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# PRODUCTION OF AMINO ACIDS FROM ALBUMIN BY SUB-CRITICAL WATER HYDROLYSIS

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Amino acids find several industrial applications and are especially widely-used in animal husbandry as well as in pharmaceutical and food industry. In this work an attempt was made to determine the best experimental conditions for the recovery of amino acids as protein degradation products by sub-critical water hydrolysis. *Bovine Serum Albumin* (BSA) was used as sample protein. Experiments were performed continuously in pipe reactors of different sizes in order to achieve different residence times varying between 4 and 900 s. Amino acid compositions were analyzed by HPLC. The results were compared with conventional acid hydrolysis in HCl for 24 h. At a residence time of 30 s the highest amount of amino acids was obtained at 583 K and 250 bar. Due to the formation of simple amino acids as alanine and glycine from complex amino acids, in particular the amount of these components increased with increasing temperature up to 583 K (29% and 18% of the values obtained by HCl hydrolysis, respectively) and decreased thereafter due to further degradation of the products. No significant influence of operating pressure (150-270 bar) could be observed at the tested temperature (523 K) and residence time (30 s). The amino acid formation increased with increasing residence time between 8 and 300 s at the tested temperature (523 K) and decreased thereafter. The addition of carbon dioxide led to carbon acid formation and to an increase in amino acid yield due to the acceleration of acid hydrolyzed catalysis steps. This protein treatment may provide a practical and economical solution for the disposal of protein-rich sources like hairs and feathers, which are considered as waste so far.

## INTRODUCTION

Natural amino acids are divided into proteinogenic (occurring in proteins) and non-proteinogenic amino acids. The proteinogenic species are subdivided into essential and non-essential. Amino acids play an important alimentary-physiological role in vivo as a preliminary stage for synthesis of a multiplicity of biomolecules and they serve as protein and peptide components. Excessive amino acids are used as combustibles in metabolism [1,2]. Essential amino acids cannot be produced by the human and animal body but have to be ingested in sufficient amounts. Hence, the main field of application of proteinogenic amino acids is the specific addition of essential amino acids in animal husbandry [3]. Furthermore, they are used as flavour additives and flavour enhancers (sodium glutamate) in food industry. Their industrial production is performed in four different ways: by means of chemical synthesis, extraction of protein hydrolyzates obtained by acid or alkaline hydrolysis, fermentation by microorganisms, and enzymatic methods.

Sub-critical water refers to water above its normal boiling temperature (373 K), but below its critical temperature, that is kept in liquid state by applying pressure. The ionic product of sub-critical water is as much as three orders of magnitude higher than under ambient conditions. Thus, the increased hydronium and hydroxide ion activity leads to an

accelerated protein hydrolysis. Especially in the sub-critical region ($T_c$ = 647 K, $P_c$ = 221 bar), in which water has a high density, ionic reaction mechanisms can preferentially be verified whereas free radical reactions only gain in importance at higher temperatures [4]. Hence, acid hydrolyzed cleavages of peptide bonds can pass off without the addition of catalysts. Furthermore, by suitable choice of the operating conditions it is possible to produce amino acids with a high selectivity, whereby parallel degradation mechanisms and further reactions do not take place. Besides the idea of using pure water as reaction medium, the addition of carbon dioxide leads to an acid hydrolyzed catalysis due to the formation of carbon acid according to Equation (1) and the connected decrease in pH of the system water / $CO_2$.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2\,H^+ + CO_3^{2-} \qquad (1)$$

However, high water temperatures must be used with caution, since thermal degradation of amino acids occurs at temperatures above 523...573 K depending on the corresponding residence time [5-8]. The advantages of sub-critical water hydrolysis in comparison to conventional techniques are the harmlessness of the solvents water and $CO_2$ as well as the short residence times in the range of minutes. The addition of further chemicals is not necessary. The objective of this work was to find out the optimal operating conditions for the production of amino acids from *Bovine Serum Albumin* (BSA).

