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Investigation of frozen protease-catalyzed peptide synthesis systems — a differential scanning calorimetry and electron microscopy approach

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Abstract

The internal structure of a protease-catalyzed frozen aqueous peptide synthesis system was studied by freeze-fracture electron microscopy. Distinct lense-like liquid microinclusions differing in size from 0.25 to 1.7 μm were observed. Differential scanning calorimetry was used to determine the amount of unfrozen water per molecule of peptide reactant. Comparison of the results with data obtained previously by examination of the same peptide synthesis system using ^{1}H NMR relaxation time technique showed that DSC is the more reliable method for this special purpose. Furthermore, the amount of unfrozen water in a frozen α-chymotrypsin solution determined by magic angle spinning NMR in previous investigations was confirmed by the calorimetric method.

Keywords: Differential scanning calorimetry; Electron microscopy; Frozen aqueous system; Peptide synthesis; Protease

1. Introduction

In enzyme-catalyzed peptide bond formation, freezing of the reaction mixture has been developed as an approach to suppress competitive hydrolytic reactions (for a review, see Ref. [1]). The yield-enhancing effect of freezing in protease-catalysed peptide synthesis has been attributed to the concentration of the reactants in the remaining liquid phase which is in equilibrium with the frozen solvent [1–3]. However, the acceptance of amino components which are completely inefficient nucleophiles at room temperature observed in a number of frozen state peptide synthesis studies [1,4–6] as well as comparative studies in ice and highly concentrated solutions at room temperature [7] strongly suggested that there are other factors besides the freeze-concentration effect which are involved in peptide yield enhancement by freezing.

The role of these factors was subject to physico-chemical studies. Recently, we reported

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on the determination of the amount of unfrozen water in chymotrypsin-catalyzed peptide synthesis systems using ^{1}H NMR relaxation time technique [7] and a study of protein–water interaction in ice by means of magic angle spinning (^{1}H MAS) NMR measurements [8].

Although differential scanning calorimetry (DSC) is a powerful and sensitive tool to determine the amount of ice and unfrozen water in frozen samples, studies concerning the amount of unfrozen water in biological samples are rather rare [9,10]. Here, we present the application of DSC in the investigation of a frozen aqueous protease-catalyzed peptide synthesis system. Furthermore, freeze-fracture electron microscopy was applied to study the internal structure of such frozen systems.

2. Experimental

2.1. Chemicals

α-Chymotrypsin was obtained from Serva (Germany) and used without further purification. H-Asp-Phe-OMe and H-Ala-Ile-OH were from Bachem (Switzerland).

2.2. Freeze-fracture electron microscopy

Stock solutions of the acyl donor ester (H-Asp-Phe-OMe), the amino component (H-Ala-Ile-OH), and α-chymotrypsin were mixed to give final concentrations of 2 mM H-Asp-Phe-OMe, 5 mM H-Ala-Ile-OH and/or 4 μM α-chymotrypsin. The final solutions were precooled to 0°C to avoid ester hydrolysis during mixing and before shock freezing. Aliquots of the samples were transferred to desk-like gold-specimen holders (diameter 3 mm) with a central pit to allow sample volumes of about 2 mm^3 and frozen in liquid nitrogen by plunging immediately. Then the samples were incubated in a freezer at −27°C for 30 min, retransferred into liquid nitrogen, and freeze-fractured in the BAF 400 T (Bal-Tec, Liechtenstein) using the microtome technique for fracturing. Fracturing and etching were performed at −120°C. Replication were done 20–30 s after fracturing using electron guns and a film thickness monitor. Platinum for staining was evaporated under an angle of 35°.

The replicas were cleaned in hot nitric acid and double distilled water, placed on electron microscopical copper grids, and examined using the EM 900 (Zeiss, Oberkochen).

2.3. DSC

We studied aqueous solutions of enzyme and peptide reactants in a molar ratio of H-Asp-Phe-OMe:H-Ala-Ile-OH:α-chymotrypsin, 2:5:0.004, to give final total concentrations of all constituents between 5 and 100 mM and a pure α-chymotrypsin solution in water (without buffer) with enzyme concentrations between 0.8 and 10 mM.

