Institut für Pharmazeutische Biologie, Universität Bonn, Germany

Stabilization and pharmaceutical use of alliinase

I. KREST and M. KEUSGEN

In recent years, numerous clinical trials were undertaken in order to elucidate the active principle of garlic (*Allium sativum* L., Alliaceae). The most prominent effect of garlic preparations is a contribution to the prevention of stroke and arteriosclerosis. Allicin [(2-propenyl)-2-propenethiosulfinate] and other sulfur containing compounds were suggested as active compounds. The extremely unstable allicin itself is liberated from the more stable alliin [*S*-(+)-2-propenyl-L-cysteine sulfoxide] by the enzyme alliinase (EC 4.4.1.4) if fresh garlic is crunched or garlic powder is moistened. Therefore, an active enzyme is required in alliin containing remedies like those prepared from garlic powder. In order to investigate enzyme stability, alliinase was isolated from garlic powder. The partially purified enzyme could be stabilized over several months by addition of sodium chloride, sucrose, and pyridoxal-5′-phosphate. Alliinase may also be freeze-dried. This allows combinations of synthetic alliin and purified alliinase as components of an acid resistant tablet or capsule. In the intestine, the pro-drug alliin would be enzymatically converted to allicin. In clinical trials, highly dosed preparations of this kind should yield a precise information about the physiological effects of allicin. In addition, alliin-homologues substances which bear a modified alkyl side chain and do not occur in nature may be tested.

1. Introduction

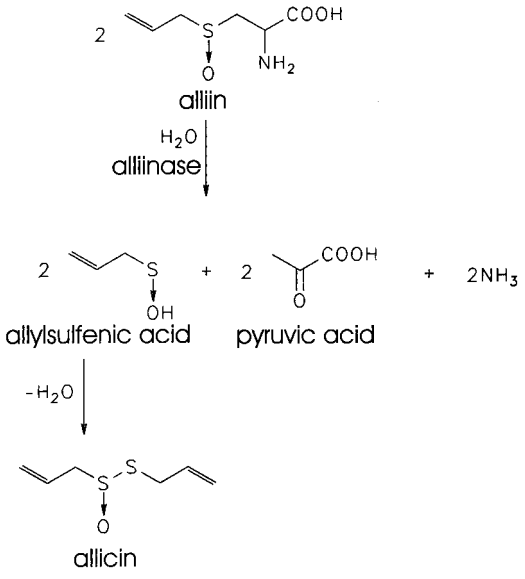
Garlic (*Allium sativum* L., Alliaceae) is used world-wide as a spice as well as a remedy in folk medicine for the treatment of infectious diseases or the prevention of stroke and arteriosclerosis [1, 2]. In addition, effects on blood coagulation, lipid- and cholesterol-lowering, blood pressure, antithrombic, and anticancer activities are under discussion. Numerous clinical trials were undertaken to elucidate the benefits of garlic [1, 2]. All these investigations were carried out either with fresh garlic, garlic powder or garlic extracts. Pure garlic compounds were never applied to patients and therefore the active principle of garlic is still under discussion. Sulfur containing compounds like the highly unstable allicin [(2-propenyl)-2-propenethiosulfinate] were suggested to be responsible for most of these effects. Allicin itself does not occur in fresh garlic but gets rapidly liberated from the precursor alliin [*S*-(+)-2-propenyl-L-cysteine sulfoxide] if cells of fresh garlic are disrupted or garlic powder is moistened. The reaction is catalysed by the enzyme alliinase (EC 4.4.1.4; Scheme) [3–5].

To answer the question of the active principle of garlic, pure compounds must be applied. Because of its instability, allicin is not suitable for a clinical trial. As an alternative strategy, the precursor alliin and stabilized alliinase may be applied as tablet or capsule. Allicin would be enzymatically liberated in the intestine and can enter the blood circuit immediately. The synthesis of alliin was investigated in recent years [6–10] and alliinase has been characterized previously [4, 5, 11, 12]. However, there are no investigations about long term stability of alliinase in order to elucidate its suitability as drug component. Furthermore, purification of the enzyme in preparative scale has not yet been reported. These are subjects of the studies presented here.

