sample treatment.

In order to simplify sample treatment and to reduce the analysis-time for single measurements, a novel biosensoric method based on immobilized alliinase (EC 4.4.1.4) in combination with an ammonia gas electrode should be developed. Alliinase catalyses the conversion of alliin to allicin, pyruvic acid, and ammonia (Scheme). The amount of enzymatically formed ammonia is proportional to the content of alliin and can therefore be used for an indirect quantification of alliin.

Other biosensors based on an ammonia gas electrode have been described in the literature in recent years [5–8]. Most of them suffered from a relative low sensitivity and long response times (T_R, defined as the time necessary for the electrode to reach 95% of its steady state potential).

Scheme



These problems are caused by the working principle of the electrode. Ammonia in samples must pass a thin membrane affecting a pH change of the inner electrolyte, which is determined by a pH glass electrode (sensing element, box Fig. 1). The pH of samples should be higher than 10. However, these conditions are incompatible with the stability properties of most enzymes. In order to get a sufficient electrode response under nearly physiological conditions (pH 6–8), the effects of temperature, pH, and flow rate on the electrode potential and on T_R were investigated.

2. Investigations and results

2.1. Apparatus

The apparatus used for experiments is shown in Fig. 1. An ammonia gas electrode was placed in a specially designed flow-through cell to allow high reproducibility of measurements. Several types of these cells were tested. Best results were achieved by a model with an inner volume of 180 µl, a height of the inner chamber of 2.0 mm, and a tangential flow direction. Experiments with an inlet placed at the center of the cell and outlets at the sides ("wall-jet

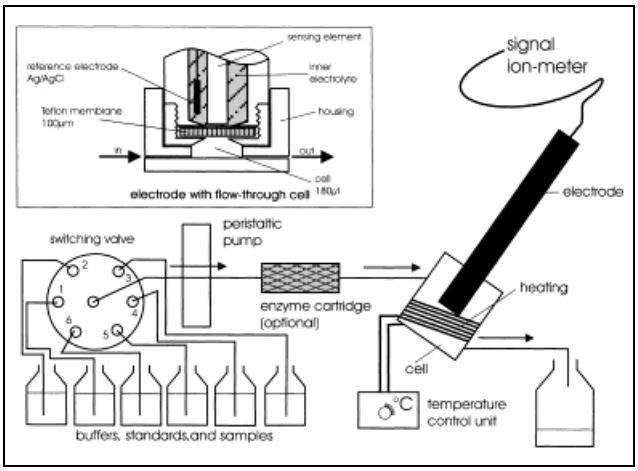


Fig. 1: Apparatus used for the determination of ammonia and alliin concentrations. Standards, samples, and buffers were selected by a switching valve and delivered by a peristaltic pump. Concentrations were determined by an ammonia gas electrode, which was combined with a flow-through cell (see cross section inside the box). For the analysis of alliin, a cartridge filled with immobilized alliinase was placed between the pump and the cell.

principle") failed because the teflon membrane probably came into contact with the pH-sensing element of the electrode. A heatable water-jacket surrounded the cell, which allowed measurements between 25 and 40 °C with a precision of 0.1 °C.

Samples, standards of ammonium and alliin as well as different buffers were selected by a switching valve and delivered to the electrode by a peristaltic pump. Flow rates were adjusted between 0.2 and 1.3 ml/min. For standard experiments, the flow rate was 1.0 ml/min. The immobilized alliinase was placed between the pump and the electrode and was removed for measurements with ammonium standards. The enzyme was found to be stable over a period of several weeks [9].

2.2. Influence of pH

As mentioned above, the ammonia gas electrode gave best results at pH-values higher than 10. In contrast, alliinase shows highest activity at about pH 7 [10, 11]. Therefore, working conditions of the electrode must be adapted to the pH-optimum of the enzyme to allow the construction of an alliin biosensor.

Electrode potentials were investigated over a pH-range from 6–8 (Fig. 2). Potentials for same ammonium concentrations were significantly decreased if the pH was increased. Calibration plots at pH 8 and 9 were almost linear over a concentration range from 5×10^{-2} M down to 5×10^{-5} M ammonium. But the latter concentration was out of the linear range at lower pH values (pH 7 and 6). It should be noted that T_R's were significantly prolonged at decreased pH-values. Measurements at pH 6 are not recommended for this reason; measurements at pH 7 gave acceptable T_R values (see below).

