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IMMUNE-BIOLOGICAL STUDY OF THE CRYSTALLINE ALCOHOL DEHYDROGENASES ISOLATED FROM CLOSELY RELATED YEAST SPECIES

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Few data [1—4] have been published in the literature concerning comparative immune-biological studies on crystalline proteins isolated from closely related species.

In the immune-biological properties of serum albumins from closely related species only quantitative differences have been found [1, 2]. Similarly, quantitative differences have only been demonstrated between the functionally homologous enzymes from closely related species of microbes [3, 4].

Although the crystalline proteins isolated from yeast have already been studied by immune-biological methods [5—8], these investigations have not been extended to a comparative examination of the immune-biological properties of crystalline enzymes isolated from different kinds of yeast.

Crystalline alcohol dehydrogenase (ADH) isolated from two subspecies of *Saccharomyces cerevisiae*, notably from baker's yeast and brewer's yeast, has been found suitable for such studies. These enzymes, homologous in function, but heterologous in species, differ in some properties [9, 10] but prove to be homogeneous in electrophoretical [9—12], solubility [9, 10] and sedimentation studies [13].

A brief summary of our investigations has already been published [14].

Materials and methods

Preparation of ADH. Crystalline ADH was prepared from baker's yeast by RACKER's method [15], and from brewer's yeast by the Racker method, as modified by KELETI [10]. Enzymes recrystallized 1 to 3 times have been used.

Protein samples dissolved in physiological NaCl and dialysed against the same solution for 4 hours under ice cooling in a shaker dialysator were used for immunisation.

Assay of enzyme activity. Enzyme activity was measured by the optical test and expressed in enzyme units (E/mg) [15]. In the reaction mixture the optimum substrate and coenzyme concentrations were employed, and the pH of the mixtures was adjusted to the optimum [9, 10].

When enzyme assay was made at the equivalence point, the quantities of enzyme and immune sera corresponded to the equivalence points shown in Tables I and II. In the presence of excess antigen, the quantities of enzyme and of immune serum corresponded to a value 2 dilution steps beyond the equivalence point.

Antibody production. Two groups of rabbits, weighing 3 to 4000 g each, were immunized intravenously with crystalline ADH, at 3-day intervals. Freshly prepared and dialysed enzyme solutions were used on each occasion, and a control test for enzyme activity was performed.

The immunization cycle lasted 4 weeks, during which period 260 mg of brewer's yeast ADH and 130 mg of baker's yeast ADH, respectively, was injected into each animal.

One week after the last injection of antigen the animals were killed by bleeding under sterile conditions. Pooled sera, sterilized by filtration through a Seitz filter, preserved with 0.1 per cent merthiolate and stored in the frozen state were used. The sera obtained from the two series were used separately and thus each result obtained is corroborated by experiments made on pooled sera from two different immunization cycles.

Precipitation test. The precipitation reaction between the homologous and heterologous enzyme antigens, respectively, and the antisera was first estimated by semiquantitative methods to determine the optimum points. Then the exact site of complete precipitation and the antigen-antibody ratio was determined by the GITLIN's [17] spectrophotometric modification of the quantitative precipitation method described by HEIDELBERGER and KENDALL [16].

Semiquantitative studies. Fourfold dilutions of baker's yeast ADH antiserum and twofold dilutions of brewer's yeast ADH antiserum were used. To 1 ml of each antiserum different amounts of enzyme dissolved in 1 ml were added (protein-N content: 0.1 to 40 μg). After 24 hours at 4° C, the precipitate was centrifuged, the supernatant was divided into two equal parts. To one part was added 1 ml of serum diluted 1 : 10, to the other, 1 ml of the enzyme solution containing 2 μg of protein-N. In this way were established the neutralization points and zones at which there is no demonstrable excess antigen or antibody in the supernatant.

Quantitative studies. The composition of the reaction mixtures was the same as in the semiquantitative tests. After allowing the tubes to stand at +37° C for 2 hours, then at +4° C for 24 hours, the precipitate formed was centrifuged, and washed three times with pre-cooled physiological NaCl. The precipitate was dissolved in 2 ml of 0.1 *N* NaOH and after two hours the protein content was determined from the extinction at 280 mμ. Calibration curves for each of the two kinds of ADH were performed on grounds of the protein-N content determined by the KJELDAHL—WINKLER method.

