STABILITY & STABILIZATION
OF ENZYMES

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Stabilization of Lipases for Hydrolysis Reactions on Industrial Scale

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Abstract
A dedicated membrane bioreactor is developed for the mild hydrolysis of chemically and thermally unstable triglycerides. This newly developed bioreactor is equipped with custom-made hydrophilic hollow-fiber membranes, increasing enzyme activity half-lives from 40 to 160 days.

1. INTRODUCTION

Immobilized enzyme systems like (membrane) bioreactors have a wide range of potential applications in enzyme engineering. Such systems have several advantages over the use of free enzymes, including the ease of enzyme recoverability and reusability, operational simplicity and enzyme stability [1-3]. Despite their favorite selective synthesis characteristics commercial exploitation of biotransformations, especially in the oleochemical and food industry has not yet reached a significant level [4,6]. High processing and down stream processing costs are often mentioned as being main obstacles for implementation of this technology. Low enzyme stability is one of the main reasons for high costs and often even prohibits large scale bioprocessing. The development of stable immobilized enzyme systems therefore has become crucially important.

It has been observed that immobilized enzymes are more stable and show higher activities than their "free" form. The extent of stabilization depends on the type of enzyme, immobilization technique (adsorptively or covalently bound, use of spacers) and the type of carrier material used. In particular, immobilization on a hydrophilic surface enhances the enzymes stability and activity. The use of hydrophobic materials appears to lead to a lower increase in enzyme stability [3,11].

ATO-DLO is developing enzyme reactor technology for the hydrolysis, esterification or modification of unusual triglycerides. These triglycerides originate from new oilseed crops and contain for example very-long chain or reactive, functionalized fatty acids (table 1). Such fatty acids are useful for oleochemical industries in the synthesis of high-value specialties like pharmaceuticals or

cosmetics [5]. The first step in the processing of these raw materials is the hydrolysis of the triglycerides of interest into fatty acids and glycerol. However, since the conventional high temperature hydrolysis processes will damage the relatively labile fatty acids, low temperature processes like enzymatic hydrolysis, are considered. Lipases are very well suited for this hydrolysis process but, although the principles of enzymatic hydrolysis are well known, the process should be optimized before industrial application can be regarded as an economic alternative. In addition, since the oils studied are highly symmetric, i.e., the fatty acids of interest are positioned predominantly on the α-positions of the triglyceride, the performance of the operation can be improved by using α-specific lipases realizing a fatty acid mixture of relatively high purity, thereby reducing and down-stream processing costs. For these applications a stable enzyme is essential. Furthermore, in-line separation technologies like membrane extraction or fractional crystallization can be used so that product inhibition is decreased, the reaction equilibrium is shifted toward increased hydrolysis and the reaction rate is increased. When a membrane bioreactor equipped with a carefully tailored membrane is used, the merits of both principles, (1) a stable, specific enzyme and, (2) in-line product removal, can be combined. Some aspects in the development of such a reactor are discussed in this paper.

Table 1.
Selected Potential Oilseed Crops.

Oilseed crop	Seed yield (tons/ha)	Oil content (% dry weight)	Major fatty acid	FA content (% of total)	Industrial applications
Crambe abyssinica (Crambe)	2.5 - 3.5	26 - 39	erucic acid	55 - 60	plastic additives, lubricants, cosmetics, fabric softeners
Limnanthes alba (Meadowfoam)	0.5 - 1.0	17 - 29	very-long-chain FA	95+	lubricants, cosmetics
Dimorphotheca pluvialis (Cape Marigold)	1.2 - 1.7	18 - 26	dimorphecolic acid	58 - 65	coatings, pharmaceuticals, flavors, lubricants, polymers
Calendula officinalis (Marigold)	1.5 - 2.5	19 - 24	calendic acid	58 - 63	paints, coatings
Euphorbia lagascae (Spurge)	1.0 - 1.5	45 - 52	vernolic acid	59 - 65	polymers, plasticizers

2. PRINCIPLE

During the last decade, membrane technology has evolved from laboratory scale to industrial applications. The constructive marriage of enzyme technology with different membrane unit operations has already lead to a number of small-scale processes [6]. One of the newest concepts is the use of a membrane hollow fiber unit as a biocatalytic reactor [4, 5]. In a hollow fiber membrane two immiscible fases are brought into contact, the interfacial area is controlled and stabilized by the membrane. The active enzyme is immobilized on the membrane surface, where it catalyzes the reaction. A schematic representation of the concept is shown in figure 1. In literature membranes made of different materials have been reported for use as enzyme carriers, but seldomly a membrane has been designed especially with biocatalytic applications in mind. With such indifferent membranes only suboptimal conditions can be realized whereas the process becomes more complicated.

