

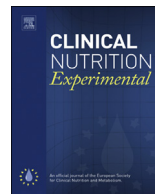


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Antisecretory Factor (AF) egg-yolk peptides reflects the intake of AF-activating feed in hens

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SUMMARY

Objective: The aim of the present investigation was to determine the level of the active form of the endogenous protein Antisecretory Factor (actAF), and also the active form of AF immunoreactive molecules in the affinity purified egg yolk.

Design: Determination of AF in affinity purified egg yolk by means of *in vivo* and *in vitro* methods.

Setting: Sahlgrenska University Hospital and Chalmers University of Technology, both units located in Gothenburg, Sweden. The farm housing the egg-laying hens is situated some 10 miles west to Stockholm.

Samples: Egg yolk collected weekly from hens subjected to AF-stimulating feed for up to 18 weeks.

Methods and main outcome measures: The methods used were:

a/ The *in vivo* rat ligated jejunal loop assay.

b/ Two variations of ELISA, i.e.

1. An indirect ELISA using a polyclonal antibody against AF-16 antibodies.

2. A competitive enzyme immunoassay for detection of the peptide AF-16 and also related immunoreactive molecules.

c/ Matrix-Assisted Laser Desorption Ionization Mass Spectrometry, (MALDI-MS).

The numeric variables registered represents:

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a/ The rat jejunal ligated loop assay demonstrates the influence of AF on the *in vivo* secretory response (mg/cm) to cholera toxin challenge. The ligated loop is some 12–15 cm long and placed on the mid part of jejunum, and the cholera toxin induced secretion is registered after a 5 h long challenge period.

b/ The indirect ELISA method demonstrates the relative concentration of immunogenic AF peptides/AF-16 peptides and also related immunoreactive compounds by means of absorbance values, while the values of the competitive immunoassay represent the concentration of AF-16 peptides including similar immunoreactive peptides in ng/ml.

c/ The MALDI-MS method provide information about the concentration of the AF-16 peptide down to nanogram per ml. levels after mass spectrometry analysis of the sample.

Results: All methods revealed similar results by demonstrating a continuous increase over time in the collected egg yolk samples. Thus, low AF activity was registered in egg yolk collected in the period of 1–10 weeks of AF-stimulated feeding, significantly higher AF values was registered in yolk collected between 10 and 15 weeks of feeding, while maximal AF concentration was determined after 15 weeks of feeding. Thus, in the period between 15 and 18 weeks of stimulated AF-feeding, no further increase of the endogenous AF activity could be registered despite continuous AF-stimulated feeding.

Conclusion: During the period of AF-stimulated feeding of the egg laying hens the registration of AF concentration in the affinity purified egg yolk samples must be continuously registered over time. The various methods used for determination of AF concentration in the affinity purified egg yolk might all serve as tools in order to achieve the optimal concentration of active AF. Together, these methods will provide information about the optimal AF concentration in the final product consisting of spray dried egg yolk (Salovum®) used for disease treatment.

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1. Introduction

Antisecretory Factor (AF) also designated S5a, is a ubiquitous occurring endogenous protein demonstrated in all tissues so far analyzed [1,2]. *In vivo* AF acts as a regulator of water and ion transport across the cellular membrane and also mediates anti-inflammatory effects [3–8]. AF mode of action *in vivo* is intimately connected to the action of the complement system, forming complexes with C3 as well as with C4 [9]. AF is a constituent of the 19S proteasome subunit and can be considered as a part of the innate host defense system [9]. AF is present in blood in an active (*actAF*) form as well as in an inactive (AF) form [10]. AF is transformed into an active form after exposure of the small intestine to bacterial toxins or after intake of certain dietary products e.g. specially processed cereals (SPC-Flakes) [11,12]. The detailed biological action behind this activating mechanisms remains to be described, but probably *actAF* is the final product of a process which include exposure of the AF peptide after binding between the proteasome and the complement [9]. The active part of the full length AF protein, i.e. the part of the protein regulating the process behind secretion and inflammation, is located in the amino terminal part [13]. Various peptides emanating from amino acids sequences in this part of the AF

protein have been demonstrated to display antisecretory as well as anti-inflammatory actions [13]. Out of these peptides, the synthetic produced AF-16 peptide, made up of amino acids 36–51 of the full length AF protein, has been demonstrated to be the most preferable to use in experimental work, and consequently also to analyze *in vivo* and *in vitro*.