2. Investigations and results

In recent investigations about alliinase, the osmolarity of the used buffers was increased by the addition of glycerol (up to 10%) to prevent inactivation of the enzyme during the purification procedure [4, 5, 10–13]. However, these enzyme preparations cannot be freeze-dried and glycerol should therefore be replaced by alternative polyalcohols like sugars. In nature, carbohydrates account for about 77% of the dry weight and are the most abundant class of compounds present in garlic bulbs [1]. The soluble polysaccharides of garlic contain up to 89% fructose and about 10% glucose; all garlic fructans embody at least one glucose unit [14, 15]. In order to mime nature, sucrose in various amounts was added to partially purified alliinase (Fig. 1). Concentrations from 10% up to 20% resulted in a stabilization over a period of 8 h (storage at 4 °C). A sucrose concentration of 5% gave no enzyme stabilization; best results were achieved at 15% and 20% sucrose. It must been noticed that enzyme solutions containing sucrose in a concentration higher than 10% are very viscous. Moreover, the influence of pyridoxal-5′-phosphate (P-5′-P) on enzyme activity was investigated because it was suggested as a co-substrate of alliinase [4, 5, 11, 12]. As shown in Fig. 2, an addition of P-5′-P is capable to enhance enzyme activity (b). P-5′-P was obviously liberated from the enzyme during 4 h of storage in blank buffer, but could be restored (c). A concentration of 25 µM was found to be sufficient. A variety of salts were also tested as enzyme stabilizing agents. Chlorides and nitrates were

Scheme



found to be most suitable. The positive effect of P-5′-P was significantly enhanced by further addition of 1% sodium chloride (a).

These substances which increased the stability of alliinase were combined with each other in order to determine the optimal combination (Fig. 3). Sodium chloride caused the most prominent effect (c, d, g–j). Sodium chloride-free preparations showed a rapid decrease in activity (a, b, e, f). Enzyme solved in blank phosphate buffer lost its activity within a few hous (a). As expected, a combination of 1% sodium chloride, 10% sucrose, and 25 μM P-5′-P gave the best results (i). After 26 h, the activity of this sample was increased by about 100% of the initial activity. Replacement of sucrose by glycerol gave a somewhat lower activity (j). Again, P-5′-P-free preparations (a, c, g, h) showed always lower activities than the corresponding P-5′-P containing samples (b, d, i, j). However, the absence of P-5′-P in puffers never caused a complete enzyme inactivation.

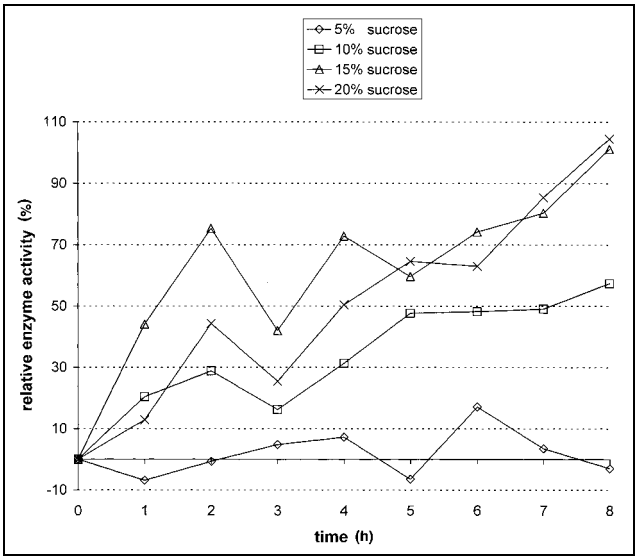


Fig. 1: Relative increase of alliinase-activity in dependence on different sucrose concentrations. Samples were stored at 4 °C between measurements. The activity of the alliinase solved in blank buffer was set as 0%

For a long term experiment, alliinase was stabilized in the optimized way described above and stored at −20 °C. Its activity was monitored over a period of 500 d (Fig. 4). No significant decrease in activity was observed. The measured values are somewhat scattered but always higher than the initial activity $(14\,\mu mol \cdot mg^{-1} \cdot min^{-1})$. The reason for this scattering is unknown. We assume that the enzyme is sensitive to thawing. Cycled thawing and freezing led to a loss in activity of about 40% for each cycle. Slow thawing at 4 °C gave the highest enzyme activities.