The DSC measurements were performed with a Perkin Elmer DSC-7 in combination with a Julabo ultra cryostat to approach a low temperature range down to −80°C. The samples were exactly weighted, filled into 50 μl aluminium pans, sealed and frozen by plunging them into liquid nitrogen. Afterwards, the frozen samples were deposited into the calorimeter at −50°C and heated with a scan rate of 0.5 K/min to room temperature (25°C). The interval under the DSC endotherm curve was integrated with respect to a polynomial baseline to obtain the ice melting heat. The DSC endotherms were treated without any smoothing or baseline correction.

3. Results and discussion

For the electron microscopy as well as for the DSC studies, an α-chymotrypsin-catalyzed peptide synthesis system was chosen which has already been used for ^{1}H NMR relaxation time studies [7]. The acyl donor ester H-Asp-Phe-OMe was coupled with the nucleophilic amino component H-Ala-Ile-OH. Whereas H-Ala-Ile-

OH proved to be a completely ineffective nucleophile at room temperature, freezing of the reaction mixture resulted in a tetrapeptide yield of 79% [7].

Fig. 1 shows the electron micrographs of a reactant solution and a mixture containing reactants and enzyme, respectively. Lens-shaped aggregations have been formed along the grain boundary (arrow head) between the ice crystals. A graining appearance is visible without remarkable differences. The well detached microinclusions differ in size from about 0.25–1.7 μm and should contain mainly the non-aqueous constituents of the mixture. In peptide synthesis experiments carried out previously with an identical reaction system the acyl donor ester H-Asp-Phe-OMe was completely consumed during the synthesis reaction [7]. Therefore, it can be concluded that enzyme-catalyzed peptide synthesis reactions in frozen aqueous systems take place in distinct cavities containing reaction media of identical composition. However, the resolution of the method cannot answer the question if there is any connection between the microinclusions along the grain boundaries. The assumption that possibly diffusion processes take place between the distinct microinclusions is supported by results of peptide synthesis studies in frozen aqueous systems catalyzed by α-chymotrypsin covalently bound to solid carriers [11]. Although it seems very unlikely to achieve a homogenous distribution of the solid enzyme-carrier preparations during shock freezing of the samples in liquid nitrogen, complete enzymatic

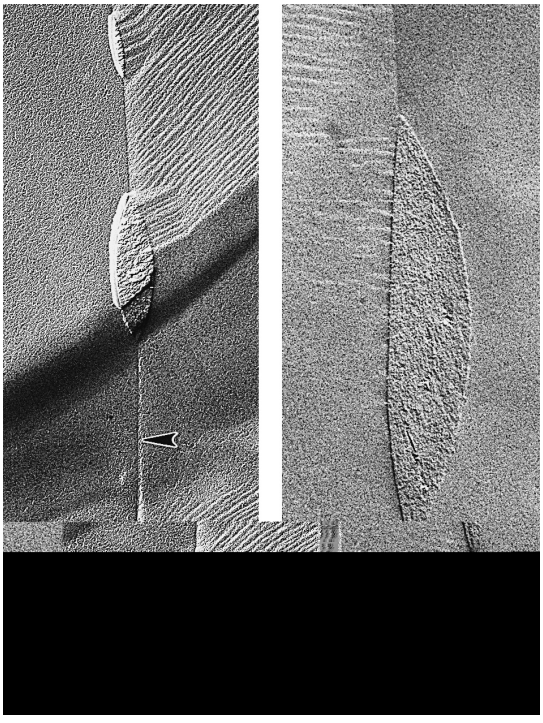

Fig. 1. Slightly etched freeze-fracture preparation of a reactant solution (a) and a reaction mixture containing enzyme and reactants (b). (a) 2 mM H-Asp-Phe-OMe, 5 mM H-Ala-Ile-OH, pH 9; (b) 4 μM α-chymotrypsin. The grain boundary is indicated by the arrow head. Micrographs are mounted with evaporation direction from bottom to top.