Salovum is a spray dried egg yolk rich in AF peptides, achieved after feeding the hens with an AF-inducing feed. The clinical use of the AF concept in the form of Salovum administration has been tested in controlled, clinical studies with patients suffering from diarrheal diseases [8,14,15], mastitis [16], Mb Crohn [7], colitis ulcerosa [14] and Meniere's disease [17]. In the studies performed so far, the regulating effects of *actAF* on secretion and inflammation have been demonstrated to significantly improve the clinical outcome of the various diseases. Furthermore, a raised intracranial pressure, commonly registered after various forms of trauma to the skull, can also be significantly counteracted and suppressed to normal levels in response to intake of Salovum [12]. The clinical influence of Salovum on the secretory and the inflammatory processes suggests a general, modulating effect of *actAF* in the first hand on neuronal tissue, but also on intestinal tissue [18]. Furthermore, improvement of the clinical outcome in the diseased patient in response to Salovum intake demonstrates a need for registration methods of AF concentration in Salovum. The validity of such methods is of major relevance for a regulated patient treatment, in order to ensure optimal and reproducible effects on the clinical outcome over time.

Thus, an improved clinical outcome has been registered in response to Salovum treatment of patients suffering from various diseases. Consequently, it is of importance to relate concentrations of AF-peptides in the egg yolk to the clinical effects achieved and registered.

The aim of this study was to analyze *in vivo* by the rat ligated loop assay the level of active AF in the affinity purified egg yolk [19]. The values of AF levels determined by this basic *in vivo* method for anti-secretory activity should thereafter be compared to the AF values achieved by the three *in vitro* methods described.

Consequently, in order to achieve reproducible and accurate clinical effects on pathophysiological variables in relation to Salovum intake, the AF concentration in the egg yolk must be determined in each one of the Salovum batches produced.

2. Material and methods

2.1. Animals

The test protocol was approved by the Regional Animal Experiments Ethical Committee in Gothenburg. All experiments were performed in accordance to guidelines for animal experiments (EC Directive 86/609/EEC). At arrival the male Sprague–Dawley rats (Nova-SCB, Sollentuna, Sweden) displayed a body weight of 180 ± 20 g. All rats were allowed a week for general adaptation in their cages before any form of experimental procedure started. The temperature and air ventilation in the animal quarters were monitored according to standard procedures.

2.2. Preparation of active AF from egg yolk

Egg-laying hens were fed an AF-stimulating diet, achieved by addition of specially processed cereals (SPC) to the ordinary hen feed. The special processing means a hydrothermal treatment of the cereals which changes the content of sugars and amino acids within defined limits. This specific alteration of sugars and amino acids in the AF-stimulating cereals has been described in detail previously [5].

The eggs were collected once a week and stored at +4 °C. The egg yolk was affinity purified before *in vivo* or *in vitro* determination of AF. AF was purified from egg yolk by affinity chromatography as previously described for human plasma [5]. The affinity purification process started by dissolving four yolks in phosphate buffered saline (sodium chloride, 145 mmol/l, buffered to pH 7.1 with 70 mmol/l phosphate, PBS), followed by centrifugation at $1000 \times g$ for 5 min, where after the supernatant was collected and passed through a 3 ml agarose column (Sephacrose 6B, GE Healthcare Biosciences AB). This process results in binding of the active AF to the agarose. PBS washing of the column followed, where after the agarose-absorbed AF was eluted from the agarose by passage of 1M α -methyl-D-glycoside. The eluate was dialyzed against PBS for 24 h at +4 °C and thereafter stored at –20 °C until use.