Alternatively, the stabilized enzyme could be freeze-dried to give a yellow, hygroscopic powder which is highly water soluble. Freeze-drying affected a 15% loss in activity. The product is stable at room temperature but should be stored at 4 °C over a longer period. The freeze-dried alliinase can either be filled into capsules or may be processed to a tablet. In order to elucidate the second possibility, freeze-dried material was examined at various pressures (Fig. 5). All the tablets obtained were highly water

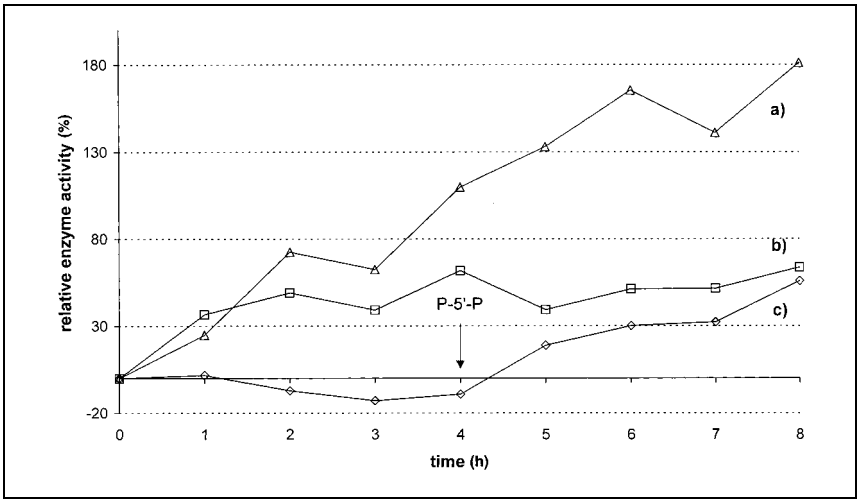


Fig. 2:
Relative increase of alliinase-activity over a period of 8 h (activity of alliinase solved in blank buffer was set as 0%). Alliinase was either solved in buffers containing 25 μmol P-5′-P and 1% NaCl (a), 25 μmol P-5′-P (b) or 25 μmol P-5′-P were added after 4 h (c)

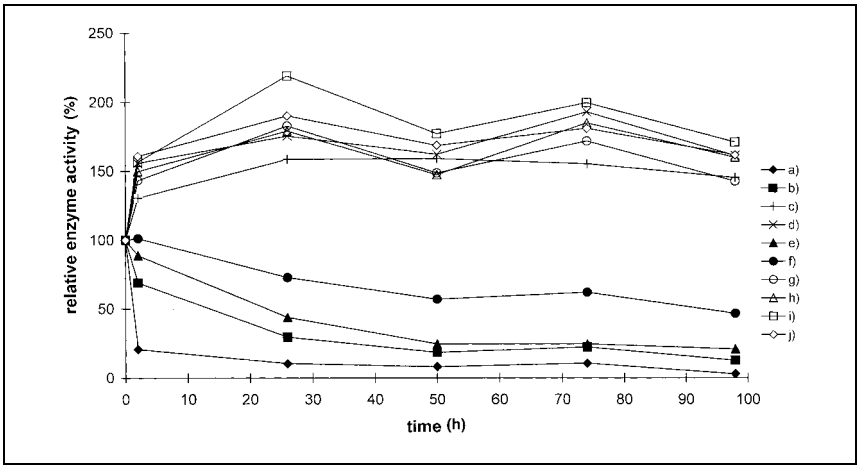


Fig. 3:
Relative activity of alliinase in dependence on sucrose (10%), glycerol (10%), NaCl (1%) and P-5′-P (25 μmol). The initial activity of all samples was set as 100%. a) blank buffer, b) P-5′-P, c) NaCl, d) NaCl + P-5′-P, e) P-5′-P + sucrose, f) P-5′-P + glycerol, g) NaCl + sucrose, h) NaCl + glycerol, i) P-5′-P + NaCl + sucrose, j) P-5′-P + NaCl + glycerol. Samples were stored at 4 °C between measurements

soluble, but the enzyme activity was significantly decreased in dependence on the applied pressure. The correlation between pressure and the reciprocal enzyme activity followed a nearly first order function up to a pressure of 500 MPa; at higher pressures, the activity was only slightly decreased to 58% of the initial activity. A similar behaviour was recently reported for butyrylcholine esterase [16]. For alliinase, the pressure-dependence of activity over the investigated range can be described by a second order function:

$$\text{relative activity}^{-1} = (-4 \times 10^{-9})\, x^2 + 10^{-5} x + 0.0103$$

$$x = \text{pressure}$$

One presupposition for an alliinase containing drug is an economical extraction and purification process of the enzyme. Partially purified alliinase can be easily obtained from garlic powder in preparative scale by extraction with phosphate buffer followed by an ammonium sulfate precipitation. This material is suitable as component of a drug; however, highly purified material would be preferable because higher enzyme activities can be achived with less protein. Complex multi-step procedures were published but can be hardly scaled up to allow an economical enzyme purification [4, 5]. For these reasons, an effective, two step chromatographic purification method for alliinase was developed (Fig. 6). In the first step, proteins were separated by gel filtration according to their molecule size. Protein elution was monitored by UV-detection. The alliinase-containing fraction was loaded on an affinity column and eluted by a step gradient of methyl-mannopyranose. The highest enzyme activity obtained by this process was