Study of the mutual dissolution. The experiments were made in 0.4 sat. $(NH_4)_2SO_4$ solution containing KCN, at +4° C, showing the quantity of protein dissolved on the basis of the extinction read at 280 mμ. The dissolution equilibrium was ensured by incubation for 24 hours, with frequent shaking. The undissolved enzyme was removed from the solution by centrifugation

Results

1. *Immunological studies*

In the first part of the experiments the serological reactions between the crystalline ADHs isolated from brewer's yeast and baker's yeast, respectively, and their respective antisera were studied by semiquantitative methods. The homogeneity of the enzymes was also established, in view of the fact that heterogenous or complex antigens have a wide neutralization zone [18]. Subsequently, we determined the quantity of homologous and heterologous antigens, respectively, reacting with the homologous serum and studied whether or not a cross-reaction ascribable to common antigenic parts or to identical antigens was present.

The investigations showed the enzymatic antigens to be immunologically homogeneous. Analysing the supernatant it has been demonstrated that the antibody combined with both the homologous and the heterologous enzymatic antigens and that the antibody and antigen, respectively, can be absorbed completely from the corresponding system both by antigens and by antisera.

It was already obvious from the semiquantitative tests that the baker's yeast ADH antiserum was capable of binding equal amounts of brewer's yeast

ADH and baker's yeast ADH. In contrast with this, brewer's yeast ADH antiserum precipitated different quantities of the two kinds of ADH.

The fact that about twice as much of brewer's yeast ADH had to be used when producing the antiserum to it and yet the antiserum could be used in twofold dilutions only, whereas baker's yeast ADH antiserum could be used in fourfold dilution, indicates that under our experimental conditions brewer's yeast ADH was a less potent antigen than baker's yeast ADH.

Table I

Precipitation test with baker's yeast ADH antiserum
(1 ml serum diluted 1 : 4)

Antigen-N added, mg	Antigen-N ppt (supernatant analysis)	Total-N ppt (determined by Gitlin's) method)	Antibody-N ppt measured, mg	Antibody-N/ antigen-N ratio in precipitate	Antibody-N ppt (calculated from formula)	Supernatant analysis
			A) + baker's yeast ADH			
0.0011	total-N	0.0289	0.0278		0.0284	Excess antibody
0.0023	,,	0.0392	0.0369	16.0	0.0334	,, ,,
0.0046	,,	0.0448	0.0402	8.7	0.0400	,, ,,
0.0069	,,	0.0472	0.0403	5.8	0.0400	,, ,,
0.0092	,,	0.0480	0.0388	4.2	0.0370	,, ,,
0.0115	nearly all	0.0512	0.0398	3.4	0.0430	∅
0.0138	partly	0.0496				Traces of antigen
0.0184	,,	0.0480				Little ,,
0.0230	,,	0.0448				Excess ,,
0.0459	,,	0.0392				,, ,,

Antigen maximum : 0.072
Antibody maximum : 0.424
Antibody-N ppt : $5.6\ x - 38.7\ x^2$

			B) + brewer's yeast ADH			
0.0011	total-N	0.0290	0.0278		0.0284	Excess antibody
0.0023	,,	0.0390	0.0367	15.9	0.0334	,, ,,
0.0046	,,	0.0400	0.0354	7.7		,, ,,
0.0069	,,	0.0448	0.0371	5.4	0.0370	,, ,,
0.0092	,,	0.0450	0.0358	3.9		,, ,,
0.0115	nearly all	0.0515	0.0400	3.5	0.0431	∅
0.0138	partly	0.0490				Traces of antigen
0.0184	,,	0.0450				Little ,,
0.0230	,,	0.0400				Excess ,,
0.0459	,,	0.0390				,, ,,

Antigen maximum : 0.072
Antibody maximum : 0.424
Antibody-N ppt : $5.6\ x - 38.7\ x^2$

The semiquantitative results have been controlled by quantitative methods, the results of which are presented in Tables I and II.

