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Short communication

Use of tryptophanase in the detection of contaminants in EMS related L-tryptophan

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1. Introduction

Eosinophilia-myalgia syndrome (EMS), a multisystemic disease, has been traced back to the intake of certain lots of L-tryptophan (Trp) of a single Japanese manufacturer, Showa Denko KK (SD) (Tokyo, Japan) [1]. The most widely accepted hypothesis is that one or more contaminant(s) in the implicated Trp-lots are responsible for EMS [2]. Since neither radiochemical and microbiological studies nor analysis for inorganic elements have detected any significant contamination [3], most investigations have concentrated on RP-HPLC/UV analysis of different lots of SD-Trp. The detector response of up to 60 contaminant peaks has been statistically compared in case associated and non-case associated SD-Trp-lots [4]. Up to now three EMS-related contaminants have been structurally elucidated: 1,1′-ethylidenebis(L-tryptophan) (1,1′-EBT) [5,6]; 3-phenylamino-L-alanine (3-PAA) [7,8] and 2-(3-indolylmethyl)-L-tryptophan [9]. However, the etiologic agent of EMS has not been identified beyond all shadow of doubt because of the lack of a valid animal or in vitro model that could show EMS-like effects [10]. To our knowledge, all investigations concerning chromatographic profiles or the identification of contaminant peaks were carried out by HPLC using different RP-18 stationary phases and various mobile phases, all containing acetonitrile as modifier and trifluoro-acetic acid (TFA) or phosphoric acid for acidification (Table 1). Hertzman et al. [3] assumed that the causative agent of EMS might not absorb in the UV-range or may be present in a peak that is hidden beneath another. We therefore examined the hypothesis that contaminant peaks might be hidden beneath the large Trp peak which elutes for at least 3 min because for an analysis of contaminant patterns the analytical column is overloaded with Trp (10 mg ml^{-1}). It may be assumed that coelutions occur since under the commonly chosen chromatographic conditions even known metabolites such as tryptamin [11], kynurenic acid and xanturenic acid would coelute with Trp [unpublished results].

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Table 1
HPLC conditions chosen for contaminant profiles of SD-Trp

Author	Detection	HPLC column	Mobile phase	
			Acidifier	Modifier
Belongia et al. [1]	UV 216 nm, Fl 280/345 nm	Biorad Hi-Pore RP-318 250·4.6 mm	0.1% TFA	MeCN
Hill et al. [4]	UV 225 nm	Whatman Partisil 5 ODS-3	0.1% TFA	MeCN
Körner and Malinka [23]	PDA 204, 216, 280 nm	Biorad Hi-Pore RP-318 250·4.6 mm	H_3PO_4 ad pH 2.3	MeCN
Müller et al. [9]	UV 220 nm	RP-C18, 5 μm, 5·250 mm	2.3% H_3PO_4	MeCN
Simat et al. [14]	UV 260 nm, Fl 290, 356	Nucleosil 120 3-C18, 250·4 mm	0.1% TFA	MeOH/MeCN
Simat et al. [11]	UV 240, 260 nm, Fl program	Nucleosil 120 3-C18, 250·4 mm	0.1% TFA	MeOH/MeCN
Toyo'oka et al. [7]	UV 280 nm, Fl (290/360)	Inertsil ODS-2 (150·4.6 mm)	0.1% TFA	MeCN
Toyoda et al. [24]	UV 280 nm	Inertsil ODS-2 (150·4.6 mm)	0.1% TFA	MeCN
Trucksess et al. [25]	UV 220, 280 nm	Delta Pak C18, 3 μm, 150 mm	0.1% TFA	MeCN

For the examination of the hypothesis our aim was to remove Trp most selectively by using the specificity of an enzyme. Tryptophanase (EC 4.1.99.1) (TRPase) was chosen because this enzyme catalyses the reversible β-elimination of Trp to yield indole, pyruvate and ammonia (Fig. 1). A quantitative conversion of Trp was reported by Ikeda and Fukui [12] using immobilised TRPase.

2. Materials and methods

Tryptophanase (EC 4.1.99.1 from *E. Coli*), 2-mercaptoethanol and pyridoxal 5-phosphate (PLP) were purchased from Sigma (Deisenhofen, FRG); ammoniumcarbonate and indole, from Merck (Darmstadt, FRG); and CNBr-activated Sepharose 4B, from Pharmacia (Freiburg, FRG). All other reagents used were of analytical grade.

