Evaluation of the Denaturation Kinetics of Two Differently Prepared Protein Concentrates from Bovine Blood Plasma

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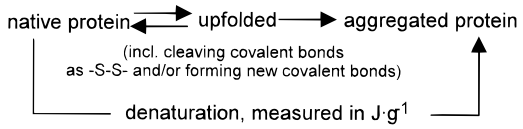
Significant damage to the blood plasma protein matrix—measured by differential thermal analysis—starts at a temperature of 50 °C. The denaturation of the plasma protein can be described by dividing the proteins into three fractions (I−III) which differ in their reaction kinetics behaviors. Two ways of producing protein concentrates have been investigated. Plasma concentrate A prepared by ultrafiltration generally shows a more sensitive behavior regarding temperature than does plasma concentrate B prepared by evaporation. Temperature shifts of up to 5 K and a decrease of the velocity constant of up to 2 decimal powers have been measured. It can be assumed that aggregation reactions dominate the upfolding reaction with increasing ionic strength of the solvent. The sharp bend (i.e. discontinuity of kinetics parameters) in the Arrhenius display of the third fraction detected in plasma concentrate A does not occur in plasma concentrate B.

Keywords: *Blood; plasma; proteins; denaturation; kinetics; thermoanalysis; ultrafiltration; concentration*

INTRODUCTION

The cost of the raw material to produce sausages could be decreased by adding blood plasma as a partial substitute for expensive meat protein while keeping or even increasing the product's quality. To combine the preservation of fresh plasma's natural quality with durability, adapted technological methods of processing are required. Here, the time and temperature of heating have to be chosen in such a way that the desired effects can be reached while negative influences on the product are minimized. To determine the time and temperature of heating of the plasma products during formation and storage, information is necessary about the kinetics of the plasma ingredients' chemical and biochemical reactions. The complexity of blood plasma's ingredients make it difficult to describe these molecular kinetics, i.e. the actual chemical, molecular mechanisms. Therefore, it is advisable to detect changes experimentally and determine formal kinetics according to these results Kessler (1988). A superimposition phenomenon of different successive reactions results in a fractional order of reactions, also called an apparent reaction order. In this context it must be kept in mind that the significance of the ascertained data depends on the definition of denaturation and its analytical registration. Many different definitions concerning denaturation are given [Anfinsen et al., 1968; Joly, 1965; Grömmer, 1990; Suelter, 1990; summarized in an overall definition by Cheftel et al. (1992)]: Every change in conformation (concerning secondary, tertiary, or quaternary structure) is called denaturation, e.g. without cleaving any peptide bonds of the primary structure. We understood the process of denaturation to be an upfolding reaction with the following aggregation:



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On the one hand, many different measuring methods have been published (Table 1) to ascertain and quantify blood plasma's protein denaturation. On the other hand, these methods focused mostly on testing denaturation phenomena in model solutions such as protein dilutions, extreme pH values, pure substances, and high sugar concentrations. These conditions strongly differ from the physiological conditions of the plasma proteins. Therefore, the structure can no longer be assumed to be a native plasma protein structure. Because of this, we investigated the reaction kinetics of plasma protein in the context of common concentrations in protein manufacturing. This paper's aim is to investigate the denaturation kinetics of plasma concentrate prepared according to different processes. With these results it should be possible to define the degree of denaturation as a function of the time and temperature of heating and of the concentration of the ingredients. Unit operations for the plasma protein's formation and storage conditions should be assessed with respect to thermal denaturation phenomena.

MATERIALS AND METHODS

Preparing Plasma Concentrate. Base material (blood from bovine, simmentaler race) obtained from the Bundesanstalt for meat research in Kulmbach was fresh plasma. It was immediately stabilized with trisodium-citrate (1%) and deep-frozen at −40 °C. Before the experiments were started, the frozen plates were thawed gently in 15 L batches in a conditioned room (12 h, 30 °C). Then the fresh plasma was tempered in a heat exchanger up to 30 or 40 °C and processed further as follows (Figure 1).

