Screening of fish extract materials for Imedeen™

in relation to patients with proven allergy for codfish

by

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Preface

The present report was prepared by Lars K. Poulsen and coworkers on request from Ferrosan A/S for the purpose of evaluating the possible cross-reactivity between raw materials used for the food supplement Imedeen and codfish allergens.

For the preparation of the report, Ferrosan A/S has granted the investigators access to confidential material relating to the production and technical details of Imedeen. Moreover, the sera from characterized codfish allergic patients have been used for the experiments. Thus the present report is to be considered confidential (but can be used in correspondence with the authorities) until further agreement about its possible publication has been reached by Ferrosan A/S and the authors.

List of Contents

Preface 2

List of Contents 3

List of Abbreviations 4

Introduction 5

Materials and Methods 6

Results 9

Conclusions 15

Raw Data 16

References 25

List of Abbreviations

EAACI	European Academy of Allergy and Clinical Immunology
EAST	EnzymeAllergoSorbentTest employing ELISA plates as immunosorbent
IgE	Immunoglobulin E
NHS	Normal Human Serum
o/n	Overnight
PBS	phosphate buffered saline
RT	Room temperature

Introduction

This report describes experiments where cross-reactivity between IgE directed to codfish and to materials related to production of Imedeen. Initially a 8-step strategy for analyses was made:

Step #1: Testing of raw materials for Imedeen in an ELISA for detection of the major codfish allergen Gad c1
A previously developed ELISA using polyclonal antibodies raised to purified Gad c1 is used to test whether the materials contain proteins, that are homologous and will cross-react with Gad c1.

Step #2: Setting up a method for detection of specific IgE for codfish
Accomplished previously to the present work.

Step #3: Setting up a method for detection of specific IgE to raw materials for Imedeen
Assuming that no sera are available to serve as positive controls, and thus the binding of the materials to the solid phase cannot be formally confirmed. As negative controls will serve normal human serum (NHS).

Step #4: Testing of targeted sera against codfish
28 selected sera (from patients reacting to codfish) are tested against codfish to confirm their IgE-binding. Negative controls are included as well.

Step #5: Testing of targeted sera against raw materials for Imedeen
The sera mentioned above are tested against the three materials: Frozen Fish Meat, Dried Fish Cartilage, and Fish Extract.

Step #6: Testing of cross-reactivity between codfish raw materials for Imedeen
Soluble raw materials are tested for inhibition of IgE-binding to codfish, i.e. preincubation of serum with the materials followed by testing of the serum with EAST-wells coated with codfish. Inhibition of codfish with itself is used as positive control.

Step #7: (if possible) Testing of cross-reactivity between materials for Imedeen and codfish
In the event that any sera are positive in step #5, the ability of codfish to inhibit serum IgE binding to solid phase coated with the raw materials.

If no positive sera materialises in step #5, the #7 will be omitted. If, on the other hand, there is experimental evidence for cross-reactivity between a codfish protein and the raw materials, other tests (western blotting) may become relevant.

Step #8: Testing of the biological activity of raw materials for Imedeen in histamine release assay using basophils that are passively sensitized with sera from codfish allergic individuals.

Materials and Methods

Patient Sera
Sera were obtained from 28 codfish allergic patients attending the Allergy Centre (Prof. Carsten Bindslev-Jensen) of Odense University Hospital, Odense, and the Allergy Clinic (Dr. Hans-Jørgen Malling) of the National University Hospital, Copenhagen, Denmark. The codfish allergy was confirmed according to the guidelines of EAACI.[1]. The patients have been further described by Hansen et al. [2].

Preparation of fish material

Sample 1: Fish Extract. Batch No. 140496. 30.03.2006
Sample 2: Fish Dry Cartilage. 20.04.2006
Sample 3: Fish Frozen Meat. 20.04.2006

Sample C: Frozen Codfish Fillet from a local supermarket

Aliquots of each fish material were dispensed with distilled water to a concentration of 100 mg/ml. The samples were processed in a stomacher for 60 seconds and allowed to extract for 60 min at 4 ºC. Thereafter the samples were centrifuged 3000 G / 10 min / 4 ºC. The supernatants were collected and used for protein determinations, specific IgE assay and histamine release.

Protein determination by amino acid analysis
Protein determination was performed by BioCentrum at the Technical University of Denmark using protein hydrolysis, followed by derivatization and amino acid analysis by HPLC.

The following protein concentrations were found:

Sample	Protein concentration
Fish Extract	25.46 mg/ml
Fish Dry Cartilage	1.2 mg/ml
Fish Frozen Meat	0.56 mg/ml
Extract of Frozen Codfish Fillet	1.108 mg/ml

Antibodies
Antisera were raised to a purified preparation of Gad c1 (Steff Koppelmann, TNO, The Netherlands) by immunization of rabbits using the adjuvant TiterMax. The IgG fraction was purified on a protein G column. A part of the IgG-fraction was biotinylated and used as catching antibody in the ELISA.

ELISA for Gad c1
The ELISA was made as a sandwich ELISA using the same antibody in catching and the detection layer,

the latter employing the biotinylated antibody [3]. For the ELISA microtiter plates (Nunc, Denmark) were coated over night at 4 ˚C with 100 l polyclonal rabbit anti Gad c1 diluted to 20microgram/ml in PBS. After three cycles of washing with PBS with 0.1 % tween 20 (PBS-T), the wells were blocked for 1 h with ELISA washing buffer and washed again. Samples and standards were diluted in PBS, and 100 microL were added to the wells of the plate and incubated for 2 hours at 37 ˚C. Biotinylated rabbit anti Gad c1 were diluted to 10 microgram/ml. The plates were washed and 100 microL of the biotinylated antibodies were added to each well and incubated for another 2 hours at 37 ˚C. Finally, the plates were incubated with enzyme-labelled avidin followed by substrate (1,2-phenylendiamine dihydrochloride), and the optical densities (OD) were measured on an ELISA reader at 490 nm with 630 nm as reference.

