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RESEARCH NOTE

Effect of supercritical carbon dioxide on arginine

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L-Arginine was converted into arginine bicarbonate by treatment with humid supercritical carbon dioxide (300 bar, 80°C, 6 h) at high pH. The influence of pH on the reaction was studied. A more sensitive modification of the 2,4-dichloro-1-naphthol/thymine/sodium hypochlorite colour reaction for arginine is presented.

INTRODUCTION

Supercritical carbon dioxide (SCC) is used in food processing for the extraction of heat-sensitive materials and is also discussed as a solvent for enzymatic reactions. A review on the influence of SCC on proteins and amino acids was recently published (Weder, 1990). After the extraction of natural materials by SCC, proteins, peptides and amino acids are generally found in the residue. If the residue is used as a food, these components should not be altered by the process or, if they are altered, they should have better nutritional or structural properties. Of seven free amino acids exposed to SCC (L-glutamic acid, L-glutamine, L-methionine, L-leucine, L-alanine, β-alanine and L-lysine), only glutamine is partially converted into 2-pyrrolidinone 5-carboxylic acid, which does not negatively influence food quality (Weder, 1984).

Weder (1980*a*, 1981) observed a loss of about 20% in L-arginine treated with carbon dioxide at a pressure of 300 bar, at room temperature and at 80°C. Since this loss was determined with colour reactions for both α-amino and guanidino groups, a possible reaction sequence to the formation of 1,3-diazacycloheptane-2-one 1-amidino 4-carboxylic acid (a compound expected to show similar properties to the ones observed) was proposed. The aim of the present work was to further study the reaction of arginine with SCC and to clarify the structure of the reaction product.

MATERIALS AND METHODS

Samples of L-arginine (Arg, biochemical grade from Merck, Darmstadt, Germany) or L-arginine hydrochloride (Arg·HCl, from Merck) were mixed in test tubes with either water or hydrochloric acid and treated with 300 bar carbon dioxide or nitrogen at 80°C or room temperature for 6 h, as previously described (Weder, 1984). Samples were designated to give the gas and the pressure used as well as the temperature and the time of treatment; for example Arg/CO_2-300/80-6 denotes arginine treated with 300 bar carbon dioxide at 80°C for 6 h.

Ninhydrin reaction of untreated and treated samples and amino acid analyses of both unhydrolysed and hydrolysed untreated and treated samples were performed as described earlier (Weder, 1984).

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Arginine colour reaction was performed with 2,4-dichloro-1-naphthol (K & K), thymine (Merck) and sodium hypochlorite (hypochlorite solution, 150–155 g active chlorine/litre, Bender & Hobein, Munich, Germany) according to Demetriou *et al.* (1974) and in a modification of this procedure, in which the 2,4-dichloro-1-naphthol solution and the thymine solution were separately added with vortexing for 10 s with the addition of each reagent.

Thin-layer chromatography (TLC) was carried out on TLC-plastic sheets (Silica gel 60, pre-coated, Merck 5748) with ethanol:water (7:3, v/v) and *n*-butanol:acetic acid:water (4:1:1, v/v). Samples were detected with ninhydrin followed by the chlorine–starch–iodide (CSI)-procedure, as earlier described (Weder, 1984), or with CSI alone.

Thin-layer electrophoresis (TLE) was performed on TLC-glass plates (Silica gel 60, pre-coated, Merck 5721) at pH 6·5 (pyridine:acetic acid:water, 25:1:225, v/v, 24°C, 400 V for 25, 75 and 120 min) and pH 10·2 (1 M ammonia:1 M acetic acid, 16:4, v/v, 26°C, 300 V for 60 min; 0·5 M ammonia:0·5 M acetic acid, 17:3, v/v, 13°C, 400 V for 75 min and 4°C, 750 V and 17 and 30 min). Samples were detected with CSI.

The carbon dioxide liberated by treatment with hydrochloric acid was estimated in a Warburg constant volume respirometer (712262, B. Braun, Melsungen, Germany), following the procedures given by Umbreit *et al.* (1964).

Chloride contents were determined in treated samples of both arginine, adjusted to a given pH, and arginine hydrochloride with chloranilic acid (mercuric salt, Merck), by slightly modifying the procedure of Fries and Getrost (1975).