Plasma Concentrate A. With an ultrafiltration operation unit (Figure 2) (outlined and constructed for this purpose) plasma was concentrated at 30 °C and 2500 hPa (effective pressure). The ultrafiltration is designed as a cross-flow filtration (membrane from polysulfone). A double-walled storage vessel (capacity of 15 L) allows the plasma to be tempered. A displacement pump provides a constant pressure (3 bar) for the feed (plasma). A Mohno pump allows stepless overflow velocities (0.8−3.0 m/s) and high fluxes. A heat-exchanger tube guarantees a constant concentrate temperature. Two bleed valves provide a concentration process without foaming.

Plasma Concentrate B Produced by Evaporation under Reduced Pressure. With a continuous flash evaporator (Büchl,

S0021-8561(95)00412-2 CCC: $12.00

Table 1. Measuring Methods To Ascertain Denaturation of Blood Plasma Proteins

method	property measured	literature
turbidimetric analysis	aggregation 600 nm	Saito and Taira (1987)
	aggregation 660 nm	Samejima et al. (1985)
	aggregation 700 nm	Hermansson (1982)
absorptiometry	free SH groups: 412 nm	Saito and Taira (1987)
fluorometry	hydrophobic interaction chromatography: excitation, 325 nm; measurement, 420 nm	Kato and Nakai (1980), Nakai (1983)
circular dichroism	secondary structure: 222 nm	Saito and Taira (1987)
viscosity	conformational change	Hermansson (1982)
differential scanning calorimetry	enthalpy of denaturation	Pfeil (1976), Gumpen (1980), Biliaderis (1983), Relkin (1990)
solubility	precipitation	Delaney (1975)
SDS−PAGE	hydrodynamic volume correlated with the molecular mass	Saito and Taira (1987)
HPLC gel filtration	hydrodynamic volume correlated with the molecular mass	Saito and Taira (1987)

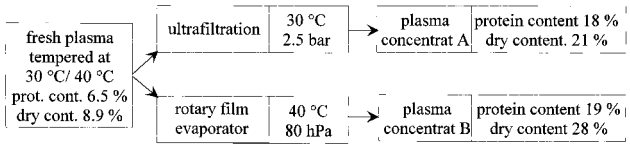

Figure 1. Method used for the preparation of plasma concentrates A and B.

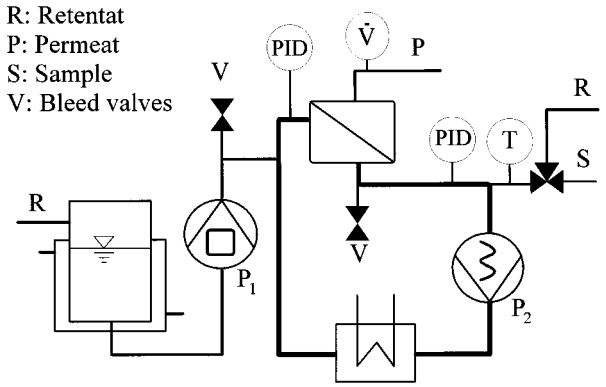

Figure 2. Ultrafiltration operation unit outlined and constructed for preparing plasma concentrate.

EL 131) plasma was concentrated at 40 °C and 70−90 hPa (effective pressure). In both cases (A and B), the plasma concentrate was split up into portions of 10 mL and stored at −50 °C.

Protein Determination. We determined the protein via Makro-N (Foss/Heraeus), according to an improved method of Dumas based on the oxidative decomposition process. The conversion factor (nitrogen → protein) is 5.73.

Dry Content Determination. We determined the dry content gravimetrically. The samples were blended with preheated sea sand in glass dishes. Then, they were dried at 105 °C to a constant weight.

Sample Preparation. Before measuring the deep-frozen (−50 °C) samples, we stored each unit for 12 h at −5 °C and then tempered it up to 20 °C. The plasma concentrates A and B were weighed into aluminum crucibles. They were then exposed to heating. Since it takes a DTA oven up to 6 min to heat and cool, we simulated the time and temperature of heating in an external water bath with an integrated thermostat. Because of turbulent circulation we reached an optimum heat transition. A partial predenaturation was simulated by selected thermal loading. We then cooled the crucibles in ethanol at 5 °C, let them air-dry for a short period of time, and then put them into the measuring cell of the DTA.