EMS associated Trp (Showa Denko lot 67236201) was made available by Centers of Disease Control (CDC, Atlanta, GA, USA); control Trp, by Degussa AG (Hanau, FRG).

Methanol (MeOH), acetonitrile (MeCN) and trifluoroacetic acid (TFA) used for chromatography were HPLC-grade (Merck, Darmstadt, FRG). Deionized water was purified by Heraeus-Destamat Bi 18E (bidistillator)(Kleinostheim, FRG).

2.1. Assay for activity of tryptophanase

Specific activity was determined using the commonly defined incubation conditions. First, 1.6 ml 0.1 M K-phosphate buffer pH 8.3 containing about 100 μg TRPase and 200 μl 0.2 mM PLP were stirred in a 4 ml vial for 10 min at 37°C, then 200 μl Trp solution (1 g l^{-1}) were added and incubated whilst stirring for 10 min at 37°C. The reaction was stopped by addition of 2 ml 0.1 M trichloroacetic acid (TCA). Under the conditions already described [13], 1 TRPase U is defined as the amount of enzyme required to form 1 μg indole/10 min. The determination of indole formation (and Trp decrease) in the diluted and membrane filtrated reaction mixture was performed by HPLC with the chromatographic conditions described in Table 2. Inhibition of TRPase by indole and pyruvate was measured by assaying the decrease of Trp concentration using the procedure described above.

The determined activity of the commercial enzyme preparation varied depending on the TRPase lot used, in a range from 15 to 100 U mg^{-1}.

2.2. Immobilisation of tryptophanase and assay for activity

CNBr-activated Sepharose (0.3 g) was swollen in 8 ml 1 mM HCl for 15 min and filled into a 10 ml BioRad Poly-Prep Chromatography column.

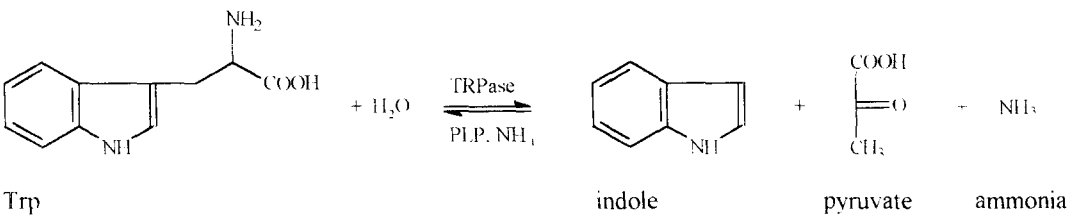

Fig. 1. Reaction catalyzed by Tryptophanase

The resulting 1 ml swollen gel was washed first with 100 ml 1 mM HCl and then with 0.1 M K-phosphate buffer (pH 7.0) until the pH of the eluate was neutral. Then, 4 mg of TRPase dissolved in 1.5 ml 0.1 M K-phosphate buffer pH 7.0 were added to the Sepharose gel and incubated by mixing on a Hecht Multi-Axle Rotating Mixer at 4°C (refrigerator) for 16–24 h overnight. After deposition of the Sepharose gel the upper aqueous layer was sucked through the column by vacuum and collected. The remaining active sites of CNBr Sepharose were blocked by addition of 5 ml 1 M ethanolamine (pH 7.0 with HCl) and using the incubation procedure as described above. The enzyme not covalently bound was finally removed by alternately washing with 5 ml either of 0.1 M K-phosphate buffer pH 7.0 or of 0.1 M acetate buffer pH 4.0, each at least 5 times. The UV_{280nm} absorption of the collected eluates after application of the enzyme solution to the Sepharose gel was measured by Perkin Elmer Lambda 2 UV/VIS spectrophotometer. The immobilised amount of TRPase was determined by substraction of the eluted amount of TRPase (calculated from UV_{280nm}-absorption) from the total applied amount. A total of 8.15 mg TRPase bound to 1g Sepharose under the described conditions. The immobilisates were stored at 4°C in 0.03% sodium azid solution.