Table II

Precipitation test with brewer's yeast ADH antiserum
(1 ml serum diluted 1 : 2)

Antigen-N added, mg	Antigen-N ppt (supernatant analysis)	Total-N ppt (determined by Gitlin's method)	Antibody-N ppt measured, mg	Antibody-N/ antigen-N ratio in precipitate	Antibody-N ppt (calculated from formula)	Supernatant analysis
		A) + brewer's yeast ADH				
0.00128	total-N	0.0256	0.0243	18.7	0.0244	Excess antibody
0.00200	,,	0.0304	0.0284	14.2	0.0290	,, ,,
0.00400	nearly all	0.0425	0.0385	9.6	0.0393	∅
0.00800	partly	0.0450	0.0370	4.6	0.0367	Traces of antigen
0.01600	,,	0.0485	0.0325			Little antigen
		Antigen maximum : 0.127 Antibody maximum : 0.242 Antibody-N ppt : $6.7\ x - 26.0\ x^2$				
		B) + baker's yeast ADH				
0.00128	total-N	0.0136	0.0123	9.45	0.0123	Excess antibody
0.00200	,,	0.0136	0.0116	5.8	0.0094	,, ,,
0.00400	,,	0.0192	0.0152	3.8	0.0160	,, ,,
0.00800	nearly all	0.0208	0.0128	1.6	0.0103	∅
0.01600	partly	0.0240	0.0080			Traces of antigen
		Antigen maximum : 0.080 Antibody maximum : 0.128 Antibody-N ppt : $6.7\ x - 200.0\ x^2$				

The following empirical formula [1] is characteristic of the differences between the single precipitation systems.

Antibody-N precipitated $= ax - bx^2$ (1)

where

x = antigen-N precipitated (added)

a and b are constants computed from experimental data :

a = the value for the linear part of the "antigen-N added — antigen : antibody ratio" curve, extrapolated graphically to 0 mg of antigen-N.

b = the ratio of the difference in the quantity of antibody-N precipitate between two given points of measurement to the difference in the quantity of antigen-N added at the same two points.

The antibody contents calculated for 1 ml of undiluted serum were :

Baker's yeast ADH antiserum = 1.704 mg

Brewer's yeast ADH antiserum = 0.484 mg

2. *Studies on mutual dissolution*

In the light of the serological evidence it has been suggested that the two kinds of crystalline protein may differ immunologically and for this reason we have examined how the proteins — in saturated solutions — dissolve in each other, employing the method of LANDSTEINER and HEIDELBERGER [19]. The results are shown in Table III.

Table III

Mutual dissolution of crystalline ADHs isolated from baker's and brewer's yeast

I. Saturated enzyme solutions tested	II. Additions to I.	III. Increase in protein content of the supernatant determined and expressed in extinction coefficient read at 280 mμ
Baker's yeast ADH	—	0.252
,,	Baker's yeast ADH	0.235
,,	Brewer's yeast ADH	0.726
Brewer's yeast ADH	—	0.550
,,	Brewer's yeast ADH	0.538
,,	Baker's yeast ADH	0.660

Saturated enzyme solutions were made in 0.4 saturated $(NH_4)_2SO_4$, containing KCN, at $+4°$ C.

3. *Enzymological studies*

It has been reported [4, 20] that the activity of certain enzymes may completely or partially be inhibited by their specific antibodies. This occurred in the case of ADH isolated from baker's yeast [7, 8]. It has been investigated whether the serological differences between the two kinds of ADH were demonstrable also in the inhibitory action of their specific antibodies.

A constant amount of baker's yeast ADH antiserum (diluted fourfold) was tested against different quantities of enzyme antigen. It has been found that the site of maximum inhibition coincides with the site of the equivalence point found in the serological experiments. This applies to both kinds of ADH. This means that the antigen-antibody ratio found at complete inhibition is identical with that found at complete precipitation. In contrast with urease [21], the amount of antibody causing complete precipitation suffices to bring about a complete inhibition of enzyme activity as well.