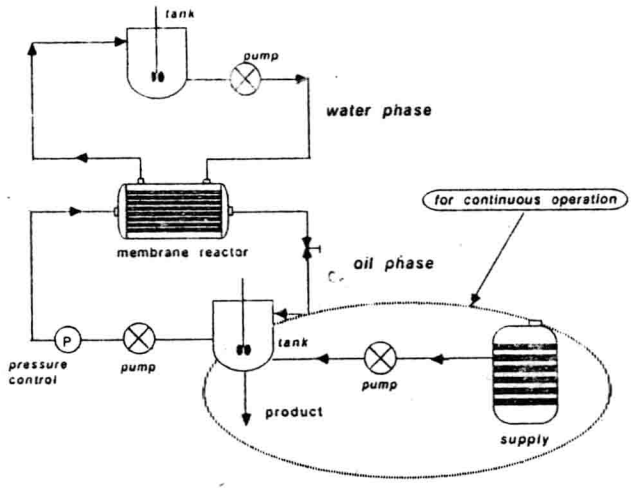

Figure 1. Schematic of the hydrolysis set up. For continuous experiments fresh oil was supplied as indicated by arrows.

A complication in operating biocatalytic membrane reactors is that vegetable oils may exhibit high viscosities and contain impurities. Furthermore, the enzyme activity towards the substrates, including inhibiting effects of products or impurities, is unclear. Therefore the hydrolytic activities of several commercially available membranes were compared, whereby *Candida rugosa* lipase was used as a model enzyme for immobilization studies.

3. REACTOR EXPERIMENTS

Experiments were done with two membrane reactor types: one containing hydrophilic, hollow cellulose fibers (Organon, 1 m^2), the other, tailor-made PSf membranes (ATO-DLO, 1 m^2) which are modified to increase hydrophilicity and lipase compatibility. *Candida rugosa* (Biocatalysts, Ltd, Pontrypridd, UK, 10^{-3} wt% aqueous solution) was immobilized adsorptively, using an ultrafiltration procedure [7]. *Rhizopus javanicus* (Biocatalysts), immobilized similarly to the *Candida rugosa* lipase, was used for α-specific hydrolysis. Edible sunflower oil was used as a model substrate for short as well as for long term studies. In long term studies the substrate was fed continuously to the reactor. GLC (Chrompack HT Simdist column) was used for degree of triglyceride hydrolysis [5].

4. RESULTS and DISCUSSION

In table 2 the results obtained with the two membranes are shown and compared to literature data. The results found with the cellulose membrane are comparable to those found by Pronk [9] who used the same type of membrane and substrate.

With an identical cellulose membrane, slightly different enzyme activity half-lives were reported, which might be attributed to the different substrate, namely triacetin. Apart from the polypropylene membrane (table 2), all the membranes are characterized as hydrophilic. The half-life increase from 2 to 15 days, found with polypropylene membranes was attributed to the high glycerol concentration during the reaction, half-life at low glycerol concentration was only 2 days. The data found with comparable substrates (Hoq, Taylor, Pronk, ATO) confirm the hypothesis that hydrophilic membrane materials have an affect on the half-life of the enzyme. Especially the enzyme half-life found with the modified polysulfone reactor, is extremely long. As shown, the initial activity of this reactor is very high: 200 molOHm $^{-2}s^{-1}$, but also the long-term activity at high conversion levels is considerable: 30-50 molOHm $^{-2}s^{-1}$ at a 70 % fatty acid conversion level.

Table 2.
The activity and stability of lipases in membrane bioreactors for hydrolysis.

reference #	membrane type	init. activity (molOH/m ^{2}s)	half life (days)
Hoq [3]	polypropylene	20	2-15
Taylor [8]	acrylic	23	30-60
Pronk [9]	cellulose	12	43
Guit [10]	cellulose	11	6
Guit [10]	p-acrylonitrile	12	6
ATO	cellulose	10	40
ATO	hydrophilized Psf	200	160

The stability of the enzymatic reactor was examined in continuous long term hydrolysis experiments with sunflower oil. Figure 2 depicts the hydrolysis rate at a 70 % fatty acid conversion level. From this graph the half-life of the enzyme activity was calculated to be 160 days, which is very much longer than the experimental values found by others (table 2).

