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THE CANAL INCLUSION COMPLEX OF ALLICIN WITH CARBAMIDE: PREPARATION, CHARACTERIZATION AND MICROBIOLOGICAL INVESTIGATION

The carbamide:allicin canal inclusion complex was prepared in the solid state. The structure of the complex obtained was characterized by x–ray crystallography, infrared spectroscopy and thermogravimetric analysis. The microbiological activities of the inclusion complex and allicin were investigated and compared with respect to fungi (Candida albicans ATCC 10231 and Aspergillus niger ATCC 16404) and bacteria (Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 9027). It was found that the inclusion complex inhibited the growth of bacteria and fungi for a longer period than allicin in the free state.

Key words: allicin, carbamide, inclusion complex, microbiological activities.

Inclusion complexes are a special group of so–called 'non–classical' complexes formed under the influence of mechanical factors, i.e. the 'space inclusion' complex component factors. The components of inclusion complexes are 'host' and 'guest' molecules. The 'host' molecules are compounds that in their crystal structure, or in their aggregates, have cavities large enough into which the molecules of the other component ('guest' molecules) can be enclosed [1]. Especially interesting is the canal inclusion complex of carbamide, which is a lattice like inclusion complex. The canals are formed by the carbamide crystal lattice. Normally, carbamide crystallizes in the form of a thick $P\bar{4}2_1$ m space group tetragonal lattice [2–4]. In the presence of a corresponding guest component, crystals can be formed where the carbamide molecules would be bonded between themselves by intermolecular hydrogen bonds and positioned hexagonally in the shape of a spiral, forming the walls of a cylindrical canal of 0.5 nm diameter [1]. These canals can receive molecules of a compound with normal chains with anti–periplanar conformation, such as n–paraffin carbohydrates and their derivatives with terminal functional groups (alcohols, aldehydes, carbon acids, amines, olefins and others), whereby complexes are readily formed when the included compound has normal chains with six or more carbon atoms. The molecule ratio of carbamide and the guest component in the inclusion complex varies and depends on the size of the included substance molecule, where 0.7 molecules of carbamide correspond to each CH_2–group of the guest molecule.[1] In reference works, carbamide complexes with alkanes (hexadecane, nonadecane, etc.) were studied [5–8].

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Allicin is a thioester of sulfenic acid (allyl thiosulfinate), formula $CH_2=CH–CH_2–S(O)–S–CH_2–CH=CH_2$. In pure condition, it is a colorless or slightly yellowish oily liquid with typical fresh garlic (*Allium sativum L.*) odor [9]. Allyl thiosulfinate is pharmacologically the most important and most active substance in fresh garlic water extract [10–12] and it is a very unstable compound, which makes its isolation, synthesis and application very difficult. In order to increase allicin stability, a canal inclusion complex of allicin with carbamide was prepared and analyzed.

EXPERIMENTAL

Material and methods

Carbamide (99%, ρ = 1.335 g/cm^3) was supplied by Merck and used without additional purification. Allicin was synthesized by the oxidation of allyl disulfide with acid hydrogen peroxide by a procedure described in the literature [13]. The method of complex formation in solution was used for the synthesis of the canal inclusion complex of allicin with carbamide. Allicin was added to a saturated carbamide solution and left to crystallize in a desiccator above a dehydrating agent at about 10°C for two to three days. The quantities of carbamide and allicin were taken to obtain a mole ratio in the complex of 4:1. The complex crystals can be visually differentiated from the carbamide crystals; they are wooly and slightly yellow colored.

Physical measurements

The compounds and free agents were characterized by the following methods:

X–ray crystallography: The initial substances carbamide and the inclusion complex carbamide:allicin were identified by the method of powder X–ray diffraction using a PHILIPS PW1350 diffractometer with CuK_α radiation and a Ni filter. The diffractograms were recorded in the 2θ range 5–60° at 298 K.

Single–crystal intensity measurements of the inclusion complex carbamide:allicin (4:1) were performed using a STOE – imagine plate detector system IPDS with MoK_α radiation and a graphite monochromator at room temperature. 2623 independent reflections were merged with R=0.0432 to 205 unique reflections. The evaluation of the systematic extinction rules resulted in the space group $P\overline{4}2_1m$. Data reduction including intensity integration, background corrections, Lorentz and polarization correction was performed with a STOE XRED program package.