2.3. Determination *in vivo* of active AF prepared from egg yolk

The test performed for determination of the total, active AF content, was done *in vivo* by means of the rat ligated loop assay using cholera toxin (CT) as the secretagogue [20]. Thus, the purified egg yolk samples were injected intravenously into the anaesthetized rat. CT challenge (1.5 ml) in the 12–15 long jejunal loop followed, where after the total, secreted volume in response to the toxin was registered after a 5 h long challenge period.

An AF preparation mediating a 50% inhibition of the CT-induced fluid secretion was assigned one “AF-unit” value of 0.5. Previous experimental diarrheal studies have shown that AF values >0.5 AF units are correlated to a significant, clinical reduction of diarrhea in man as well as in animals [5,21].

2.4. AF-16 polyclonal antibody

Rabbits were used for production of primary polyclonal antibody against the AF peptide ³⁶-VCHSKTRSNPENNVGL-⁵¹ (aP8, Innovagen AB, Lund, Sweden). The peptide was produced by means of organic chemical synthesis on solid phase [22].

2.5. ELISA test of immunogenic AF-16

The level of AF-16 in affinity purified egg yolk was determined in an Enzyme-Linked Immunosorbent Assay (ELISA). The egg yolk samples were titrated in Maxisorp microtiter plates (Nunc) and incubated overnight at +4 °C. After blocking with 0.2% bovine serum albumin (BSA) in PBS for 45 min at 37 °C, the plates were washed with PBS + 0.05% Tween 20. The polyclonal antibody aP8, raised against the AF-16 peptide (see above), was thereafter added (diluted 1/500 in PBS + 0.05% Tween 20 + 0.2% BSA), followed by 2 h incubation of the plates at room temperature. After washing, alkaline phosphate (AP)-conjugated goat anti-rabbit immunoglobulin IgG (Jackson ImmunoResearch Europe Ltd.) was added for 1 h at room temperature. After washing, the substrate 4-Nitrophenyl phosphate (Sigma–Aldrich Sweden AB) in diethanolamine buffer (pH 9.8) with 1 mM MgCl₂, was added to the plates, and the bound enzyme was registered by reading the absorbance at 405 nm. Rabbit pre-immune serum was used as background. The pre-immune 405 absorbance values were subtracted from the sample absorbance which resulted in net absorbance values.

2.6. Competitive enzyme immunoassay of AF-16 in egg yolk

A commercial AF-16 Enzyme Immunoassay (EIA) kit was used (BMA Biomedicals, Augst, Switzerland). The laboratory procedure followed Protocol III in the enclosed Peptide EIA kit manual. The standards S1–S6 (0.2–200 ng/ml) of AF-16 included in the kit, H-VCHSKTRSNPENNVGL-OH, was serially diluted in EIA buffer + 0.25% BSA. The standards or the affinity purified egg yolk samples, i.e. eggs collected before start of the feeding experiments, or eggs from hens treated for 5 weeks, 10 weeks or 18 weeks with AF-stimulating feed were added to the 96-well plate. This plate had been coated with polyclonal antibodies against AF-16 diluted in EIA buffer + 0.25% BSA. The samples were analyzed in duplicates. After 1 h of incubation at room temperature, a dissolved biotinylated tracer was added for 2 h followed by 1 h incubation with streptavidin-HRP. The substrate was then added and the bound enzyme developed. The reactions were terminated by adding 2 M HCl per well, where after the absorbance was read at 450 nm within ten minutes. The concentration (ng/ml) of AF-16 was calculated in the egg yolk samples after a standard curve was plotted of the 450 nm absorbance reading (minus the absorbance values of the EIA buffer in the blank wells).

2.7. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis

Purified solutions of egg-yolk were prepared using affinity-chromatography as described above. α -Cyano-4-hydroxycinnamic acid (HCCA), trifluoroacetic acid (TFA) and acetonitrile (ACN) were all purchased from Sigma Aldrich. Deionized water (18.4 M Ω) was prepared using a Millipore Milli-Q system (Bedford, MA). Standard AF-16 peptide was purchased from Ross-Pedersen AS, Copenhagen.