Determination of the Denaturation Enthalpy. We determined the residual denaturation enthalpy Φ_{res} of the plasma proteins with a DTA System TA 3000 (Mettler) (Figure 3). We chose a 150 μL aluminum crucible as sample crucible. We used benzophenone p.a. (melting point T_1 = 48.0 °C), indium p.a. (melting point T_2 = 156.6 °C), and lead p.a. (melting point T_3 = 327.4 °C) to calibrate the heat flow rate. We minimized the error in temperature measurement by

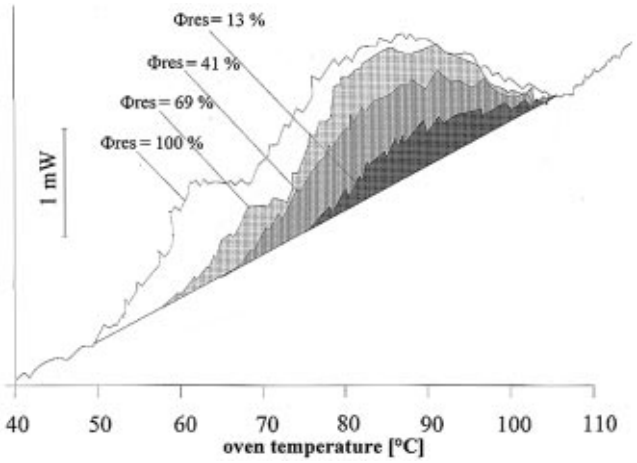

Figure 3. Example of curves obtained for the determination of the denaturation enthalpy.

Widmann (1984)

$$\Delta T_{error} = 2S_r[2(T - T_2)/(T_3 - T_1)]^2 \quad (1)$$

to 0.33 °C at temperatures of 30 °C and to 0.06 °C at temperatures of 100 °C (given S_r as a device-specific value by the DTA manufacturer). The time lag we measured between the temperature T_o of the oven and the temperature T_r of the reference was quantified by τ_{lag}. Sample (plasma concentrates A and B) and reference (silica sand) were in the same oven. Only the temperature of the oven was measured directly. Therefore, we had to determine the temperature of reference as follows:

$$T_r = T_o - \Delta T_{lag} = T_o - \tau_{lag}\beta \quad (2)$$

We were able to determine the temperature of the sample

$$T_s = T_o - \tau_{lag}\beta + \Delta T_{sr} \quad (3)$$

by measuring the difference of the temperatures of the sample and reference

$$\Delta T_{sr} = T_s - T_r$$

RESULTS AND DISCUSSION

We investigated the denaturation kinetics of two plasma concentrates A and B that differ from each other with regard to their content of substances with small molecular weights. We achieved an exact correlation between the time and temperature of heating and the denaturation degree. Therefore, we had to quantify the time required to heat and to cool. Depending on the chosen temperature, between 15 and (at most) 30 s was required to reach the bulk temperature in the crucible.

Table 2. Reference Magnitude for Thermally Untreated Plasma Concentrate A

measured quantity	av value X	standard deviation σ
dH (mJ/mg)	17.4	0.4
T_{onset} (°C)	51.1	0.4
T_{peak} (°C)	72.6	1.0
T_{endset} (°C)	88.0	1.0

Table 3. Residual Denaturation Enthalpy Φ_{res} of the First and Second Plasma Fractions

temp of heating (°C)	extrapolated Φ_{res} (%)
55	80.4
58	81.4
60	79.3
av value	80.3

To minimize the relative error caused by different heating times, we most often used a minimum time of 1 min.