RESULTS AND DISCUSSION

Two samples of L-arginine (0·5 and 5·0 g) were exposed to humid supercritical carbon dioxide (300 bar, 80°C) for 6 h (Arg/CO_2-300/80-6 (I and II), see Table 1). In parallel, arginine samples were also treated with 300 bar carbon dioxide at room temperature (Arg/CO_2-300/RT-6 (I and II)) and with 300 bar nitrogen at room temperature (Arg/N_2-300/RT-6). Arginine contents of the treated and untreated samples were determined by ninhydrin reaction, automated amino acid analysis of unhydrolysed and hydrolysed samples, and an arginine colour reaction.

The absorbances obtained with the modified arginine colour reaction (successive addition of 2,4-dichloro-1-naphthol and thymine instead of mixing prior to addition) showed that the modified version is 10-fold more sensitive than the procedure described by Demetriou *et al.* (1974). Molar absorbancy coefficients at 515 nm for the modified method and that given in the literature were $2{\cdot}24 \times 10^4$ and $2{\cdot}20 \times 10^3$, respectively. The reproducibility of the modified version was found to lie between 0·6 and 3·4%; the correlation of the calibration curve set with L-arginine hydrochloride was 0·998.

The arginine samples treated with 300 bar carbon dioxide, either at 80°C or room temperature, exhibited a decrease in arginine content of about 20% for the smaller and about 10% for the larger samples (Table 1), whereas exposure to nitrogen caused only minor alterations. Thus, the alterations were obviously caused in the presence of carbon dioxide.

In theory, the amino group of arginine may react with carbon dioxide to yield products with a covalently bound carbon dioxide moiety, e.g. a urea derivative, a carbamic acid or a carbamate (Weder, 1980*b*). The formation of the urea derivative would not explain the decrease in the arginine colour reaction, because the guanidino group is free. Free carbamic acids as well as the carbamates of amino acids are unstable; the latter decompose at room temperature, even in solution, with liberation of carbon dioxide (Bailey, 1950). But the carbamic acid may cyclize to yield the 1,3-diazacycloheptane-2-one 1-amidino 4-carboxylic acid. This product would not respond to the ninhydrin reaction and probably also not to the arginine colour reaction, since creatinine which also has the one nitrogen of the guanidino group substituted twice, does not give this reaction.

To separate possible reaction products from unaltered arginine, Arg/CO_2-300/80-6, Arg/CO_2-300/RT-6 and Arg/N_2-300/RT-6 were examined by TLC on silica gel. After developing with ethanol:water (7:3, v/v) or *n*-butanol:acetic acid:water (4:1:1, v/v), and visualizing with ninhydrin and/or CSI, only arginine and traces of other compounds, which were also present in the untreated arginine, were found (results not shown). The two systems separated creatine, creatinine, citrulline and ornithine; the second system also separated ornithine from arginine. All the compounds were detected by the CSI reagent.

In parallel, Arg/CO_2-300/80-6, Arg/CO_2-300/RT-6 and Arg/N_2-300/RT-6 were also examined by TLE on silica gel. Electrophoreses at pH 6·5/400 V and pH 10·2/300, 400 and 750 V for various times detected no difference between treated and untreated samples (results not shown).

Since the existence of a reaction product that was not separated from arginine by all of the systems used cannot be completely ruled out, treated and untreated arginine samples were incubated with arginase. Following high voltage TLE of the reaction products, the arginine spot had totally disappeared, and a new spot that migrated in a manner similar to ornithine was visible (Hegarty, private communication). Thus, arginine was completely converted into ornithine by arginase, and no other compound that migrated like arginine was present. These results clearly demonstrate that the urea derivative, the carbamic acid, the carbamate and the diazacycloheptane derivative were not formed.