Thus, one μL of α -Cyano-4-hydroxycinnamic (HCCA) acid solution (10 mg/mL in 50% ACN, 47.5% H_2O , 2.5% TFA) were mixed with one μL of the standard AF-16 peptide solution and the affinity purified egg yolk solutions, respectively. The mixtures were then vortexed, centrifuged and dropped onto the MALDI ground steel plates and allowed to air dry.

All the mass spectrometry data (mass range of 400–3000 Da) were acquired with an UltrafleXtreme MALDI TOF/TOF Instrument (Bruker Daltonics GmbH) running in reflective positive (RP) mode at 1 kHz laser repetition rate, and with the laser beam focus set to medium. The peptide calibration standard mixture in a mass range between 1000 Da and 3500 Da were used to calibrate the spectra externally. An autoXecute method in Flex control (v 3.0 Bruker, Daltonics GmbH, Germany) were used to collect 90,000 satisfactory laser shots in 300 shot steps from each spot on the MALDI MTP 384 ground steel BC target plate with a hexagon raster movement. Flex analysis (v 3.0 Bruker, Daltonics GmbH, Germany) and Origin (v. 8.1 OriginLab, Northampton, MA) were used for data analysis. Average spectra were exported as ASCII file from Flex Analysis. Peak areas were then calculated using a Gaussian function in nonlinear curve fitting analysis mode.

2.8. Statistics

Graphs were constructed using Excel 2010. One-way Student's t-test was used for comparing mean values in order to calculate the p values of significance. Thus, statistics are presented using the mean value \pm SEM unless otherwise stated.

3. Results

3.1. Active AF in egg yolk tested in vivo by the rat ligated loop assay

Egg-yolk samples were tested for AF activity by means of the rat ligated loop assay. The eggs had been collected each week from hens during their intake of an AF-stimulating diet. Each week three or four rats were used for ligated loop test of affinity purified AF prepared from four pooled egg yolks. Thus, the AF activity tested represents the mean AF activity extracted from the four pooled egg yolks. The values of AF activity presented in Table 1 represent values from the beginning, middle part and end of the experiment, i. e. after a total of 18 weeks of feeding with the AF stimulating diet. The results in Table 1 demonstrate stimulated AF activity for up to 14–18 weeks of diet intake. However, the continuous increase of AF activity terminated after some 15 weeks, and in the period between 15 and 18 weeks no further stimulation of AF activity could be registered by the ligated loop assay. Consequently, the weekly egg collection ended after 18 weeks of AF stimulating feeding. In the yolk from eggs collected during the last weeks of AF-stimulating feed intake, a significant AF activity was determined in samples diluted up to 1:1000 (Table 1).

3.2. ELISA for test of AF-activity in purified egg yolk

The results in Fig. 1 demonstrate increasing immunogenic AF-16 values in egg yolk samples obtained from hens fed AF-stimulating feed for up to 18 weeks. The ELISA absorbance levels was low in

Table 1

AF activity in purified egg-yolk samples collected from hens feeding an AF-inducing diet between 2 weeks and up to 18 weeks. A value of >0.5 AF-units correlates to a significantly inhibited intestinal secretion in response to cholera toxin challenge.

Test group	Weeks of AF-inducing diet	N	AF-units means \pm SEM*	Purified egg yolk dilution in test
A	2–3	6	0.2 \pm 0.01	–
B	8–9	6	0.6 \pm 0.02	–
C	14–18	8	0.5 \pm 0.02	1:1000

- Significance: A vs. B, $P = 0.0025$.
- A vs. C, $P = 0.0123$.
- B vs. C, $P = 0.001$, (the sample was diluted 1:1000 before test).