Results Concerning Plasma Concentrate A [Prepared by Ultrafiltration (UF)]. Thirty-two independent measurements of thermally unloaded plasma concentrates A produced the magnitude of reference (Table 2) by which we determined the residual denaturation enthalpy Φ_{res}. Small changes of the limits of integration partly resulted in great changes in the reaction enthalpy. Therefore, in preliminary tests, fixed borders of integration had to be determined: $T_{start} = 52.2$ °C and $T_{end} = 103.4$ °C. At a chosen temperature of heating at 54 °C (30 min) the residual denaturation enthalpy (Φ_{res}) was 88%, at 52 °C (30 min) Φ_{res} was 94%, and at 50 °C (30 min) Φ_{res} was 96%. Below 50 °C the reduction of the residual denaturation enthalpy fell below the identification limit of the measuring method. Heated to a maximum of 60 °C the samples showed a significant decrease of the residual denaturation enthalpy down to $\Phi_{res} = 57\%$ with increasing heating time. There was no further significant damage in this temperature range if the heating time was prolonged. At temperatures above 60 °C the plateau of residual denaturation enthalpy $\Phi_{res} = 57\%$ disappeared. Thus, we can conclude that there is a stable protein fraction up to a temperature of 60 °C. Furthermore, the onset temperatures, corrected by eqs 2 and 3, confirmed this result. While the onset temperatures had been measured at 51.1 °C for thermally unloaded samples, they rose to 60 °C after a short heating time. If the initial temperature is 60 °C, a complete denaturation of the thermally sensitive proteins can be concluded.

Regarding complete denaturation of thermally sensitive proteins (i.e. significant thermal denaturation below 60 °C), it was impossible (despite variation of the reaction's order) to linearize the measured values over the whole time range. Assuming a reaction of first order, there are two time domains that can be linearized (Figure 4). The first range is marked by a sharp reduction of the residual denaturation rate up to 3 min (at 58 °C). The following decrease of the residual denaturation rate is much smaller. We thus conclude that the thermally sensitive proteins can be further divided into dissimiliar proteins. Up to 3 min the superposition of both denaturations results in a great decrease of the residual denaturation rate. The first fraction is completely denaturated in about 3 min (at 58 °C). Now a second less thermally sensitive fraction II determines any further denaturation. We can relate the measuring data to both of the different thermally sensitive fractions I and II by extrapolating the less thermally sensitive fraction II back to a heating time of 0 min (Figure 4). The extrapolation of the residual denaturation enthalpy is 80%, obtained as an average of three independent measurements at different temperatures (Table 3). The plasma protein matrix can, therefore, be subdivided into three parts with different behaviors of denaturation. On the one hand, we measured a significant denaturation of the first and second fractions at an onset temperature of 50 °C. On the other hand, a significant denaturation of the third fraction starts at a temperature of 60 °C (Figure 5; Table 4).

Albumin is stabilized through 17 covalent disulfide links (Cheftel et al., 1992). This explains the plainly higher specific enthalpy of albumin (Table 4). Fibrinogen is known as a very temperature sensitive protein (Kaboth and Begemann, 1977). After testing with pure substances under identical conditions regarding ionic strength and protein concentration (Table 5), we concluded the following, on the basis of an arithmetic comparison of the mass proportions and enthalpy proportions (Table 4): The plasma proteins fibrinogen and α- and β-globulins describe the first fraction, while