EAST and EAST-inhibition

Enzyme-AllergoSorbent Test (EAST) was performed as a modification of the previously RAST technique by Poulsen et al [4]. Microtiter plates were coated over night at 4 ˚C with 100 microL 2 microgram/mL of the relevant antigen preparation (i.e. codfish extract or extracts of Imedeen products) diluted in PBS. Plates were washed three times in between all incubation steps with PBS with 0.1 % tween (PBS-T). The coated wells were blocked with PBS-T including 2 % v/v rabbit serum for 1 hour at room temperature on an orbital shaker. In the inhibition assay appropriate dilutions of samples of the inhibiting antigen extract were mixed with serum from codfish allergic individuals diluted in PBS-T with 2 % rabbit serum and preincubated over night at 4 ˚C on an orbital shaker. 100 microL of the serum were added in duplicate to the blocked microtiter plates and incubated for 3 hours at 37 ˚C. Finally, the plates were incubated with HRP-labelled rabbit anti human IgE (Dako, Denmark) diluted in PBS-T with 2 % rabbit serum at a concentration of 1.3 microgram/mL at 37 ˚C for 2 hours, followed by incubation with OPD-substrate at room temperature. Optical densities were determined by an ELISA-reader.

The inhibition was calculated in percent as the OD value for the inhibited sample divided with the value of the uninhibited.

Histamine release

Histamine release experiments were performed on buffy coat cells passively sensitized with patient sera. The passive sensitization was in brief performed according to the following method:

1 Four blood bank buffy coat were screened prior to the experiments. The selected buffy coat shall fulfill the following criteria: Anti-IgE induced histamine release > 30 % and no release reactions to 10 inhalation allergens, 10 food allergens and the codfish extract.
2 Mononoclear cells from the buffy coat were isolated by Lyphoprep gradient centrifugation.
3 The PBMC's containing 1-2 % basophils were then exposed to pH 3.6 for 5 min at 4 ºC. By this procedure basophil bound IgE dissociate from the surface IgE-receptor – i.e. the cells are stripped.
4. The stripped cells are then incubated with each of the 28 patient sera for 60 min at 37 ºC. By this procedure the stripped basophils will bind IgE from the patient sera.
5. Thereafter the sensitized cells are incubated with the above mentioned fish samples. The strongest concentration is 1:10 of the extracts and dilution factor is 3.5. Each fish sample is tested in 6 concentrations.
6. Unspecific histamine release can be seen in high concentrations of a protein extract. To exclude this possibility each extract was tested on cells sensitized to a non-allergic serum.

7. After incubation of patient sensitized cells and control sensitized cells, histamine release was determined by the HR-Test system from RefLab (accredited according to ISO/EN 17025).
8. Responses are classified according to the lowest sample dilution inducing > 10 % histamine release. This system implies that cells responding to the lowest concentration will be a class 6 reaction and cells only responding to the highest concentration will be a class 1 reaction. No reaction to any of the dilutions is a class 0 reaction.

Results

Step #1: Testing of raw materials for Imedeen in an ELISA for detection of the major codfish allergen Gad c1

The materials were tested in the ELISA using purified Gad c1 as standard. It can be seen that the codfish extract is positive, with a potency roughly 10 times of Gad c1, corresponding to roughly 10 % of the protein in the extract being Gad c1. The extract of the frozen meat gives a strongly positive response, but right-shifted three decades compared to Gad c1, i.e. corresponding to a Gad c1 homologue content of around 0.1 %. I should be noted however, that the slope of the dilution curve of the frozen meat is strikingly different from those of Gad c1 and codfish, which could suggest interference with the ELISA, when testing under almost undiluted conditions (neat, 1:2, 1:4, and 1:8). The dried cartilage is negative and the extract border line positive[1], with a potency that is at least ten million times lower than Gad c1. Due to the high level of total protein (25 mg/ml) and the low response of the undiluted sample, it cannot be concluded with certainty that the sample actually contains immunoreactive material, but if this is the case, it would amount to less than 0.32 ng/ml Gad c1 equivalent protein corresponding to less than 13 parts per billion of the protein content.

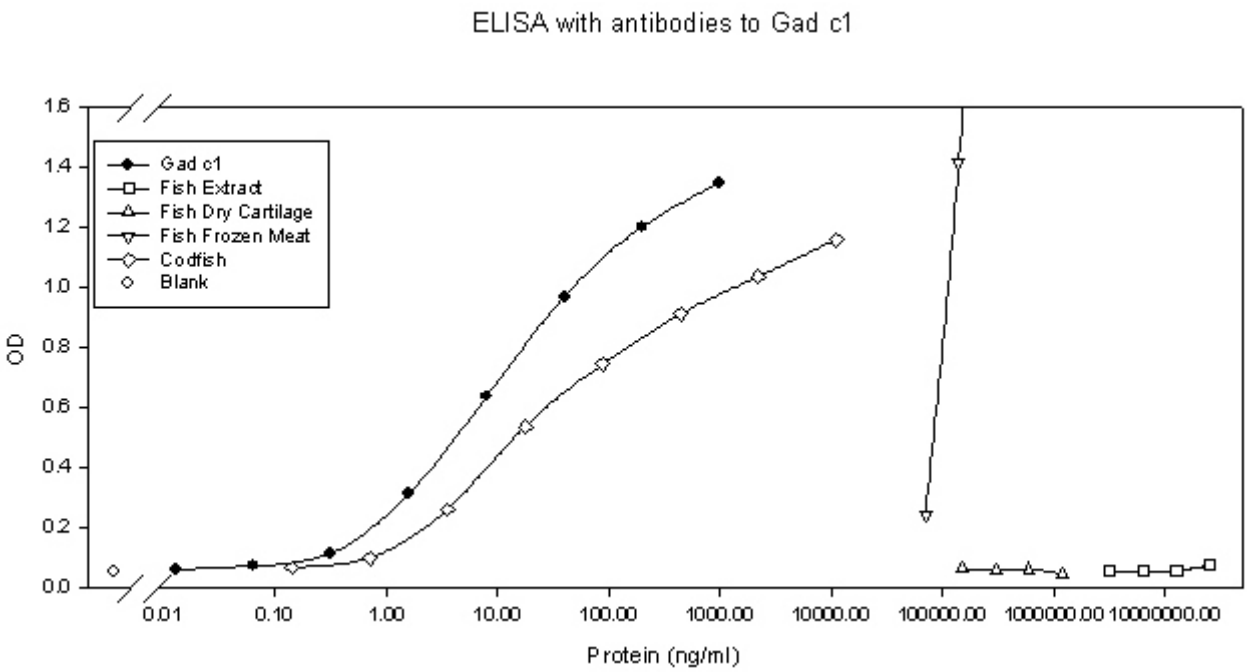

Fig. 1 ELISA dilution curves

[1]Limit of detection for the ELISA was defined as the mean blank + 3 times the standard deviation of the blank. When run in tetraplicates the mean = 0.0560, std dev. = 0.0017, and thus L.O.D. = 0.0626 O.D. units. Only the undiluted sample but not 1:2, 1:4 or 1:8 dilutions of Fish Extract exceeded this value (O.D. = 0.0760). A similar finding was found when running the ELISA a second time.