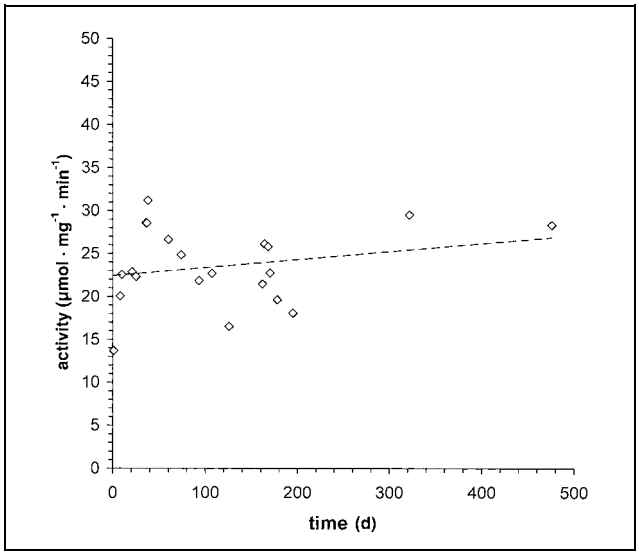


Fig. 4: Activity of alliinase over a period of nearly 500 days (initial activity in blank buffer: 14 µmol · mg^{-1} · min^{-1}). Samples were stored at −20 °C before measurements

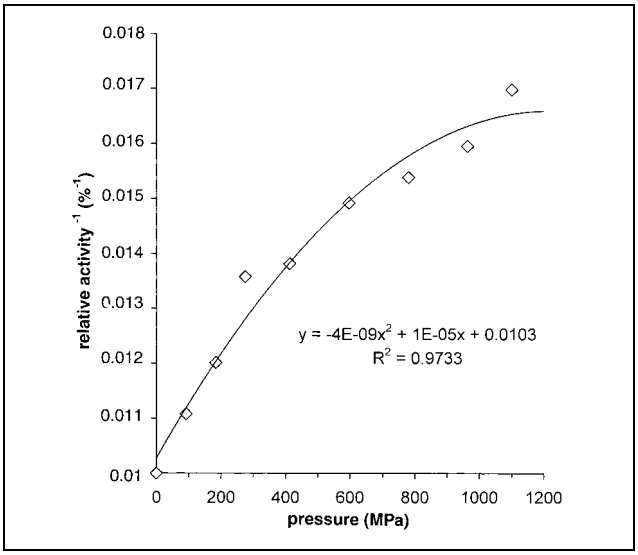


Fig. 5: Activity of stabilized, freeze-dried alliinase in dependence on different pressures. Reciprocal activity values are depicted as a function of pressure