## MATERIALS AND METHODS

**Reactor system.** Aqueous solutions of 1% (w/w) BSA (purchased from Merck, Germany) in deionized water were hydrolyzed in the continuous-flow apparatus depicted in Figure 1. The piping and the reactor consisted of non-corroding and heat-resistant Cr-Ni-stainless steel 1.4404 with an inner diameter of 3.05 mm (outer diameter 6.35 mm). The feed suspension was pressurized to an overpressure of 2 bar with nitrogen in order to increase the pump´s inlet pressure and to prevent the feed from contact with air oxygen. The albumin solution was delivered to the preheater by the feed pump and heated up to a temperature lower than 453 K in order to avoid the start of the reaction. Pure water was pumped from the water reservoir by another pump and heated. The feed solution was mixed with the hot water flow at the mixing point. Thus, the reaction mixture was rapidly heated to the desired reaction temperature. Straight after the leaving the reactor the medium was cooled down in order to

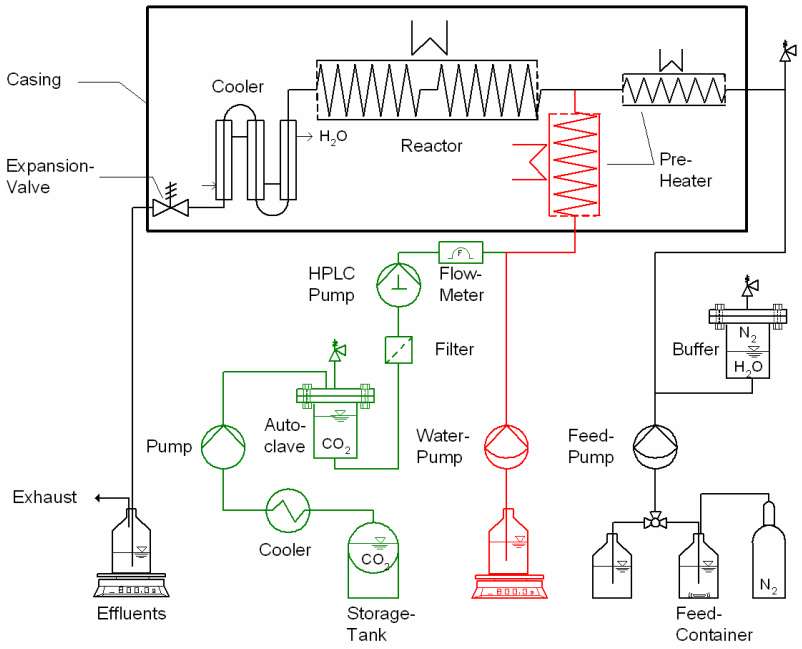


**Figure 1**: Experimental setup of the hydrolysis reactor

obtain a well-defined reaction time. For this purpose the suspension was passed through a pipe-in-pipe heat exchanger. After quenching the medium was expanded so that the samples could be taken. For monitoring the experimental conditions, temperatures in the preheaters as well as before and after the reactor were measured by a temperature control unit. The system pressure was adjusted by an overflow valve.

**Analysis.** The quantitative determination of the amino acids was performed by reversed phase chromatography after derivatization to isoindol derivatives with o-phthaldialdehyde (OPA) in the presence of mercaptoethanole. The instrumentation consisted of a pump (PE 250), a sample device (AS 465, Bio Tek), a thermostat (self-made), a Superspher 4 RP column (18.4 mm * 250 mm, Merck) and a fluorescence detector (1520, Bio-Tek/Iaesco). After diluting, the samples were treated with protein precipitation reagent and centrifuged at 16000 g. The supernatants were mixed with a borate buffer. 15 µL of this mixture were pipetted in a flange vessel and put into the sample device. After the automatic addition of OPA reagent and a reaction time of 2.5 min, a 15 µL sample of this mixture was injected. The wavelength was set to 340 / 420 nm at a temperature of 313 K. The non-proteinogenic amino acid β-alanine was used as internal standard.