Step #2: Setting up a method for detection of specific IgE for codfish
This step has been developed previously, and in Fig. 2 is shown dilution curves (1:10, 1:50, 1:250) for ten of the strongest reacting sera.

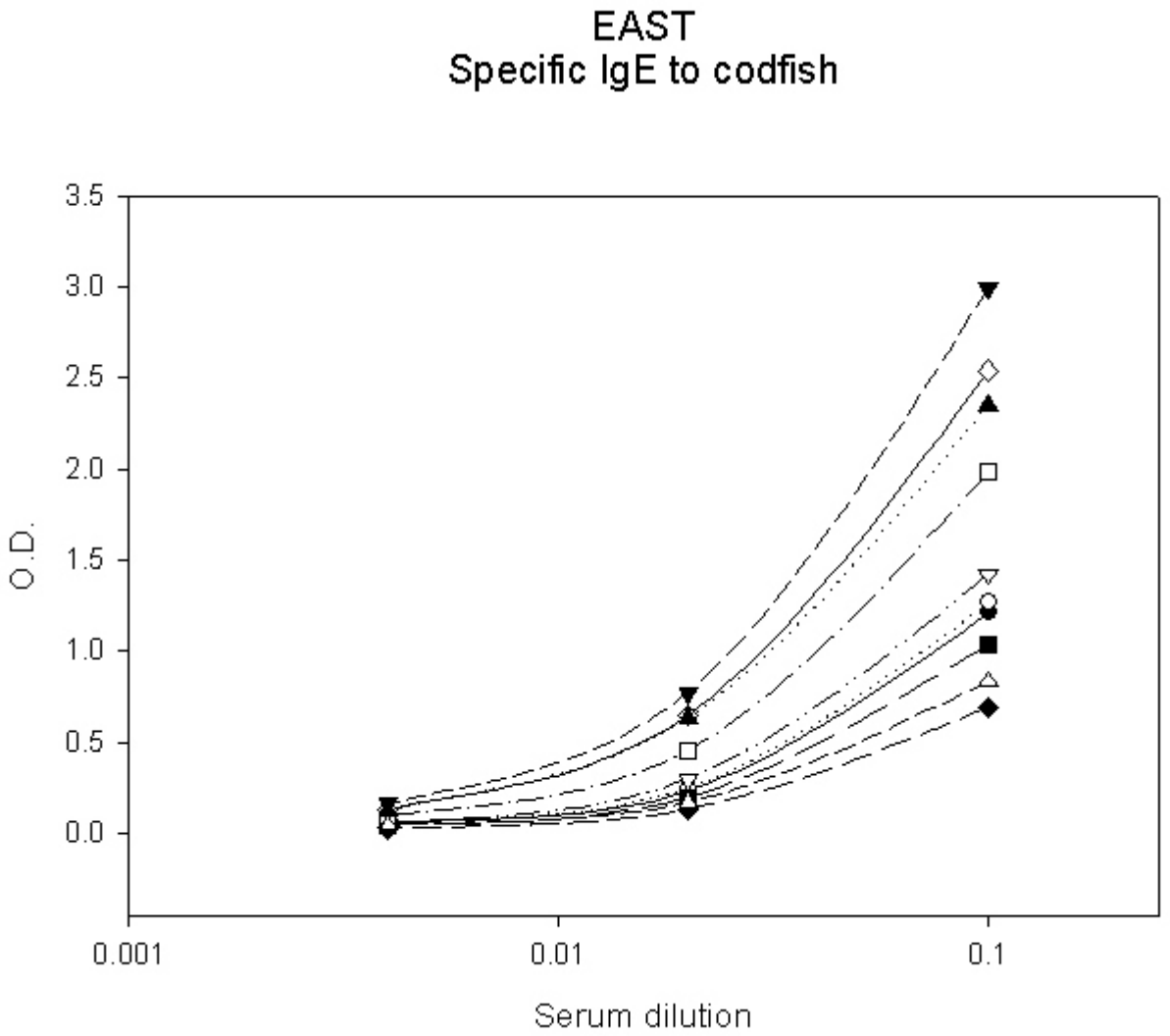

Fig. 2 Serum dilution curves in a direct EAST, where codfish extract served as the allergosorbent.

Step #3: Setting up a method for detection of specific IgE to raw materials for Imedeen.
Since there were no antibodies with known specificity to the raw materials for Imedeen, the extracts were used in the same (standard) concentrations as were used for codfish extract, i.e. 2 microgram/ml for coating. All sera were tested, cf. step #5.

Step #4: Testing of targeted sera against codfish
The EnzymeAllergoSorbentTest (EAST) for detection of specific IgE to codfish extract was applied to all sera in a dilution of 1:10. As shown in Fig. 2 some sera reacted strongly, whereas some sera only

tested slightly positive (Fig. 3), and thus only sera with an OD above 0.2 were used for the subsequent EAST inhibition experiments in step #6.