Infrared spectroscopy (IR): FT–IR spectra were obtained by using a MB Series Bomem Hartmann & Braun FT–IR spectrophotometer. The FT–IR spectrum of the synthesized and purified allicin was made between KBr plates with 0.1 μm film thickness, and for the carbamide:allicin inclusion complex on a KBr pellet (2.6 mg sample, 140 mg KBr) in the wavelength range from 4000 to 400 cm^{-1}.

Thermogravimetric analysis (TG): Thermogravimetric curves were recorded on a Bomem TG/plus apparatus in the 303–673 K range with linear temperature increments of 10 K/min. The sample mass was 10 mg.

Antimicrobial test: The microbiological activities of free allicin and of allicin bonded in the complex were investigated by the diffusion method for various time intervals. The active substance quantity on a 12.7 mm dia. disk was 180 μg. The samples were incubated at 37°C (bacteria) and 25°C (fungi) for 24 hours. The bacteria *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, and fungi *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 were tested. The *B–1 Bacto antibiotic medium 1 dehydrated* (Difco Laboratories, Detroit, U.S.A.) substrate was used for bacteria growth, and *Tripton soya–agar* (Torlak Institute for Immunology and Virusology, Belgrade) for fungi.

RESULTS AND DISCUSSION

X–ray analysis: Pure carbamide and the carbamide:allicin canal inclusion complex are clearly crystalline and the diffractograms of their powder samples are shown in Figure 1 (A and B). Peak indexing was made on the basis of comparison with the carbamide values (hkl) from the JCPDS–28–2015 data base [14].

Analysis of the complex diffractograms indicates the presence of an amorphous peak, a change of intensity, and a slight position shift of the peaks for the complex samples as compared with the peaks for pure carbamide. The change in intensity of the index peaks could hint at allicin presence in some carbamide crystal lattice plane, while the peak shifts could serve to determine the parameters of the unit cell of the inclusion complex crystal lattice as compared to carbamide (Table 1).

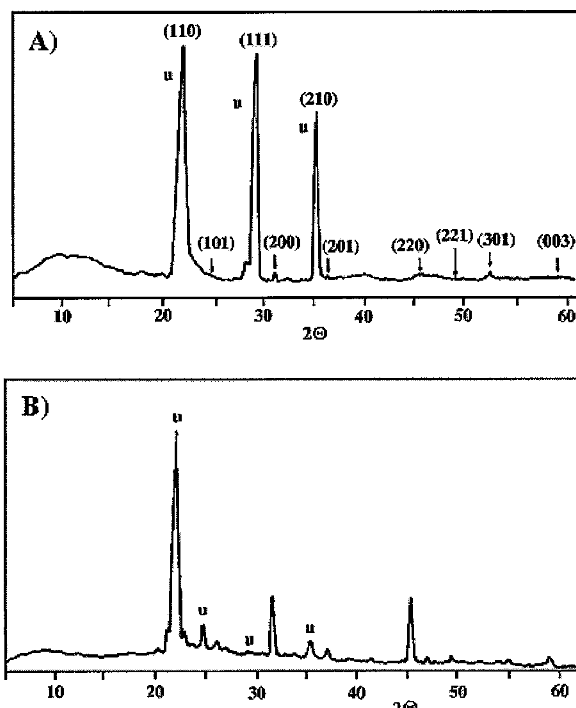

Figure 1. Diffractograms of carbamide (A) and the carbamide:allicin (4:1) inclusion complex (B)

Based on the determination of the exact position of the known index peaks (200) and (101), the values of parameters **a** and **c**, of the crystal unit cell, were determined. The calculated values are given in Table 1.

Table 1. Parameters of the elementary cells of carbamide and the carbamide:allicin inclusion complex determined from powder diffractograms at room temperature

Compound	a (Å)	c (Å)	D_x (mg/cm^3)	V ($Å^3$)
Carbamide $CO(NH_2)_2$, JCPDS 28–2015	5.645	4.704	1.331	149.90
carbamide:allicin, 4:1	5.686	4.739	1.317	151.12
carbamide	5.663	4.700	1.334	150.73

At the same time, the volume of the crystal unit cell, and the roentgen (calculated) density of the samples was calculated. It can be concluded that the presence of allicin causes an increase in the unit cell volume and, at the same time, a decrease of the roentgen density.