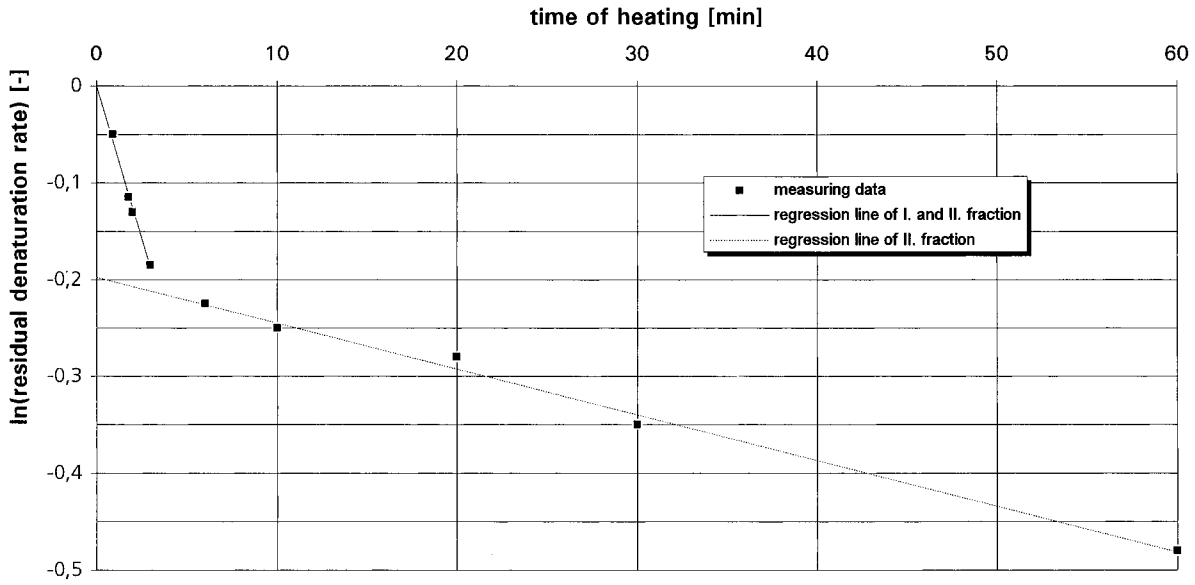

Figure 4. Characterization of the heat sensitive plasma proteins in two time ranges (first and second fractions) as a reaction of first order (for a temperature of heating at 58 °C),

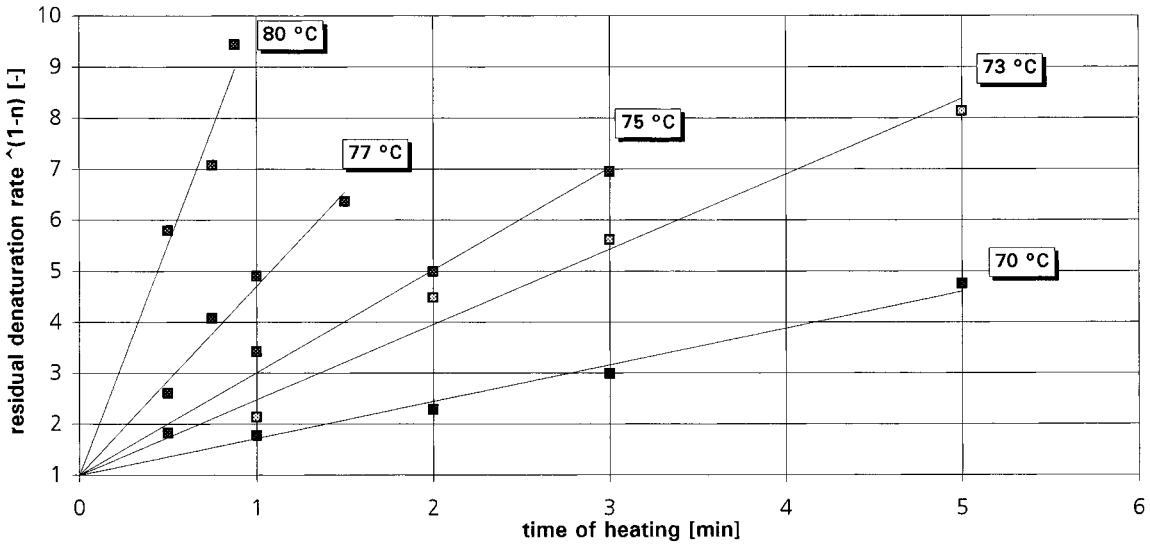

Figure 5. Characterization of the stable plasma protein fraction (third fraction) as a reaction of 2.6 order.

Table 4. Mass Proportion of the Plasma Fractions with Different Thermal Stabilities

no.	fraction	*T* range (°C)	mass proportion (%)	enthalpy proportion (%)	specific enthalpy[a] (J/g)	reaction order
I	fibrinogen and α,β-globulin	<60	28[b]	20	12.4	1.0
II	γ-globulin	<60	30[b]	23	13.3	1.0
III	albumin	>60	42[b]	57	23.6	2.6

[a] Respecting plasma concentrate A (18.0% protein content); specific enthalpy = 17.4 J/g (enthalpy proportion/mass proportion). [b] The mass proportion of the fractions is referred to the publication of Kolb (1967).