For determination of the specific activity, 4.3 ml of 0.1 M K-phosphate buffer pH 8.3 were added to 1.7 ml of the immobilised enzyme preparation. Of the diluted gel, 200 μl were transferred into a 10 ml column; 400 μl 0.1 M phosphate buffer pH 8.3, 200 μl 0.2 mM PLP and 10 μl 0.5 M mercaptoethanol were added. After 10 min incubation at 37°C (heating oven) on the rotating mixer, 200 μl of a Trp solution (1 g l^{-1}) were added and incubated at 37°C for 10 min. After deposition of the gel the eluate was diluted and indole formation was determined by HPLC (Table 2). The immobilised TRPase exhibited an activity of about 80 U ml^{-1} (corresponding to 34 U mg^{-1} bound TRPase, activity of the free enzyme: 90 U mg^{-1}).

2.3. Cleavage of Trp for determination of contaminants

2.3.1. Free enzyme

About 2 mg of Trp (SD 67236201 or Degussa) were incubated at 37°C for 76 h with 300 U free TRPase, in 40 ml 0.1 M ammoniumcarbonate buffer containing 0.4 mg PLP. For incubation the mixture was divided on four threaded culture tubes (20 ml) and stirred on the rotating mixer for 76 h at 37°C.

2.3.2. Immobilised enzyme

About 2 mg Trp (SD 67236201 or Degussa) were incubated at 37°C for 76 h with 2 ml Sepharose bound tryptophanase (about 160 U), in 30 ml 0.1 M ammoniumcarbonate buffer containing 0.4 mg PLP and 0.2 ml 0.5 M mercaptoethanol. For incubation the mixture was divided on four threaded culture tubes (20 ml) and stirred on the rotating mixer for 40 h at 37°C. After that the immobilisate was filtered off.

The resulting incubation solutions (free or immobilised enzyme) were freeze-dried six times

Table 2
HPLC-conditions

Equipment	Merck L 6200 pump, Merck L-4000 UV detector (Darmstadt, FRG), Shimadzu RF-530 fluorescence detector (Duisburg, FRG), Bruker Chromstar software (Bremen, FRG)				
Column	Spherisorb ODS 2 (C-18, 5 μm), 250×4 mm, precolumn 20×4 mm[a] Nucleosil 120 3-C18, 250×4 mm, precolumn 20×4 mm[b]				
Mobile phase A	0.1% TFA in water				
Mobile phase B	Methanol				
Mobile phase C	Acetonitrile				
Gradient profile	Min	A (%)	C (%)	Gradient[a]	
	0	85	15	Linear	
	15	55	45	Step	
	18	55	45	Linear	
	20	85	15	Step	
	Linearity (retention time)		Ind 0.05–4 mg l^{-1} (7.3 min) Trp 0.6–50 mg l^{-1} (15.5 min)		
	min	A (%)	B (%)	C (%)	Gradient[b]
	0	95	5	0	Linear
	10	86	14	0	Linear
	40	46	14	40	Linear
	42	26	14	60	Linear
	46	95	5	0	Step
Flow-rate	1.2 ml min^{-1}[a] 1.0 ml min^{-1}[b]				
Temperature	Ambient[a] (35°C)[b]				
Injection volume	20 μl				
Detection	Fl, 280/356 nm[a] UV, 260 nm[b]				

[a]For determination of Trp and Ind (enzymatic activity).
[b]For determination of the contaminant pattern of Trp and digested Trp.

(and each time refilled with bidistilled water) to remove the volatile ammonium carbonate buffer. The last lyophilisate was dissolved in 200 μl 0.1% TFA/MeOH (95:5, v/v) under ultrasonic agitation. After membrane filtration the solution was chromatographed as described previously (Table 2) [14]. Peak identification for peaks 1–4 (Fig. 2 and Fig. 3) was done by comparison of retention times with those of standard substances and by standard addition. Peaks 5–8 were identified by comparison of the RP-HPLC typical SD-Trp contaminant pattern with the characterization reported by Müller et al. [9].

3. Results and discussion

3.1. Choice of buffer

In order to analyse microcontaminants of Trp it was necessary to select a volatile buffer that could be removed after incubation. Since monovalent cations, especially ammonium or potassium ion, have an activating effect on TRPase [16], we compared TRPase-activity in a volatile 0.1 M ammoniumcarbonate buffer with the widely used 0.1 M K-phosphate buffer [12,13], both at pH 8.3. The activity of free TRPase was elucidated to be

about 20% lower at 37°C in the carbonate buffer than in the K-phosphate buffer. This proved to be satisfactory since the ammonium carbonate buffer could be removed without residue by repeated freeze-drying. The freeze-drying procedure should not alter the pattern of contaminants since most of them are supposed to be not volatile because SD-Trp had to pass anion- and cation exchangers during the purification process and was dried after crystallisation [15]. Hitherto, most of the identified contaminants proved to be amphoteric substances [11].