To define the carbamide:allicin complex crystal lattice and structure, X–ray diffraction analysis on a single crystal sample was used. The structure was solved by direct methods using the SHELXL86 [15] program. A full–matrix least–squares refinement of the fractional coordinates and anisotropic atom displacement parameters for non–hydrogen atoms was performed with SHELXL93 [16]. The position of the H atoms was refined isotropically. The final R–factor was R=0.0339 for 21 parameters and 205 reflections. The highest and lowest peaks in the final difference map

Table 2. Crystal data and structure refinement (CCDC 239749)

Empirical formula	C H_4 N_2 O +allicin
Molecular weight	60.06
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system, Space group	tetragonal, $P42_1m$
Unit cell dimensions	a=5.6577(11) Å $\alpha=90^o$ b=5.6577(11) Å $\beta=90^o$ c=4.7042(8) Å $\gamma=90^o$
Volume	150.58(5) $Å^3$
Z, Density (calculated)	2, 1.325 Mg/m^3
Absorption coefficient	0.114 mm^{-1}
F(000)	64
θ– range for data collection	4.33 do 27.98°
Index ranges	–7<=h<=7, –7<=k<=7, –5<=l<=5
Reflections collected / unique	2623 / 205 [R(int) = 0.0432]
Refinement method	Full–matrix least–squares on F^2
Data / restraints / parameters	205 / 0 / 21
Goodness–of–fit on F^2	1.184
Final R indices [I>2σ (I)]	R1 = 0 .0 339; wR2 = 0 .0 798
R indices (all data)	R1 = 0.0394; wR2 = 0.0837
Absolute structure parameter	–1(4)
Largest diff. peak and hole	0.120 and –0.111 e/$Å^3$

were 0.120 and –0.111 $eÅ^{-3}$ and these values did not support the presence of any new atoms in the crystal structure. The basic crystallographic data, as well as the structure of the refinement details are given in Table 2.

The final atomic coordinates and the equivalent isotropic parameters in the carbamide:allicin complex (4:1) show no difference as compared to carbamide [2] and they are given in Table 3.

The defined carbamide:allicin complex crystal structure obtained by the single crystal method confirmed that the crystal structure was that of carbamide. On the basis of the defined atom positions, it was confirmed that the carbamide molecule packing in the carbamide unit cell was as presented in Figure 2 and

Table 3. Fractional atomic coordinates ($x10^4$) and equivalent isotropic displacement parameters ($Å^2x10^3$). U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

Atom	x	y	z	U(eq)
O	5000	0	9021(3)	53(1)
C	5000	0	11664(4)	42(1)
N	3588(2)	1412(2)	13162(4)	69(1)
H(1)	3620(20)	1380(20)	14840(70)	55(5)
H(2)	2650(40)	2350(40)	12110(50)	83(8)

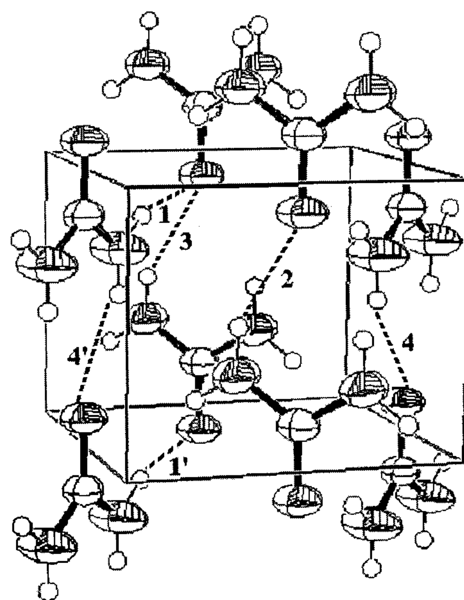

Figure 2. The crystal packing for the unit cell projected along the c–axis. The dash line denotes hydrogen bonds of the N–H···O type. The H····O distances are: 1 and 1' 2.182 Å; 2 2.256 Å; 3 2.256 Å; 4 and 4' 2.256 Å

that it was stabilized by a hydrogen bond network between oxygen from the CO–group and the H(1) and H(2) protons from the NH_2–group in the carbamide molecules.

It can be said that all the crystallographic parameters indicate the 'guest' presence. These are, first of all, data which can be obtained by powder diffractrogram analyses, as an increase of the unit cell volume in the investigated complex as compared to the unit cell volume of pure carbamide [2,17].

