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RESIDUE OF HORSE SERUM PURIFICATION AS A NITROGEN SOURCE FOR GROWTH OF MICROORGANISMS

1 – CLOSTRIDIA

by

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Many commercial firms and vaccine producers desire to use cheap and inexpensive materials for preparation of biological products.

For many years at Razi Institute, Iran, large quantities of by-products obtained from purification and concentration of hyperimmune horse plasma against tetanus and diphtheria toxins have been discarded. To use these materials as a source of nitrogen, attempts were made to evaluate it for large scale production of human and veterinary vaccines.

This preliminary report presents some experiments carried out for preparation of Cl. perfringens type D epsilon toxin, by using horse serum residue peptic digest (H.S.R.P.D.).

MATERIALS AND METHODS

Protein source :

Horse albumin and other non-specific protein resulting from purification and concentration of antidiphtheria and antitetanus hyperimmune horse plasma was mostly used as protein source (Pope 1930). These materials which in the course of serum purification remained on the large filter, were freed from ammonium sulphate by washing continuously in running tap water. Then the protein material was dried or kept at 4°C if it was wet.

Method of digestion :

Two kg. of dried or 10 kg. of wet protein prepared as above was mixed with 20 litres of water. The mixture was heated to 80°C, then 20 litres of cold water

was added to it. The reaction of the mixture was then adjusted to pH 8.0 by means of anhydrous sodium carbonate, and the temperature was brought to 50°C. (Pope and Smith 1932). The digestion produced for 6 hours during which 150 ml. of trypsin emulsion was added to the mixture at half hourly intervals. The temperature was kept at 50°C and the mixture was frequently stirred throughout the procedure. After digestion 600 ml. of glacial acetic acid was added and the mixture brought to boiling point. Vigorous boiling was continued for 30 minutes and the digest was then left overnight in the cold room. During the storage period, undigesed maerial was precipitated. This was separated from the supernatant. To the supernatant, i.e. pancreatic digest of proteins, 200 g. activated charcoal (Darco 60) was added for decoloration. The mixture was left for 30 minutes and then it was first filtrated for clarification, then passed through a Seitz filter for sterilization. Total nitrogen was estimated by the microkjeldahl method, and the amino-acid content by the method of Sorensen.

Preparation of medium :

Horse serum residue peptic medium was prepared according to the following four formulae. :

(*) (A) 1-Pancreatic digestion of protein ....300 ml.

2-$PO_4HNa_2, 7H_2O$ .....7 g.
3-NaCl ..... 2.5 g.
4-Water ....1000 ml.

(B) Trace Element Solution (T.E.S.)

$FeSO_4, 7 H_2O$ 0.05%
$CuSO_4, 5 H_2O$ 0.02%
$ZnSO_4, 7 H_2O$ 0.02%
$MnCl_2, 4 H_2O$ 0.008%
$MgSO_4, 7 H_2O$ 4%

(*) Concentration of the total nitrogen for 1 litre of the medium is 3 g. per litre.

(C) Vitamin Solution.

Biotin	0.05	mg.	per	cent
Thiamine	50	"	"	"
Nicotinic acid	50	"	"	"
Pyridoxine	50	"	"	"
Vitamin B12	0.0025	"	"	"

(D) Glucose Solution

Glucose	50 g.
Distilled water	100 ml.

Medium (A) was sterilized by autoclaving at 110°C for 30 minutes. Solutions B, C and D were sterilized by filtration through Seitz filters.

To prepare the medium, solutions B, C and D were added at the rate of 5, 2, and 20 ml. respectively per litre to A.

To evaluate the usefulness of this medium, two other common media i.e. papain digest of meat and peptone media, were used simultaneously in each experiment.

Papain digest of meat was prepared according to the technique described previously (Rafyi and Ardehali 1961).

Peptone medium was produced following the procedure recomended by Smith and Matsucka (1954).

Production of Epsilon toxin

Strain. Cl. perfringens type D strain designated D-6 was used in this investigation. This strain had been shown to be a relatively good epsilon toxin producer.

Growth. It has been shown that the maximum yield of epsilon toxin is obtained 20 hr. after incubation (Janson 1960), thus the cultures were grown for 20 hr. in 800 ml. flasks and 15 litres bottles. Each bottle and flask was inoculated with 2% starter of Cl. perfringens type D. After active growth started the pH of the growing culture was readjusted to pH 7.5 with sterile 10% NaOH two times during incubation.