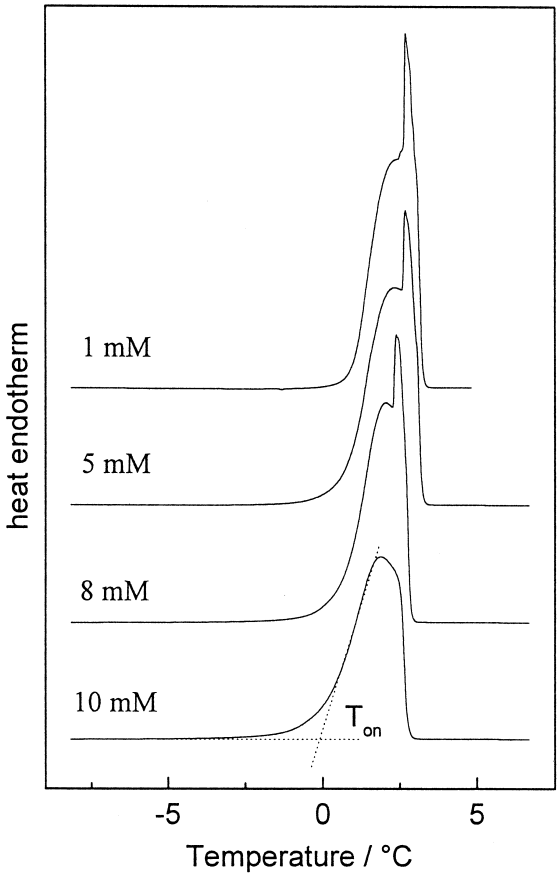

Fig. 2. Representative DSC heating curves of ice melting in frozen aqueous systems containing different concentrations of α-chymotrypsin (heating rate 0.5 K/min). The onset temperature T_{on} was determined from the intersection of two tangents (baseline and low temperature shoulder).

conversion of the acyl donor esters used in these experiments was observed.

DSC measurements were performed on the reaction system of the α-chymotrypsin-catalyzed synthesis of H-Asp-Phe-Ala-Ile-OH as well as on pure aqueous solutions of the enzyme catalyst. The difference between the expected melting enthalpy of the total amount of water in the solutions with the calorimetrically measured melting enthalpy gives the amount of unfrozen water which has no contributions to the melting enthalpy:

$$m_{\mathrm{W}}^{\mathrm{unfrozen}} = m_{\mathrm{W}}^{\mathrm{total}}\left(\frac{\Delta H^{\mathrm{ice}} - \Delta H^{\mathrm{meas.}}}{\Delta H^{\mathrm{ice}}}\right) \qquad (1)$$

($\Delta H^{\mathrm{ice}} = 333.7$ J/g is the melting enthalpy of pure ice and $\Delta H^{\mathrm{meas.}}$: the calorimetrically measured enthalpy. $m_{\mathrm{W}}^{\mathrm{unfrozen}}$ and $m_{\mathrm{W}}^{\mathrm{total}}$ are the masses of the determined unfrozen water and the total amount of water according the concentration of the sample, respectively.)

A series of samples with different concentrations were examined where the solubility of the substances defined the extreme of possible concentrations. A representative collection of DSC scans for the enzyme–water system is given in Fig. 2. In general, only a small shift of the onset melting temperature of ice is observable at different α-chymotrypsin concentrations.

Fig. 3a visualizes the dependence of the measured enthalpy of ice melting in the peptide synthesis system on the reactant concentration. A linear decrease of the melting enthalpy per gram ice was detected with increasing concentrations of the reaction components. The extrapolation of ΔH^{ice} to a total concentration of