Although the solution of the single–crystal structure of the inclusion complex had not confirmed the presence of the allicin molecule in the host crystal lattice it can be concluded that the inclusion of allicin molecules in the host crystal lattice was possible in a limited, non–stoichiometric and disordered molar ratio.

Infrared spectroscopy: Figure 3 shows the IR spectra of carbamide, synthesized and purified allicin and the carbamide:allicin (4:1) inclusion complex.

In the IR spectrum of the carbamide:allicin canal inclusion complex there are differences compared to the IR spectra of allicin and carbamide. In the 3000–4000 cm^{-1} range, the complex has two carbamide peaks unified into one wider and more complex peak with a weak inflexion the maximum of which was shifted to a somewhat lower frequency value, which is characteristic of the hydrogen bond, and the peak at about 3224 cm^{-1} was more clearly formed. This region covers the N–H valence vibrations, ν(N–H), from the carbamide molecules and the C–H valence vibrations, ν(C–H), from allicin. The 'amide band I' originating from ν(CO), was shifted towards lower frequency values in the complex,

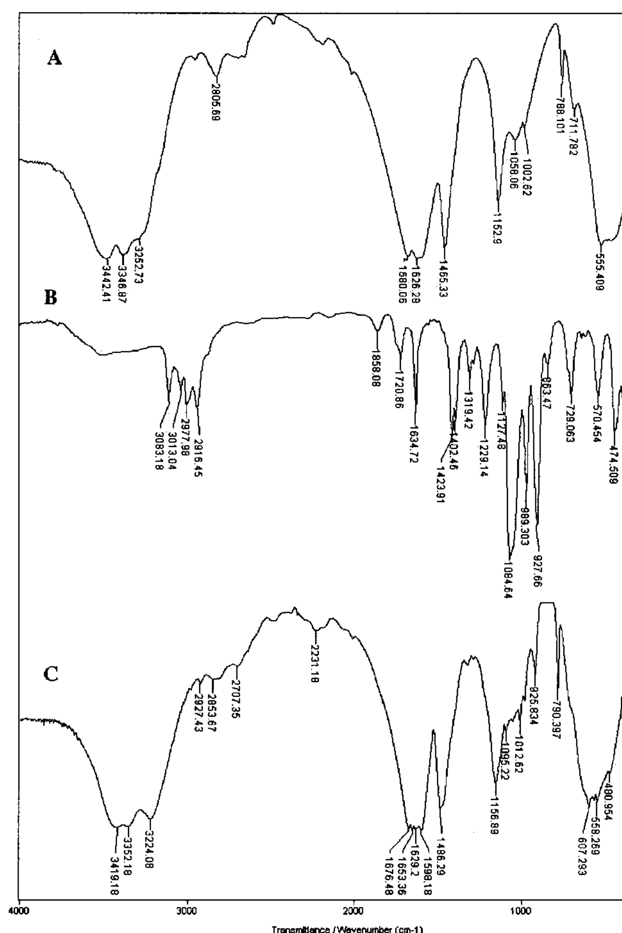

Figure 3. IR spectra of carbamide (A), allicin (B) and the carbamide:allicin canal inclusion complex (C)

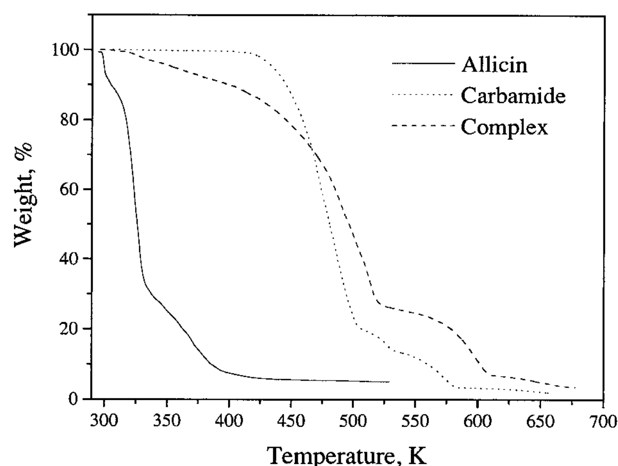

Figure 4. TG curves for allicin (——), carbamide (·····) and the carbamide:allicin canal inclusion complex (– – –)

and was found at 1676 cm^{-1}, indicating a hydrogen bond in the complex. The bending vibrations, δ(N–H), of the NH_2 group in the complex occurred at 1653 cm^{-1}, i.e. at a slightly higher frequency than for pure carbamide, most probably due to intermolecular hydrogen bonded between allicin and carbamide in the complex. Confirming this opinion, the shift of the 'amide band III' was towards higher frequency values, at 1486 cm^{-1}, and the 'amide band II', which, in the complex, appeared as a complex and intensive band with a peak at 607 cm^{-1}.