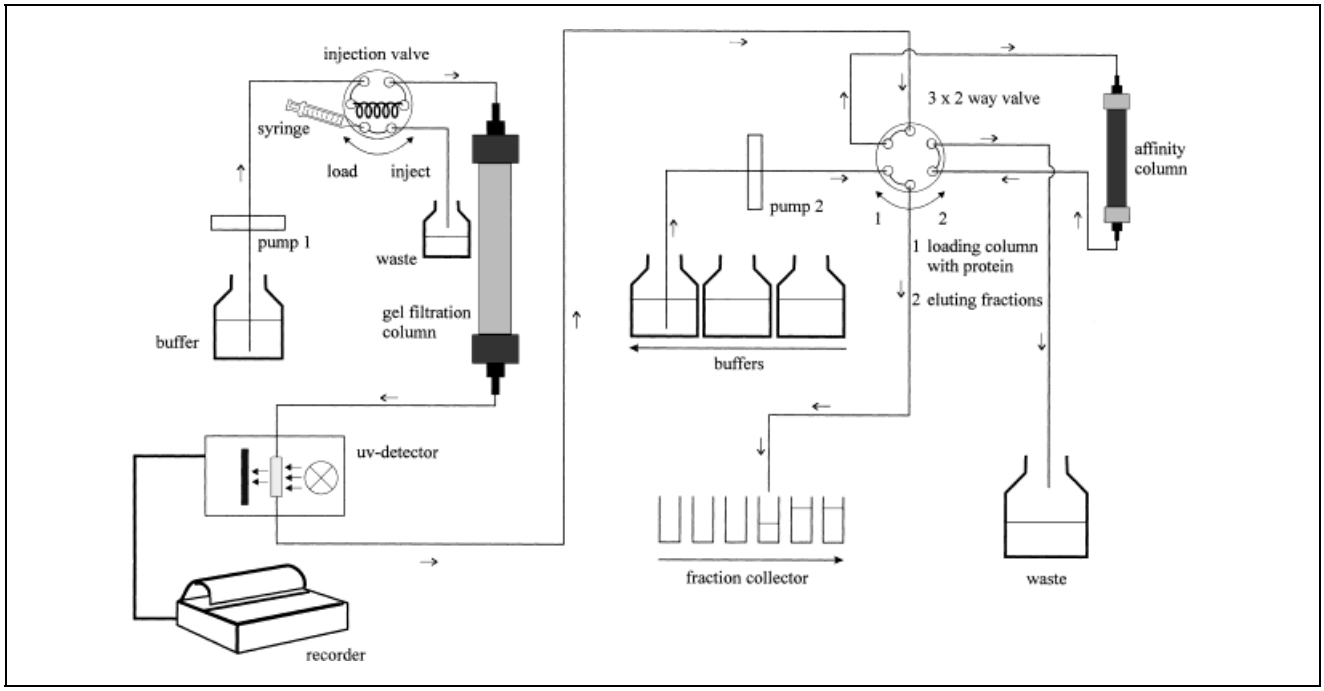


Fig. 6: Combined gel filtration/affinity chromatography for the purification of alliinase. Samples were firstly subjected to gel filtration and proteins traced by UV-absorption (280 nm). Fractions containing alliinase were directly loaded on an affinity column

at 202 μmol · mg^{-1} · min^{-1} which is close to literature values [5, 11, 12]. It must be noticed that highly purified alliinase is less stable than the partially purified enzyme. This problem is under current investigation. First experiments gave evidence that this material is much more sensitive to a loss of P-5′-P.

3. Discussion

We have demonstrated that alliinase preparations can be stabilized and enzyme activity can be conserved for several months. A freeze-dried enzyme is sensitive to pressure, but the remaining activity was found to be always higher than 50% of the initial activity. This opens the possibility to develop an alliin/alliinase containing drug. Medicinal effects caused by allicin can be elucidated in clinical trials and may lead to a novel type of enzyme-based, semisynthetic drug. Alliinase also shows activity towards further cysteine sulfoxides allowing combinations with synthetic, not naturally occurring homologous of alliin in order to give more effective drugs [4, 17].

Rabinkow et al. recently discussed the production of allicin by recombined alliinase [11, 18]. These results are very promising. But from an economic point of view, the isolation of alliinase from natural sources is the more favourable way because high quality garlic powder is inexpensive, available at all times over the year and can be stored at room temperature over several months. Additionally, alliinase makes up 10% of the total protein amount of a garlic clove allowing an effective enzyme isolation [1].

The most suitable way for the production of an allicin pro-drug is the preparation of small, separate alliin and alliinase pellets which can be filled into an acid resistant capsule (Fig. 7). In the intestine, alliinase would be activated by water resulting in a formation of allicin. As an advantage of this method, high levels of allicin may be obtained.

Our investigations may lead to a nature-derived, chemically defined drug for prevention and treatment of diseases related to the blood circuit system. The development of such remedies is recommended because the number of patients with arteriosclerosis and hard stroke steadily increased during the last decades. Therefore, this new type of drug is expected to be a considerable progress in the treatment of these diseases and represents an alternative to crude, chemically not defined garlic-containing remedies.