The high reaction rates and enzyme stability of the specially prepared reactor can be attributed to the specific hydrophilic nature of the modified polymers used. The influence of polymer nature has been surveyed in literature [1,11], and although results appear to be somewhat equivocal, the hydrophilicity of the material seems to have a major effect on the aqueous conditions, or water activity, near the active site of the enzyme thus prolonging the enzyme stability.

Apart from its high activity and prolonged stability the newly developed reactor can be used in combination with crude preparations of 1,3-specific lipases. It has been shown that a polymer which is often used in membrane reactors, i.e.,

cellulose, is readily attacked by cellulases present in crude enzyme preparations [7]. The polysulfone membranes used in this study are not susceptible to this cellulolytic activity.

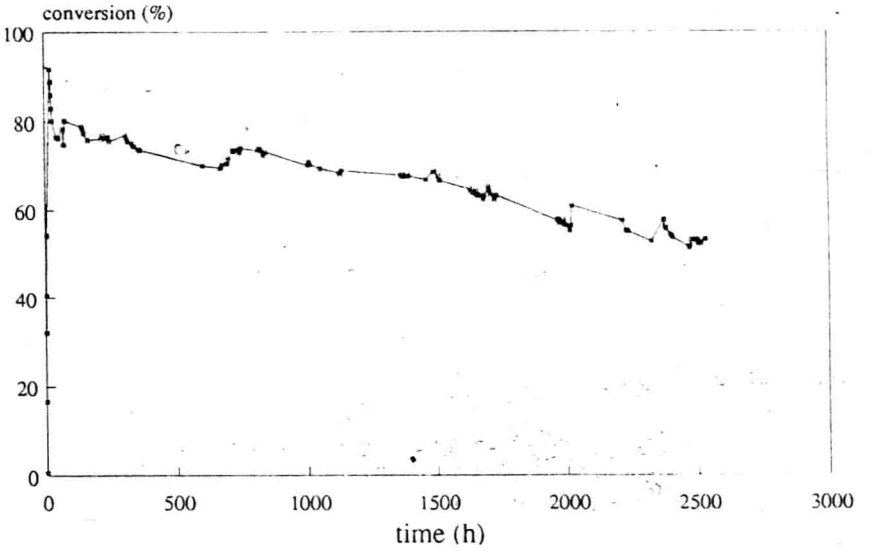

Figure 2. Hydrolysis rate during a long term experiment with a dedicated membrane reactor using sunflower oil as a substrate.

5. CONCLUSIONS

A special membrane bioreactor is developed which is very suitable to mildly hydrolyze chemically and thermally unstable triglycerides. Enzyme activity half-lifes up to 160 days were observed with the newly developed bioreactor, whereas reactors described in literature, with the same substrate were limited to 40 days. The membrane reactor is not susceptible for cellulase activity, hence crude enzyme preparations can be used. Other potential utilizations of the reactors include chiral synthesis or separation of racemic mixtures.

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Cold denaturation of proteins as investigated by subzero transverse temperature gradient gel electrophoresis

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Abstract

Cold denaturation of RNase A was studied by subzero temperature gradient gel electrophoresis in a temperature range from -2° to -6°C. This new approach allows to measure the transition temperature of protein cold denaturation and to detect unfolding intermediates. Under our experimental conditions, Bovine Pancreas RNase A exhibited two transitions : 1)- a continuous transition with a midpoint temperature of -14°C corresponding to the rapid equilibrium between the native enzyme state and a "predenaturated state" ; 2)- a discontinuous transition at -22.5°C from "predenaturated" to denaturated state. Electrophoretic patterns suggested that the intermediate state is more compact than the native and unfolded ones.

1. INTRODUCTION

More than 25 years ago, Brandts predicted cold denaturation of chymotrypsinogen based on a long extrapolation of indirect data (1). For RNase A, Brandts and Hunt (2) postulated that the shape of ΔG vs. T is an indication that at low temperature a denaturation process occurs, but this cold-induced transition was inaccessible to experiment. This second process was termed "cold denaturation". There have been many attempts since the initial statement to confirm the existence of cold denaturation of proteins by direct experiments (for a review, see ref.3). Numerous methods are used to investigate structural transitions of proteins, but so far there was no method for the direct observation of their cold denaturation.