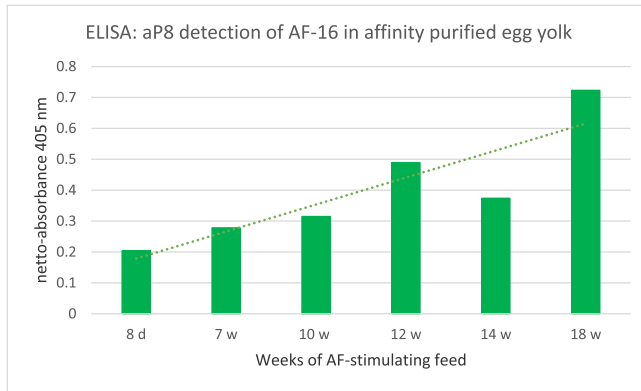


Fig. 1. ELISA test by use of antibody aP8 in pooled (N = 4) egg yolk samples prepared weekly during AF-stimulated feeding of the hen.

the samples prepared from the 10 week yolks, but rose to a more than doubled levels after 18 weeks of feeding. The continuous rise of immunogenic AF-16 values in response to feeding tentatively suggests that the AF yolk values directly reflect the biological response of the hen to the AF-stimulating feed.

3.3. EIA for test of AF-activity in purified egg yolk

A commercial kit for determination of yolk AF-16 concentration demonstrates constant increase of absorbance values over time (Fig. 2). After 10 weeks of AF-stimulating feed intake, the AF-16 concentration was four times higher when compared to 5 weeks (3 and 12 ng/ml, respectively), and after 18 weeks the AF-16 concentration demonstrated 24 ng/ml. The AF concentration in the egg yolk did not, however increase in the period between 15 and 18 weeks of feeding.

3.4. Relative quantification of AF-16 in purified egg yolk determined by MALDI-MS

MALDI-MS was used for the relative quantification of the AF-16 peptide in purified egg yolk samples (Fig. 3). The autoXecute method showed an increasing normalized signal intensity in egg yolk collected between 3 weeks and up to 18 weeks of AF-stimulating feed intake. Since HCCA was used as the matrix solution, the signal intensities of AF-16 were normalized to the matrix signal at m/z 568.2 [3HCCA + H]. The results in Fig. 3 demonstrate a more than threefold increase in AF-16 signal in the yolk samples collected between 3 weeks and up to 18 weeks after intake of AF-stimulating feed.

The relative amount of AF-16 peptide in egg yolk was determined by means of a dilution series of the AF-16 peptide added to purified egg yolk samples and followed by MALDI-MS analyses (Fig. 4). Normalizing to the three different matrix signals all gave similar results. These results tentatively suggest that this method is applicable to relative quantification of the egg-yolk AF-16 peptides down to ng/ml levels.

The AF-16 concentrations in purified egg yolk samples were determined by means of MALDI-MS (Fig. 5). The numeric AF-16 values of concentration (ng/ml) were determined by comparing to the values calculated in the standard curve demonstrated in Fig. 4. After 18 weeks of feeding, the AF-16 concentration could be determined to 1.5 ng/ml.

4. Discussion

A regulated intake of Salovum has been tested in controlled, clinical studies on patients suffering from various diseases dominated by secretory imbalance and inflammation [8,14,15,23]. The clinical influence of Salovum demonstrated on this broad variety of pathophysiology suggests a general, modulating effect of *actAF* in the first hand on neuronal tissue, but also on intestinal tissue [18].

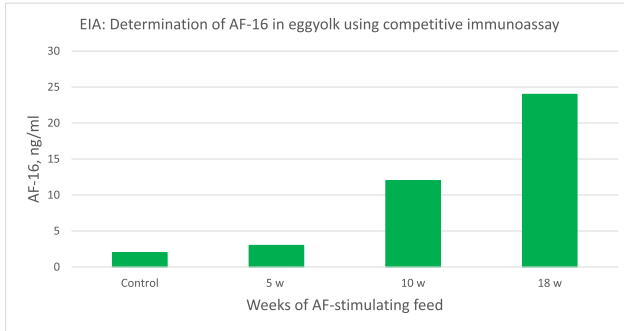


Fig. 2. Competitive enzyme immunoassay of AF-16 concentration performed on purified egg yolk obtained from hens fed AF-stimulating feed ($N = 4$). The ng/ml concentrations values were determined by means of calculating AF-concentrations in the standard curve.