## RESULTS AND DISCUSSION

**Total hydrolysis.** Acid hydrolysis of BSA with 6.6M HCl was performed at 383 K for 24 h in order to determine the theoretical amount of amino acids actually verifiable. The theoretical achievable amount could be calculated by the help of the known number of the particular amino acid in the BSA molecule [9]. In fact, yields of 1 [$g/g_{theor.}$] should arise for all amino acids since it is supposed to be total hydrolysis. However, as can be seen from Figure 2, hat this is not the case. This could originate from the hydrolysis method itself, because some amino acids can suffer losses due to partial or complete destruction (Ser, Thr, Tyr, Met, Cys). The hydrolyses of amino acids containing aliphatic side chains (Val, Ile) are often incomplete after 24 h. The values for the other components are possibly too high due to contaminations [9]. The common names of the amino acids identified in this work are given in Table 1.

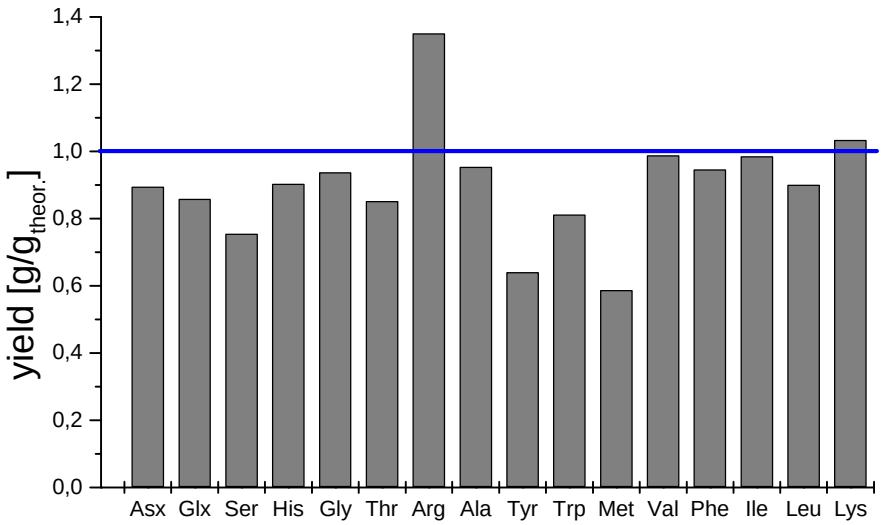


**Figure 2**: Total hydrolysis of BSA with 6.6M HCl at 383 K for 24 h

**Dependence on reaction temperature.** Figure 3 shows the effect of the reaction temperature on the amino acid yield at $\tau$ = 30 s and P = 250 bar. The total amount of amino acids increases with rising reaction temperature until 583 K. For temperatures higher than 583 K, the yield decreases because of amino acid decomposition. Asx seems to be thermally labile since its yield decreases at temperatures higher than 523 K. It is conspicuous that in particular alanine (Ala) is produced in significant amounts. This could be due to the formation of alanine as degradation product of other complex amino acids.

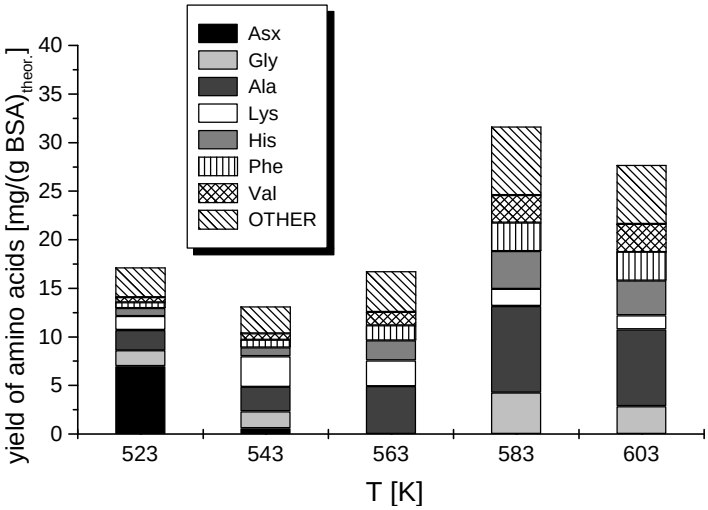


**Figure 3**: Effect of temperature on amino acid production, $\tau$ = 30 s, P = 250 bar

It should be mentioned that the "other" fraction contains other proteinogenic amino acids, which could only be identified in small amounts. Proline (Pro) and cysteine (Cys) could not be detected by the used analysis method and are thus not included in the "other" fraction.