Here, we report the first method for direct investigation of protein unfolding at subzero temperature. This new experimental technique has proved to be a convenient approach : 1- to

detect cold denaturation of proteins, 2- to monitor size/conformation changes accompanying this process, 3- to distingish between fast and slow transitions and 4- to determine the transition temperature(s) of unfolding.

The present report describes the use of subzero temperature gradient gel electrophoresis (TGGE) for the study of low temperature-induced transitions of RNase A. RNase A was chosen as a model protein because its structure is well known and because some information on its stability and structural features at subzero temperatures are available (4).

2. MATERIALS AND METHODS

All reagents and biochemicals were obtained from commercial sources : Bovine pancreatic RNase A (type XII A) and N,N'-methylene bis-acrylamide were purchased from Sigma (St Louis, USA), acrylamide (Electran grade1) from BDH (Dorset, England), and ethylacrylate from Fluka (Buchs, Switzerland). The cryosolvent, used as buffer for gels and electrode tanks, was a mixture of 50% ethyleneglycol / 50% aqueous buffer (v/v). The aqueous buffer was 0.1M Tris/acetate pH 3.7 at 20°C.

2.1. TGGE apparatus

The apparatus was basically similar to the device described by Riesner et al. (5). The electrophoresis unit was a LKB Multiphor fatbed (Pharmacia LKB) and the temperature gradient plate was a laboratory-made copper block of 180 x 150 x 5mm covered in electric insulating and connected with two thermocryostats. Thermal insulation of the electrophoresis unit with expanded polystyrene blocks allowed to minimize thermal exchanges between the electrophoresis system and its surrounded environment.

2.2. Gel polymerisation

Gels were prepared as described by Perella et al. (6). Copolymerisation of acryl-containing monomers on GelBond films was achieved at -15°C for 2 days by adding 0.16% (v/v) TEMED and 0.05% (w/v) ammonium persulfate to a solution of acrylamide, ethylacrylate, and bisacrylamide in the hydroorganic buffer. Gel composition was T% = 8.13 g/100ml (where g is grams of acrylamide + ethylacrylate + crosslinker) and C% = 1.85. The acrylamide/ ethylacrylate molar ratio was 1.57. Urea was introduced at low concentrations (3 or 4M) into the gel mixture as a protein destabilizing agent (7).

2.3. Subzero TGGE

Protein samples for electrophoresis were in solution in the buffer-EGOH mixture having the same composition as the gel phase. Urea (final concentration 3 or 4 M) was added to RNase A solution (0.68 mg prot./ml). Then, sucrose was added to the samples to increase their density. Pyronin Y was used as a tracking dye, but since it binds to native RNase A, its presence in gel was also useful to check the functionality of RNase A dye binding site.

2.3.1. Preelectrophoresis

The first step of TGGE was a standard vertical slab gel electrophoresis. The protein solution was layered in a continuous line across the top of the gel. RNase A migrated into the gel at constant temperature (+15°C). Migration was stopped when the protein has entered the gel matrix. The running buffer was identical to the gel buffer. Preelectrophoresis was carried out at 30 mA for 90 min in a Hoeffer vertical electrophoresis unit.

2.3.2. Tranverse temperature-gradient electrophoresis

The second step was analogous to TGGE using a temperature gradient (t > 25°C) as designed by Riesner (5) : the direction of migration was perpendicular to that of the temperature gradient. After preelectrophoresis, the gel on GelBond film was laid on the temperature-gradient plate. Proteins were allowed to reach their equilibrium conformation at each temperature of the gradient for 30 min. Then, TGGE was carried out for 3 days at 1800 V and a current of about 2 mA. The applied electric field was 130 V/cm.

Temperature gradient in gels were measured over the whole plate using a thermistor (Comark 2001; England) ; a surface probe was placed on the gel and moved along the gradient at 1cm intervals.

pH measurements were performed at different temperatures (from 5 to 30°C) with a digital pHmeter (Orion 701A). The faint variation of pH vs. temperature of the cryosolvent was linear within the temperature range ; it was assumed that the linear dependence of pH on temperature continues at subzero temperatures (8). Thus the apparent pH values of the cryosolvent at subzero temperatures were determined by linear extrapolation.

2.3.3. Protein staining

Before staining, pictures of the gels were taken to determine the position of free and bound Pyronin Y.