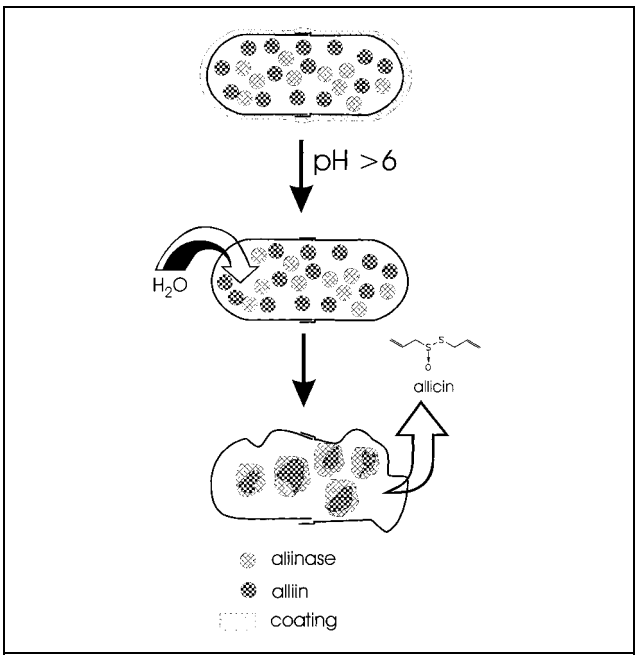


Fig. 7: Application of alliinase as component of a drug. An acid resistant capsule is filled with pellets of alliin and alliinase. In the intestine, alliin and alliinase are dissolved and allicin is liberated. Alternatively, alliin may be replaced by a homologous sulfoxide

4. Experimental

4.1. Chemicals and plant material

All chemicals were purchased from Merck or Fluka unless otherwise specified and were purified by standard procedures. Calibration proteins were obtained from Promega. Polyacrylamide and Bradford reagent were purchased from Sigma. Racemic L-alliin was prepared from L-cysteine by sulfur alkylation followed by oxidation of the sulfur atom [6–10]. L-(+)-Alliin was purified by fractionated recrystallization to a final purity of 98% [9–11]. All fractions were subjected to TLC and HPLC [19, 20]. Analytical data for alliin are given in the literature [10]. Powder of Chinese garlic was supplied by the Finzelberg GmbH, Andernach, Germany (Prod. No. 0105800, Lot. No. 1690050 and Lot. No. 2690039).

4.2 Purification procedure

Unless otherwise mentioned, phosphate buffer (60 mM, pH 7.0) was used for all operations. Garlic powder (100 g) was extracted with 400 ml of an ice cold buffer additionally containing 10% glycerol, 5% NaCl, 0.5% PVP, 0.05% 2-mercaptoethanol, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The suspension was mixed with an Ultra Turrax® device (IKA, Staufen, Germany) for 5 min. Cell fragments were removed by repeated centrifugation (3000 × g and 61000 × g) and proteins were precipitated by $(NH_4)_2SO_4$ (45% rel. saturation). The residue was resolved and dialysed over night in phosphate buffer containing 1% NaCl, 10% sucrose, 25 mM pyridoxal phosphate (P-5′-P), and 0.02% NaN_3. This partially purified alliinase (138 ml, 18 mg protein per ml) was stored at −20 °C. For further purification, portions of 2 ml each were subjected to gel filtration on a Sephadex S 200 HR® column (300 × 15 mm, Pharmacia, Uppsala, Sweden). Protein was detected by UV-absorption at 280 nm and alliinase-containing fractions were loaded on a column filled with concanavalin-A-derivatized agarose (120 × 8 mm). Alliinase was eluted by a step gradient using phosphate buffer containing 1% NaCl, 0.02% thiomersal and subsequently 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM methylmannopyranose (10 ml each). The yield after combined gel filtration and affinity chromatography was at 23%; the purified enzyme showed a specific activity of 202 μmol · mg^{-1} · min^{-1}) for L-(+)-alliin. Partially purified alliinase was freeze-dried to weight constancy and used for performing tablets (100 mg each) on a Perkin Elmer hydraulic press (Überlingen, Germany Ser.-No. 584).

4.3 Enzyme characterisation

Protein concentrations of alliinase preparations were determined alternatively according to the methods of Bradford [21] and Lowry et al. [22] using BSA as standard. Protein samples from all stages of the purification procedure were subjected to dissociating SDS electrophoresis according to Lämmli [23]. A polyacrylamide concentration of 12% was used throughout all experiments. Proteins were visualised by staining with Coomassie blue and relative molecular masses were determined by calibration proteins. Enzyme activity was deduced from the amount of enzymatically formed pyruvate following the procedure firstly described by Schwimmer and Mazelis [24]. The standard reaction mixture contained 18 U LDH, 0.4 μmol NADH and 2 μmol L-(+)-alliin. The volume was adjusted with buffer to give 1.00 ml. The decrease of NADH was traced at 340 nm. All experiments were performed at 25.0 °C.

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Michael Keusgen
Institut für Pharmazeutische Biologie
Nußallee 6
D-53115 Bonn
M.Keusgen@uni-bonn.de