**Dependence on residence time.** The results for the production of amino acids in dependence on the residence time in the reactor are shown in Figure 4. At a constant reaction temperature an increase in residence time to $\tau$ = 300 s tends to result in an increase in amino acid yield. At residence times higher than 300 s the degradation of the products is faster than their production. This can also be verified in Figure 5, which shows the effect of residence time on glycine (A) and alanine (B) production at different temperatures. An increase in temperature from 523 to 563 K leads to a shift of the optimum from 300 to 50 s for glycine and 90 s for alanine, respectively. In this case, after 300 s the bulk of these amino acids is already degraded into other products. A further increase in reaction temperature (603 K) leads to a decrease in amino acid yield. Degradation of the amino acids is much faster than production of these components. Thus, it appears that a reaction temperature of 603 K is definitely unsuitable for amino acid production even at low residence times, because the maximal amino acid yield is much lower than at other temperatures.

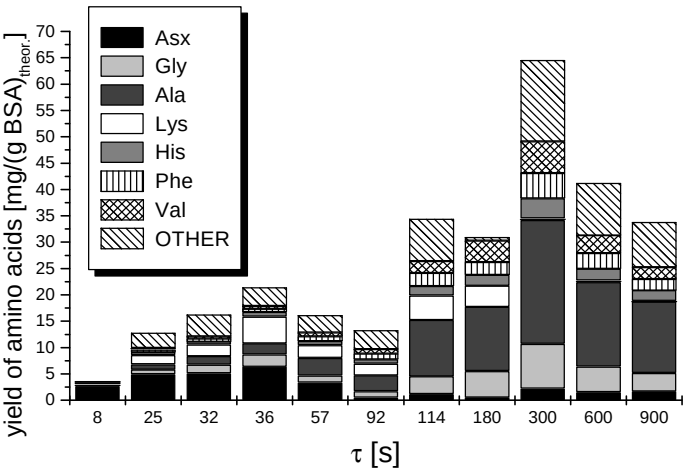


**Figure 4**: Effect of residence time on amino acid production, T = 523 K, P = 250 bar

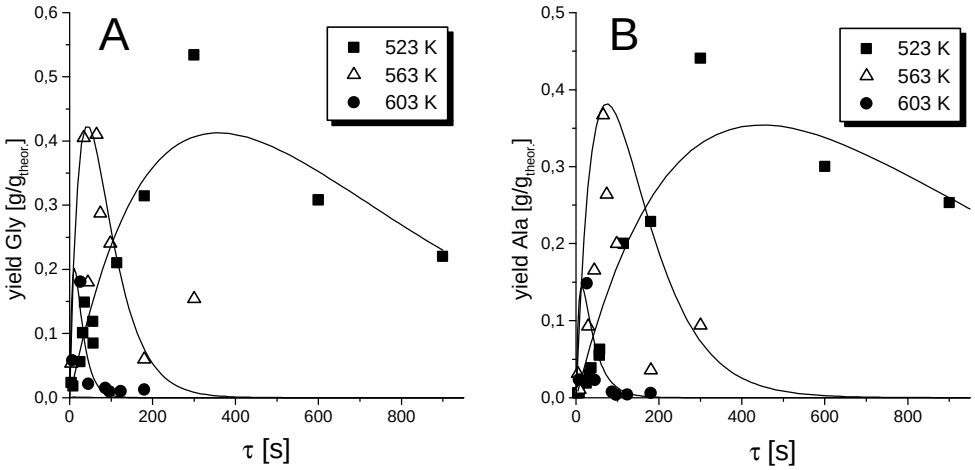


**Figure 5**: Effect of residence time on glycine (A) and alanine (B) production for different temperatures, P = 250 bar

**Dependence on $CO_2$ addition.** Figure 6 shows the effect of carbon dioxide if added to sub-critical water. The solubility of $CO_2$ in water increases significantly in the sub-critical regime. With known $CO_2$ solubilty under reaction conditions, different degrees of saturation (wt.-%) could be calculated and adjusted. Reaction temperature was kept constant at 523 K in these experiments. Especially at the highest tested residence time (300 s) it can be seen that the addition of $CO_2$ has a positive effect on the amino acid production. The effect becomes smaller at lower residence times. The experiments show that the addition of $CO_2$ results in a decrease in pH and thus favours acid hydrolyzed catalysis. Hence, it is possible to break the peptide bonds in an acid media without the addition of further acids. The advantage of this method is that $CO_2$ is easily separated from the reaction mixture by depressurizing it to ambient conditions.

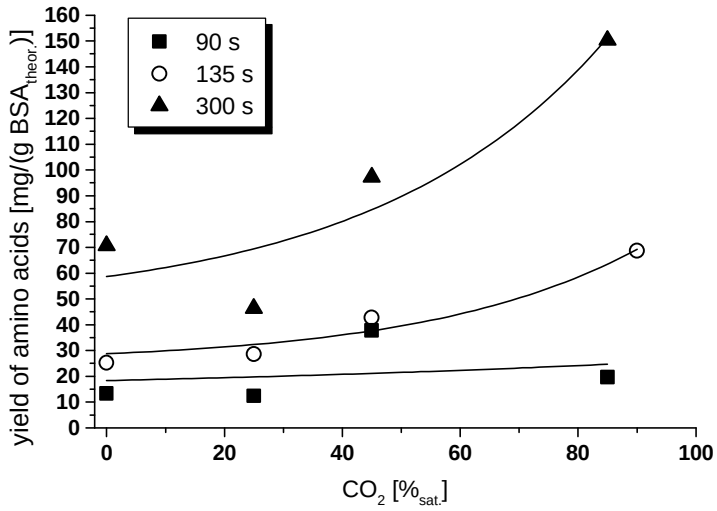