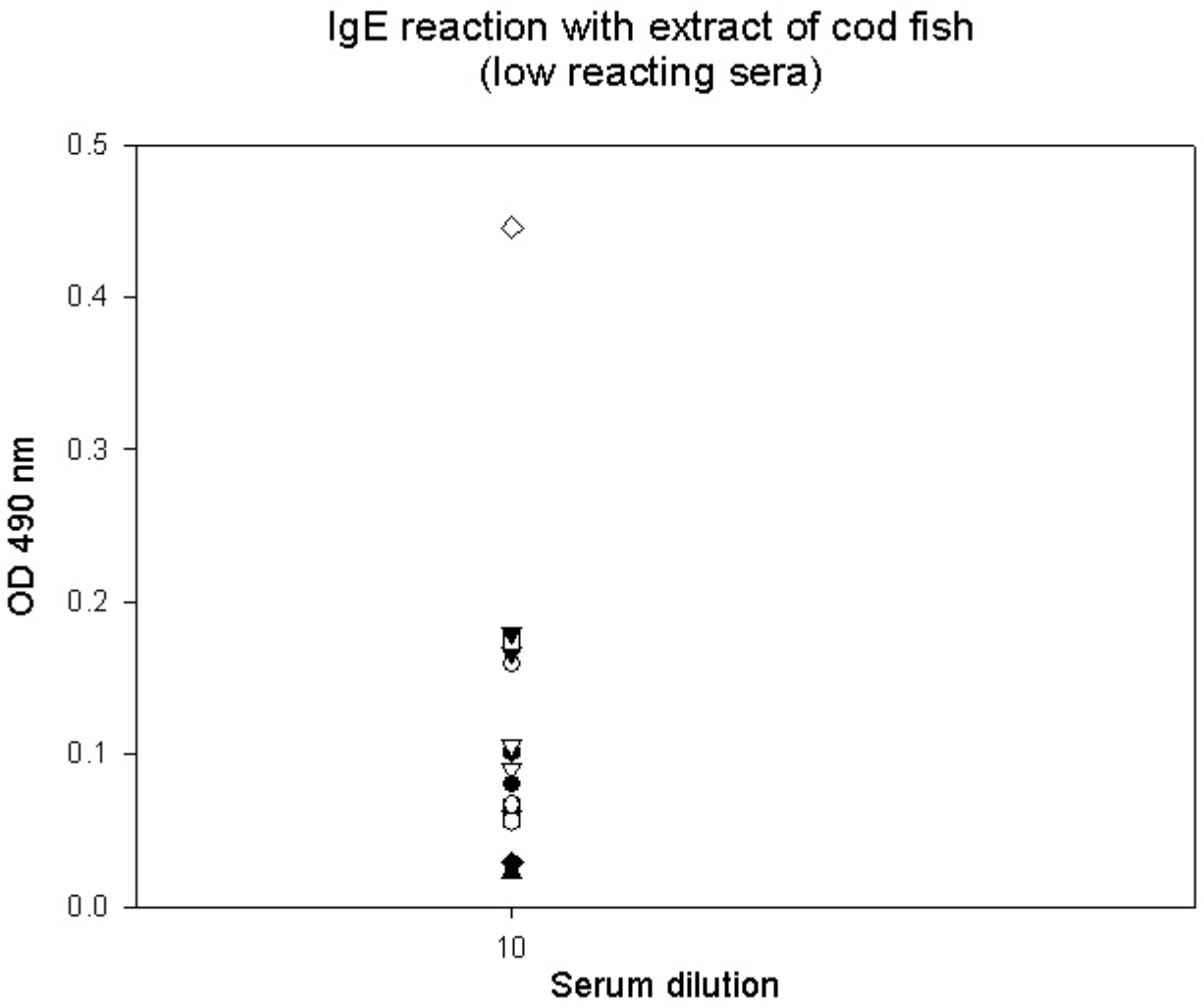

Fig. 3. Direct EAST with codfish extract acting as allergosorbent. Low reacting sera.

Step #5: Testing of targeted sera against raw materials for Imedeen

Sera from the 28 patients were tested in EAST where wells had been coated with 2) Fish Extract, 3) Fish Dry Cartilage, and 4) Fish Frozen Meat in comparison to 1) Codfish extract. In no case did the OD-values of the Imedeen raw materials exceed 0.1, i.e. there were no IgE reactions to any of the three materials.

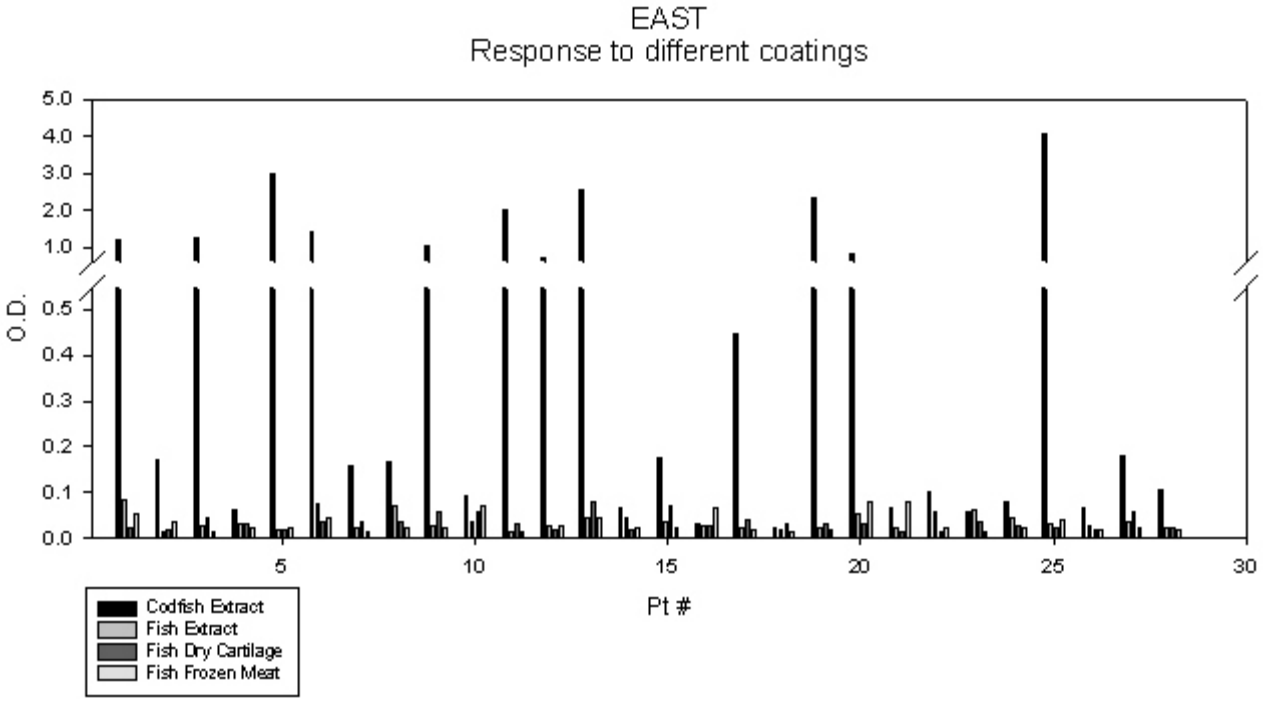

Fig. 4 Direct EAST. Comparison of IgE binding to codfish extract with raw materials for Imedeen.