Staining of RNase A was carried out with Coomassie Brillant Blue R250. Gels were shaken slowly for 30 min in solution A (20% Ethanol and 5% Acetic Acid), then for 20 min in the staining solution, followed by several rinses in the solution A.

3. RESULTS

Subzero TGGE was carried out at 4 M urea concentration. Indeed, study of urea-induced denaturation of RNase A by transverse urea gradient gel electrophoresis at pH4.0 (9) showed that the transition midpoint of denaturation is at this concentration. In our experiments, urea was added both to the gel solution and to the sample buffer to destabilize RNase A and to shift the cold denaturation transition to an experimentally accessible temperature range. Thus, adding 4 M urea made observable the cold denaturation of RNase A. In the temperature range of -11 to -24 ± 0.5°C (Fig.1) two transitions were observed : 1- a faint continuous transition zone at around -14°C and, 2- a discontinuous transition zone at a lower temperature (-22.5°C).

The dramatic mobility decrease of RNase A at around -22.5°C suggested the existence of a temperature-dependent protein conformational state which was retarded by the polyacrylamide gel sieving effect during electrophoresis. The form of decreased mobility appeared to have a higher hydrodynamic volume than the native enzyme.

After preelectrophoresis, the RNase A band was linear over the temperature range. The distance of migration measured after preelectrophoresis compared to the values obtained after subzero TGGE showed that the cold denaturated protein did not migrate at all. Indeed, its migration distance from the top of the gel corresponded to the preelectrophoresis migration distance.

To check whether the observed phenomenon was trivial or not, different measurements were performed. It was found that the pH variation vs. temperature was negligible. Morever subzero TGGE carried out with and without Pyronin Y showed no differences in mobility of RNase A. On the other hand, as shown in Fig.2, the use of the reducing agent dithiothreitol (DTT) at 10mM caused an increase of about 4°C in the transition temperature corresponding to the discontinuous transition. These temperatures with and without DTT were -18.5°C and -22.5°C, respectively. 10mM DTT increased the transition temperature toward high temperatures due to the reduction of disulfide bridges (10). In these two conditions, transitions observed were similar in shape and no definite intermediate was detected. So, we concluded that the observed phenomenon is strictly dependent on temperature.

Conversely, when subzero TGGE was carried out in the presence of 3M urea in a temperature range from -6 to -19.5°C (results not shown), only the first continuous transition zone was observed. This transition corresponds very likely to the above-mentioned transition at -14°C.

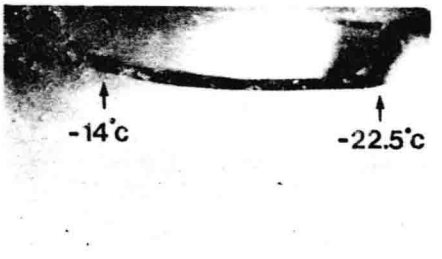


Figure 1. Subzero TGGE of RNase A. The temperature gradient was linear from 11°C (left) to -24°C (right).

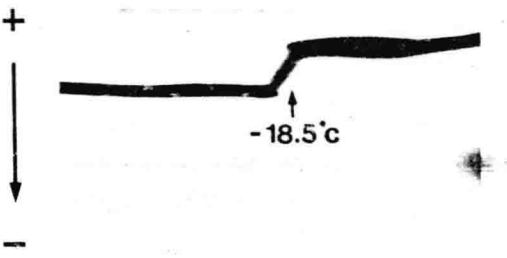


Figure 2. Subzero TGGE in the presence of DTT. The linear temperature gradient was from -12.5 to -23.5°C.

In parallel, "cold denaturation" of RNase A was investigated by UV spectrophotometry. The RNase A concentration was 10 mg/ml. All other components of the electrophoresis medium including gel comonomers were added in the same concentrations. As shown in Fig.3, the absorbance at 286nm vs. subzero temperatures showed a significant inflexion of the curve at around -20.5°C. When DTT was added to the sample solution, the inflexion point was shifted to -16°C (Fig.4).

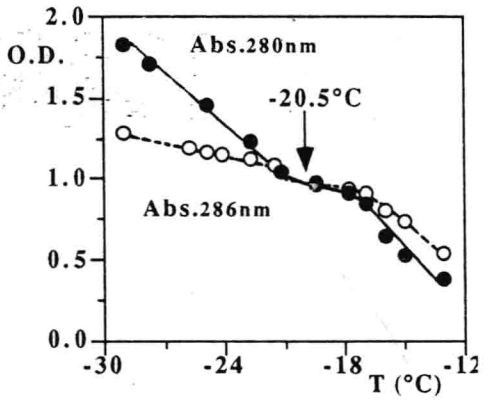


Figure3. RNase A absorbance at 280 and 286 nm vs. subzero temperatures.