**Figure 6**: Effect of degree of $CO_2$ saturation on amino acid production for different residence times, T = 523 K, P = 250 bar

**Table 1**: Common names and molar masses of amino acids identified in this work

| abbr. | amino acid | molar mass | abbr. | amino acid | molar mass | abbr. | amino acid | molar mass |
|---|---|---|---|---|---|---|---|---|
| Asx | asparagine / | 133.10 | Gly | glycine | 75.07 | Met | methionine | 149.21 |
| | aspartic acid | 133.10 | Thr | threonine | 119.12 | Val | valine | 117.15 |
| Glx | glutamine / | 146.15 | Arg | arginine | 174.20 | Phe | phenylalanine | 165.19 |
| | glutamic acid | 147.13 | Ala | alanine | 89.09 | Ile | isoleucine | 131.17 |
| Ser | serine | 87.00 | Tyr | tyrosine | 181.19 | Leu | leucine | 131.17 |
| His | histidine | 155.16 | Trp | tryptophan | 204.23 | Lys | lysine | 146.19 |

## CONCLUSIONS

This study focuses on the production of amino acids from proteins using sub-critical water hydrolysis. Samples of BSA were treated in a continuous flow reactor under different reaction conditions. It was observed that the production of amino acids depends preliminary on reaction temperature, with an optimum temperature being found at 583 K. At relatively mild conditions (523 K) the amino acid yield increases up to a residence time of 300 s and decreased thereafter due to decomposition reactions. In contrast, a change in pressure in the range between 150 and 270 bar had no significant effect on the reaction (results not shown). Considerable quantities of glycine and alanine in particular could be produced because of formation from complex amino acids, whereas other amino acids could only be found in traces. Besides the experiments with pure water as reaction medium it was proved that the addition of carbon dioxide resulted in higher yields due to acid hydrolysis of the peptide bonds. Thus, sub-critical water hydrolysis can be an efficient process for recovering amino acids from organic protein-rich waste-materials such as hairs and feathers.

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