Step #6: Testing of cross-reactivity between codfish raw materials for Imedeen

Eight sera with an reactivity corresponding to an OD-value > 0.2 were tested in EAST-inhibition, where the binding to the codfish extract was attempted inhibited by codfish extract (autologous inhibition) or with 1) Fish Extract, 2) Fish Dry Cartilage, and 3) Fish Frozen Meat (heterologous inhibition).

For all eight sera a substantial inhibition was seen in autologous inhibition with codfish extract undiluted, 1:10, and 1:100. For the six highest reacting sera inhibitions > 80% were consistently observed in all concentrations of codfish extract. For the two lowest reacting sera (OD values between 0.2 and 0.25) autologous inhibition were 60-80 % most likely due to the fact that % inhibition was not corrected for the blank values. Assuming an average blank value of 0.030 a correction would in most cases increase autologous inhibition of these sera to > 80%, but this was not done due the large relative variation of background values.

In two cases an inhibition caused by the Fish Extract was observed. This was however observed sporadically and only for the 1:100 dilution, whereas no inhibition was seen with the same extract in the undiluted or the 1:10 dilution. Thus the results were ascribed to outlayers.

In one serum a dose-dependent inhibition was seen with the Fish Dry Cartilage and the Fish Frozen Meat. The response of these extracts undiluted was lower than to codfish 1:100, indicating a potency of less than 1 % of the codfish extract. Two other sera demonstrated significant inhibition by a single concentration of Fish Frozen Meat, undiluted and 1:10, respectively.

Taking into account the apparent cross-reactivity between codfish and Fish Frozen Meat as demonstrated in the Gad c1 ELISA it is likely the two or three sera have a real cross-reactivity between Fish Frozen Meat and codfish, and one serum for Fish Dry Cartilage. Even using undiluted Fish Extract with a final protein concentration of 12 mg/ml it has not been possible to cause any significant inhibition. In the autologous system a strong inhibition could be observed with a final concentration of 65 microgram/ml of codfish extract.

Step #7: (if possible) Testing of cross-reactivity between materials for Imedeen and codfish
Since no sera reacted positively to the three Imedeen-related materials #7 was impossible to perform and was omitted.

Step #8: Testing of the biological activity of raw materials for Imedeen in histamine release assay using basophils that are passively sensitized with sera from codfish allergic individuals
In the Table below is shown the response in passively sensitized basophils to the three previously tested materials, and codfish extract used as a positive control. In correspondence with the results from Step #4, not all sera are positive in this test despite the clinical reactivity to codfish.

Patient#	Fish Extract	Fish Dry Cartilage	Fish Frozen Meat	Codfish
1	0	0	0	5
2	0	0	0	0
3	0	0	0	1
4	0	0	0	0
5	0	0	0	6
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	6
10	0	0	0	0
11	0	0	0	6
12	0	0	0	1
13	0	0	0	4
14	0	0	0	4
15	0	0	0	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	6
20	0	0	0	5
21	0	0	0	0
22	0	0	0	6
23	0	0	0	0
24	0	0	0	0
25	0	0	0	6
26	0	0	0	4
27	0	0	0	6
28	0	0	0	5
Negative Control	0	0	0	0

None of the samples related to Imedeen raw materials were positive. It should be remarked that when a serum displays a reaction of 5 or 6 it means that it reacts to a codfish concentration that is 330 or 1000 times lower, than the concentration that did not induce a reaction for the Imedeen induced samples.

Conclusions

The study has demonstrated that by use of rabbit antibodies, the activity of the Fish Extract which is directly used for production of Imedeen has less than 10^{-7} times the activity of Gad c1, the major allergen from codfish.

The direct IgE-tests (EAST) using the three raw materials as coatings demonstrated no significant binding, suggesting that no or very small amounts of allergens are present in the preparations.

The inhibition experiments demonstrated a possible low activity in a few sera for the Frozen Meat and the Dry Cartilage, but no consistent inhibition for the Fish Extract.

Finally, passive sensitization of basophils with sera from codfish allergic patients, which is considered to be the most sensitive allergen detection method, did not show any reaction to neither Dry Cartilage nor Fish Extract.

Raw Data

ELISA

	Conc. (ng/ml)	O.D.
25.46 mg/ml	**25460000**	**Fish Extract (1)**
Undiluted	25460000	0.076
1:2	12730000	0.0575
1:4	6365000	0.0555
1:8	3182500	0.058
1.2 mg/ml	**1200000**	**Fish Dry Cartilage (2)**
Undiluted	1200000	0.0465
1:2	600000	0.062
1:4	300000	0.059
1:8	150000	0.065
0.56 mg/ml	**560000**	**Fish Frozen Meat (3)**
Undiluted	560000	3.193
1:2	280000	2.858
1:4	140000	1.4165
1:8	70000	0.245
	5-fold dilutions	
1.108 mg/ml dil. 1:100	**1108000**	**Codfish Extract (5)**
Undiluted	11080	1.159
1:5	2216	1.036
1:25	443.2	0.912
1:125	88.64	0.744
1:625	17.728	0.5365
1:3125	3.5456	0.261
1:15625	0.70912	0.0995
1:78125	0.141824	0.0675
1 ug/ml	**1000**	**Gad c1**
Undiluted	1000	1.347
1:5	200	1.2015
1:25	40	0.9695
1:125	8	0.637
1:625	1.6	0.313
1:3125	0.32	0.115
1:15625	0.064	0.0725
1:78125	0.0128	0.0595

blank#1	0.0545
blank#2	0.0565
blank#3	0.0535
blank#4	0.058
mean	0.056
std.dev	0.00174553000547109
mean + 3 x std dev =	0.0626071200218844