Toxin titration. Samples were taken from each flask and bottle for determination of minimum lethal doses and flocculation tests. Samples were centrifuged at 3000 r.p.m. and 0.25 per cent of trypsin (Difco: 1.250) was used for convertion of

prototoxin to epsilon toxin. The mixtures were incubated at 37°C. for 45 minutes and diluted immediately for determination of M.L.D. in mice as described by Batty and Glenny (1947).

EXPERIMENTS AND RESULTS

Experiment No. 1. The purpose of this experiment was to compare the use of H.S.R.P.D. medium with papain digest of meat and peptone medium for production of Cl. perfringens type D toxin. In this experiment 800 ml. of each medium kept in one liter flasks was used. Each flask of medium was inoculated with 16 ml. (2%) of actively growing culture of Cl. perfringens type D incubated at 37°C. After a total 20 hr. period of incubation and adjustment of the pH, a sample was taken of each flask for determination of the M.L.D. and the flocculation test. Five different tests as above were made and the results are tabulated in Table I.

TABLE I

Comparison of different culture media in preparation of Cl. perfringens type D, epsilon toxin

Type of medium	Batch No.	M.L.D./ml.	Lf/ml.
Horse serum residue peptic digest medium.	1	9000	75
	2	6000	60
	3	8000	70
	4	7000	65
	5	5000	50
Papain digest of meat.	1	8000	70
	2	5000	50
	3	6000	60
	4	4000	50
	5	7000	65
Commercial peptone.	1	5000	60
	2	1500	20
	3	3000	40
	4	11000	90
	5	8000	80

Experiment No. 2 This experiment was done to confirm the result of experiment No. 1 The experiment was performed in the same manner as the previous experiment, except that 15 litres of each medium were distributed in 20 litres bottles

for toxin production. The M.L.D. and Lf values of toxin were determined as mentioned above. The result summarized in Table II.

TABLE II

Comparison of different culture media in large scale production of Cl. perfringens type D, epsilon toxin.

Type of medium	Batch No.	M.L.D./ml.	Lf/ml.
Horse serum residue peptic digest medium.	1	6000	60
	2	5000	50
	3	7000	65
	4	4000	40
	5	7500	70
Papain digest of meat.	1	3000	35
	2	5000	50
	3	4000	45
	4	2000	20
	5	1500	20
Peptone medium	1	8000	90
	2	7000	80
	3	11000	100
	4	5000	50
	5	2000	30

The result of these experiments showed that the horse serum residue peptic digest medium produces a maximum yield of 8000-9000 M.L.D. of toxin which is comparable to the toxin produced in peptone medium and somewhat higher than production from papain meat digest medium. This medium gives a more homogenous results comparing with peptone or papain digest media.

DISCUSSION

Pulpy kidney disease is a fatal disease of lambs and sheep. It was diagnosed in Iran, in 1938 (Rafyi and Ardehali 1963). Because of the economical importance of the disease, the need for an effective vaccine has been increasing every year. More than 7,000,000 doses of vaccine using papain meat digest medium was prepared and used in 1966. As the result of increasing requests for the vaccine, some other cheap but effective source of nitrogen for medium preparation has been sought for many years.

At the Razi Institute large quantities of by-products obtained from purification and concentration of hyperimmune horse plasma against tetanus and diphheria toxins have been discarded in the past. Attempts were made to use this by-product as a source of nitrogen. The results of preliminary experiments were promising and a more precise experiment proved that this could be used as a relatively rich source of protein for preparation of pulpy kidney vaccine. The maximum titre obtained using this new medium, i.e. 7000-9000 M.L.D, was comparable to that of papain medium which is known, to be one of the best media for pulpy kidney vaccine preparation. More than 3,000,000 doses of vaccine have now been prepared using this new medium with satisfactory result.

SUMMARY

Horse albumin and other non specific protein resulting from purification of antidiphtheria and antitetanus sera were used as a medium for pulpy kidney vaccine production. The medium was found to be comparable to peptone medium and rather superior to papain meat digest which had been used for many year at the Razi Institute for pulpy kidney vaccine preparation. The technique of medium preparation is described in detail.

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