The decrease of the valence vibration frequencies and the increase of the bending vibration frequencies are due to the formation of hydrogen bonds and the weakening of covalence bonds in the acceptor (>S=O) and the donor (>N–H), i.e. a decrease of the force constants of their valence vibrations.

Thermogravimetric analysis: The TG curves for the carbamide, allicin and carbamide:allicin canal inclusion complex are shown in Figure 4.

The TG curve for allicin shows high instability, mass loss is initiated already at room temperature and the greatest thermal destruction is achieved at 393K. A slight curve inflexion between 533 and 673 K can be ascribed to the thermal destruction of the residual impurities present in the synthesized allicin. The carbamide TG curve shows greater stability than that of allicin, and its thermal decomposition occurs in several stages. The first stage of the carbamide pyrolysis is between 420 and 509 K, where the greatest mass loss is achieved. In this stage destruction of carbamide:allicin canal inclusion complex is slower and occurs within the temperature range of 378 to 541K. Also, the part of the complex TG curve that could correspond to allicin has a slower mass loss, and complete mass loss is achieved only at temperatures higher than 400 K, different to pure allicin, i.e. the canal inclusion complex shows a greater thermal stability as compared to the pure compounds. These results also indicate that there is a connection between the host and guest molecules due to intermolecular action, which is in compliance with the previous results.

Microbiological analysis: The fungicidal and bactericidal properties of allicin and the carbamide:allicin canal inclusion complex were investigated in various time intervals after synthesis, since allicin is known to be unstable and decomposes with time and loses its microbiological properties. The results of these investigations are given in Table 4.

The carbamide:allicin canal inclusion complex shows greater stability and microbiological activity on all the microorganisms tested compared to allicin. Experimentally determined a minimal microorganisms growth inhibition zones were reached 30 days after synthesis in allicin, while the complex conserved its activity to a significant extent after 60 days. The most susceptible microbe was Candida albicans, and the least susceptible *Pseudomonas aeruginosa*. These investigations verify the formation of a new supramolecular complex, carbamide:allicin, which conserves its stability, microbiological activity and gives allicin a longer shelf life.

Table 4. Microbiological activity of allicin and the carbamide:allicin canal inclusion complex with respect to the microorganisms tested

t, day	Inhibition zones, mm									
	C. albicans		*A. niger*		*E. coli*		*S. aureus*		*P. aeruginosa*	
	A	C	A	C	A	C	A	C	A	C
0	45	45	30	30	30	31	32	32	15	15
8	35.6	42.5	23	28	27.5	30	30	31	14	15
23	26	38	14	25	14	24.5	20	28	12.8	13.5
40	18.8	27	14	23.5	14	22.5	16	24.5	–	–
52	15.4	23	–	18.7	–	18	14	20	–	–
62	–	20	–	18.5	–	17.5	–	18	–	–

A – allicin, C – complex carbamide:allicin

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IZVOD

KANALSKI INKLUZIONI KOMPLEKS ALICINA SA UREOM: PRIPREMA, KARAKTERIZACIJA I MIKROBIOLOŠKO ISPITIVANJE

(Naučni rad)

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Pripremljen je kanalski inkluzioni kompleks urea:alicin u čvrstom stanju. Struktura dobijenog kompleksa okarakterisana je primenom Rendgenske kristalografije, infracrvene spektroskopije, i termičke analize. Ispitana je i upoređena mikrobiološka aktivnost inkluzionog jedinjenja i alicina na gljive (*Candida albicans* ATCC 10231 i *Aspergillus niger* ATCC 16404) i bakterije (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027). Utvrđeno je da inkluzioni kompleks inhibiše rast bakterija i gljiva u dužem vremenskom periodu od alicina u slobodnom stanju.

Ključne reči: alicin, karbamid, inklusioni kompleks, mikrobiološka aktivnost.