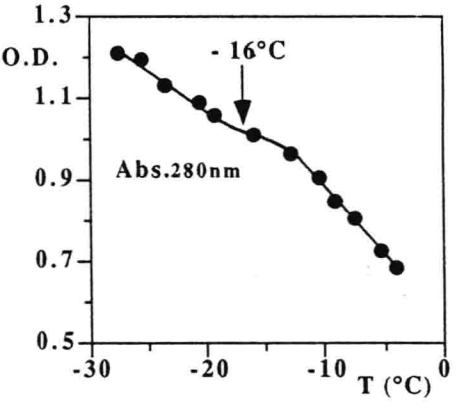


Figure4. RNase A absorbance at 280 nm vs. subzero temperatures in the presence of DTT.

4. DISCUSSION

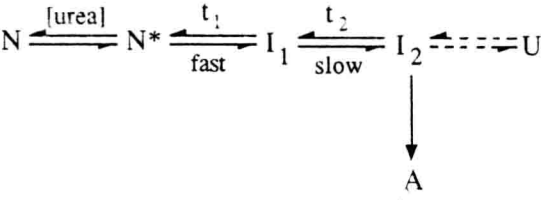
Under our experimental conditions, Bovine Pancreas RNase A exhibited two transitions (Fig.1) : a first continuous transition zone with a midpoint temperature at t_1 = -14°C and a second discontinuous transition zone at t_2 = -22.5°C.

The particularity of the first one lies in the fact that between -14°C and -22.5°C, the RNase A molecular population migrated with a mobility slightly higher than that of the enzyme in its native state. Electrophoretic patterns suggested that the apparent hydrodynamic volume was smaller than that of the native enzyme. At temperatures higher than -22.5°C, RNase species can be observed without staining since Pyronin Y binds to RNase. On the other hand, at lower temperatures, it was found that RNase looses its binding property and Coomassie Blue staining was necessary to observe the RNase bands in gels. Binding of Pyronin Y to RNase A shifted the denaturation discontinuous transition zone toward lower temperatures, suggesting that the dye acts as a protein stabilizer. Because Pyronin Y interacted with RNase A at temperatures higher than -22.5°C, and because the difference in mobility around -14°C was very small (Fig.1), it may be assumed that the functional and structural properties of these two states are relatively close. In addition, since the observed transition is continuous, one has to conclude that these states are in fast equilibrium.

The low-temperature discontinuous zone is due to a slow transition between two states. At temperature lower than the transition zone, the equilibrium is shifted toward the open conformational states which consists of protein molecules in unfolded conformation. This transition temperature observed by subzero TGGE was correlated with spectroscopic studies. The difference in temperature transitions obtained by these two methods was very small (≈2°C). But, since the absorbance curve showed only a discrete modification in the absorbance profile, one may hypothesize that the structural modification due to cold denaturation is very limited. Considering that the distance of migration of the unfolded state was the same before and after subzero TGGE and that the change in electrophoretic pattern of RNase on both sides of -22.5°C was relatively important, it may be suggested that the "denaturated" protein is aggregated. Aggregate formation of unfolded proteins is a particular case of irreversible unfolding transitions. Aggregation as well as inclusion bodies formation is dependent on protein concentration ; this may occur during electrophoresis, since the protein concentration in the moving zone is very high.

At higher temperature, most of the RNase molecular population was supposed to correspond to the above-mentioned "intermediate state". Thus, one may propose a hypothetical reaction scheme for the RNase unfolding transition where the intermediate state was supposed more compact than the destabilized native enzyme (N*). The pathway for the cold-induced unfolding

of RNase may be depicted as follows :



where I_2, I_1 and N* are the unfolded, intermediate and destabilized native states, respectively. A is I_2 aggregated form. In fact, subzero TGGE which operates in destabilizing conditions did not show the real native state (N) because protein was urea-destabilized before electrophoresis into N*.