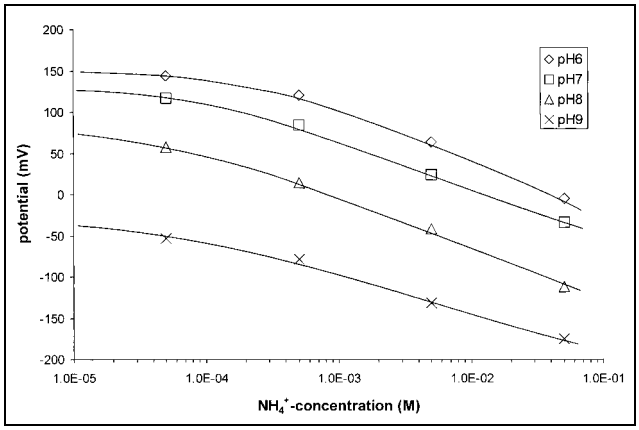


Fig. 2: Correlation between pH and electrode potential at different ammonium concentrations (35 °C)

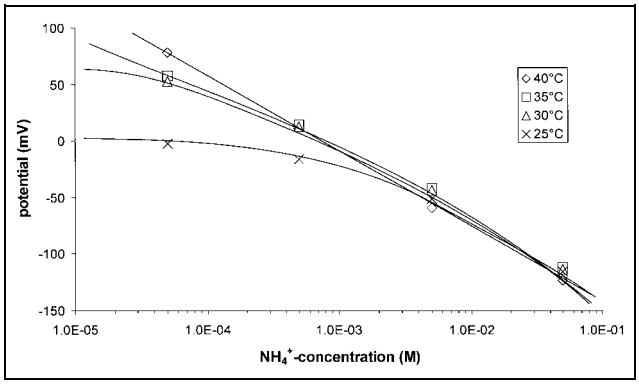


Fig. 3: Correlation between temperature and electrode potential at different ammonium concentrations (pH 8)

2.3. Influence of temperature

In order to determine the influence of temperature on the electrode, 4 ammonium standards were analyzed in a temperature range from 25–40 °C (Fig. 3). For all measurements, the pH was adjusted to 8.0. The temperature exhibited a strong influence on electrode potentials at ammonium concentrations lower than 10^{-3} M. The calibration plot recorded at 25 °C was curved over the whole range; measurements between 30 °C and 40 °C gave acceptable results. A linear plot over the whole concentration range was achieved at 40 °C. However, the inner electrolyte of the electrode slightly evaporates at this temperature. Consequently, all following experiments were carried out at 35 °C. Analysis of ammonium-standards at 35 °C and pH 7 also gave an acceptable linearity of the calibration plot. These settings are in good agreement with the conditions reported for highest alliinase activity [10, 11].

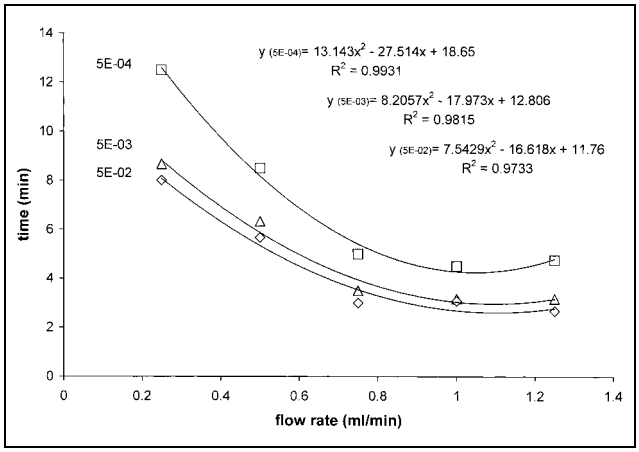


Fig. 4: Dependence of the response time T_R on the flow rate at different ammonium concentrations of standards (35 °C, pH 7)

2.4. Influence of flow rate

The apparatus depicted in Fig. 1 was operated at varying flow rates between 0.2 and 1.3 ml/min and T_R values for three different ammonium standards were recorded (Fig. 4). The electrode was flushed with an ammonium-free buffer before each set of measurements. Ammonium standards were analyzed from the lowest to the highest concentration. Measurements in the opposite direction are not recommended because the T_R values were dramatically increased.

As expected, the longest T_R occurred at the lowest flow rate (0.25 ml/min). An increased flow rate (0.6 ml/min) affected a cut by half of T_R. The best response was achieved between 0.8 and 1.2 ml/min. For all ammonium concentrations, the correlation between flow rate and T_R can be described by a second order function over the investigated range.

The geometry of the flow-through cell has also a significant effect on the electrode response. In general, a reduced cell volume affected a faster response. On the other hand, laminar flow inside the cell can be easily disturbed inside low-volume cells. This might be the reason for slightly increased T_R values at flow rates higher than 1.0 ml/min.