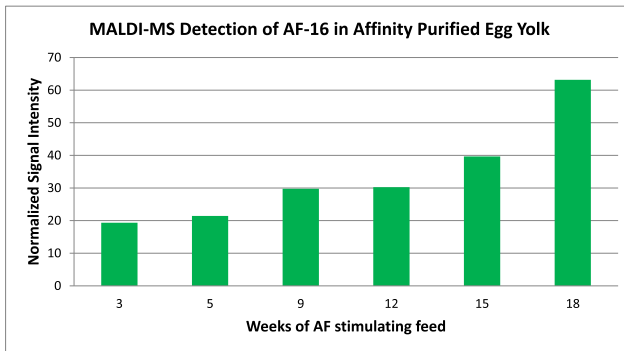


Fig. 3. 90,000 satisfactory laser shots collected from each sample showing the average normalized signal intensity of the AF-16 peptide from pooled, purified egg yolk samples ($N = 4$) ranging from 3 weeks and up to 18 weeks after intake of AF stimulating feed. The experiment was repeated 3 times and with similar results at each occasion.

Furthermore, improvement of the clinical outcome in response to Salovum treatment demonstrates the need for a method capable of quantification of the *actAF* concentration in Salovum. The validity of such methods is of major relevance for a regulated patient treatment, in order to ensure constant and reproducible effect on the clinical outcome over time on the diseased patient. Consequently, the AF concentration in the egg yolk must be determined in each one of the Salovum batches produced.

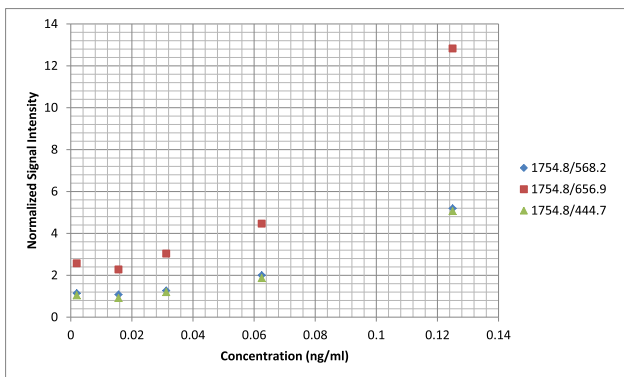


Fig. 4. MALDI-MS analysis of the relative amount of the AF-16 peptide in purified egg yolk samples ($n = 3$).

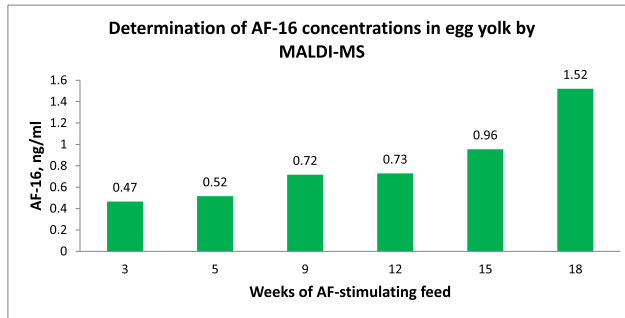


Fig. 5. MALDI-MS analysis of the AF-16 concentration in purified egg yolk samples. The numeric values in ng/ml were determined by comparing to the values calculated in the standard curve visualized in Fig. 4 (n = 3).

The present results of AF determinations in egg yolk emanates from use of four different methods, performed *in vivo* as well as *in vitro*. Thus, the rat ligated loop assay is performed *in vivo*, and the secretory response to the challenging agent injected into the loop is registered without any interference of anesthetic drugs. The absence of anesthetic drugs during the secretory response is of importance, since most forms of these drugs in various ways influences the transport of water and ions across the cellular membrane. The intestinal secretory response to cholera toxin after intravenous injection of affinity purified egg yolk represent the net sum effect of all forms of antisecretory molecules present in the yolk. Previous works demonstrate that almost all of such peptides emanates from the amino terminal part of the AF protein [13]. However, antisecretory influence mediated by low concentrations of other peptides than the AF-related peptides, cannot be excluded.

ELISA was used for test of immunogenic AF-16, which also includes related immunogenic molecules, and the polyclonal antibody used in this test has been raised in rabbit.