Table 5. Kinetic Data of the Three Fractions of the Plasma Protein Denaturation

	first fraction	second fraction	third fraction	third fraction
T range (°C)	55−60	55−60	60−70	70−80
activation energy (E_A) (kJ/mol)	200	270 (290)[a]	490	190 (195)[b]
frequency factor (ln k_0) (−)	70	90	170	60
n (−)	1.0	1.0	2.6	2.6

[a] 5.4% γ-globulin solution (Fluka, no. 29030) including the same salt composition and ionic strength as plasma concentrate A. [b] 7.6% albumin solution (Fluka, no. 05470) including the same salt composition and ionic strength as plasma concentrate A.

the complex group of γ-globulins is identified as the second fraction. We also identified albumin as the representative of the third fraction. We managed to linearize the residual denaturation rate as a function of the heating time due to an appropriate choice of the reaction's order (Figure 6). To determine *exactly* the order of reaction, the ordinate intercept of the three regression lines (of the first, second, and third fractions) were calculated by extrapolating back to a time of heating of 0 min. By assuming the correct order of reaction, the respective ordinate intercept is 1.00. This means the residual denaturation enthalpy Φ_{res} of the first, second, and third fractions, respectively, is 100% at a time of heating of 0 min.

The order of reaction was calculated for 12 different temperatures. From this we concluded an apparent reaction order of 2.6 for the third fraction and a reaction order of 1.0 for the first and second fractions. Seventy-seven independent measurements produced 16 velocity constants that describe the three fractions in an Arrhenius display (Figure 6). To clarify the correlation $\ln(k) = 1/T$, the term t(50% denaturation) = $f(T)$ was chosen. This term represents the half-life of plasma protein denaturation for a given temperature (Figure 6). For the first and second fractions we determined a linear correlation between the logarithm of the velocity constants and the reciprocal of the absolute temperature in the temperature range from 55 up to 60 °C.

The E_a was determined by estimation of the slope value $(E_a/R) = \Delta \ln k(1/T)/\Delta(1/T)$. The phenomenon of a discontinuity of kinetics parameters (sharp bend of the third fraction in the Arrhenius display) at a temperature of 70 °C is caused by two consecutive reactions that differently dominate in their respective temperature ranges. In the range between 60 and 70 °C the upfolding reaction (including cleaving covalent bonds; $E_a = (3-4) \times 10^2$ kJ; Kessler, 1988) dominates, while at temperatures between 70 and 80 °C the aggregation reaction ($E_a = (1-2) \times 10^2$ kJ) determines the velocity of the entire denaturation.

In Table 5 the kinetic data for the three fractions are summarized. To demonstrate the quality of the kinetic parameters obtained, we exemplarily compared data of the plasma protein denaturation from the third fraction. For three different temperatures the data obtained by measurement were compared to those obtained by calculation (Figure 7).

Results Concerning Plasma Concentrate B (Prepared by Evaporation under Reduced Pressure). The procedure explained for plasma concentrate A was repeated with plasma concentrate B. The results are presented in Figure 8 and Table 6. We note that the sharp bend of the third fraction (in the Arrhenius display) found for plasma concentrate A did not occur. Therefore, we conclude that for high salt concentrations the aggregation not only limits the velocity of reaction for higher temperatures (>70 °C) but generally bottlenecks protein denaturation. Thus, there exists a strong correlation between the ionic strength of the solvent and the temperature-induced aggregation. The kinetics parameters measured for plasma concentrate B are listed in Table 6. However, the upfolding reaction, also