Moreover, the flow rate significantly influenced the standard deviation of ammonium analysis (Fig. 5). Particularly

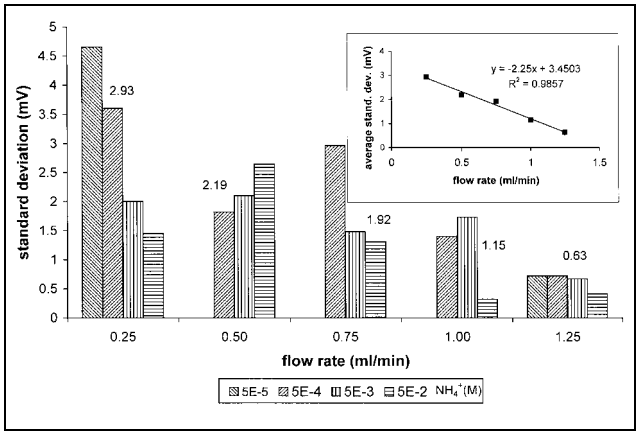


Fig. 5: Dependence of absolute standard deviations on the flow rate at different ammonium concentrations of standards (n = 3). The average standard deviation of each series is indicated at the top of each set of columns. Values for average standard deviation as a function of flow rate are depicted inside the box (35 °C, pH 7)

low ammonium concentrations showed a high standard deviation at low flow rates (0.25 ml/min). Differences in the standard deviation of individual ammonium measurements were less significant at higher flow rates. The average standard deviations exhibited a nearly linear correlation as a function of flow rate over the investigated range (Fig. 5, box). Best results were achieved at 1.25 ml/min but also those obtained at 1.0 ml/min were acceptable. A problem with these experiments was that the measured potential for the lowest ammonium concentration (5×10^{-5} M) was found to be ambiguous, which was probably caused by ammonium-contaminated chemicals. Taking into account the results shown in Fig. 4, a flow rate of 1.0 ml/min appeared most suitable and was chosen for the following experiments.

2.5. Determination of alliin

For the analysis of alliin standards (10^{-6} M–10^{-2} M), the apparatus depicted in Fig. 1 was operated at optimized working conditions described above. A cartridge (1 ml) filled with immobilized alliinase was placed between the pump and the electrode. Alliin was converted to allicin, pyruvate (pyruvic acid) and ammonium (ammonia) by the immobilized enzyme (Scheme). The obtained calibration plot was nearly linear in the range from 3×10^{-5} M to 1×10^{-3} M alliin and showed a satisfactory correlation coefficient of greater than 0.99 (Fig. 6). The detection limit was at 5×10^{-6} M. Four samples of garlic dry extract were also analysed. The results were in good correlation with those obtained by a spectroscopic FIA method (Table) [12]. It should be noted that the values were corrected by the amount of native ammonium of each sample. Ammonium levels prior to alliinase activity may give some information about storage and process conditions of garlic material [12].

Table: Alliin concentrations in different samples of garlic dry powder

Sample No.	NH_4^+ (%)	Alliin by FIA [12] (%)	Alliin by electrode (%)
1	0.13	3.41	3.62
2	0.09	2.20	2.25
3	0.15	4.86	4.77
4	0.09	2.31	2.48

The results are compared with those obtained by a FIA-method. Alliin concentrations were corrected by the amount of native ammonium.

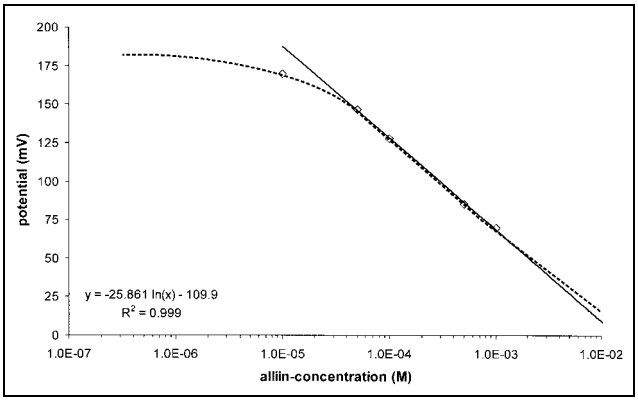


Fig. 6: Typical calibration plot (broken line) and linear regression graph (full line), which was obtained by five alliin-standards (35 °C, pH 7)

3. Discussion

It has been demonstrated that the ammonia gas electrode can be operated at pH-values lower than 10. Satisfactory results were obtained at an increased temperature (precision of ±0.1 °C) of the electrode. Because the equilibrium between ammonium and ammonia at pH 7 is strongly shifted to the ionic form, an ammonium ion sensitive electrode may also be used. The T_R values of this electrode are better than those of an ammonia gas electrode but an external reference electrode is necessary. Moreover, an ammonium electrode interferes with some ions like sodium or potassium. For the latter reason, we favour the use of an ammonia gas electrode.