Subsequently, we have examined the kinetics of the antigen-antibody reaction on the basis of the reduction of enzymatic activity. Two minutes incubation sufficed to bring about complete reaction, *i. e.* no enzymatic activity was observable at the equivalence point.

It has been described that when the antibody has an inhibitory effect on the enzymatic activity of the antigen, preincubation of the enzyme with substrate sometimes diminishes this inhibitory effect [4, 6, 20, 22, 23]. In the case of enzymes possessing coenzyme, this protective effect may be taken over by the coenzyme [4, 5, 20]. We found that preincubation of the enzyme with alcohol or diphosphopyridine nucleotide caused no diminution in the inhibitory capacity of antibody, neither at the equivalence point, nor in the presence of excess antigen (Table IV).

Table IV

Effect of baker's yeast ADH antiserum (after 10 minutes of incubation) on the activity of brewer's yeast and baker's yeast ADHs preincubated with alcohol and DPN, respectively

(Increase in optical density per minute, at 340 m μ)

Preincubated with	Baker's yeast ADH		Brewer's yeast ADH	
	at equivalence point	at antigen excess	at equivalence point	at antigen excess
—	0.204	0.220	0.320	0.310
Immune serum	0.000	0.070	0.000	0.100
Alcohol	0.000	0.070	0.000	0.100
DPN	0.000	0.070	0.000	0.100

Reaction mixture 0.1 ml ADH solution (from a mixture containing ADH in a quantity corresponding to the equivalence point and antigen excess, respectively and immune serum, with an ADH content of 1.5 μ (g/m) + 0.1 ml DPN (0.2 per cent) + 0.5 ml $M/2$ phosphate buffer (pH 8.5) + 0.7 ml destilled water + 0.1 ml alcohol (4.5 M). Final volume: 1.5 ml.

When the enzyme is preincubated simultaneously with alcohol and DPN, *i. e.* when the antibody is added to the functioning enzyme, the inhibitory effect of the specific antibody is to some extent diminished. This protective effect is hardly appreciable at the equivalence point (Table V). In presence of excess antigen the inhibition of enzyme activity is protracted (Fig. 1).

Table V

Effect of baker's yeast ADH antiserum (after 10 minutes of incubation) on the activity of ADHs isolated from baker's yeast and brewer's yeast

(The quantities of ADH correspond to the equivalence point. Increase in optical density per minute, at 340 mμ)

Preincubated with	Baker's yeast ADH	Brewer's yeast ADH
—	0.204	0.320
Immune serum	0.000	0.000
Alcohol + DPN	0.024	0.010

Reaction mixture: as in Table IV.

We have investigated whether the antiserum competes with the substrate. Experiments were performed with baker's yeast ADH antiserum reacting with baker's yeast and brewer's yeast ADH, respectively (Figs. 2 and 3). As demon-

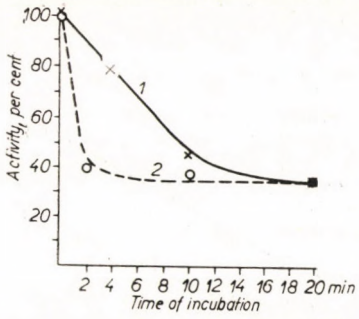

Fig. 1. Requirement of time for baker's yeast ADH antiserum inhibition (in the presence of excess antigen) of baker's yeast ADH preincubated with alcohol + DPN.

1.: Excess baker's yeast ADH preincubated with DPN and alcohol for 1 minute + baker's yeast ADH antiserum.

2.: Excess baker's yeast ADH preincubated with baker's yeast ADH antiserum for 1 minute + DPN and alcohol.

The incubation time shown in the curve means the duration of incubation after the system had been completed. The activity values measured at the single points show the true activity measured after the addition of fresh substrate and coenzyme.

Reaction mixture: 0.1 ml ADH solution (from a mixture containing a quantity of ADH corresponding to the excess antigen and immune serum, with an ADH content of 1.5 μg/ml) + + 0.1 ml DPN (0.2 per cent) + 0.5 ml $M/2$ phosphate buffer (pH 8.5) + 0.7 ml distilled water + + 0.1 ml alcohol (4.5 M). Final volume: 1.5 ml.