3.2. Immobilised TRPase

Our results differed from those of Ikeda and Fukui [12] who succeeded in using immobilised TRPase coupled with lactate dehydrogenase for a Trp-assay, as our immobilisate did not keep its entire activity. We observed a loss of more than

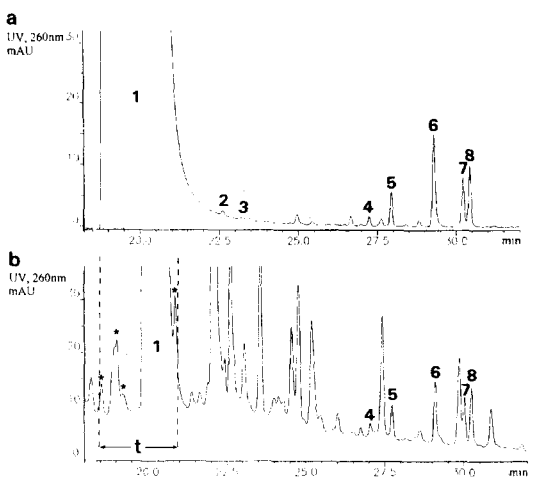

Fig. 2. HPLC chromatogram obtained from an EMS related Trp lot (Showa Denko 67236201) (a) prior to digestion with TRPase (10 mg ml^{-1}); (b) after digestion with TRPase, t: time period Trp peak elutes without removal by enzymatic digestion, *: Trp coeluting substance peaks; conditions for separation see Table 2. Peaks: 1 = Trp, 2 = 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 3 = 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 4 = 1,1'-EBT, 5 = 2-[2,3-dihydroxy-1-(3-indolyl)-propyl]-L-tryptophan, 6 = 2-(3-indolylmethyl)-L-tryptophan, 7 and 8 = 1-(3-indolylmethyl)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (diaster-eomers).

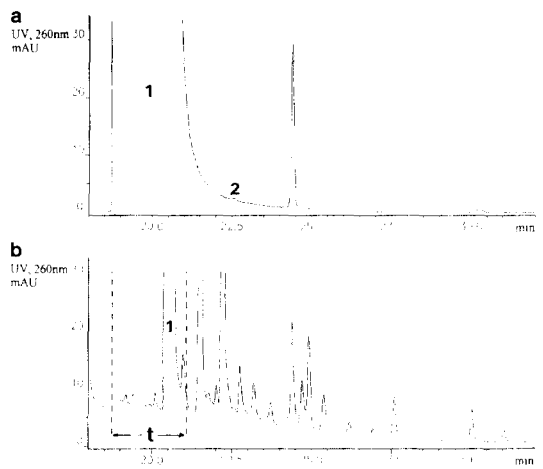

Fig. 3. HPLC chromatogram obtained of Trp from Degussa, (a) prior to digestion with TRPase (10 mg ml^{-1}) and (b) after digestion with TRPase, t: time period Trp peak elutes without removal by enzymatic digestion conditions for separation see Table 2, for peak identification see Fig. 2.

90% of activity after 17 days storage at 4°C (in K-phosphate buffer or in 0.03 M sodium azid) and about 80% loss after 24 h incubation at 37°C (K-phosphate buffer). Protection and reactivation of TRPase was described with mercapto compounds such as glutathione [13,16], cysteine [13], mercaptoethanol [16] and dithiothreitol [16]. Preincubation with mercaptoethanol or addition of this thiol to the reaction mixture reactivated the immobilisate almost to its previous activity.

3.3. Optimisation of Trp-cleavage

TRPase is described as being stable up to 60°C (measured for 10 min) with temperature-increasing reaction rate [17]. Considering the increase of side reactions of Trp with increasing temperature, we limited temperature during the incubation to 37°C.