The developed method shows the following advantages in comparison to conventional chromatographic methods:

- the required equipment is inexpensive;
- no pre-column derivatization is required;
- duration from extraction to the final result is reduced, which allows a complex calibration at the beginning of each set of samples;
- analysis can be carried out over concentrations of two orders of magnitude;
- only inexpensive aqueous solutions are required as carrier.

However, some critical points must be considered:

- about 5–10 ml sample volume was needed for a single measurement requiring 50–500 mg of a garlic sample for a three- to five-fold measurement. Taking into account that the method was designed for the purposes of quality control, size of samples should be a minor problem.
- The immobilized enzyme should be handled with care. Blank water or alcohol may inactivate the alliinase. However, its stability was analysed extensively. Immobilized alliinase, which was stored at pH 7.0 under optimized conditions, is stable over one year and can be used over a period of several weeks [9, 12].

A topic of current investigation is the improvement of the system described above. For example, reduction of the size of the enzyme cartridge and a reduction of sample volumes are of special interest.

4. Experimental

4.1. Chemicals

Chemicals were purchased either from Merck or Fluka (unless otherwise mentioned) and were purified by standard procedures. Water for preparing buffers and standards was purified by a Millipore-Q-Reagent-system (Millipore, Bedford, MA, USA).

4.2. Operating conditions of the ammonia gas electrode

An Orion (Beverly, Mass., USA) ammonia gas electrode, type 95-10, combined with a WTW (Weilheim, Germany) pMX 3000 ion meter was used for all experiments. The inner electrolyte of the electrode was diluted with H_2O (1 : 9) according to Liu et al. [5]. Signals were traced by a chart recorder (type Erba, W + W, Basel CH). A flow-through cell allowing a tangential flow of solvents and an inner volume of 180 µl was manufactured from polypropylene and mounted in an angle of 35° to avoid disturbances by air bubbles inside the cell (Fig. 1). Electrode and cell were surrounded by a heatable double water jacket and temperature (25–40 ± 0.1 °C; for standard experiments 35.0 °C) was adjusted by an IKA (Heitersheim, Germany) Combimag RCB combined with an electronic thermometer. Standards, samples and buffers were selected by means of a Knauer (Berlin, Germany) A 0617 switching valve with motor drive and delivered by an Ismatec (Glattbrugg, CH) MC-MS/CA 4/8 peristaltic pump. A flow rate of 1.0 ml/min was used for standard experiments. Citrate-phosphate buffer (pH 7.0, 0.1 M) was used as solvent for most experiments. The pH sensitivity of the electrode was determined by citrate-phosphate buffers (pH 6.0, 0.08 M, and pH 7.0, 0.1 M), phosphate buffer (pH 8.0, 0.2 M) and borate buffer (pH 9.0, 0.05 M). Ammonium sulfate suprapur® (Merck, Darmstadt, Germany) was solved in the corresponding buffers as described above to give final concentrations in a range between 5×10^{-5} M and 5×10^{-2} M NH_4^+. At the beginning of each experiment, the cell was rinsed with blank buffer for at least 30 min. Standards were measured beginning at the lowest ammonia concentration up to the highest. Values were taken if the potential of the electrode was stable for at least 1 min (+/−0.1 mV), which was comparable with the T_{95} value.

4.3. Determination of alliin concentrations

Synthetic L-(+)-alliin was prepared following the procedure already described [13–17]. The purity of alliin was determined by HPLC [3]. Alliinase was isolated from garlic powder (Finzelberg, Andernach, Germany Prod. No. 0105800) and purified following procedure described elsewhere [18, 19]. The enzyme was immobilized in HiTrap®cartridges (Pharmacia, Uppsala, Sweden) with a volume of 1 ml according to the manufacturer's instructions. Standards (10^{-6} M–10^{-2} M) and samples were solved in Sørensen phosphate buffer (pH 7.0, 0.06 M) containing 1% NaCl. The electrode was operated in the same way as described above. Samples of garlic dry powder (280 mg each, item. no. 0105301, Finzelberg GmbH, Andernach, Germany) were solved in 25 ml buffer.

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