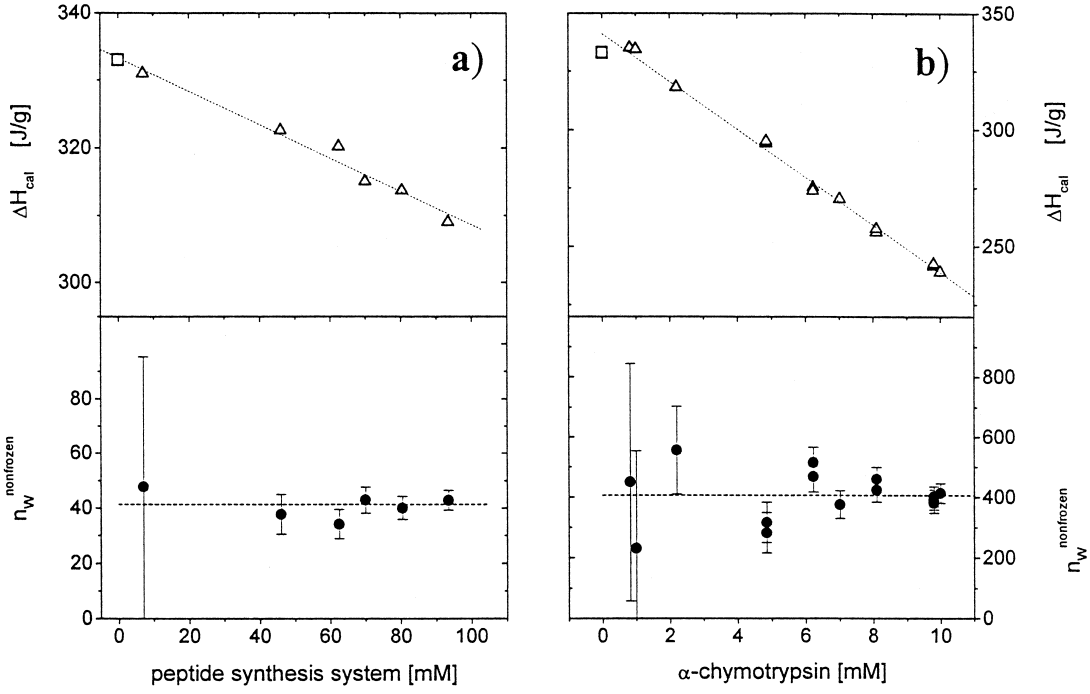

Fig. 3. (a) Top: Absolute endothermic enthalpies of ice melting per gram water in α-chymotrypsin-catalyzed condensation of H-Asp-Phe-OMe and H-Ala-Ile-OH. The molar ratio of H-Asp-Phe-OMe:H-Ala-Ile-OH:α-chymotrypsin was 2:5:0.004 to give the final overall concentrations. Bottom: Mol of unfrozen water per mol peptide synthesis system calculated according to Eq. (1) at different concentrations of the reaction components. The large error values result from the very small enthalpy differences between pure ice and the peptide synthesis system at high water excess (1 mM: $>99\%$ ice and $<1\%$ unfrozen water). (b) Top: Absolute endothermic enthalpies of ice melting per gram water in frozen aqueous systems containing different concentrations of α-chymotrypsin. Bottom: Molar ratio of unfrozen water:α-chymotrypsin calculated according to Eq. (1) at different enzyme concentrations.

0 mM (pure water) provided a value of 334 J/g (see Fig. 3a, top, open square). This is in excellent agreement with the literature value for ice melting of 333.7 J/g [12].

The absolute weight of unfrozen water in the sample (determined using Eq. (1)) was recalculated into molar values. The linear fit in Fig. 3a (bottom, dashed line) provides a constant value for the amount of unfrozen water of 41 molecules per molecule reactant independently of the total peptide concentration. In this calculation, α-chymotrypsin was neglected. That was possible because the enzyme contributes only 0.06% to the overall molar concentration (for example 7 mM peptide to 7.004 mM peptide and enzyme). Starting from ^{1}H NMR relaxation time results we obtained for the same reaction system previously [7], an amount of 169 molecules of unfrozen water per molecule of the peptide reactants can be calculated. This difference is attributed to the difficulty to separate water relaxation times from relaxation times of peptide and protein segments with sufficient mobility even in the frozen state [8]. Therefore, DSC proved to be the more reliable method for this special purpose.

The amount of unfrozen water per molecule α-chymotrypsin was determined applying the same procedure as described above to water solutions containing different concentrations of α-chymotrypsin only (Fig. 3b). A constant value of 400 molecules of unfrozen water per enzyme molecule was obtained, independently of the enzyme concentration. This result is in excellent agreement with MAS NMR studies carried out recently [8].

In conclusion, using freeze-fracture electron microscopy, the internal structure of such systems could be visualized for the first time. DSC proved to be a useful tool to determine the amount of unfrozen water in frozen aqueous peptide synthesis systems.

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