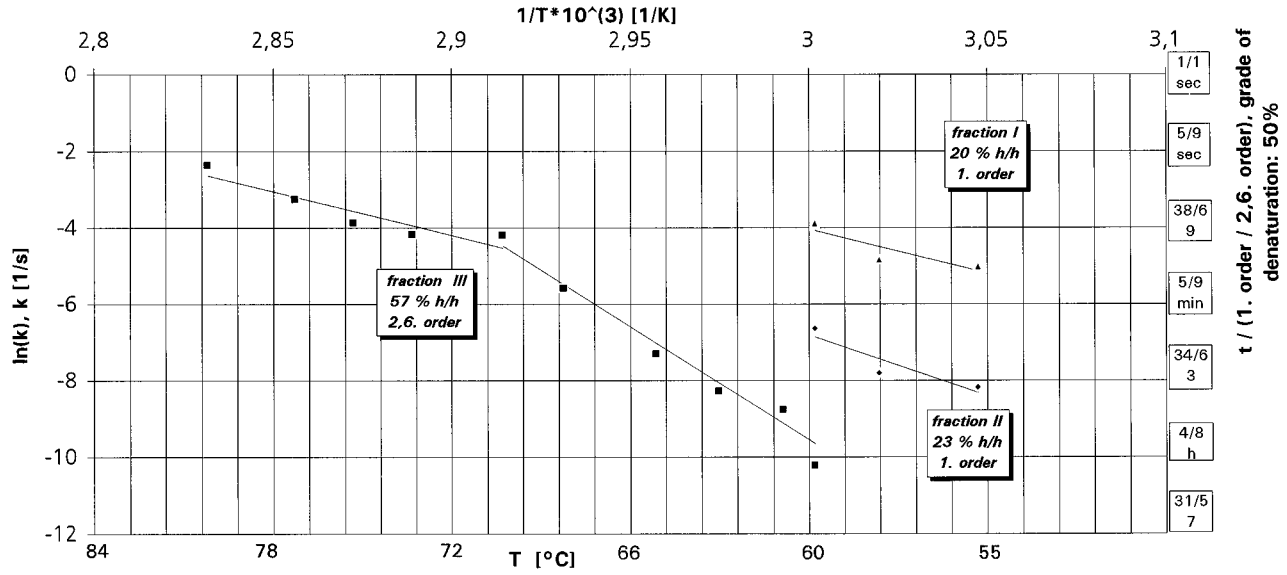

Figure 6. Arrhenius display referred to the kinetic of plasma protein denaturation (plasma concentrate A).

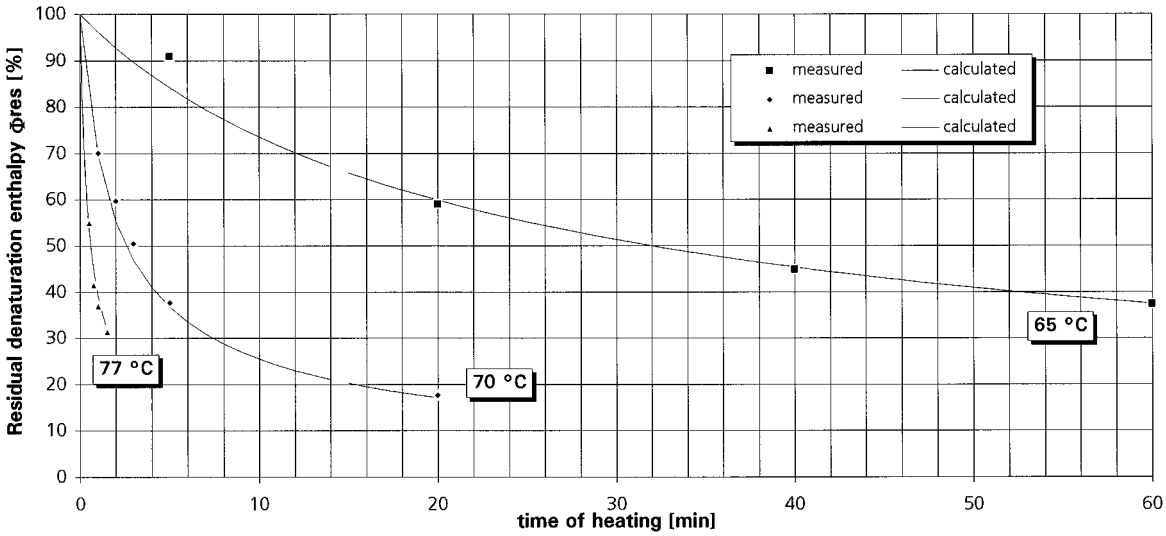

Figure 7. Calculated residual denaturation enthalpy Φ_{res} and measuring data of the third plasma fraction.