Indole, ammonia and pyruvate are the products of Trp cleavage (Fig. 1). Indole is reported to decrease the activity of TRPase [13,18,19]. In accordance with Morino and Snell [19], we found a decrease of the initial velocity of the TRPase-reaction (measured for 10 min) of more than 50% when indole was added to the mixture (Trp:indole, 2:1). Addition of pyruvate resulted in

only a slight loss of initial velocity. A procedure to avoid the inhibiting effect of the indole formed might be to layer an organic solvent such as toluene over the surface of the reaction mixture [20,21]. However, lipophil Trp contaminants such as tetrahydro-β-carbolines might be extracted and falsify the result of the digestion. As an alternative we tried to strip the indole out of the reaction mixture by bubbling a gentle nitrogen stream through it. However, only half of the indole content of an aqueous solution (5 mg indole l^{-1}) could be removed after 20 h of leading nitrogen through it by a stainless steel frit at 37°C (30 ml N_2 min^{-1}). Moreover it caused a pH-shift in incubation solutions of the volatile ammonium carbonate buffer. An increase of cleavage rate could not be achieved.

Besides the reaction products, several amino acids are known to inhibit TRPase. Competitive inhibitors which are known to be present in Trp raw materials are oxindolylalanine (Oia) and kynurenine (Kyn) [16]. Oia is known to be a competitive inhibitor of TRPase and reacts irreversibly with pyridoxal phosphate [22]. The amount of Oia and Kyn present in Trp raw-material is very low compared to Trp (up to 100 ppm [11,14]) so that a competitive inhibition of TRPase by these compounds may be disregarded.

3.4. Detection of Trp-coeluting contaminants

Cleavage of EMS related SD-Trp and blind sample (Degussa, FRG) was monitored by HPLC. After 76 h of incubation with free TRPase, 85–95% of the initial Trp had been cleaved. Inhibition of TRPase by the indole formed is likely to be the reason for the slow and incomplete cleavage. Blind incubations of buffer, TRPase and PLP revealed that no interfering peaks occurred within the time range during which Trp elutes (18.5–21.5 min). All substance peaks originating from the commercial TRPase, PLP and the buffer eluted prior to 18 min.

In Fig. 2 and Fig. 3 chromatograms of digested and untreated Trp material are compared. Several peaks were eluted in the time range (t) previously hidden by Trp in both presented Trp materials. In the EMS related Trp several more peaks could be detected eluting prior to the residual Trp-peak that were not present in the 'blind' Trp. Past 21 min, several new by-products were detected that were not present in the Trp before digestion or in the TRPase, PLP or buffer used. They are likely to be formed during incubation and could perhaps be avoided if the enzymatic digest could be accelerated. Although digests were performed in duplicate and gave similar results it cannot be completely excluded that the detected Trp-hidden peaks are incubation artifacts of Trp or Trp-contaminants.

TRPase is known to cleave not only Trp but also 4-, 5-, 6- and 7-substituted Trp-analogues and other amino acids substituted with electronegative groups in β-position. (R = -Cl, -Me). Their reaction rate is much lower than that of Trp [16]. The major contaminants in SD-Trp (Fig. 2, peaks 4–8) known as 'Peaks A–E', which were structurally elucidated by Müller et al. [9], were not markedly degraded during incubation (Fig. 2b).

The aim of incubation with the immobilised TRPase was to avoid the contamination of the Trp solution by the enzyme itself and by-products present in the commercial preparation. However, after 40 h incubation a part of the bound TRPase seems to have been cleaved from the Sepharose judging from the loss of activity and the pattern of peaks in the chromatograms. Additionally, mercaptoethanol which had been added to restore the activity of the immobilised TRPase led to more incubation artefacts eluting after Trp.

4. Conclusions

Trp could be removed selectively from a solution by the use of TRPase. Essential for this investigation was, in addition to the application of a volatile buffer (ammonium carbonate) and the formation of volatile reaction products (indole), the specificity of TRPase for Trp in the presence of mainly Trp-derived contaminants. However, the introduced method has the drawback of product inhibition of the used enzyme and the resulting long incubation times leading to the generation of new artefacts.

This investigation has shown for only one EMS-related Trp-sample the presence of Trp-coeluting peaks that were not present in one examined blind-Trp-sample from a different supplier. No conclusions can be drawn as to whether the detected substances might be related to EMS. Nevertheless, gradient RP-HPLC on C-18 phases using acidic modifiers may not succeed in recognising contaminants coeluting with Trp. This investigation indicates that a different chromatographic principle of separation should be applied to some SD-Trp case and none-case lots to confirm that no decisive contaminant has been overseen to date.

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