EAST, coating with codfish

	OD490 nm						OD490 nm					
	Fort. 1:10		**Fort. 1:50**		**Fort. 1:250**		**Fort. 1:10**		**Fort. 1:50**		**Fort. 1:250**	
Prøve	**1**	**2**	**1**	**2**	**1**	**2**	Mean	CV	Mean	CV	Mean	CV
11	1.264	1.159	0.235	0.230	0.079	0.047	1.2115	4.33%	0.2325	1.08%	0.063	25.40%
13	1.267	1.260	0.254	0.245	0.063	0.060	1.2635	0.28%	0.2495	1.80%	0.0615	2.44%
16	2.951	3.033	0.770	0.777	0.164	0.160	2.992	1.37%	0.7735	0.45%	0.162	1.23%
17	1.425	1.424	0.309	0.289	0.054	0.052	1.4245	0.04%	0.299	3.34%	0.053	1.89%
20	1.029	1.045	0.202	0.190	0.059	0.035	1.037	0.77%	0.196	3.06%	0.047	25.53%
22	1.997	1.978	0.459	0.450	0.096	0.097	1.9875	0.48%	0.4545	0.99%	0.0965	0.52%
23	0.687	0.699	0.135	0.132	0.028	0.030	0.693	0.87%	0.1335	1.12%	0.029	3.45%
24	2.558	2.523	0.641	0.655	0.131	0.132	2.5405	0.69%	0.648	1.08%	0.1315	0.38%
31	2.348	2.359	0.631	0.638	0.133	0.130	2.3535	0.23%	0.6345	0.55%	0.1315	1.14%
32	0.835	0.832	0.168	0.173	0.042	0.051	0.8335	0.18%	0.1705	1.47%	0.0465	9.68%
969	4.007	4.061	1.915	1.975	0.466	0.489	4.034	0.67%	1.945	1.54%	0.4775	2.41%
12	0.184	0.156					0.17	8.24%				
15	0.067	0.055					0.061	9.84%				
18	0.162	0.156					0.159	1.89%				
19	0.171	0.162					0.1665	2.70%				
21	0.029	0.151					0.09	67.78%				
26	0.071	0.059					0.065	9.23%				
27	0.184	0.163					0.1735	6.05%				
28	0.034	0.024					0.029	17.24%				
29	0.444	0.447					0.4455	0.34%				
30	0.023	0.023					0.023	0.00%				
33	0.067	0.066					0.0665	0.75%				
972	0.102	0.101					0.1015	0.49%				
971	0.056	0.056					0.056	0.00%				
970	0.078	0.082					0.08	2.50%				
968	0.058	0.075					0.0665	12.78%				
967	0.165	0.195					0.18	8.33%				
966	0.110	0.101					0.1055	4.27%				

Coating with Fish Extract

	OD490 nm						OD490 nm					
	Fort. 1:10		**Fort. 1:50**		**Fort. 1:250**		**Fort. 1:10**		**Fort. 1:50**		**Fort. 1:250**	
Prøve	**1**	**2**	**1**	**2**	**1**	**2**	Mean	CV	Mean	CV	Mean	CV
11	0.091	0.073	0.026	0.031	0.025	0.016	0.082	10.98%	0.0285	8.77%	0.0205	21.95%
13	0.035	0.021	0.018	0.011	0.032	0.019	0.028	25.00%	0.0145	24.14%	0.0255	25.49%
16	0.013	0.018	0.029	0.035	0.041	0.026	0.0155	16.13%	0.032	9.38%	0.0335	22.39%
17	0.082	0.068	0.054	0.061	0.031	0.018	0.075	9.33%	0.0575	6.09%	0.0245	26.53%
20	0.021	0.034	0.012	0.014	0.021	0.023	0.0275	23.64%	0.013	7.69%	0.022	4.55%
22	0.014	0.016	0.011	0.023	0.017	0.012	0.015	6.67%	0.017	35.29%	0.0145	17.24%
23	0.018	0.034	0.025	0.021	0.041	0.043	0.026	30.77%	0.023	8.70%	0.042	2.38%
24	0.031	0.054	0.021	0.026	0.024	0.023	0.0425	27.06%	0.0235	10.64%	0.0235	2.13%
31	0.031	0.015	0.026	0.017	0.064	0.048	0.023	34.78%	0.0215	20.93%	0.056	14.29%
32	0.056	0.051	0.042	0.036	0.071	0.068	0.0535	4.67%	0.039	7.69%	0.0695	2.16%
969	0.036	0.024	0.021	0.022	0.031	0.020	0.03	20.00%	0.0215	2.33%	0.0255	21.57%
12	0.010	0.013					0.0115	13.04%				
15	0.030	0.034					0.032	6.25%				
18	0.024	0.020					0.022	9.09%				
19	0.074	0.062					0.068	8.82%				
21	0.039	0.032					0.0355	9.86%				
26	0.041	0.050					0.0455	9.89%				
27	0.041	0.027					0.034	20.59%				
28	0.021	0.029					0.025	16.00%				
29	0.014	0.031					0.0225	37.78%				
30	0.019	0.017					0.018	5.56%				
33	0.023	0.024					0.0235	2.13%				
972	0.049	0.062					0.0555	11.71%				
971	0.057	0.064					0.0605	5.79%				
970	0.046	0.040					0.043	6.98%				
968	0.027	0.024					0.0255	5.88%				
967	0.034	0.036					0.035	2.86%				
966	0.016	0.026					0.021	23.81%				

Coating with Fish Dry Cartilage

	OD490 nm						OD490 nm					
	Fort. 1:10		**Fort. 1:50**		**Fort. 1:250**		**Fort. 1:10**		**Fort. 1:50**		**Fort. 1:250**	
Prøve	**1**	**2**	**1**	**2**	**1**	**2**	Mean	CV	Mean	CV	Mean	CV
11	0.016	0.027	0.019	0.028	0.041	0.024	0.0215	25.58%	0.0235	19.15%	0.0325	26.15%
13	0.036	0.054	0.070	0.052	0.045	0.023	0.045	20.00%	0.061	14.75%	0.034	32.35%
16	0.024	0.013	0.026	0.048	0.031	0.013	0.0185	29.73%	0.037	29.73%	0.022	40.91%
17	0.038	0.034	0.028	0.031	0.018	0.011	0.036	5.56%	0.0295	5.08%	0.0145	24.14%
20	0.057	0.059	0.042	0.030	0.062	0.064	0.058	1.72%	0.036	16.67%	0.063	1.59%
22	0.039	0.026	0.024	0.036	0.027	0.020	0.0325	20.00%	0.03	20.00%	0.0235	14.89%
23	0.023	0.014	0.011	0.015	0.013	0.028	0.0185	24.32%	0.013	15.38%	0.0205	36.59%
24	0.086	0.072	0.060	0.043	0.049	0.027	0.079	8.86%	0.0515	16.50%	0.038	28.95%
31	0.035	0.024	0.046	0.044	0.035	0.032	0.0295	18.64%	0.045	2.22%	0.0335	4.48%
32	0.034	0.024	0.043	0.038	0.017	0.014	0.029	17.24%	0.0405	6.17%	0.0155	9.68%
969	0.024	0.023	0.040	0.034	0.021	0.027	0.0235	2.13%	0.037	8.11%	0.024	12.50%
12	0.018	0.015					0.0165	9.09%				
15	0.036	0.024					0.03	20.00%				
18	0.041	0.028					0.0345	18.84%				
19	0.035	0.034					0.0345	1.45%				
21	0.056	0.061					0.0585	4.27%				
26	0.019	0.013					0.016	18.75%				
27	0.075	0.063					0.069	8.70%				
28	0.029	0.024					0.0265	9.43%				
29	0.039	0.043					0.041	4.88%				
30	0.034	0.026					0.03	13.33%				
33	0.015	0.013					0.014	7.14%				
972	0.017	0.012					0.0145	17.24%				
971	0.028	0.042					0.035	20.00%				
970	0.030	0.023					0.0265	13.21%				
968	0.015	0.024					0.0195	23.08%				
967	0.054	0.057					0.0555	2.70%				
966	0.024	0.021					0.0225	6.67%				