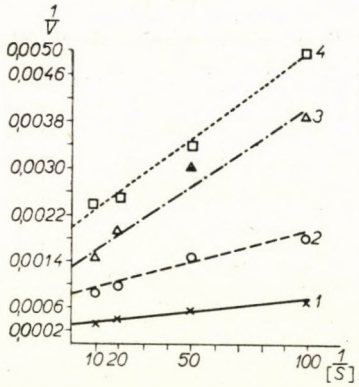

Fig. 2. LINEWEAVER—BURK plot showing non-competitive inhibition of baker's yeast ADH antiserum on baker's yeast ADH.

1: without immune serum
2: 1 ml enzyme + 0.5 ml immune serum
3: 1 ml enzyme + 1.0 ml immune serum
4: 1 ml enzyme + 1.33 ml immune serum

Reaction mixture: 0.1 ml ADH solution or ADH + immune serum (ADH content: 15 μg/ml) + 0.1 ml DPN (0.2 per cent) + 0.5 ml $M/2$ phosphate buffer (pH 8.5) + 0.7 ml distilled water + 0.1 ml alcohol (different concentrations). Final volume: 1.5 ml.

strated by the method of LINEWEAVER and BURK [24], the inhibition is not competitive in either case.

It should be mentioned that on the addition of substrate a dissociation of the antigen-antibody complex is observable. An example is presented in Table VI.

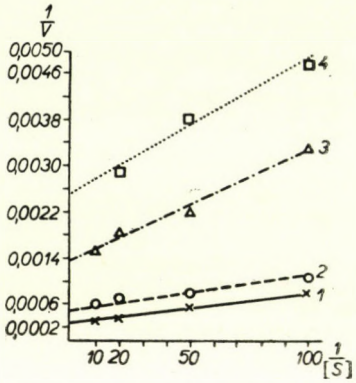

Fig. 3. LINEWEAVER—BURK plot showing non-competitive inhibition of baker's yeast ADH antiserum on brewer's yeast ADH.
1: without immune serum
2: 1 ml enzyme + 0.5 ml immune serum
3: 1 ml enzyme + 1.0 ml immune serum
4: 1 ml enzyme + 1.33 ml immune serum
Reaction mixture: as in Fig. 2.

Table VI

Dissociation of antigen-antibody complex after the addition of substrate

(Increase in optical density at 340 mμ)

Measurement time	Baker's yeast ADH		Brewer's yeast ADH	
	without immune serum	with immune serum	without immune serum	with immune serum
1 minute	0.370	0.000	0.598	0.000
2 minutes	0.578	0.012	0.812	0.022
3 minutes	0.706	0.030	0.902	0.058
4 minutes	0.806	0.047	0.950	0.090

Reaction mixture: 0.1 ml ADH solution or ADH + immune serum (40 μg/ml) + 0.1 ml DPN (0.2 per cent) + 0.5 ml *M*/2 phosphate buffer (pH 8.5) + 0.7 ml distilled water + 0.1 ml alcohol (4.5 *M*). Final volume: 1.5 ml.

The data obtained with brewer's yeast ADH antiserum were the same as those obtained with baker's yeast ADH antiserum. Preincubation of the enzyme antigen with alcohol or with DPN caused no reduction in the inhibitory action

of the antibody, either at the equivalence point, or in the presence of excess antigen.

Preincubation simultaneously with substrate and coenzyme also had a protective effect against the inhibition with antibody (Fig. 4).

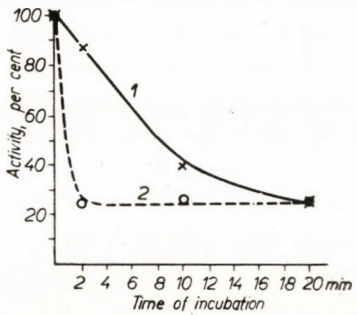

Fig. 4. Requirement of time for brewer's yeast ADH antiserum inhibition (in the presence of excess antigen) brewer's yeast ADH preincubated with alcohol + DPN.