Table 1. Alterations of arginine exposed to supercritical carbon dioxide

Sample	pH	Differences between treated and untreated samples (%)					H_2CO_3 (%)	HCl (%)
		a	*b*	*c*	*d*	Mean[e]		
Arg/CO_2-300/80-6 (I)[f]	11	−23·1	−20·5	−19·9	−16·5	−20·0	21·7	n.d.[g]
Arg/CO_2-300/80-6 (II)[h]		−8·6	−16·5	−13·4	−11·3	−12·4	12·1	n.d.
Arg/CO_2-300/RT-6 (I)[f]	11	−24·3	−22·5	−23·0	−19·0	−22·2	22·1	n.d.
Arg/CO_2-300/RT-6 (II)[h]		−6·3	−13·2	−7·5	−11·7	−9·7	13·7	n.d.
Arg/N_2-300/RT-6 (I)[i]	11	−2·2	−0·5	0·0	−4·7	−1·8	1·9	n.d.
Arg(pH 9)/CO_2-300/80-6[j]	9	−18·8	−23·7	−15·5	−12·0	−17·5	2·4	9·0
Arg(pH 7)/CO_2-300/80-6[j]	7	−16·5	−25·2	−18·2	−14·5	−18·6	0·6	15·1
Arg·HCl/CO_2-300/80-6[k]	5·7	−0·1	−2·4	−4·3	−3·5	−2·6	0·5	17·1
Arg(pH 3)/CO_2-300/80-6[j]	3	−18·6	−26·0	−16·4	−14·8	−18·9	1·7	17·0

[a] Ninhydrin reaction; means of at least four values.
[b] Amino acids analysis; means of at least two values.
[c] Amino acid analysis of hydrolysed samples; means of at least two values.
[d] Arginine colour reaction; means of at least four values.
[e] Means of different determinations for each treated sample.
[f] L-Arginine (0·5 g) mixed with 2·5 ml distilled water.
[g] Not determined.
[h] L-Arginine (5·0 g, different lot) mixed with 10·0 ml distilled water.
[i] L-Arginine (0·1 g) mixed with 1·0 ml distilled water.
[j] L-Arginine (1·0 g) mixed with 2·0 ml distilled water and adjusted to the pH given in brackets with 6 N HCl.
[k] L-Arginine hydrochloride (1·0 g) mixed with 2·0 ml distilled water.

Furthermore, arginine and carbon dioxide can form a bicarbonate or a carbonate, depending on pH, where carbon dioxide is not covalently bound. To test this possibility, the amount of gas liberated from Arg/CO_2-300/80-6, Arg/CO_2-300/RT-6 and Arg/N_2-300/RT-6 by hydrochloric acid was determined respirometrically. To prove it was carbon dioxide, the gas was shown to be completely absorbed by potassium hydroxide solution. The amounts of carbon dioxide liberated from each sample and calculated as bicarbonate are given in Table 1. The bicarbonate moieties corresponded to the differences in arginine content between the treated and the untreated samples. The formation of arginine carbonate would maximally decrease arginine content by 15·1%, if all the arginine was converted into the carbonate. Thus, arginine bicarbonate was formed under the reaction conditions used (Arg/CO_2-300/80-6 and Arg/CO_2-300/RT-6). From the maximum possible bicarbonate content of 26·3%—that is, if all the arginine has reacted—it can be calculated that 76–84% of the arginine had reacted in the smaller samples (I in Table 1) and 37–52% in the larger samples (II).

To study the influence of pH on the reaction between arginine and carbon dioxide, arginine was treated with 300 bar carbon dioxide at 80°C and at various pH values for 6 h. The results are listed in the lower part of Table 1. The differences in arginine content were mainly caused by the amount of hydrochloric acid to adjust the pH. The hydrochloric acid is bound to arginine to form arginine hydrochloride and some arginine dihydrochloride at pH 3, which was stable during lyophilization. In accordance with these results, the Arg·HCl/CO_2-300/80-6 sample, where arginine hydrochloride was used and no hydrochloric acid was added, did not show significant differences between treated and untreated sample.

Only small amounts of carbon dioxide were detectable in these samples (Table 1). Even at a pH 9 the amount of carbon dioxide bound as bicarbonate was only 2·4%. Thus, only 10% of the arginine was converted into arginine bicarbonate at that pH. These results show that the α-amino group of arginine must be unprotonated to react with carbon dioxide, and to be converted into arginine bicarbonate.

Out of the various reactions between amino groups and carbon dioxide discussed in the literature, bicarbonate formation was shown to be responsible for the observed loss in arginine content after treatment of free arginine with humid SCC. No other reaction products could be demonstrated. This reaction requires an unprotonated α-amino group; that is, a pH above 9. Thus, the reaction is not of any importance at the usual pH values of foods.

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