Coating with Fish Frozen Meat

	OD490 nm						OD490 nm					
	Fort. 1:10		**Fort. 1:50**		**Fort. 1:250**		**Fort. 1:10**		**Fort. 1:50**		**Fort. 1:250**	
Prøve	**1**	**2**	**1**	**2**	**1**	**2**	Mean	CV	Mean	CV	Mean	CV
11	0.053	0.054	0.010	0.011	0.051	0.053	0.053	0.94%	0.01	5.00%	0.052	1.92%
13	0.012	0.014	0.041	0.045	0.037	0.043	0.013	7.69%	0.043	4.65%	0.04	7.50%
16	0.026	0.016	0.023	0.025	0.054	0.066	0.021	23.81%	0.024	4.17%	0.06	10.00%
17	0.040	0.044	0.051	0.055	0.042	0.043	0.042	4.76%	0.053	3.77%	0.042	1.19%
20	0.021	0.021	0.020	0.024	0.018	0.022	0.021	0.00%	0.022	9.09%	0.02	10.00%
22	0.069	0.073	0.060	0.066	0.048	0.058	0.071	2.82%	0.063	4.76%	0.053	9.43%
23	0.010	0.012	0.021	0.025	0.032	0.038	0.011	9.09%	0.023	8.70%	0.035	8.57%
24	0.021	0.029	0.040	0.042	0.015	0.019	0.025	16.00%	0.041	2.44%	0.017	11.76%
31	0.013	0.019	0.014	0.016	0.051	0.053	0.016	18.75%	0.015	6.67%	0.052	1.92%
32	0.079	0.081	0.012	0.020	0.033	0.037	0.08	1.25%	0.016	25.00%	0.035	5.71%
969	0.031	0.047	0.039	0.043	0.021	0.025	0.039	20.51%	0.041	4.88%	0.023	8.70%
12	0.031	0.039					0.035	11.43%				
15	0.022	0.026					0.024	8.33%				
18	0.012	0.014					0.013	7.69%				
19	0.015	0.029					0.022	31.82%				
21	0.043	0.049					0.046	6.52%				
26	0.021	0.025					0.023	8.70%				
27	0.020	0.022					0.021	4.76%				
28	0.061	0.067					0.064	4.69%				
29	0.015	0.017					0.016	6.25%				
30	0.011	0.013					0.012	8.33%				
33	0.073	0.089					0.081	9.88%				
972	0.019	0.023					0.021	9.52%				
971	0.011	0.013					0.012	8.33%				
970	0.017	0.025					0.021	19.05%				
968	0.014	0.017					0.0155	9.68%				
967	0.014	0.026					0.02	30.00%				
966	0.014	0.022					0.018	22.22%				

EAST inhibition. Sample 1 = Fish Extract, Sample 2 = Fish Dry Cartilage, Sample 3 = Fish Frozen Meat, Sample 5 = Codfish Extract. The 8 sera with the strongest binding to codfish were used. U.f. = Undiluted.

	Prøve 11		Mean	C.V. %	% inhib
Uinhib	1.252	1.058	1.155	16.80%	0.00%
1, u.f.	1.194	1.095	1.1445	8.65%	0.91%
1, 1:10	1.212	1.192	1.202	1.66%	-4.07%
1, 1:100	0.341	0.316	0.3285	7.61%	71.56%
2, u.f.	0.95	0.912	0.931	4.08%	19.39%
2, 1:10	1.123	1.115	1.119	0.71%	3.12%
2, 1:100	1.167	1.164	1.1655	0.26%	-0.91%
3, u.f.	0.848	0.859	0.8535	1.29%	26.10%
3, 1:10	0.984	0.954	0.969	3.10%	16.10%
3, 1:100	1.043	1.002	1.0225	4.01%	11.47%
5, u.f.	0.023	0.037	0.03	46.67%	97.40%
5, 1:10	0.046	0.076	0.061	49.18%	94.72%
5, 1:100	0.088	0.087	0.0875	1.14%	92.42%
	Prøve 13		Mean	C.V. %	% inhib
Uinhib	0.93	0.976	0.953	4.83%	0.00%
1, u.f.	1	0.926	0.963	7.68%	-1.05%
1, 1:10	1.121	1.075	1.098	4.19%	-15.22%
1, 1:100	1.102	1.091	1.0965	1.00%	-15.06%
2, u.f.	1.034	0.934	0.984	10.16%	-3.25%
2, 1:10	1.088	1.088	1.088	0.00%	-14.17%
2, 1:100	1.101	1.081	1.091	1.83%	-14.48%
3, u.f.	0.935	0.999	0.967	6.62%	-1.47%
3, 1:10	0.831	0.893	0.862	7.19%	9.55%
3, 1:100	0.958	1.049	1.0035	9.07%	-5.30%
5, u.f.	0.045	0.039	0.042	14.29%	95.59%
5, 1:10	0.023	0.043	0.033	60.61%	96.54%
5, 1:100	0.046	0.05	0.048	8.33%	94.96%
	Prøve 16		Mean	C.V. %	% inhib
Uinhib	0.229	0.233	0.231	1.73%	0.00%
1, u.f.	0.215	0.216	0.2155	0.46%	6.71%
1, 1:10	0.259	0.256	0.2575	1.17%	-11.47%
1, 1:100	0.267	0.251	0.259	6.18%	-12.12%
2, u.f.	0.247	0.244	0.2455	1.22%	-6.28%
2, 1:10	0.245	0.245	0.245	0.00%	-6.06%
2, 1:100	0.255	0.27	0.2625	5.71%	-13.64%
3, u.f.	0.277	0.271	0.274	2.19%	-18.61%
3, 1:10	0.204	0.219	0.2115	7.09%	8.44%
3, 1:100	0.251	0.266	0.2585	5.80%	-11.90%
5, u.f.	0.049	0.084	0.0665	52.63%	71.21%
5, 1:10	0.049	0.043	0.046	13.04%	80.09%
5, 1:100	0.038	0.048	0.043	23.26%	81.39%