5. CONCLUSION

Fink and Painter (11) predicted that RNase should be 50% unfolded at -45°C at pH3, in 70% methanol, due to the cold denaturation. However, their preliminary investigations to detect directly cold denaturation failed to reveal any evidence for unfolding. The hypothetical explanation they proposed was that the rate of unfolding under these conditions is sufficiently slow so that the native state is effectively stabilized.

The use of destabilizing agents allows to shift the stability curve, i.e. to shift cold denaturation temperature to the temperature range of experimental accessibility. In our experiments, RNase A destabilization by urea was necessary to observe its "cold denaturation". Though changes in UV spectrum vs. temperature correlate with electrophoresis data, spectroscopic studies are not sufficient to confirm our hypothetical reaction scheme for RNase unfolding process. Our results have to be validated by more sophisticated techniques operating in the same destabilizing conditions. However, this first application of subzero TGGE demonstrates that this technique is of interest for the study of low temperature-induced unfolding transitions.

6. ACKNOWLEDGEMENT

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Genetic algorithms as a new tool to study protein stability

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Abstract

Genetic algorithms use recombination and mutation of solution trials to derive near optimal solutions for problems involving complex and large state spaces. Protein engineering represents such a demanding task and we illustrate how genetic algorithms are a promising new design tool to identify optimal amino acid mutations and to study protein stability in a variety of applications. A first example illustrates optimal substitutions in the core of a protein. The next example searches for mutations in a long protein sequence which improve protein stability by several criteria which do not compromise the critical features of the starting wild type sequence. A three-dimensional lattice simulation achieves a compact and stable fold for a simple model protein composed of four beta strands. Loop length and overall hydrophobicity prove to be most important for the stability of the fold. In grid-free simulations the strength of different beta strand propensities assigned to extended residues can be simulated to test their effect on stability. The influence of hydrophobicity on helix stability is studied in grid-free simulations of four helix bundles: strong hydrophobicity in a few particular residues stabilizes more effectivly in the simulations than using hydrophobic residues distributed over larger regions of the helices.

1. INTRODUCTION

Genetic algorithms in engineering applications are only metaphors of life borrowed for the purpose of optimization [1]. In contrast, we have begun to apply genetic algorithms to analyze and improve protein stability. The following aspects of genetic algorithms promise to offer advantages over earlier approaches to model proteins in a simplified way.

(i) The genetic technique can be more realistic as it models the evolution of an adapted protein structure by mutation, recombination and selection in a natural way.

(ii) Genetic algorithms process in each generation and in parallel many structures and even more schemata [1] in search of the global minimum for a protein conformation.

(iii) The simulations should be achieved computationally faster than with the use of full molecular dynamics [2], another frequently utilized technique to minimize a protein's conformational energy.
(iv) The confomational space is searched in a unique way such that two "bad" solutions (as judged by the parameters given for the selection) which have only partial regions of optimal structure may recombine to yield a much better ("fitter") new structure.
(v) Complex parameters may be incorporated into genetic algorithms. The increase in calculation time is relatively modest as even a rough estimate of each parameter is sufficient to drive selection [1].

In the following we illustrate by simple examples for a variety of applications how these advantages can be used to simulate and identify mutations and design criteria which should enhance protein stability.

2. MATERIALS AND METHODS

Simulation programs were written in VAX-PASCAL using modified versions of the simple genetic algorithm [1]. The fittest individuals from several (0-24) selection runs ("epochs", typically runs of 120 generations, population sizes of 500 individuals) were collected for a final competition run against a random background.

In protein structure simulations it is critical to encode the structure in an efficient way.
(i) In the sequence models, amino acid sequences were encoded in each individual by six-bit tupels according to the genetic code.
(ii) In the three-dimensional grid simulations, coordinates were calculated according to the four possible directions (two-bit tupels) of the C_α atom trace on a tetrahedral lattice.
(iii) For the grid-free simulations standard conformations were encoded by tupels and coordinates calculated from these according to their ϕ and ψ dihedral angles.

Genetic algorithms also allow discrimination amongst many different parameters known to be important in protein structural stability and folding (hydrophobicity, accessibility, charges, amino acid content, helical propensity, etc.). The optimal fitness function made from such parameters and their relative weights which efficiently drives selection to realistic and observable structures contains the crucial parameters necessary to select and fold a particular structure. In practice it is important to be as simple as possible and to introduce or alter only one parameter at a time to understand its effect. The refined fitness functions used here are sketched in the examples given. Details of the protein engineering program and the grid-bound simulation are described in [3].