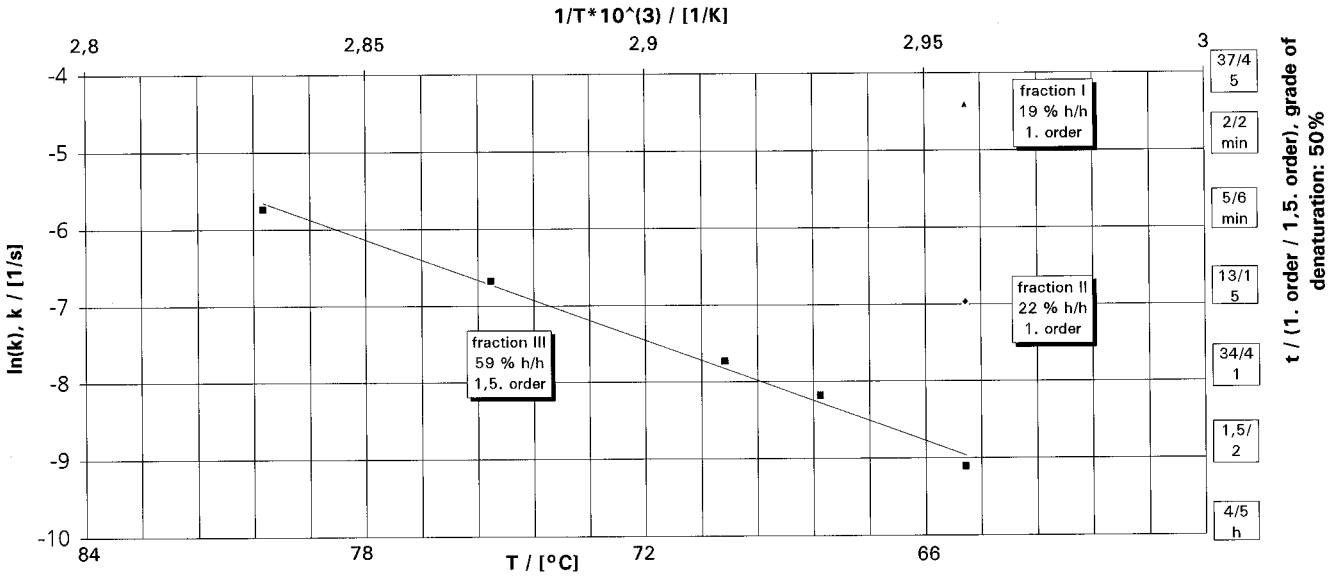

Figure 8. Arrhenius plot of the kinetic of plasma protein denaturation (concentrate B).

induced by temperature, is closely correlated with the ionic strength and, therefore, superposed by the aggregation reaction with increasing ionic strength of the solvent. Moreover, the stability of all three fractions increases: the velocity constants decrease by up to 2 decimal powers, and the denaturation onset tempera-

Table 6. Kinetic Data of the Three Fractions of the Plasma Protein Denaturation

	first fraction	second fraction	third fraction
T range (°C)	<65	<65	≥65
E_A (kJ/mol)	−[a]	−	220
ln k_0 (−)	−	−	75
n (−)	1.0	1.0	1.5

[a] −, not measured.

ture is increased by 5 K. Hereby the stabilizing influence of high salt concentrations on the stability of plasma proteins can be concluded. The apparent reaction order of 2.6 (third fraction) changed to 1.5, while the reaction order of the first and second fractions remained consistent at 1.0.

Summary. The reaction kinetics of plasma proteins by enthalpy measurements using a differential thermal analyzer were investigated. The measurements were made in the context of common concentrations in protein manufacturing and within the range of normal processing temperatures used in sausage production. Thus, the results provide measurable and meaningful denaturation characteristics of plasma applicable to manufacturing conditions. With these results it is possible to define the amount of denaturation as a function of the time and temperature of heating and of the concentration of the ingredients. Unit operations for the plasma protein's formation and storage conditions can be assessed with respect to thermal denaturation phenomena.

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