	Prøve 17		Mean	C.V. %	% inhib
Uinhib	0.883	0.83	0.8565	6.19%	0.00%
1, u.f.	1.044	1.004	1.024	3.91%	-19.56%
1, 1:10	1.164	1.178	1.171	1.20%	-36.72%
1, 1:100	1.158	1.142	1.15	1.39%	-34.27%
2, u.f.	1.066	1.07	1.068	0.37%	-24.69%
2, 1:10	1.148	1.124	1.136	2.11%	-32.63%
2, 1:100	1.207	1.206	1.2065	0.08%	-40.86%
3, u.f.	0.929	0.888	0.9085	4.51%	-6.07%
3, 1:10	0.801	0.806	0.8035	0.62%	6.19%
3, 1:100	1.125	1.112	1.1185	1.16%	-30.59%
5, u.f.	0.055	0.058	0.0565	5.31%	93.40%
5, 1:10	0.033	0.042	0.0375	24.00%	95.62%
5, 1:100	0.141	0.141	0.141	0.00%	83.54%
	Prøve 22		Mean	C.V. %	% inhib
Uinhib	0.356	0.375	0.3655	5.20%	0.00%
1, u.f.	0.376	0.49	0.433	26.33%	-18.47%
1, 1:10	0.481	0.506	0.4935	5.07%	-35.02%
1, 1:100	0.48	0.514	0.497	6.84%	-35.98%
2, u.f.	0.379	0.474	0.4265	22.27%	-16.69%
2, 1:10	0.469	0.488	0.4785	3.97%	-30.92%
2, 1:100	0.492	0.477	0.4845	3.10%	-32.56%
3, u.f.	0.424	0.471	0.4475	10.50%	-22.44%
3, 1:10	0.213	0.492	0.3525	79.15%	3.56%
3, 1:100	0.475	0.522	0.4985	9.43%	-36.39%
5, u.f.	0.074	0.067	0.0705	9.93%	80.71%
5, 1:10	0.031	0.038	0.0345	20.29%	90.56%
5, 1:100	0.058	0.069	0.0635	17.32%	82.63%
	Prøve 24		Mean	C.V. %	% inhib
Uinhib	0.767	0.774	0.7705	0.91%	0.00%
1, u.f.	0.611	0.634	0.6225	3.69%	19.21%
1, 1:10	0.666	0.68	0.673	2.08%	12.65%
1, 1:100	0.693	0.414	0.5535	50.41%	28.16%
2, u.f.	0.195	0.096	0.1455	68.04%	81.12%
2, 1:10	0.34	0.342	0.341	0.59%	55.74%
2, 1:100	0.591	0.513	0.552	14.13%	28.36%
3, u.f.	0.245	0.52	0.3825	71.90%	50.36%
3, 1:10	0.355	0.386	0.3705	8.37%	51.91%
3, 1:100	0.641	0.404	0.5225	45.36%	32.19%
5, u.f.	0.062	0.07	0.066	12.12%	91.43%
5, 1:10	0.031	0.034	0.0325	9.23%	95.78%
5, 1:100	0.045	0.121	0.083	91.57%	89.23%

	Prøve 31		Mean	C.V. %	% inhib
Uinhib	0.224	0.183	0.2035	20.15%	0.00%
1, u.f.	0.193	0.169	0.181	13.26%	11.06%
1, 1:10	0.121	0.142	0.1315	15.97%	35.38%
1, 1:100	0.19	0.211	0.2005	10.47%	1.47%
2, u.f.	0.273	0.196	0.2345	32.84%	-15.23%
2, 1:10	0.182	0.196	0.189	7.41%	7.13%
2, 1:100	0.207	0.196	0.2015	5.46%	0.98%
3, u.f.	0.226	0.342	0.284	40.85%	-39.56%
3, 1:10	0.185	0.12	0.1525	42.62%	25.06%
3, 1:100	0.176	0.21	0.193	17.62%	5.16%
5, u.f.	0.067	0.08	0.0735	17.69%	63.88%
5, 1:10	0.049	0.037	0.043	27.91%	78.87%
5, 1:100	0.052	0.062	0.057	17.54%	71.99%
	Prøve 973-04		Mean	C.V. %	% inhib
Uinhib	0.899	0.927	0.913	3.07%	0.00%
1, u.f.	0.89	0.759	0.8245	15.89%	9.69%
1, 1:10	0.896	0.854	0.875	4.80%	4.16%
1, 1:100	0.886	0.752	0.819	16.36%	10.30%
2, u.f.	0.756	0.713	0.7345	5.85%	19.55%
2, 1:10	0.793	0.812	0.8025	2.37%	12.10%
2, 1:100	0.913	0.869	0.891	4.94%	2.41%
3, u.f.	0.258	0.592	0.425	78.59%	53.45%
3, 1:10	0.854	0.816	0.835	4.55%	8.54%
3, 1:100	1.161	0.891	1.026	26.32%	-12.38%
5, u.f.	0.073	0.053	0.063	31.75%	93.10%
5, 1:10	0.043	0.04	0.089	33.71%	90.25%
5, 1:100	0.104	0.074	0.0415	7.23%	95.45%

References

(1) Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Bjorksten B, Moneret-Vautrin D et al. Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee. Allergy 1995; 50:623-35.

(2) Hansen TK, Poulsen LK, Skov PS, Hefle SL, Hlywka JJ, Taylor SL et al. A randomized, double-blinded, placebo-controlled oral challenge study to evaluate the potential of commercial, food-grade gelatin. Food Chem Toxicol 2004; 42:2037-44.

(3) Poulsen LK, Pedersen MH, Platzer M, Madsen N, Sten E, Bindslev-Jensen C et al. Immunochemical and biological quantification of peanut extract. Arbeiten aus dem Paul-Ehrlich-Institut 2004; 94:97-106.

(4) Poulsen LK, Pedersen MF, Malling H-J, Søndergaard I, Weeke B. Maxisorp RAST. A sensitive method for detection of absolute quantities of antigen-specific IgE. Allergy 1989; 44:178-89.