After 18 weeks of AF-stimulated feeding, quantification using MALDI-MS of the AF-16 concentration in the egg yolk samples resulted in a calculated AF-16 concentration value of 1.5 ng/ml. This is in contrast to the 24 ng/ml value achieved by use of the competitive immunoassay. The discrepancy between these two concentration values might partly be explained by the immunological reactivity in binding of AF antigens. Thus, such immunological cross reactivity will inevitably produce high ELISA absorbance values. In contrast, the MALDI-MS method determines solely the exact AF-16 concentration in the samples, due to the fact that these values are based on molecular weight data obtained by mass spectrometry from repeated laser shots into the medium.

Quantitative analysis using the MALDI-MS technique has previously been viewed as very difficult [24]. This is based on the fact that the matrix crystallization seldom yields a uniform distribution of the analyte and matrix on the analyzed target, resulting in regions with high and low signal intensity [25]. Thus, in the present experiments we therefore applied a standardized sample preparation protocol, careful calibration, replicated measurements and normalization of the ion intensities to several different matrix peak [24,26,27]. Thereby we achieved quantification when comparing intensities to known concentrations of AF-16 analyzed in the egg yolk medium. Consequently, we could create a calibration curve for the real egg yolk samples.

Amongst the methods used for AF determinations in the present paper the MALDI-MS technique must so far be considered as the most accurate technique for determination of the AF-16 concentrations in the purified egg yolk samples. The *in vivo* rat ligated loop assay demonstrates the net sum of all forms of peptides mediating antisecretory activity in the affinity purified egg yolk. Consequently, the effects achieved by this *in vivo* method also include antisecretory effects mediated by molecules not related to AF, but also affinity purified by the methods described. However, a similar form of significant increase of values over time in the 18 weeks long experimental feeding period was demonstrated by all of the four methods used. Thus, low values were registered at the beginning of the feeding period, while increasing values were registered for up to 15 weeks. Thereafter a plateau was reached with no further increase achieved by any of the methods. Consequently, the ligated loop assay does serve as the

basic assay for determination of the total, *in vivo* antisecretory capacity of the substance injected intravenously. However, in clinical routine, the *in vivo* ligated loop assay cannot be used for AF determinations due ethical reasons, performance difficulties, time and cost. Thus, all forms of AF analyses in clinical work must be performed by means of *in vitro* methods.

The *in vitro* methods, however, all serve with a more limited, but more specific determination of the antisecretory peptides.

Consequently, the observed correlation over time between the *in vivo* and *in vitro* methods strongly suggests that the three *in vitro* methods all can be used for determination of anti-secretory and anti-inflammatory potency of the future Salovum batches produced. Furthermore, the inhibited increase of yolk *actAF* in response to feeding in the 15–18-week period suggests the existence of a “regulatory feed-back” system for the interaction between plasma *actAF* concentration in relation to intestinal stimulation of AF. This form of interaction needs further experiments to explain.

The presented methods for determination of AF concentrations in the affinity purified egg yolk will, when performed together, hopefully provide a more exact quantitative description of the *actAF* concentration and potency determined in the various Salovum batches to be produced. For the medical doctor, a reproducible description of the yolk quality is a prerequisite for a documented, standardized and repeatable treatment of the patient. Following tests of the clinical potency and effects of the present batch of Salovum produced, has been performed. Thus, significant reduction of pediatric diarrhea has been documented from Lahore, Pakistan following oral Salovum treatment of the diseased children (Prof. Shakila Zaman, pers. comm). In Sweden, oral Salovum® treatment of neonatal piglet diarrhea significantly inhibit diarrhea secretion, while at the same time eliminate death commonly registered in relation to dehydration (dr. Leif Göransson, pers. comm.)

However, there is still a need for a continuous development of refined chemical analysis of the egg yolk, preferably specifically pointing out the anti-secretory and anti-inflammatory potency. This work must be continuously ongoing in order to ensure the Salovum-treated patient to reach an optimal clinical outcome.

Conflict of interest

None.

Acknowledgements

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