1: Excess brewer's yeast ADH preincubated with DPN and alcohol for 1 minute + brewer's yeast ADH antiserum.

2: Excess brewer's yeast ADH preincubated with brewer's yeast ADH antiserum for 1 minute + DPN and alcohol.

The incubation time shown in the curve means the duration of incubation after the system had been completed. The activity values measured at the single points show the true activity measured after the addition of fresh substrate and coenzyme.

Reaction mixture: 0.1 ml ADH solution (from a mixture containing a quantity of ADH corresponding to excess antigen and immune serum, with an ADH content of 1.5 μg/ml) + + 0.1 ml DPN (0.2 per cent) + 0.5 ml $M/2$ phosphate buffer (pH 8.5) + 0.7 ml distilled water + + 0.1 ml alcohol (4.5 M). Final volume: 1.5 ml.

Discussion

Immune-biological studies on protein antigens with enzymatic function may allow correctly to interprete the correlation between the groups determining the properties of the antigen and those responsible for enzymatic function[4, 20].

We have studied crystalline ADHs isolated from different yeast species. Under the experimental conditions employed both kinds of ADH proved to be antigens. Immune-biological studies revealed only quantitative differences between the two kinds of ADH, partly in the antibody production, and partly in the antigen-antibody reaction.

The two kinds of ADH antisera are exhausted equally with heterologous and homologous antigens. The antibody titres are different, notably brewer's yeast ADH antiserum contains less antibody than baker's yeast ADH antiserum, which had been produced with less antigen. The difference, which exceeds the physiological range of variations, is based on the different antigenic powers of the two kinds of the ADH.

In the case of antigens with enzymatic function the effect of the specific antibody (antienzyme) on the enzymatic activity varies from enzyme to enzyme.

With both kinds of ADH the antienzyme containing serum inhibits the activity of the enzyme. The maximum of the inhibitory action agrees closely with the optimum of the zone of precipitation. Similarly to cross-precipitation, inhibition, too, can be produced equally with the homologous and heterologous antisera.

A similar effect has been described to occur with the lecithinases isolated from *Cl. bifermentans*, *Cl. welchii*, as well as from other Clostridia [3, 4].

It is known that after incubating the enzymatic antigen with its substrate or coenzyme the inhibition caused by the antiserum will often be less marked than the inhibition resulting when the substrate or coenzyme is added to the system after the addition of antiserum. We have found that preincubation with the substrate or coenzyme separately had no effect on the inbitory action of the specific antisera on either kind of the ADH studied. When the enzymatic antigen is preincubated simultaneously with substrate and coenzyme, *i. e.* if we add the antiserum to the functioning enzyme, the inhibition of enzymatic activity sets in slower.

The fact that preincubation with substrate alone has no effect on the enzymatic inhibition of the antiserum as well as the observation that the antibodies are not competing with the substrate, indicate that the antibody combines with the enzymatic antigen at a different point of the protein surface than the substrate.

The differences in structure and kinetical properties we have found to exist between the two kinds of enzyme in our previous investigations [9, 10] corroborate the quantitative differences demonstrated in the present immune-biological studies.

SUMMARY

It has been found that crystalline alcohol dehydrogenases isolated from baker's yeast and from brewer's yeast have antigenic properties. In precipitation tests with the specific immune sera quantitative, but no qualitative differences have been demonstrated.

The crystalline enzymes prepared from the two kinds of yeast and their specific antisera give cross-precipitation, indicating the existence of common antigenic properties. The two kinds of protein dissolve in each other's saturated solution, supporting the view that the two proteins differ somewhat in structure.

The antisera inhibit the activity of both enzymes and the maximum of inhibition coincides with the optimum found in the precipitation system.

Preincubation with substrate or coenzyme separately does not reduce the inhibitory power of the antisera. However, when the antisera are added to the functioning enzymes, the antigen-antibody reactions are slowed down.

The inhibitory action of the antisera does not compete with the substrate.

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